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Development of Materials and Electrodes for Microbial Bioelectrolysis Systems and Monitoring of Microbiologically Influenced Corrosion

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Abstract

The global energy transition toward carbon neutrality demands innovative technologies that integrate renewable electricity with sustainable carbon management strategies. This doctoral thesis addresses critical challenges in Power-to-X systems by investigating three interconnected research areas: oxygen evolution reaction (OER) electrocatalysis for efficient hydrogen production, microbiologically influenced corrosion (MIC) in biological methanation environments, and bioelectrochemical CO₂ conversion using alternative inocula from geothermal emissions.

The first part focuses on reducing precious metal demand in proton exchange membrane water electrolysis by rational catalyst design. A core-shell IrO₂@TiO₂ catalyst was developed by depositing ultrathin IrO₂ layers onto rutile TiO₂ supports, achieving electrochemical performance comparable to commercial IrO₂ while drastically lowering iridium loading. Crystallographic compatibility between rutile phases enabled superior electronic percolation and iridium utilization, demonstrating that support-catalyst engineering is an effective strategy for precious metal reduction.

The second part investigates the complex interplay between metallic (i.e., copper and iron alloys) cathode materials, microbial inocula, and corrosion phenomena in bioelectrochemical systems. Systematic comparison of biotic, thermally treated, and sterile conditions applied to a biomethanation inoculum revealed that complete sterilization consistently led to the most severe corrosion in all tested metal coupons, while controlled thermal treatments of the inoculum enabled selective enrichment of microorganisms capable of protective biomineralization. Across all materials, corrosion mitigation was governed primarily by solution chemistry, particularly carbonate and phosphate availability, rather than by microbial activity itself, highlighting the transition from microbiologically influenced corrosion to microbially mediated corrosion inhibition.

The third part demonstrates proof-of-concept bioelectrochemical CO₂ conversion using alternative inocula from geothermal emissions at the Monte Amiata volcanic complex. Reactor performance was strongly controlled by sulfate concentration in the inoculum, with high sulfate suppressing methanogenesis through competitive sulfate reduction. Stable methane production was achieved only under low-sulfate conditions, forming an acetogenic-methanogenic consortium sustained by continuous electrochemical polarization.

This integrated investigation provides fundamental insights into the critical challenges facing Power-to-X deployment. For anodic water oxidation, crystallographic engineering of support-catalyst interfaces enables dramatic precious metal reduction while maintaining performance. For cathodic biomethanation systems, understanding MIC mechanisms reveals that solution chemistry, particularly carbonate and phosphate availability, controls passivation more than microbial presence, with thermal treatment severity determining whether protective or detrimental surface films form. Finally, bioelectrochemical CO₂ conversion using geothermal emissions is fundamentally viable but requires rigorous inoculum selection to minimize sulfate-driven metabolic competition, with sustained polarization serving dual functions of driving productive electrosynthesis while preventing electrode passivation. These findings establish a foundation for developing integrated carbon capture and utilization systems that couple renewable electricity with biological CO₂ reduction at geothermal sites, while identifying critical materials and operational challenges that must be addressed for practical deployment.

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List of symbols and abbreviations

ACN: Acetonitrile	Spectroscopy
AcM: Acetoclastic Methanogens	EPS: Extracellular Polymeric Substance
AD: Anaerobic Digestion	HER: Hydrogen Evolution Reaction
AEL: Alkaline Electrolyzer	IC: Ion Chromatography
AEM: Anion-Exchange Membrane	ICP-MS: Inductively Coupled Plasma –
ASV: Amplicon Sequence Variant	Mass Spectroscopy
BEP2M: Bioelectrochemical Power-to-	ICP-AES: Inductively Coupled Plasma
Methane	Atomic Emission Spectroscopy
BES: Bioelectrochemical Systems	IET: Indirect Electron Transfer
CCS: Carbon Capture and Storage	IIET: Indirect Interspecies Electrons
CCU: Carbon Capture and Utilization	Transfer
CV: Cyclic Voltammetry	IPA: Iso-Propyl Alcohol
DAC: Direct Air Capture	LCA: Life Cycle Assessment
DET: Direct Electron Transfer	LOM: Light Optical Microscopy
DI: Deionized	LPR: Linear Polarization Resistance
DIET: Direct Interspecies Electron Transfer	LSV: Linear Sweep Voltammetry
DME: Dimethyl Ether	MEC: Microbial Electrolysis Cell
DVB: Divinylbenzene	MES: Microbial Electrosynthesis
ECSA: Electrochemically Active Surface	MET: Mediated Electron Transfer
Area	MIC(I): Microbiologically Influenced
EDS: Energy-Dispersive X-ray	Corrosion (Inhibition)
Spectroscopy	OCP: Open Circuit Potential
EIS: Electrochemical Impedance	OER: Oxygen Evolution Reaction

PEIS: Potential-Controlled Electrochemical Impedance Spectroscopy	Bacteria
PEM: Proton-Exchange Membrane	SEM: Scanning Electron Microscopy
PFSA: Perfluorosulfonated Acid	SNG: Synthetic Natural Gas
PtC: Power-to-Chemicals	SOEC: Solid Oxide Electrolysis Cell
PtG: Power-to-Gas	SRB: Sulfate-Reducing Bacteria
PtH ₂ : Power-to-Hydrogen	STEM: Scanning Transmission Electron Microscopy
PtM: Power-to-Methane	TEM: Transmission Electron Microscopy
PtP: Power-to-Power	TDA: 1-tetradecylamine
PtX: Power-to-X	TGA: Thermogravimetric Analysis
PWG: Global Warming Potential	TRL: Technology Readiness Level
QAPS: Quaternary Ammonia Polysulfone	TTIP: Titanium (IV) isopropoxide
RDE: Rotating Disk Electrode	UHP: Ultra High Purity
ReSOC: Reversible Solid-Oxide Cell	VFA: Volatile Fatty Acid
RES: Renewable Energy Source	XPS: X-ray Photoelectron Spectroscopy
RHE: Reversible Hydrogen Electrode	XRD: X-ray Diffraction
RWGS: Reverse Water-Gas Shift	YSZ: Yttria-Stabilized Zirconia
RSD: Relative Standard Deviation	
SAOB: Syntrophic Acetate-Oxidizing	

Preface

The research presented in this doctoral thesis was conducted between 2022 and 2025 at the Department of Chemistry of Università degli Studi di Milano, under the supervision of Professor Gian Luca Chiarello and Doctor Pierangela Cristiani of RSE S.p.A. The work was carried out within the framework of Next Generation EU and Ricerca sul Sistema Energetico - RSE S.p.A (CUP n. G43C22002300004), addressing fundamental challenges in Power-to-X technologies for sustainable energy storage and carbon utilization.

Climate change mitigation demands transformative approaches to energy conversion and carbon management. This thesis investigates three interconnected research areas that collectively address critical bottlenecks in deploying bioelectrochemical systems for renewable energy storage: reducing precious metal dependency in water electrolysis through rational catalyst design, understanding microbiologically influenced corrosion in biological methanation environments, and, demonstrating bioelectrochemical CO₂ conversion using geothermal emissions.

CHAPTER 1 and CHAPTER 2 introduce Power-to X technologies, with particular focus on water electrolysis, biological methanation, and the associated challenges.

CHAPTER 3 details the experimental procedures and analytical techniques used throughout the research.

CHAPTER 4 focuses on developing low-loading iridium oxide electrocatalysts for oxygen evolution reaction (OER) in proton exchange membrane water electrolysis. By exploiting crystallographic compatibility between rutile TiO₂ supports and rutile IrO₂, core-shell architectures were synthesized that achieve commercial-level performance at substantially reduced iridium loadings, addressing the scarcity and cost challenges limiting large-scale hydrogen production.

CHAPTERS 5-9 investigate microbiologically influenced corrosion (MIC) on cathode materials relevant to bioelectrochemical systems. Using microbial consortia from a biological methanation pilot plant operated by RSE S.p.A., systematic corrosion studies were performed on copper, brass, ARMCO iron, and carbon steel under controlled thermal treatments. Paradoxically, complete sterilization consistently produced worse corrosion outcomes than selective microbial enrichment, revealing that certain thermoresistant populations actively promote surface passivation through

biomineralization processes. These findings challenge conventional corrosion mitigation strategies and demonstrate that solution chemistry, particularly carbonate and phosphate speciation, governs passivation more than microbial metabolic activity.

CHAPTER 10 demonstrates proof-of-concept for bioelectrochemical methane production using CO₂ from geothermal emissions at the Monte Amiata volcanic complex (Tuscany, Italy). Soil microbial communities adapted to elevated CO₂ environments were successfully enriched in acetogenic-methanogenic consortia (*Sporomusa*, *Methanobrevibacter*) capable of sustained electromethanogenesis. However, inoculum geochemistry, particularly sulfate content, fundamentally determined success or failure, with high-sulfate soils supporting only sulfate-reducing bacteria that outcompeted methanogens despite continuous cathodic polarization.

The integration of these research areas reveals a unifying principle: solution chemistry exerts primary control over both corrosion processes and microbial community assembly, superseding the direct effects of microbial metabolic activity. This insight has profound implications for the design of bioelectrochemical systems, suggesting that operational strategies should focus on controlling carbonate, phosphate, and sulfate speciation rather than pursuing complete sterilization or tolerating uncontrolled microbial growth.

INTRODUCTION

Chapter 1

1 Power-to-X (PtX)

1.1 State of the art

The challenge of achieving deep decarbonization of the global energy system is both urgent and complex. Energy demand and consumption have steadily increased over the past decades, and this trend is expected to continue.¹ Currently, most of the global energy supply is derived by fossil fuels such as oil, coal, and natural gas.¹ In 2024, energy-related greenhouse gases emissions (Fig. 1.1) reached unprecedented levels (37.8 Gt CO₂ eq.), with carbon dioxide (CO₂) as the main contributor.^{2–5}

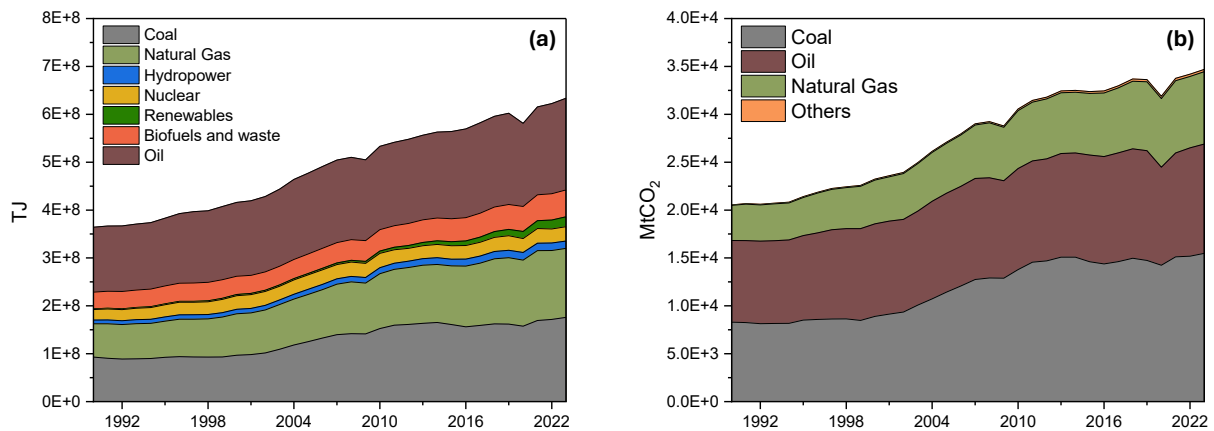


Figure 1.1. Overview of the global (a) total energy supply by source and (b) CO₂ emissions by energy source.

To mitigate climate change and reduce greenhouse gas emissions, fossil-based energy resources are gradually being replaced by renewable alternatives, including hydroelectric, geothermal, wind, solar, biofuels, and biomass, which currently account for approximately 30% of the global energy mix.^{6,7} Projections suggest that achieving net-zero emissions by 2050 will require renewable energy production to exceed fossil fuels, reaching a share of nearly 90% in the global energy mix.^{3,8} Renewable energy sources (RES) such as solar and wind are pivotal for this transition. However, their intrinsic intermittency and temporal mismatch with energy demand

pose significant challenges to ensuring a stable and fully renewable energy supply.⁹ In this context, Power-to-X (PtX) technologies have emerged as critical pathways to integrate and couple electricity with the chemical, transport, heating, and industrial sectors. PtX enables the conversion of surplus or dedicated renewable electricity into storable and dispatchable energy carriers, fuels, or chemical feedstocks, thereby enhancing system flexibility and facilitating decarbonization across multiple sectors (Fig. 1.2). The key advantages of these carriers lie in their ability to be stored for extended periods, transported through existing infrastructure, and reconverted back into electricity (i.e., fuel cells) or other end-use energy forms. Fundamentally, PtX acts as a chemical bridge linking the electricity sector and the wider energy economy, coupling electricity, heat, transport, and industrial processes through exchange of electrons and molecules.

The cornerstone of the PtX network is Power-to-Hydrogen (PtH₂). Hydrogen represents the simplest and most versatile energy vector, produced primarily by water electrolysis powered by renewable electricity.^{10–12} Four technological families dominate PtH₂ field: alkaline electrolyzers (AEL), a mature and robust option suitable for steady operation; proton-exchange membrane (PEM) electrolyzers, that offer rapid dynamic response and high hydrogen purity but at higher cost; anion-exchange membrane (AEM) electrolyzers, a relative new technology that couple AEL and PEM technologies; and solid oxide electrolysis cells (SOECs), capable of reaching electrical efficiencies exceeding 80% by exploiting high-temperature operation and waste-heat integration.¹³ Emerging approaches such as microbial electrolysis cells (MECs) or low-energy urea electrolysis highlight a growing trend toward integrating electrochemical and biological processes, where hydrogen generation intersects with waste valorization.^{14–16}

Once produced, hydrogen can either be used directly, as a fuel in fuel-cell electric vehicles or stationary fuel-cell systems, or act as a platform molecule for the synthesis of other energy carriers. This latter role defines hydrogen as the central node in the broader PtX framework. By reacting with captured carbon dioxide (CO₂), hydrogen enables the Power-to-Gas (PtG) pathway, yielding synthetic natural gas (SNG) through chemical or biological methanation.¹⁷ The exothermic Sabatier reaction has been widely demonstrated both in thermocatalytic reactors, achieving efficiencies between 70% and 85%, and in biological systems, where methanogenic archaea catalyze the same transformation under milder conditions with near-complete selectivity, albeit at lower production rates.^{18–20}

Importantly, PtG establishes a functional bridge between electricity and gas networks, allowing the enormous storage potential of Europe's natural-gas infrastructure (estimated at ~94 million m³) to absorb fluctuations in renewable electricity supply.²¹ Demonstration projects such as

HELMETH, BioPower2Gas, and Hychico have proven that thermal integration between high-temperature electrolysis and methanation can push system efficiencies above 75% on a higher-heating-value basis, showcasing the technical viability of large-scale chemical energy storage.^{22–}

24

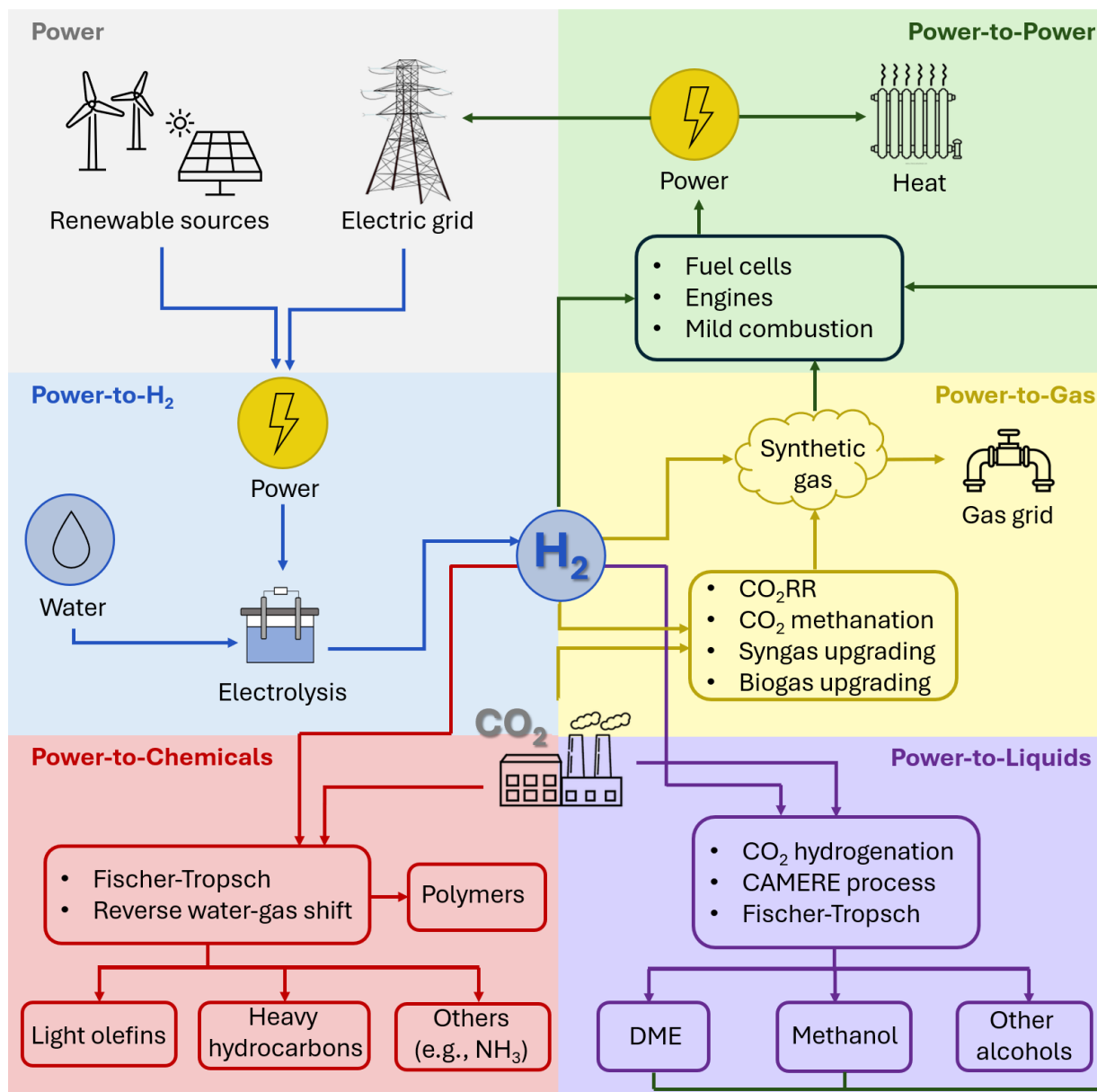


Figure 1.2. Overview of conversion pathways in Power-to-X technology.

Beyond methane, hydrogen and CO₂ can also serve as precursors for liquid fuels, establishing the Power-to-Liquids (PtL) pathway. The hydrogenation of CO₂ to methanol (CH₃OH), typically carried out through Reverse Water-Gas Shift (RWGS) reaction followed by methanol synthesis, as in the CAMERE process,²⁵ can achieve CO₂ conversions above 60%. The resulting methanol serves as a platform molecule, enabling downstream synthesis of dimethyl ether (DME), ethanol,

or higher hydrocarbons. These molecules offer multiple advantages, including high volumetric energy density, compatibility with existing distribution systems and engines, and suitability for immediate use in aviation and heavy transport, where electrification remains challenging. DME has emerged as a clean-burning fuel with potential as both a diesel substitute and a hydrogen carrier for fuel-cell systems.²⁶ All these CO₂ + H₂ pathways are carbon-neutral when powered by renewables and can even achieve net-negative emissions if the CO₂ feedstock originates from direct air capture or biogenic sources.^{27,28}

The PtX concept further extends into Power-to-Chemicals (PtC), in which captured CO₂ becomes a feedstock for the synthesis of value-added products such as light olefins (e.g., ethylene, propylene) the building blocks of petrochemistry.²⁹ Through sequential RWGS and modified Fischer-Tropsch reactions, renewable hydrogen and CO₂ can be transformed into hydrocarbons and chemical intermediates traditionally derived from fossil feedstocks. Catalysts such as potassium-doped, Zn/Cu-promoted Fe systems have demonstrated enhanced CO₂ conversion and selectivity toward C₂-C₃ olefins, paving the way for electrified carbon-neutral chemical manufacturing.³⁰ Compared to carbon capture and storage (CCS), CO₂ valorization through PtC offers both equivalent carbon abatement and additional economic value, accelerating the industrial uptake of renewables.

The final link in the chain, Power-to-Power (PtP), closes the cycle by reconvertng stored chemical energy back into electricity. Hydrogen or methane can be oxidized in fuel cells or reversible solid-oxide cells (ReSOCs) that alternate between electrolysis and generation modes within the same device.³¹ This dual functionality lowers system complexity and capital requirements, while improving round-trip efficiency. In both urban and remote contexts, PtP units can operate as distributed polygeneration hubs, providing electricity, heat, and grid-balancing services simultaneously.³²

From a broader perspective, Power-to-X technologies outline a vision of a circular, interconnected energy system in which electrons and molecules flow without boundaries between different sectors. Their advantages are substantial: they provide grid stability through long-term energy storage, valorize captured CO₂, and exploit existing infrastructures for energy transport and use. However, significant barriers remain. Multi-step conversion processes inevitably incur efficiency losses that can reduce overall round-trip efficiencies to ~45-55%. In addition, the high cost of electrolysis and synthetic-fuel synthesis limits competitiveness relative to fossil-based alternatives.³³ Furthermore, hydrogen storage and transport technologies must evolve beyond energy-intensive compression or liquefaction to ensure economic and environmental viability.

Despite these challenges, the trajectory of recent research and demonstration projects, ranging from electrochemical materials to integrated system modelling, indicates that PtX is moving beyond its role as a support technology for renewable integration, and it is instead emerging as a keystone of the post-carbon energy economy. By converting surplus renewable energy into molecular carriers and subsequently reconverting them when needed, Power-to-X redefines energy storage as an active and circular process that continuously recycles both energy and carbon, thereby coupling technological innovation with long-term sustainability.

Given these considerations and recognizing the pivotal role of hydrogen as a platform molecule for the large-scale implementation of Power-to-X, the following section (Section 1.2) will focus on Power-to-H₂ technologies. The PtH₂ pathway represents the core process of the entire Power-to-X process: without cost-effective, robust, and scalable hydrogen production from renewable electricity, downstream conversion processes cannot fully realize their potential.

1.2 Power-to-H₂: the electrolytic H₂ generation

Hydrogen has emerged as a central energy carrier in the ongoing transition towards a carbon-neutral energy system,^{34–37} especially because it can be generated at high purity and subsequently used either directly as a fuel or converted into other chemical energy carriers.³⁸ Electrolytic hydrogen production can be accomplished via four main technological pathways (Table 1.1): alkaline electrolysis (AEL), proton exchange membrane electrolysis (PEM), anion exchange membrane (AEM), and solid oxide electrolysis cells (SOEC).³⁹

Alkaline electrolysis (AEL), the most established technology, operates at 20–80 °C in concentrated KOH electrolyte where hydroxide ions (OH[−]) act as charge carriers. The typically delivering efficiencies are of about 59–70% and current densities in the range of 200–500 mA cm^{−2}.⁴⁰ Nickel-based electrodes are employed, with Ni, Co or Fe oxides for the oxygen evolution reaction and nickel-based alloys for the hydrogen evolution side, separated by porous diaphragms.⁴¹ Its advantages include technological maturity, long operating experience, and relatively low cost, but drawbacks arise from sluggish dynamic response, limited load-flexibility, electrode corrosion, and comparatively low hydrogen purity.

Proton exchange membrane (PEM) electrolysis, in contrast, operates under acidic conditions with protons (H⁺) migrating across a solid perfluorosulfonated acid (PFSA) membrane such as Nafion.^{42,43} The working temperatures range between 20–200 °C and the electrode configurations commonly involve Ir- or Ru-based metal oxides as anodic catalysts for the oxygen evolution reaction (OER), coupled with Pt at the cathode for the hydrogen evolution reaction (HER). PEM

systems supports current densities above 2 A cm^{-2} and can reach electrical efficiencies approaching 65-82%.⁴⁴ PEM systems offer high hydrogen purity (99.999%), compact design, and rapid response to fluctuating loads, making them particularly compatible with renewable integration⁴⁴. However, their reliance on scarce noble metals such as Pt, Ir, and Ru drives up system costs and limits scalability.

Anion exchange membrane (AEM) electrolysis is a relatively new approach that combines features of both alkaline (AEL) and PEM systems. AEM system operates under alkaline conditions at moderate temperatures (20-200 °C), where hydroxide ions (OH^-) act as the charge carriers across a solid polymer membrane functionalized with quaternary ammonium groups.⁴⁵ In terms of electrocatalytic materials, it appears as a low-cost alternative compared to the other technologies, employing electrodes such as Co_3O_4 or other transition metal oxides for OER, and Ni cermet for HER. A key advantage lies in the possibility of employing inexpensive, earth-abundant catalysts such as nickel or cobalt instead of platinum group metals, potentially lowering system costs. Nevertheless, present AEM electrolyzers exhibit lower efficiency ($\approx 40\text{-}45\%$), modest current densities, and limited membrane durability, highlighting the need for significant advances in membrane chemistry and electrode integration before large-scale deployment can be realized.⁴⁶

Solid oxide electrolysis cells (SOEC) represent a third pathway, operating at elevated temperatures (700-1000 °C), it employs ceramic electrolytes such as yttria-stabilized zirconia (YSZ), where oxide ions (O^{2-}) serve as the charge carriers. Part of the energy demand can be provided as heat, yielding thermodynamic efficiencies that can reach 85-99%.⁴⁷ The oxygen electrode is often based on perovskite-type oxides, while the hydrogen electrode typically uses Ni/YSZ, ensuring high catalytic activity under reducing conditions. Furthermore, SOECs allow co-electrolysis of H_2O and CO_2 , enabling the production of syngas as a precursor to synthetic fuels.⁴⁸ The drawbacks are equally pronounced: high-temperature operation accelerates material degradation, requires long start-up times, and imposes engineering challenges in terms of sealing, thermal cycling, and durability. Beyond water electrolysis, alternative hydrogen production routes include biomass gasification, thermochemical water splitting cycles at 800-2000 °C, and photocatalytic water splitting under solar irradiation. While these offer intriguing pathways, particularly in regions with abundant solar or biomass resources, they remain less mature and face significant efficiency and stability barriers compared to electrolysis.^{49,50} Despite their different operating principles, electrolytes, and catalytic materials, all these routes (i.e., AEL, PEM, AEM, and SOEC) are fundamentally based on the same process of water splitting, i.e. the

electrochemical decomposition of H₂O into hydrogen and oxygen. The reaction limitations and potential improvements will be further addressed in the following section (Section 1.2.1), where both the oxygen and hydrogen evolution reactions will be examined, with particular emphasis on PEM systems due to their significant room for optimization.

Table 1.1. Key elements and technological routes of water electrolysis for hydrogen generation.
41,52,53

Specification	AEL	PEM	AEM	SOEC
TRL	Mature	Commercialized	R&D	R&D
Operating Temperature (°C)	20-80	20-200	20-200	700-1000
Operating pressure (bar)	25-30	30-80	< 35	< 30
Operating pH	14	0	14	-
Current density (mA cm ⁻²)	200-500	800-2500	200-500	300-1000
Cell voltage (V)	1.8-2.4	1.8-2.2	1.8-2.2	0.95-1.3
Hydrogen purity (%)	99.5-99.9998	99.9-99.9999	99.9-99.9999	99.9
Efficiency (%)	59-70	65-82	-	85-99
Electrolyte	20-30% KOH	PFSA	DVB	YSZ
Separator	Asbestos, NiO, ZrO ₂	PFSA	Solid electrolyte	QAPS
Charge carrier	OH ⁻	H ⁺	O ²⁻	O ²⁻
Anode (OER)	Ni, Co, Fe oxide	Ir, Ru oxide	Ni, Co oxide	Perovskite
Cathode (HER)	Ni-alloys	Pt	Ni-cermets	Ni/YSZ

1.2.1 Water splitting reaction

Water splitting through electrolysis represents the basis of sustainable hydrogen production, offering a direct route to convert renewable electricity into a storable, carbon-free fuel. All four technologies discussed in the previous section (AEL, PEM, AEM and SOEC) are based on the same overall reaction of water splitting (Fig. 1.3). However, the electrolysis of water is governed

by complex electrochemical dynamics, which differ depending on the electrolyte environment, the nature of the charge carriers and the operating conditions⁵¹ (Table 1.1).

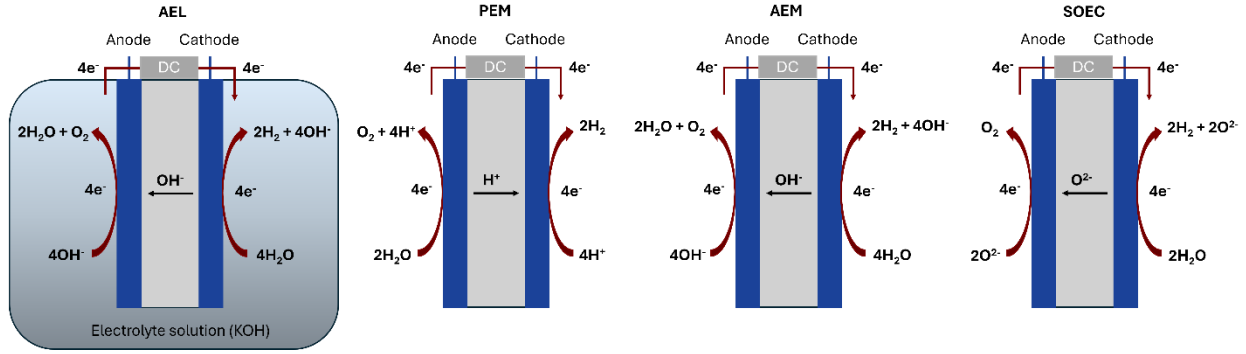
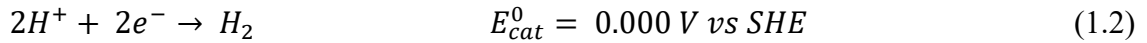
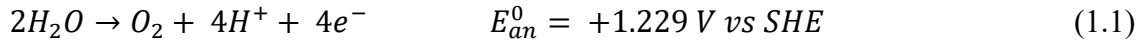


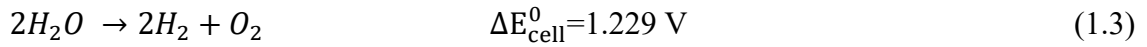
Figure 1.3. Overview of electrolyzers technology (AEL, PEM, AEM and SOEC).

Additionally, the anodic and cathodic equilibrium potentials vary depending on both electrolyte pH and operating temperature, even though the overall cell potential remains determined by the Gibbs free energy of water splitting.

For instance, in the acidic environment of PEM electrolyzers,⁵² the anodic oxygen evolution reaction (OER, Eq. 1.1) and cathodic hydrogen evolution reaction (HER, Eq. 1.2) proceed as follows:



The overall water splitting reaction (Eq. 1.3) is therefore:



Alkaline and AEM electrolyzers, both operate under strongly basic environments ($pH \approx 14$), which shifts the equilibrium potentials of the hydrogen and oxygen electrodes according to the Nernst equation.⁵² Under such conditions, the hydrogen evolution reaction occurs at $E_{cat}^0 = -0.828 \text{ V}$, while the oxygen evolution reaction occurs at $E_{an}^0 = +0.401 \text{ V}$, maintaining the same overall cell potential of 1.229 V but with redistributed electrode potentials. In contrast, SOECs operate at elevated temperatures (typically 700-1000 °C), where a fraction of the reaction free energy is supplied as heat rather than electricity.⁵³ Additionally, the Gibbs free energy of water splitting decreases with temperature, reducing the minimum electrical potential required. As a result, the

reversible potential decreases from 1.229 V at 25 °C to ~1.0 V at 800 °C, while efficient operation is often targeted around the thermoneutral voltage of 1.229 V.⁵⁴ These variations highlight how electrolyte pH and operating temperature fundamentally modulate the thermodynamics of water splitting, even though the global reaction remains invariant.

The theoretical minimum voltage of 1.229 V at 25 °C corresponds to the Gibbs free energy of water splitting under standard conditions (i.e., pH 0). In practice, the applied voltages (Table 1.1) are significantly higher due to overpotentials and ohmic losses, typically 1.8-2.4 V for AEL, 1.8-2.2 V for PEM and AEM, 0.95-1.3 V for SOEC. Indeed, the main limitation of this process lies in the anodic oxygen evolution reaction (Eq. 1.1), whose kinetics are hindered by the complex four-electron transfer required for the reaction to proceed.⁵⁵ The sluggish kinetics of OER result in substantial overpotentials ($\eta_{\text{anode}} > \eta_{\text{cathode}}$), thereby increasing energy consumption.⁵⁶ For this reason, the development of highly active, durable, and cost-effective OER catalysts is the central challenge for advancing electrolyzer technologies.

1.2.2 The oxygen evolution reaction in acidic environment

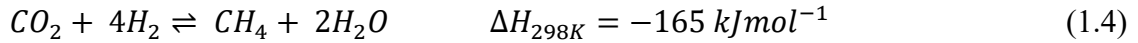
Current state-of-the-art OER electrocatalysts are primarily based on ruthenium and iridium oxides, with IrO₂ representing the benchmark in acidic environments due to its superior stability.^{57–60} The compromise between activity and durability across different iridium catalyst surfaces remains a key challenge of research in the community, with amorphous iridium oxide exhibiting higher activity but lower stability than its rutile counterpart.^{61–63} Both structures exhibit sufficiently good properties for implementation in water electrolyzers devices. However, the scarcity and cost of these precious metals prompt a drastic reductions in catalyst loadings.⁶⁴ Indeed, the target loadings for these metals have been drastically reduced over time, from current values of 2-3 mg_{Ir} cm⁻² to the US Department of Energy's target of 0.125 mg_{Ir} cm⁻².⁶⁵ To overcome this challenge, research efforts are therefore focusing on engineering electrodes with catalyst materials that exhibit favorable morphologies (e.g. core-shell architecture), thereby improving both utilization and inter-particle contact even at low loadings. In unsupported IrO₂ catalysts, reducing the loading typically causes in-plane conductivity losses and limits iridium utilization.⁶⁴ A promising approach involves optimizing the catalyst geometry by enlarging the active layer volume through the incorporation of semi-conductive supports. This strategy promotes the formation of a continuous three-dimensional conductive network, thereby increasing the surface-to-volume ratio of exposed iridium within the active layer. Across all approaches, the balance between activity, stability, and material cost remains delicate.

Ultimately, the efficiency and scalability of water electrolysis, and thus the viability of large-scale power-to-hydrogen deployment, hinge on overcoming the OER barrier. Advances in catalyst design, membrane durability, and system integration with renewable power sources are critical to bridging the gap between laboratory performance and industrial viability.

1.3 Power-to-Gas: focus on CO₂ methanation (PtM)

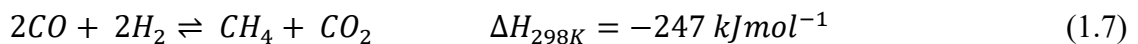
The global transition towards net-zero carbon emissions is driving the development of technologies that not only capture CO₂ but also value it as a feedstock for energy carriers and chemicals. Among these, the Power-to-Gas (PtG) approach has emerged as a particularly attractive option. PtG couples renewable hydrogen production via water electrolysis (Section 1.2) with CO₂ utilization, ultimately yielding methane (PtM) that can be readily injected into the existing natural gas infrastructure. Different reactor configurations are reported in the literature depending on the carbon dioxide source used in the methanation stage (biogas, syngas or pure CO₂).^{66–68}

The cornerstone of PtG is the CO₂ methanation also called Sabatier reaction (Eq. 1.4):^{69,70}

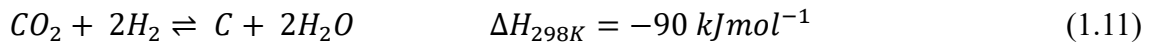
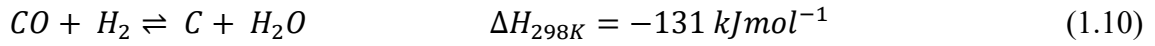
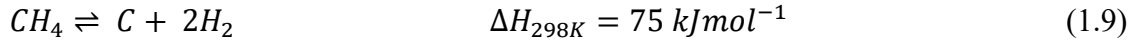
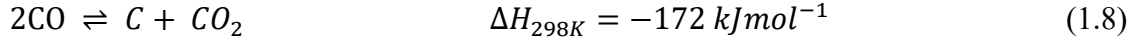


This reaction is strongly exothermic and presents several drawbacks related to the high energy demand. Indeed, catalytic methanation reactors are typically operated at temperatures between 200 °C and 600 °C and pressures ranging from 5 to 100 bar.^{71,72}

However, the reaction pathway of CO₂ methanation is not always straightforward. It can proceed via a direct route (Eq. 1.4), or through a sequence of two steps: a Reverse-Water-Gas-Shift, RWGS (Eq. 1.5), followed by a CO methanation (Eq. 1.6), or alternatively a reverse dry reforming reaction (Eq. 1.7).^{73,74}



A key challenge of CO₂ methanation process is catalyst selection, since a great variety of side reactions (Eqs. 1.8-1.12) can occur, affecting both reaction selectivity and catalyst deactivation (i.e., coking).



The most common catalysts are based on Ni, Ru, Rh and Co, with Ni being the most used thanks to its activity, selectivity and low cost.^{69,70,75–78} Current research efforts focus both on catalyst design and reactor engineering. Nickel-based systems remain the industrial benchmark due to their cost-effectiveness and relatively high activity, but their tendency to coke and sinter has motivated the use of noble metal promoters such as Ru and Rh.^{77,78} Additionally, CO₂ streams should be of high purity to prevent catalyst deactivation. The main challenges ahead include efficient heat management, catalyst stabilization, and reactor scale-up.

1.3.1 Catalytic biogas methanation

The economic rationale of methanation is debated, as methane has a lower market value than hydrogen. However, when accounting for the direct and indirect costs of CO₂ emissions and the challenges of deploying a hydrogen infrastructure, the process can be attractive under specific conditions.

A notable case is the methanation of biogas derived from the anaerobic digestion (AD), which has recently been proposed as a second-generation upgrading route. This technology exploits microbial decomposition of biomass under oxygen-free conditions (i.e., enzymatic catalysis), while heterogeneous catalysis can be integrated to further enhance reaction performance. A wide range of feedstocks can be employed, including agricultural residues, animal manure, and organic waste. However, when combining both catalytic and enzymatic technologies, it should be taken into account catalyst deactivation due to the impurities present in biogas streams. In fact, the resulting biogas typically contains 35-65% CH₄ and 15-50% CO₂, with variable amounts of impurities such as H₂S, water and NH₃ depending on the source of organic waste and the operating conditions of the anaerobic digester.^{79,80} For applications such as combined heat, power generation

and injection into the natural gas grid, the gas must be upgraded to biomethane. Conventional upgrading processes rely on the partial removal of CO_2 and impurities to yield a purified CH_4 -rich stream (up to 92% CH_4), commonly referred to as biomethane.⁸¹ Once purified, this gas can either be injected into the natural gas grid or directed to a methanation plant (indirect catalytic route), where nearly complete conversion (99%) of CO_2 to methane can be achieved (Fig. 1.4). In the first case, the methane content should be high enough to meet the required standards for direct injection into the natural gas distribution network. For instance, Italian legislation establishes that the natural gas grid must contain 92% methane by volume, according to Legislative Decree 257/2016, Article 10. However, this process requires energy-intensive scrubbing technologies that are highly demanding in terms of costs. On the contrary, direct hydrogenation of raw biogas enriches methane content while avoiding energy-intensive scrubbing technologies.⁷⁹

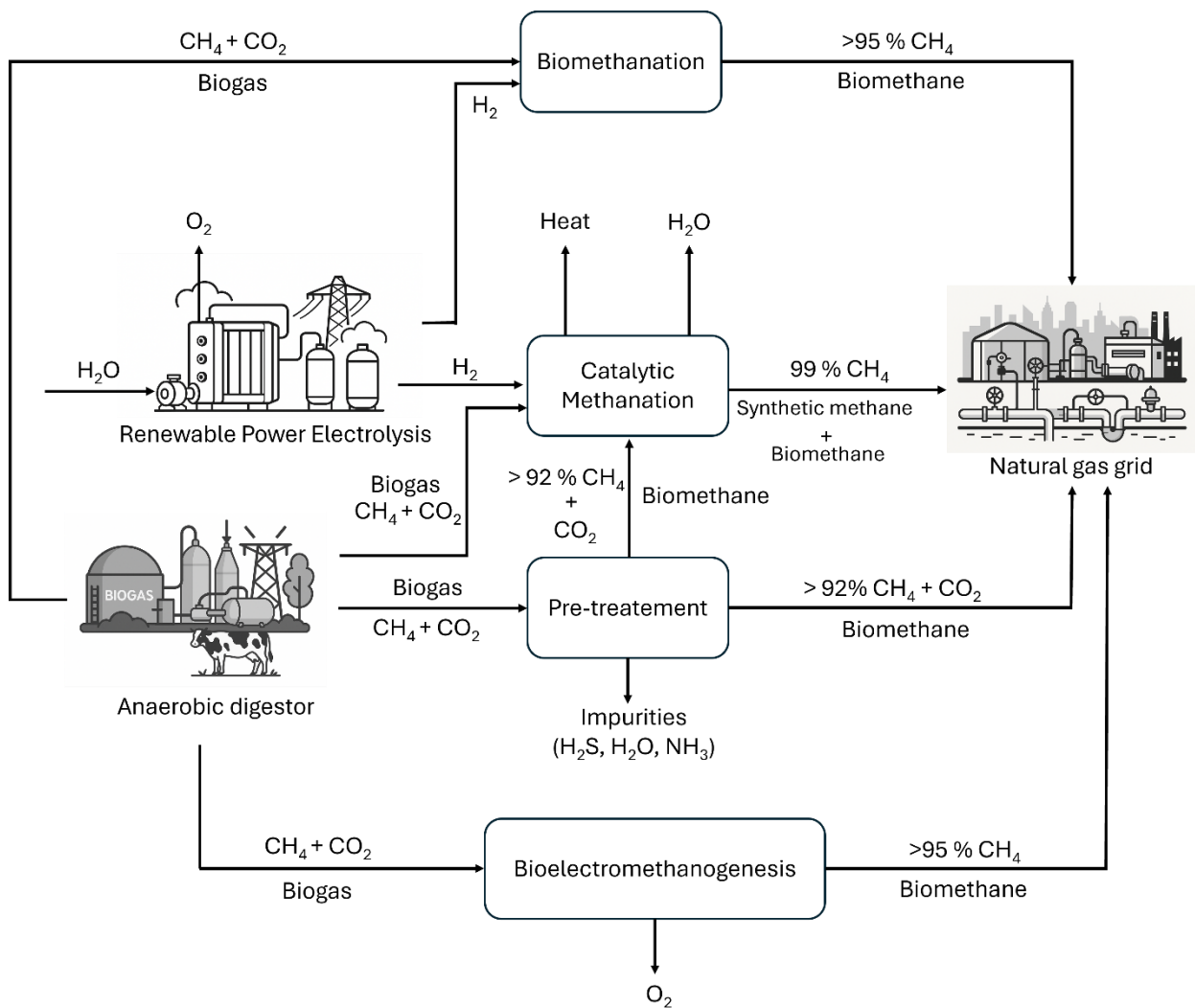


Figure 1.4. Conceptual process flow diagram of Power-to-Methane.

Laboratory-scale studies have demonstrated direct biogas methanation over both unpromoted and Ru-promoted Ni catalysts, showing negligible differences in activity with increasing CH₄ concentrations in the feed.⁸² A fluidized bed reactor was successfully operated for over 1100 h with real biogas feed, demonstrating process feasibility and highlighting the role of steam addition in mitigating coke deposition.⁸³ At intermediate scale, a setup treating 10 Nm³/h of biogas achieved stable operation for more than six months, with a sustained CO₂ conversion of ~90%.⁸⁴

Comparisons between direct biogas methanation and the separation/hydrogenation of pure CO₂ have also been reported. The former, often referred to as second-generation biogas upgrading, has proven technically feasible, though concerns remain for large-scale deployment. In particular, residual CO and H₂ concentrations in upgraded biomethane are critical parameters for direct grid injection, with threshold values depending on local regulations.^{85,86}

To limit CO formation, a two-step methanation strategy has been proposed, involving intermediate removal of reaction-produced steam to drive equilibrium toward complete CH₄ formation. Steam removal can be achieved via condensation.⁸⁷ Alternatively, in situ steam adsorption using materials such as zeolite 5A has been tested; however, its practical application is hindered by limited adsorption capacity and the need for frequent adsorbent regeneration.⁸⁸

Despite the high CH₄ content of biogas, its direct methanation is not automatically economically viable.⁸⁹ Nevertheless, the strongly exergonic nature of the Sabatier reaction and its near-complete equilibrium conversion at low temperatures make it attractive from a thermodynamic point of view. The main challenges are associated with process scale-up: the intense exothermicity, reactor design constraints, and the need for efficient heat management render industrial implementation highly complex.^{89,90} Although the catalytic pathways (direct and indirect) are technically feasible, they remain highly energy and cost intensive, which raise concerns about their large-scale applicability.

1.3.2 Biogas upgrading technologies

Nowadays, increasing attention is being directed toward strategies that avoid highly energy consuming processes, including elevated temperature and pressures required by conventional heterogeneous catalysis, by exploiting both microbial metabolism of methanogens and hybrid strategies that combine microbial metabolic pathways with electrochemical processes.⁹¹ Indeed, within the broader field of PtM, two distinct approaches to biogas upgrading can be identified (Fig. 1.4): i) *biomethanation*, where renewable H₂ is generated via electrolysis and subsequently fed to anaerobic digesters (in-situ approach) or to a biomethanation plant (ex-situ approach) to

reduce CO₂ from biogas and increase CH₄ production;⁹² ii) *bioelectromethanogenesis*, where methanogenic archaea receive electrons directly from cathodes, bypassing hydrogen intermediates, and thereby catalyzing the conversion of CO₂ to CH₄.⁹²

Biomethanation represents a promising strategy to enhance CH₄ content of biogas by simply exploiting the metabolic activity of methanogenic Archaea as biocatalyst. This upgrading can be implemented through in situ, ex situ, or hybrid configurations. In in-situ biomethanation, H₂ is directly injected into the anaerobic digester, where it is consumed by hydrogenotrophic methanogens to convert CO₂ into CH₄, thereby enriching the methane fraction of the effluent biogas. For example, Wahid et al. reported a decrease in CO₂ concentration from 33.3% to 3.1% and a corresponding increase in CH₄ from 66.7% to 94.5% compared with an untreated control.⁹³ Despite these encouraging results, in situ systems are often challenged by operational limitations, including excessive pH increases (>8.5) caused by bicarbonate consumption, volatile fatty acid (VFA) accumulation, and inhibition of methanogenesis due to elevated H₂ partial pressures.⁹⁴ In ex situ biomethanation, raw biogas and externally supplied green H₂ are introduced into a dedicated reactor enriched with hydrogenotrophic methanogens. This separation of functions ensures stable operation of the anaerobic digester while avoiding inhibitory effects of H₂ on VFA-degrading microbial communities. Ex situ systems therefore offer improved process control, higher conversion efficiency, and have been suggested as a more robust solution than in situ configurations to overcome both biological and technical challenges, particularly those linked to H₂ accumulation.^{95,96} A hybrid biomethanation configuration combines these two modes. In this approach, H₂ is initially injected into the anaerobic digester to partially upgrade the biogas, which is subsequently fed into a secondary ex situ reactor where the remaining CO₂ is converted to CH₄. Although hybrid systems can achieve high methane contents in the final product, they are limited by reduced H₂ solubility and shorter gas residence times in the second stage due to the already high CH₄ concentration entering the ex situ unit.⁹⁷ However, bioelectromethanogenesis emerges as one of the most promising applications of biogas upgrading, providing an alternative to costly electrolyzers, which are often the most expensive unit in PtG plants, and additionally, can reach coulombic efficiencies above 90%.^{98,99} Indeed, catalytic, enzymatic and hybrid routes for biogas upgrading share a common limitation: the reliance on H₂ generated via renewable power electrolysis. While this pathway supports the transition toward zero-emission energy systems, the high cost of catalysts for H₂ production, and in some cases the additional expense of operating at elevated temperatures and pressures (as required in SOEC technology), challenge its economic

viability. In this context, bioelectromethanogenesis emerges as a promising alternative, as microorganisms can directly utilize electrons, thereby bypassing hydrogen as an intermediate.

Chapter 2

2 Biological Methanation

Independently of the technology used, biological conversion of H_2 and CO_2 in CH_4 represent the terminal step of anaerobic digestion. In practice, AD systems predominantly operate with mixed microbial consortia rather than pure cultures. While pure cultures would, in principle, facilitate process control and enhance selectivity by avoiding competition with hydrogen-consuming homoacetogens or hydrogen-sensitive fermenters, their application is constrained by the requirement for sterility, strict anaerobic conditions, and high operational costs.¹⁰⁰ Moreover, pure cultures are particularly vulnerable to environmental perturbations (e.g., pH variations). By contrast, mixed microbial communities provide several practical advantages: they are inherently robust, tolerate fluctuations in pH, temperature and current density, and eliminate the need for sterile operation. Importantly, the presence of facultative anaerobes in mixed cultures can serve as O_2 scavengers, thereby protecting strictly anaerobic methanogens from oxidative inhibition.¹⁰¹ Indeed, methanogens are highly sensitive to even trace amounts of oxygen; nevertheless, mixed consortia have demonstrated resilience, maintaining CH_4 production under O_2 -rich feed gases (up to 4.7% O_2 in steel mill off-gas), and rapidly recovering activity once anoxic conditions were restored.¹⁰¹

Mixed communities also benefit from syntrophic interactions and direct or indirect interspecies electron transfer (DIET and IIET, Section 2.3), which facilitate efficient electron delivery to methanogenic archaea.¹⁰² However, mixed cultures may introduce challenges associated with the formation of by-products and impurities, necessitating downstream separation and purification, and may reduce electron recovery efficiency due to competing metabolic reactions.¹⁰³ Thus, the choice between pure and mixed cultures reflects a trade-off between robustness and product specificity.

Methanogenesis proceeds via three main pathways: i) hydrogenotrophic, ii) acetoclastic, and iii) methylotrophic. Of these, hydrogenotrophic and acetoclastic routes dominate biomethane production, whereas methylotrophic methanogenesis becomes significant under sulfate-reducing conditions, owing to the thermodynamic inhibition of the other two pathways by sulfate reducers.¹⁰⁴ Acetoclastic methanogens (AcM) utilize acetate as both energy and carbon source, relying on acetate generated by autotrophic acetogens via the Wood-Ljungdahl pathway.¹⁰⁵ In this process, acetate is first activated to acetyl-CoA and subsequently cleaved to yield a methyl group

for methane formation. Syntrophic associations between acetogens and methanogens thus play a central role.^{106,107} Syntrophic acetate-oxidizing bacteria (SAOB) can also transform acetate into H_2/CO_2 , providing an alternative route that couples with hydrogenotrophic methanogenesis. The balance between AcM and SAOB is strongly influenced by environmental conditions: AcM are highly sensitive to acetate concentrations, whereas SAOB are more competitive under elevated ammonium levels.¹⁰⁸

Hydrogenotrophic methanogens, including *Methanobacterium*, *Methanoculleus*, *Methanomicrobium* and *Methanothermobacter*, directly reduce CO_2 with H_2 (Eq. 1.4, Section 1.3). They often dominate biogas upgrading systems, outcompeting AcM such as *Methanosarcina*.¹⁰⁸ Methanogens also exhibit metabolic plasticity under substrate limitation. In such conditions, cells may redirect resources from biomass synthesis to energy conservation, ensuring survival.¹⁰⁹ Moreover, certain *Methanosarcinales* are metabolically versatile, capable of both acetoclastic and hydrogenotrophic methanogenesis. Due to the low solubility of H_2 in water, hydrogen availability frequently limits methanogenesis, prompting substrate redirection. For instance, when H_2 partial pressure decreases, acetogens such as *Thermacetogenium phaeum* may shift towards syntrophic acetate oxidation.¹¹⁰

Substrate and product fluxes further determine microbial growth yields. Early studies estimated that approximately 0.3-0.4% of H_2 and 3% of CO_2 are assimilated into biomass,¹¹¹ whereas later findings suggested that up to 8.5% of CO_2 contributes to archaeal growth.¹¹² These results highlight the intricate balance between catabolic and anabolic processes shaping methanogenic community dynamics.

2.1 Bioelectrochemical power-to-methane

Bioelectrochemical Power-to-Methane (BEP2M) represents an innovative extension of the PtG concept in which living microorganisms act as the biocatalysts mediating electron transfer between renewable electricity and carbon dioxide.¹⁸ In contrast to catalytic methanation, which requires high temperatures (200-600 °C) and pressures (1-80 bar), bioelectrochemical systems (BES) operate under mild conditions, typically 20-80 °C and near-atmospheric pressure, significantly lowering the energy footprint of the process. Additionally, advantages of BEP2M include tolerance of biocatalysts to impurities such as hydrogen sulfide (H_2S), easy integration with AD systems without the requirement of expensive gas scrubbing, renewable nature of the catalysts themselves, and simpler overall plant management. Additionally, literature reports that bioelectromethanogenesis has a higher CO_2 reduction efficiency (96%) compared to metal-

catalyzed electrodes (10-57%).¹¹³ Moreover, diverse studies reports methane purity via bioelectromethanogenesis up to 95%.¹¹⁴⁻¹¹⁶ From an environmental perspective, biological Power-to-Methane has also shown superior performance. Life cycle assessment (LCA) studies estimated an impact of 26 kg CO₂-eq per MWh of CH₄ produced via biological pathways.⁹⁸ In comparison, catalytic PtM fueled by renewable electricity exhibited significantly higher global warming potential (GWP): 147 ± 48 kg CO₂-eq/MWh CH₄ when powered by solar energy and 75 ± 48 kg CO₂-eq/MWh CH₄ when powered by wind.¹¹⁷ Depending on the CO₂ source, electricity mix, and system boundaries, catalytic PtM has been reported to range widely from 22 up to 1076 kg CO₂-eq/MWh.¹¹⁷ Depending on electrolyzers technology, CO₂ origin (air, cement flue gas, or biogas), and the 2019 energy scenario literature reported even higher values, between 650 and 815 kg CO₂-eq/MWh CH₄.¹¹⁸ Collectively, these findings suggest that biological P2M generally results in a lower environmental burden, particularly in terms of GWP, than catalytic alternatives.⁹⁸

2.2 CO₂ sources

The advantage of BEP2M systems rely on the less stringent requirements regarding the purity of CO₂ sources compared to catalytic methanation, that can face catalysts deactivation. Indeed, methanogenic microorganisms can tolerate either pure CO₂ or a CO₂-containing gas mixture.^{119,120} The composition of this feed must be carefully regulated, since oxygen is toxic to anaerobic archaea, while other gases such as H₂S must also be kept at very low concentrations to avoid process inhibition.¹²¹⁻¹²³ The main sources of CO₂ for BEP2M include: i) industrial off-gas treatment within carbon capture and utilization strategies, ii) direct air capture, iii) biological anaerobic processes, and iv) natural CO₂ emissions from geothermal reservoirs or geothermal power plants. The latter source has received relatively little attention despite its abundance: several regions worldwide exhibit significant natural geothermal CO₂ vents, often associated with volcanic activity, geothermal systems, or tectonic settings. Italy, in particular, is one of the richest areas globally in geothermal manifestations and is notable for its high-enthalpy geothermal fields, which enabled the world's first large-scale geothermal power development, especially in the Larderello district of Tuscany.^{124,125} Unlike industrial CO₂ emissions, the fluxes released from natural geothermal vents have not yet been precisely quantified, even though they may represent the second largest global source after industrial processes. Additional contributions arise from biological and agricultural activities. However, these latter emissions are typically characterized by low CO₂ concentrations and are widely distributed sources, which makes targeted capture or

recovery technologically and economically unfeasible. For this reason, biological and agricultural emissions are generally not considered viable for CO₂ recovery or utilization strategies.

Industrial and power sectors are responsible for about 65% of global CO₂ emissions.^{126,127} These activities represent large-volume point sources that could be exploited as feedstock for microbial electrosynthesis (MES), thereby mitigating emissions, reducing environmental impact, and enabling the production of value-added chemicals and fuels. Nevertheless, industrial and power plant off-gases are generally characterized by low gas purity, containing contaminants such as oxygen, which is toxic for anaerobic archaea, and other inhibitory components like H₂S, which must be carefully minimized to allow efficient BEP2M operation.¹²⁸

Industrial CO₂ emissions can be classified into combustion-related, produced by fuel oxidation, and process-related, arising from chemical transformations.¹²⁹ Capture strategies can be implemented at different stages of combustion (i.e., pre-, oxy- or post-combustion) producing streams with CO₂ purities ranging from 4 to over 90%, although only a few technologies are commercial while most remain at low TRL. Direct Air Capture (DAC) represents an emerging alternative, offering location-independent recovery of high-purity CO₂ from both point and diffuse sources, but the technology is still limited by high energy demand and costs.^{130,131}

Another issue concerning industrial exhaust gases is that they are typically released at elevated temperatures. For instance, flue gases from cement production can reach 200-400 °C,^{132,133} while those from steel manufacturing vary between 100 and 350 °C; in steel rolling processes, off-gas temperatures can even exceed 600 °C.¹³⁴ This thermal profile makes it essential to implement systems for both heat recovery and gas cooling in order to reach temperatures compatible with microbial activity in BES processes. Although waste heat is already reused within industrial systems, additional cooling steps would be required for BES integration, introducing complexity, energy losses, and extra costs.^{135,136}

Another major challenge in coupling industrial off-gases with BES is the variability in gas composition and flow rates across sectors, which makes standardization difficult and may require case-specific engineering solutions. To fully exploit the potential of such integration, comprehensive thermal flow modeling, cost-benefit analyses, and pilot-scale demonstrations are needed. The steel industry accounts for about 5% of global CO₂ emissions, with exhaust streams containing up to 23% CO₂ and variable oxygen concentrations, sometimes exceeding 16%, which substantially reduces methane productivity compared to pure CO₂ feeding.¹³⁷ Cement production is responsible for 5-7% of global emissions, with off-gases containing 14-33% CO₂ and 8-14%

O₂ together with other impurities such as SO₂, NO_x, CO, and particulates, making them particularly challenging for electromethanogenesis.^{138,139} Beyond anthropogenic sources, natural geothermal emissions also represent a significant and underexplored CO₂ supply, with Italy being one of the richest areas worldwide in geothermal manifestations. In regions such as the Amiata volcanic geothermal area, CO₂ concentrations of 92-96% have been reported with low O₂ levels and daily fluxes exceeding 17000 t.^{124,125} While impurities such as water vapor, H₂S, and trace metals may require pre-treatment, the non-condensable gas fraction of geothermal power plants offers a promising CO₂ source, as it typically contains 85-95% CO₂ and can be further purified with abatement systems. Overall, both industrial and geothermal CO₂ sources present promising opportunities for BEP2M, but their integration requires careful management of gas purity, cooling, and inhibitory components, as well as robust assessments of technical and economic feasibility.

2.3 Microbiology of BEP2M systems

Electromethanogenesis and biological methanation are catalyzed by seven orders of methanogenic microorganisms that belong to the Archaeal domain: *Methanopyrales*, *Methanococcales*, *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales*, *Methanocellales* and *Methanomassiliicoccales*.¹⁴⁰ These microorganisms inhabit a wide range of anoxic environments, from hydrothermal vents and marine sediments wetlands, landfills, animal digestive tracts and engineered anaerobic digesters.¹⁴¹ Their physiology reflects remarkable versatility: species differ in temperature, pH and salinity optima, as well as in substrate utilization and methanogenic pathways, although most display optimal growth at near-neutral pH (6-8) under strict anaerobic conditions.¹⁴² Methanogenic archaea have long been exploited in anaerobic digestion for biogas production, a technology commercialized for more than half a century, whereas their application in bioelectrochemical systems such as BEP2M is a more recent development of the past decade.¹⁴³

The fundamental difference between conventional anaerobic digestion and electromethanogenesis lies in the source of electrons for CO₂ reduction. In anaerobic digestion, methanogens rely on H₂ and acetate generated by fermentative bacteria (i.e., acetogenesis) as electron donors. By contrast, in electromethanogenesis electrons are supplied directly from a cathode, driven by an applied voltage, and transferred to the microbial biofilm through distinct mechanisms (Fig. 1.5).^{144,145}

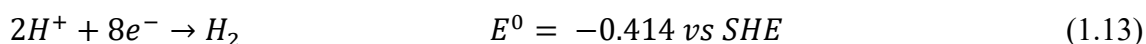
Direct electron transfer (DET) involves the uptake of electrons from the electrode via electroactive cell components, such as c-type cytochromes, conductive pili or protein nanowires, with *Methanosarcina* and *Methanothrix* representing the best-studied examples.^{145–148} Although DET provides a straightforward route for CO₂ reduction (Eq. 1.12), its efficiency is limited by

several factors that influence different aspects of the process. For instance, electrode surface area and material properties (e.g. roughness, electrical conductivity) can affect microbial attachment and electron transfer rates.¹⁴⁹ Mass transport limitations also play an important role, as the accumulation of products on the electrode surface can hinder CO₂ reduction.¹⁵⁰ In addition, pH increases at the cathode may lead to carbonate fouling and even corrosion, ultimately deactivating the electrode.¹⁵¹ Finally, in mixed microbial communities, such as those typically present in digestates, competition for electrons among different species can reduce methane selectivity and alter community composition, often in ways that do not favor DET.



Indirect electron transfer (IET) relies on the syntrophic production or electrochemical generation of intermediates such as H₂, acetate or methylated compounds (e.g., methanol or methylamine), which then serve as electron carriers between the electrode and methanogens.^{145,152} A related mechanism, **mediated electron transfer (MET)**, employs secreted redox-active molecules including flavins, quinones or phenazines as electron shuttles.¹⁵³ Finally, in mixed microbial communities, syntrophic interaction between different microorganisms can occur. In **direct interspecies electron transfer (DIET)**, electrons are relayed between partners via conductive pili, whereas in **indirect interspecies transfer (IIET)**, electroactive microbes generate intermediates that are subsequently consumed by other microorganism to yield the final product.^{145,154} For instance, certain electroactive microorganisms at the cathode can use electrons to reduce proton to H₂, which is then consumed by hydrogenotrophic methanogens for methane production.

Hydrogenotrophic methanogenesis, which is widespread among all methanogenic orders except Methanomassiliicoccales, represents the dominant pathway in these systems. In the latter case, H₂, produced either biotically or abiotically (Eq. 1.13), is used to reduce CO₂ to CH₄ via the Wolfe cycle, a central metabolic route that involves the bifurcation of electrons derived from hydrogen to ferredoxin and heterodisulfide reduction.¹⁵⁵ The thermodynamics of these processes are well established, with standard redox potentials of -0.414 V vs SHE for H₂ production (Eq. 1.13) and -0.244 V for CO₂ reduction to CH₄ (Eq. 1.12), highlighting both the energetic feasibility and the delicate balance required for efficient bioelectrochemical methanogenesis.



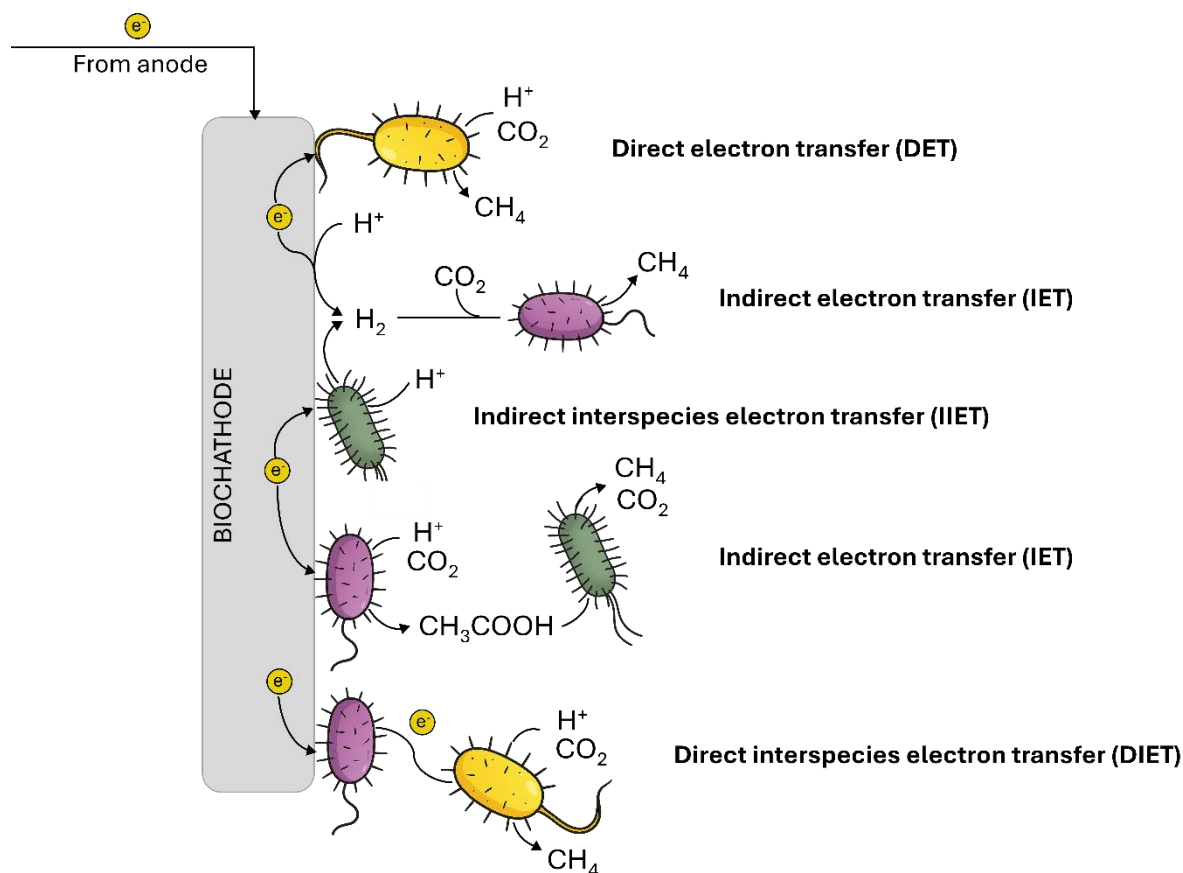
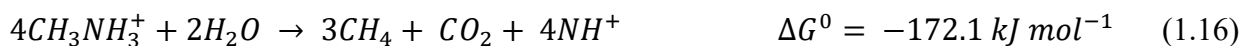


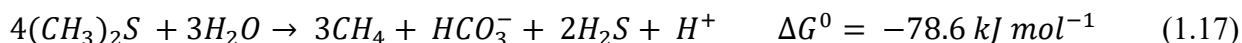
Figure 2.1. Overview of the different electron transfer mechanisms in bioelectromethanogenesis.

These microorganisms usually establish syntrophic relations with acetate-oxidising, which generate CO_2 and H_2 as metabolic by-products. Acetoclastic (or acetotrophic) methanogens, such as those belonging to the genera of *Methanotrix* and *Methanosarcina*, convert acetate into CO_2 and CH_4 , as illustrated in Equation 1.14.^{117,156}



Methylotrophic methanogens, predominantly members of the *Methanosarcinales* and *Methanomassiliicoccales*, utilize methylated substrates such as methanol (Eq. 1.15), methylamines (Eq. 1.16), or methylated sulfur compounds like methylthiol and dimethyl sulfide (Eq. 1.17) as electron donors for methane production:





The underlying mechanism involves the transfer of a methyl group from the substrate to coenzyme M (CoM-SH) via substrate-specific methyltransferases, forming methyl-SCoM. For every four methyl groups processed, one is oxidized to CO₂, generating the reducing equivalents required for the reduction of the remaining three methyl-SCoM molecules to CH₄.^{117,157,158}

2.4 Critical operational parameters in BEP2M

Bioelectrochemical power-to-methane (BEP2M) systems rely on the coordinated function of electrochemical interfaces and methanogenic microorganisms to convert renewable electricity and CO₂ into methane. Their performance is governed by a tightly interdependent set of physicochemical parameters that influence electron transfer pathways, microbial activity, and long-term system integrity. Among these, pH, temperature, bioreactor configuration, and microbial corrosion represent the most consequential operational factors. Understanding how these parameters interact is essential for advancing BEP2M technologies beyond laboratory conditions and towards industrial-scale energy storage.

pH exerts one of the most immediate and pervasive influences on BEP2M operation because it simultaneously affects methanogenic physiology, electrolyte conductivity, gas solubility, and cathodic reaction kinetics. Methanogens generally operate most efficiently in a narrow, near-neutral to slightly alkaline environment (6.8-7.5), where intracellular proton gradients remain stable and enzymatic pathways for CO₂ reduction proceed at maximum turnover rates.¹⁵⁹ Even mild acidification impairs key steps in the methanogenesis pathway by imposing energetic stress on membrane-bound cofactors and stimulating competing fermentative or acetogenic processes.¹⁶⁰ At the electrochemical interface, pH determines the availability of protons for hydrogen evolution and therefore influences whether methane formation proceeds via direct extracellular electron uptake or via H₂-mediated pathways. Acidic conditions can accelerate abiotic hydrogen evolution, shifting the microbial community toward hydrogenotrophic metabolism at the cost of coulombic efficiency.¹⁶¹ In contrast, alkaline conditions reduce proton conductivity and can lead to localized accumulation of hydroxyls near the cathode surface, creating microenvironments that inhibit microbial colonization. Effective BEP2M operation therefore requires tight pH regulation, often achieved through bicarbonate buffering, CO₂ dosing, or adaptive voltage control that constrains cathodic pH excursions.

Temperature plays an equally central role by dictating microbial metabolic rates, membrane fluidity, kinetic barriers for electron transfer, and the physicochemical behavior of electrolytes. Mesophilic operation remains the standard because of the robustness and diversity of the microbial consortia that develop under these conditions. Methanogenic activity, biofilm stability, and interspecies electron transfer pathways are generally more resilient in mesophilic environments, which helps stabilize performance under fluctuating electrical input. However, thermophilic operation can deliver significantly higher methane production rates due to enhanced enzymatic kinetics and improved gas-liquid mass transfer. The advantages of elevated temperatures are offset by challenges such as increased material degradation, reduced stability of some electroactive biofilms, and changes in electrolyte composition that affect charge transport. Because BEP2M systems often integrate into processes that produce waste heat, temperature control is not simply a biological consideration but a systems-level engineering challenge. Uniform thermal management is essential, as temperature fluctuations can rapidly disrupt syntrophic interactions between hydrogen producers, methanogens, and other members of the bioelectrochemical community.

Bioreactor configuration exerts a more structural influence on BEP2M performance by shaping electron transfer pathways, mass-transport characteristics, and the spatial organization of microbial communities. Single-chamber reactors offer the simplest design and often achieve low internal resistance due to minimal electrode spacing, but they are susceptible to oxygen crossover from the anode, which can suppress strict anaerobic methanogens. Dual-chamber systems, typically separated by ion-exchange membranes, enable precise control over the chemistry of each compartment and support strictly anaerobic cathodic conditions. These advantages, however, come with increased ohmic resistance, membrane fouling, and long-term drift in pH gradients across the separator. Beyond simple single- or dual-chamber designs, stacked modular architecture is becoming increasingly relevant for scale-up. Stacks enable higher volumetric productivity but require careful management of electrical potential distribution, hydraulic homogeneity, and inter-electrode interference. Across all configurations, electrode material and architecture represent a critical design axis. Carbon-based materials dominate modern BEP2M systems because of their chemical stability, high surface area, and natural biocompatibility, while surface functionalization can further enhance direct electron uptake by methanogens. Reactor architecture ultimately acts as the physical scaffold that determines whether the system operates via direct electron transfer, hydrogen-mediated pathways, or a hybrid regime shaped by both bioelectrochemical and ecological factors.

A fourth and increasingly recognized challenge is microbial corrosion, which poses significant risks to the longevity and safety of BEP2M reactors. At reducing potentials, many anaerobic microorganisms, including methanogens and sulfate-reducing bacteria, can directly access electrons from metallic surfaces, effectively using reactor materials as electron donors. This “electrical” form of microbial corrosion accelerates metal dissolution and undermines the structural integrity of cathodes, current collectors, and auxiliary components. Indirect corrosion mechanisms also play a role: metabolic by-products such as sulfides, organic acids, and extracellular polymers create microenvironments with steep redox or pH gradients, promoting localized pitting. The applied cathodic potential itself can exacerbate corrosion by altering thermodynamic favorability of metal oxidation or by enriching microbial guilds capable of exploiting the electrode material. Addressing microbial corrosion requires integrating materials science with microbiome engineering, for example, using corrosion-resistant carbon electrodes, applying protective coatings, adjusting operating potentials, or shaping microbial community composition to suppress corrosive taxa without compromising methanogenic activity.

Together, pH, temperature, reactor architecture, and microbial corrosion form a tightly interdependent network of operational constraints and opportunities. Their interactions determine not only methane production rates and coulombic efficiency but also the stability, scalability, and economic viability of BEP2M systems. A comprehensive understanding of these parameters reveals that optimizing BEP2M is not merely a matter of tuning individual variables; it requires an integrated approach in which microbial ecology, electrochemistry, and engineering design co-evolve. As future systems incorporate adaptive control frameworks, advanced electrode materials, and real-time microbial monitoring, BEP2M technologies are poised to become a central component of renewable energy storage and carbon utilization infrastructures.

2.5 Microbiological influenced corrosion

Corrosion is an irreversible reaction that occurs at the interface between a material (metal, ceramic, polymer) and its surrounding environment.¹⁶² It generally results in either the degradation of the material itself or the dissolution of an environmental component into the material. From an electrochemical perspective, a corroding metal surface functions as a micro-galvanic cell in which oxidation (Eq. 1.18) and reduction (Eq. 1.19) reactions occur at anodic and cathodic sites, respectively.¹⁶³



Equation 1.19 represents the predominant cathodic reaction occurring in the absence of oxygen. The nature of the corrosion products depends on the availability of oxygen. In aerated environments, electrons released during the oxidation of the metal in its zero-valent state (M^0) reduce molecular oxygen, resulting in the formation of rust or other metal oxides. Under anoxic conditions, the electrons produced by metal oxidation instead reduce protons derived from water dissociation. However, in strictly oxygen-depleted environments, metal oxidation coupled solely with proton reduction is typically limited. Microorganisms can drastically accelerate this process: anaerobic corrosion frequently arises from microbial activity or from the metabolic end products they generate.¹⁶⁴ Most microorganisms, characterized by negatively charged outer membranes, adhere to metal surfaces and secrete extracellular polymeric substances (EPS) that constitute the structural matrix of biofilms. Corrosion induced by sulfate-reducing bacteria (SRB) represents the most extensively studied form of microbiologically induced corrosion. Since the early “electrochemical depolarization” hypothesis,¹⁶⁵ which identified hydrogen film formation on metal surfaces as an electron source subsequently exploited by hydrogenotrophic SRB, numerous revisions have been proposed and remain under debate.^{166–168} The overall framework of microbiological corrosion is illustrated in Figure 1.6, which shows how microorganisms can survive under diverse environmental conditions within a structured biofilm on a solid metal surface.

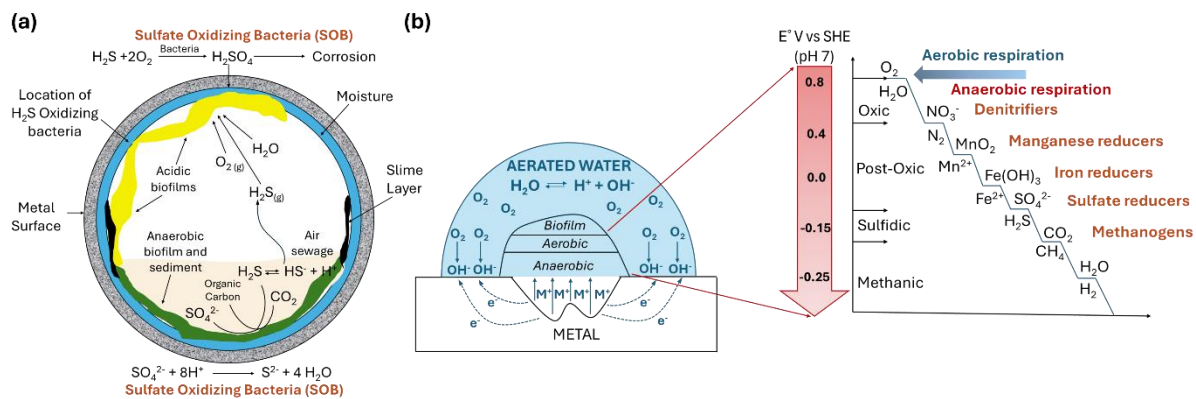


Figure 2.2. Schematic representation of the microbial corrosion system within a biofilm: (a) the sulfur cycle in a wastewater pipeline under flow conditions; (b) the metabolic pathways associated with aerobic and anaerobic biofilm communities that contribute to the corrosion of the underlying metal substrate.¹⁸

The entire sulfur cycle, operating in oxygen-rich, anaerobic, or microaerophilic environments alike (such as within wastewater or biogas treatment pipelines), is commonly associated with the most severe cases of microbiologically influenced corrosion (Fig. 1.6a). Figure 1.6b further

illustrates how aerobic and anaerobic bacterial populations coexist synergistically within the biofilm, adapting to environmental redox gradients and to the progressive depletion of oxygen near the metal surface. In the absence of oxygen, the availability of alternative electron acceptors and nutrients critically shapes both the evolution of the redox potential and the composition of the microbial community within the biofilm. The most relevant electron acceptors, in order of decreasing reduction potential, include nitrogen species, manganese oxides, oxidized iron compounds, and finally dissolved carbon dioxide.

EXPERIMENTAL SECTION

Chapter 3

3 Experimental part

3.1 Experimental methodologies for evaluating OER performance

3.1.1 Materials

Titanium (IV) isopropoxide (TTIP, for synthesis, Technipure[®]) 1-tetradecylamine (TDA, 95%), methanol (MeOH, $\geq 99.5\%$, Emplura[®]) and acetonitrile (ACN, $\geq 99.9\%$) were purchased from Sigma Aldrich. Iso-propyl alcohol (IPA, American Chemical Society grade) was purchased from Macron Fine Chemicals. Perchloric acid solution (70% HClO₄, 99.999%, Omnipur) for electrochemical measurements was purchased from Sigma Aldrich. All chemicals were used as received without further purification. Liquid nitrogen is collected from a 22 psi tank from Airgas with 100% purity. Oxygen (ultra high purity, UHP) and 5% H₂/Ar were purchased from Airgas. Unsupported rutile iridium oxide (TEC77110) was obtained from Tanaka Precious Metals (TKK). Deionized (DI) water was delivered by a Milli-Q Ultrapure Water System and had a resistivity of 18.2 MW cm at 25 °C.

An aqueous H₂IrCl₆ solution (IrCl₆²⁻) was prepared in-house by digesting IrCl₃·xH₂O (99.8%, Sigma Aldrich) in freshly prepared aqua regia (3:1 v/v mixture of HCl/HNO₃ both ACS reagent grade). The digestion was carried out by heating the suspension until the majority of the solid was dissolved. The mixture was then filtered through a 100 nm syringe filter to remove any remaining insoluble species. The resulting clear filtrate was subsequently evaporated until no orange NO_x fumes were released, even upon addition of excess HCl. The use of a scrubber system is recommended during this step. After cooling to room temperature, an aliquot of the solution was analyzed by ICP-MS to determine its iridium concentration. The batch employed for the synthesis described herein had a concentration of 45.6 mmol_{Ir}/L.

3.1.2 Synthesis of TiO₂ supports

TiO₂ spheres were synthesized through a sol-gel protocol adapted from previously reported procedures.^{169–171} In a typical procedure, 5 mmol of deionized water was introduced into a 7:3 v/v

mixture of MeOH/ACN mixture (25 mL) contained in a glass vial. Subsequently, 1 mmol of TDA was dissolved in the mixture and gently stirred at room temperature for 10 min. The vial was then sealed with a rubber septum, and 1 mmol of TTIP was added dropwise via syringe under vigorous stirring. As hydrolysis and condensation proceeded, the solution gradually turned opaque, indicating the formation of TiO₂ spheres. After stirring at room temperature for 24 h, the resulting milky suspension was centrifuged at 6000 rpm for 20 min, washed with MeOH, and centrifuged again under the same conditions. The solid was then washed with water and centrifuged once more. The resulting gel-like product was redispersed in water, rapidly frozen in liquid nitrogen, and subsequently dried under vacuum at room temperature.

Finally, the obtained powder was calcined under a 5% H₂/Ar flow (300 sccm) at 550 °C (TiO₂-AR) and 800 °C (TiO₂-R) using a heating rate of 5 °C min⁻¹ for 6 h. The materials were allowed to cool naturally under the same reducing atmosphere prior to exposure to air.

3.1.3 Synthesis of supported iridium oxide electrocatalysts

30 mg of the TiO₂ support were added to 4 mL of deionized (DI) water and dispersed in an ultrasonic bath for 15 minutes. To further improve dispersion of the TiO₂ spheres, the suspension was additionally treated with a sonication horn for 5 minutes using a pulsed regime (2s on, 3s off) at 20% amplitude. To achieve a nominal Ir loading of 8 wt. %, 263 µL of the previously prepared IrCl₆²⁻ solution was introduced into the dispersion using a micropipette (corresponding to 8.77 µL of solution per mg of TiO₂ support). The mixture was immediately returned to the ultrasonic bath for an additional 15 minutes and then dried overnight in an oven at 70 °C.

Once fully dried, the resulting powder was collected and calcined in air at 500 °C for 30 min using a heating rate of 5 °C min⁻¹. For the TiO₂-AR support, a preliminary calcination step at 550 °C under a H₂/Ar atmosphere was carried out prior to the air treatment. The solid was subsequently washed with 300 mL of boiling water, filtered, and dried at 70 °C.

3.1.4 Physical characterization

Transmission electron microscopy (TEM), scanning transmission electron microscopy (STEM), and energy-dispersive X-ray spectroscopy (EDS) mapping were carried out on a JEOL 2800 microscope operated at 300 kV. The core-shell morphology of the IrO₂@TiO₂ particles was evaluated by STEM, and the thickness of the IrO₂ shell was determined from EDS line-scan analyses.

X-ray diffraction (XRD) patterns were collected using a Rigaku SmartLab diffractometer equipped with a 2.2 kW Cu K α radiation source ($\lambda = 0.15405$ nm) and a K β filter. Samples were mounted on a zero-background holder. Diffraction peaks corresponding to rutile TiO₂, anatase TiO₂, and rutile IrO₂ were assigned using ICSD entries 80843, 63711, and 84577, respectively.

Nanoparticle shape and morphological uniformity were assessed by scanning electron microscopy (SEM) using an FEI Magellan 400 XHR microscope operated at 10 kV. TiO₂ particle size distributions were obtained by analyzing at least 100 nanoparticles in SEM images using ImageJ software.

Thermogravimetric analysis (TGA) was performed on a Netzsch TG 209 F1 Libra instrument. Samples were heated to 800 °C at 3 °C min⁻¹ in an alumina crucible under a synthetic air flow (10 mL min⁻¹) to remove chemisorbed species and fully oxidize the TiO₂ surface, yielding a white powder.

X-ray photoelectron spectroscopy (XPS) measurements were conducted on a Kratos AXIS Supra instrument equipped with a monochromatic Al K α source and an integral charge neutralizer. For each sample, a survey spectrum (600-20 eV binding energy, 1 eV step size) was recorded, ensuring that the C 1s main peak appeared at 28 ± 1 eV. High-resolution spectra were acquired with a 0.1 eV step size, 200 ms dwell time, and averaged over ten scans. The Ir 4f and O 1s core levels were probed over 58-72 eV and 527-537 eV, respectively. Spectral fitting was performed using CasaXPS.

The nominal IrO₂ loading was quantified by inductively coupled plasma–mass spectrometry (ICP-MS) following sample digestion. A 76 μ L aliquot of catalyst ink (see preparation below) was deposited into a 100 μ L alumina crucible, dried on a hot plate, and yielded 0.373 mg of catalyst per crucible. Thirty microliters of a saturated KOH/KNO₃ aqueous solution were added and evaporated. The crucibles were then subjected to the following heat-treatment protocol under air: (1) 10 °C min⁻¹ to 300 °C, 10 min dwell; (2) 3 °C min⁻¹ to 360 °C, 4 h dwell; (3) 5 °C min⁻¹ to 425 °C, 15 min dwell. This procedure melted the KOH/KNO₃ mixture and converted iridium and titanium species into forms soluble in aqua regia. The crucibles were placed in glass vials containing 3 mL of aqua regia and heated on a hot plate at 190 °C until complete dissolution was achieved. The resulting solution was collected, and the vial was rinsed to ensure quantitative transfer. The total recovered solution mass was recorded, and the appropriate volume was analyzed using a PerkinElmer NexION 5000 ICP-MS.

3.1.5 Electrochemical characterization

Electrochemical measurements were conducted in 0.1 M perchloric acid using a three-electrode glass cell and a rotating disk electrode (RDE) from Pine Research. All experiments were performed on a Bio-Logic VSP-300 potentiostat. A glassy carbon plate served as the counter electrode, and a HydroFlex reversible hydrogen electrode (RHE, Gaskatel) was used as the reference electrode. Electrocatalysts were deposited onto a 0.196 cm² gold electrode tip at a nominal loading of 0.02 mg_{IrO₂} cm⁻². Mass activities were later corrected using the actual IrO₂ loadings determined by ICP-MS. Catalyst inks were prepared in IPA at a concentration of 204 g_{catalyst} μL⁻¹. For each measurement, 10 μL of the ink dispersion was drop-cast onto the freshly polished gold tip. No ionomer binder was added, and no catalyst detachment was observed during the experiments. The electrolyte was replaced between measurements, and the reference electrode was immersed only for the minimum duration required, as contamination by catalyst residues could cause substantial shifts in the RHE potential and artificially inflate activity estimates. The reference electrode was calibrated against a fresh, identical RHE in the same electrolyte before and after each experiment. All reported potentials are referenced to the RHE scale.

Each measurement sequence began with potential-controlled electrochemical impedance spectroscopy (PEIS) at 1.0 V, collected over a frequency range of 100 MHz to 10 Hz. The high-frequency resistance was obtained from the intercept of the Nyquist plot with the real axis, and an 85% iR compensation was applied to all subsequent measurements. Electrodes were then conditioned by 50 cyclic voltammetry (CV) cycles between 0.8 and 1.6 V at 100 mV s⁻¹ under 2000 rpm rotation. The capacitive behavior was assessed by recording three CV cycles between 1.0 and 1.3 V at scan rates of 100, 50, and 20 mV s⁻¹. Finally, linear sweep voltammetry (LSV) was performed from 1.3 to 1.6 V at 2 mV s⁻¹ under 2000 rpm rotation to ensure efficient removal of evolved O₂ from the electrode surface.

3.2 Experimental methodologies for assessing MIC

3.2.1 Materials

Four different materials (two copper alloys and two iron alloys) were employed to evaluate microbial corrosion: copper (Cu), duplex OT60 brass (Cu-Zn alloy), ARMCO[®] iron (Fe) and S355 carbon steel (CS). The different chemical composition (wt. %) of the four alloys is reported in Table 3.1. Coupons of copper, duplex OT60 brass, and Fe ARMCO[®] were cut from rectangular plates into smaller sizes of 7.4 cm² area. Carbon steel coupon S355 was cut from a rod into a small plate of 4 cm² area. For the electrochemical measurements, the surface of each sample was

polished using different SiC abrasive papers (from 800 to 2500-grit). The samples were then coated with a non-conductive polymeric resin to expose an area of 1.4 cm² to the test solution. For electrochemical purposes, electrical contact was obtained by soldering the metal samples with copper wires and insulating them with liquid rubber. To avoid any contamination from the surrounding environment, coupons were rinsed with distilled water, degreased with ethanol (70% v/v), and subsequently exposed to UV light (254 nm) for 40 min under a sterile hood.

Table 3.1. Overview of the composition (wt. %) of the metal alloys tested.

Material	Composition (wt. %)												
	Zn	Sn	C	Mn	Si	P	S	Cr	Ni	N	Mo	Cu	Fe
Fe (ARMCO®)	-	0.010	0.02	0.2	-	0.015	0.015	-	-	0.007	-	0.06	bal.
CS (S355)	-	-	0.14	1.24	0.19	0.020	0.002	0.23	0.19	-	0.06	0.31	bal.
Cu	-	-	-	-	-	-	-	-	-	-	-	1.00	-
Cu-Zn (OT60)	0.4	-	-	-	-	-	-	-	-	-	-	0.6	-

3.2.2 Electrolyte

The electrolyte used for microbial corrosion tests was isolated from a biological methanation pilot plant sludge (RSE S.p.A.). Microbial composition of both bacteria and Archaea hydrogenotrophic methanogens, was characterized by Illumina sequencing following the procedure described in Section 3.2.6 and results are presented in Figure 5.1 of Section 5.1. Ion Chromatography (IC) and Inductively Coupled Plasma - Atomic Emission Spectroscopy (ICP-AES) analysis (Section 3.2.7) of the pool are presented in Table 5.1 and 5.2 of Section 5.2. Conductivity and pH of the bacterial pool were assessed via a conductivity cell (AMEL 134) and a pH meter (AMEL 334-B) and measured 4.04 ms cm⁻¹ and 6.96, respectively. To maintain conditions representative of the actual methanation environment, the culture medium used for corrosion testing replicated the chemical composition of the plant's aqueous phase. The culture solution used in this work contained the following nutrients: Na₂HPO₄·12H₂O (896 mg L⁻¹); NH₄Cl (424 mg L⁻¹); KH₂PO₄ (216 mg L⁻¹); MgCl₂·6H₂O (80 mg L⁻¹); CaCl₂·2H₂O (60 mg L⁻¹); FeCl₂·4H₂O (16 mg L⁻¹); CoCl₂·6H₂O (6.7 mg L⁻¹); MnCl₂·4H₂O (3.3 mg L⁻¹); NiCl₂·6H₂O (0.7 mg L⁻¹); H₃BO₃ (0.3 mg L⁻¹); ZnCl₂ (0.3 mg L⁻¹); Na₂SeO₃ (0.3 mg L⁻¹); CuCl₂ (0.2 mg L⁻¹); Na₂MoO₄·2H₂O (0.1 mg L⁻¹). The phosphate system (Na₂HPO₄/ KH₂PO₄) provides buffering capacity to maintain pH stability during microbial

metabolic activity, as employed in operational methanation facilities. Since the inoculum was collected from a methanation plant operating with this nutrient composition, the microbial consortium was pre-adapted to these phosphate-rich, buffered conditions. CO₂, dosed as described in Section 3.2.3, served as the primary carbon source for autotrophic and mixotrophic members of the community, consistent with methanation process requirements. This medium composition reflects the nutrient supplementation, buffering requirements, and process chemistry typical of methanation plants, ensuring both environmental and practical relevance of the experimental conditions.

Different thermal treatments were applied to the microbial pool before the starting of the test and subsequently tested for corrosion behavior: i) boiling for 120 minutes; ii) double autoclaving at 121 °C for 20 minutes; and iii) autoclaving at 121 °C for 60 minutes followed by filtration through a 0.22 µm membrane and UV irradiation at 254 nm for 40 minutes (sterile reference). The results were compared with the untreated biotic solution.

3.2.3 Experimental setup

Experimental setup consisted of a 250 mL single glass vessel. Each cell was filled with approximately 200 mL of electrolyte and tightly sealed with a rubber stopper, which accommodates gas inlet and outlet lines. Electrochemical measurements were carried out using a three-electrode configuration (Fig. 3.1) consisting of a working electrode WE (tested alloy), a reference electrode RE (saturated KCl Ag/AgCl electrode, $E = + 0.197$ vs SHE, AMEL Srl, mod. 373/12) and a counter electrode CE (DSA[®]). The RE was positioned as close as possible to the WE, so that no compensation for the ohmic drop (IR) was necessary. Tests were carried out at 45°C in a thermostatic sand bath with temperature control and under deaerated conditions by initial insufflation of CO₂ for 30 minutes (44.5 mmol dosed). No temperature variations were detected during the test. The following temperature (45 °C) represents the optimal temperature for thermophilic methanogenic microorganisms, representative of operational conditions in biological methanation plants.¹⁷² Complete immersion of the cells in a thermostatic sand bath with temperature control ensured dark conditions throughout the experiment, excluding phototrophic microbial activity.

All the glassware and electrodes were previously washed with bleach, rinsed with water, and washed with 70% v/v ethanol, then sterilized in an oven at 121 °C for 60 minutes. The entire setup was sterilized under a vertical laminar flow UV hood (254 nm, 45 minutes). Additionally, the test

preparation was carried out under the same UV hood to maintain a sterile environment and prevent any potential contamination.

Four identical reactors were prepared for each condition: biotic, boiled, autoclaved and sterile (Section 3.2.2). The evolution of microbiologically induced corrosion (MIC) was documented over 15 days. For each condition, four brass coupons were tested simultaneously under identical conditions. Two coupons were dedicated to electrochemical measurements (Section 3.2.4) throughout the 15-day exposure period. The other two coupons were used for post-test characterization (Sections 3.2.5 and 3.2.6): one coupon was used for surface characterization without altering the corrosion products morphology, while the other was used for both molecular analyses and surface characterization after biofilm removal from a defined area (0.7 cm^2) of the exposed surface (1.4 cm^2). No tests were conducted with thermally treated medium without inoculum, as the research objective focused on the corrosion behavior resulting from incompletely sterilized methanation inocula. The sterile condition served as the abiotic control, representing brass corrosion in the absence of viable microbial activity.

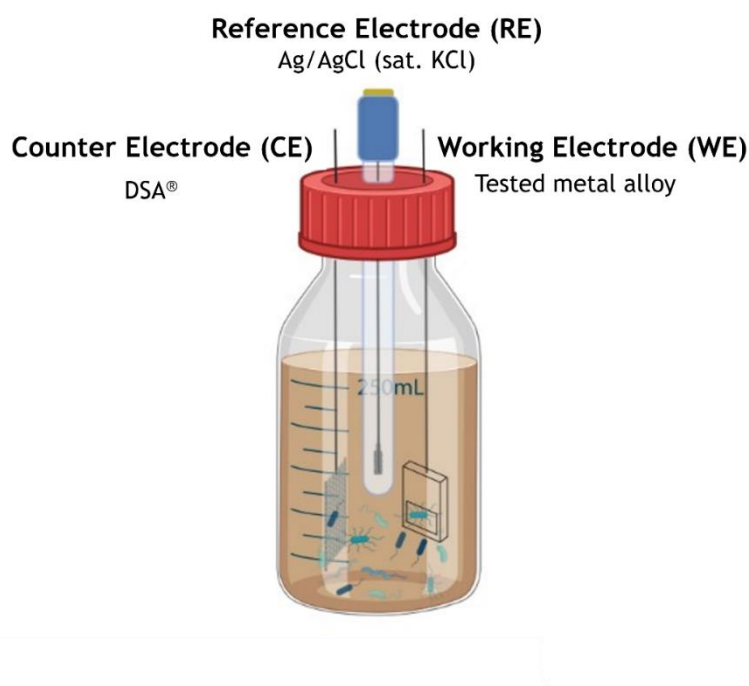


Figure 3.1. Three-electrode cell setup used for electrochemical measurements.

3.2.4 Electrochemical characterization

Electrochemical analyses were carried out on duplicates using a Gamry potentiostat/galvanostat (1000 Interface) and a three-electrode cell configuration (Section 3.2.3 and Figure 3.1).

Open circuit potential (OCP) was monitored over 15 days for each condition (i.e., biotic, boiled, autoclaved and sterile).

Linear Polarization Resistance (LPR) was performed after 1 and 15 days. The potential scan ranged from -0.020 V vs. OCP to 0.020 V vs. OCP at a scan speed of 0.167 mV/s. The polarization resistance (R_p) was calculated from the E vs. I curve using a linear regression method.

Electrochemical Impedance Spectroscopy (EIS) measurements were performed with an amplitude of 10 mV vs OCP in the frequency range of 100 kHz to 0.01 Hz and a density of 10 points/decade. Impedance spectra were recorded after 1, 7 and 15 days. The obtained data were normalized to the exposed sample surface area expressed in cm^2 . The most representative data were selected for each condition and discussed.

All electrochemical data presented in the manuscript correspond to the average of duplicate measurements for each condition, with error bars indicating standard deviation where applicable.

3.2.5 Surface characterization

Corrosion tests were performed in duplicate to characterize both the morphology of the corrosion products and the composition of the biofilm. Accordingly, one coupon was used for surface characterization without altering the corrosion products morphology. The other replicate was used for both molecular analyses and surface characterization of the biofilm. In this latter case, the biofilm was removed from a defined area (0.7 cm^2) of the exposed coupon surface (1.4 cm^2). The remaining area exposed to the inoculum was then dried in an oven for 15 minutes at 100°C under air atmosphere to enable surface characterization of the biofilm. Morphological observations and chemical characterizations of the corrosion products were performed on both surface and the cross section of the studied replicates.

Corrosion products morphology was assessed using: i) *Light Optical Microscope* (Nikon Eclipse MA2000); ii) *Field Emission Scanning Electron Microscope* (FE-SEM) Tescan Mira II XMH. Chemical composition was studied by Energy-dispersive X-ray Spectroscopy (EDS) using a Bruker XFlash 5010 detector. An acceleration voltage of 20 kV was used; iii) *Raman Spectroscopy* Horiba XploRA PLUS system equipped with an Olympus BX43 microscope and a long working distance $50\times$ ($\text{NA}=0.50$) objective. A 532 nm solid state laser and 2400 line/mm diffraction grating were used to collect the spectra, that combined with the resolution (1024×256 pixels, pixel dimension $26\times 26 \mu\text{m}^2$) of air-cooled Peltier CCD detector ensure $\approx 1 \text{ cm}^{-1}$ spectral sensitivity. To avoid altering the nature of the corrosion products, special filters were used to reduce the laser

power (from 25 % to 1 %). To have a more complete view of the Raman shift of our samples, all the spectra was collected in the range from 100 to 2000 cm^{-1} .

Cross-sections of metal coupons were prepared to assess the distribution of the corrosion products, as well as to identify the possible presence of biofilm on the surface. The samples were mounted in cold-curing epoxy resin and polished using silicon carbide (SiC) papers up to 1200 grit, followed by a mirror finish with diamond paste down to 1 μm , in accordance with the ASTM E3–01 standard procedure.

3.2.6 Molecular analysis

Samples for molecular analyses were collected from the material surface (i.e., biofilm) with sterile swabs. Bulk solution (post-test) and inoculum (pre-test) were collected with 10 mL syringes and stored in Falcon tubes at -18 ± 4 °C. Samples were obtained for all four conditions (i.e., biotic, boiled, autoclaved, sterile). DNA was extracted from biofilm swabs using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). Each swab was cut with sterile scissors into a Lysing Matrix E tube, and DNA extraction was performed following the manufacturer's instructions. DNA from the bulk solution and inoculum was obtained with the same kit after filtration through nitrocellulose membranes (0.22 μm); half of each filter was cut with sterile scissors and processed for extraction. DNA quality was assessed through a first PCR on the extracted DNA and on its dilutions 1:10 and 1:100. Sequencing was performed by MiSeq Illumina (Illumina, Inc., San Diego, CA, USA) on the 16S rRNA gene of both bacteria and archaea.

The bacterial community was characterized by sequencing the V5-V6 hypervariable region of the 16S rRNA gene with the 783F (5'-CAGGATTAGATACCC-3') and 1027R (5'-CGACRRCCATGCANCACT-3') primers. PCR amplification was performed with an initial denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 50 s, 47 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 5 min. Reactions (25 μL) contained 25 μL GoTaq® Green Master Mix (Promega, Madison, WI, USA), 1 μM of each primer, and 2.5 μL of DNA. Both primers carried homemade barcodes and Illumina adapters fused to the 5' end. Amplicons were purified from 1.5% agarose gels using the Wizard SV Gel and PCR Clean-Up System (Promega) and quantified with Qubit® (Life Technologies, Carlsbad, CA, USA). Sequencing was performed on an Illumina MiSeq platform (2 × 300 bp paired-end, Illumina Inc., San Diego, CA, USA). Demultiplexed sequences were processed with the dada2 pipeline to infer Amplicon Sequence Variants (ASVs), which were taxonomically classified using the RDP classifier (confidence > 80 %).

Singletons were removed for the following analyses performed with R (version 4.3.1). Cluster analysis was performed on the Hellinger transformed dataset to evaluate similarities between samples. The number of ASVs was evaluated as a diversity index on rarefied datasets to compare the four different treatments (i.e., biotic, boiled, autoclaved, and sterile) for each substrate (i.e., biofilm, solution, and inoculum). The samples from the four substrates were rarefied separately, each at a different level, because the comparison was between treatments (i.e., biotic, boiled, autoclaved, and sterile) and not between substrates (i.e., biofilm, solution, and inoculum). The process of rarefaction of the entire dataset would have resulted in a loss of information. For instance, the range of sequences in biofilm samples was from 800 to 6,038. Consequently, the cutoff point for rarefaction was established at 800 sequences. In contrast, the range of sequences in inoculum samples was from 3238 to 30829. As a result, it was possible to retain a greater number of sequences. ASV of the most representative genera of the different treatments were further investigated by NCBI blast (<https://www.ncbi.nlm.nih.gov/>).

qPCR analyses were carried out to quantify bacteria populations in the collected samples. The abundance of total bacteria was quantified using qPCR with specific primers for targeting the 16S rRNA. Each reaction, including samples, standards and a negative control, was conducted in a final volume of 10 μ L using the Luna Universal qPCR Master Mix 2X (NEB, MA, USA) with 0,3 μ M primers concentration. Calibration curves were made using an appropriate standard with 5 consecutive dilution steps and all assays were performed in triplicates on a QuantStudio Real-Time PCR System. The primers selected to quantify bacteria populations were 331F 797R and the cycling conditions included an initial denaturation at 95 °C for 4 min, followed by 40 cycles of denaturation at 95 °C for 15s, annealing at 60 °C for 30s, and extension at 72 °C for 15s. To obtain dissociation curves, a final step at 95 °C for 15s, 55 °C for 15s, and 95 °C for 15s was performed. 16S rRNA gene copy numbers were normalized against volume (ml).

3.2.7 Solution characterization

Chemical analyses of the solution were conducted at the beginning and at the end of the experiment for each condition (i.e., biotic, boiled, autoclaved and sterile control), to measure the ionic release of the main alloying elements (Cr, Fe, Ni, Cu, Zn, S and P) and the evolution in concentration of Volatile Fatty Acids, VFAs (oxalates, formates, acetates, propionates) and carbonates.

Inductively Coupled Plasma - Atomic Emission Spectroscopy (ICP-AES) analytical technique (Analytik Jena Plasma Quant, PQ 9000) was performed to determine the amount (ppm) of ions in solution using 1200W.

Ionic Chromatography (IC, Dionex ICS 1000) equipped with an S9-SC column and an AMMSIC chemical suppressor was used for analysis of the anionic species in the solution. A 10 mL sample was withdrawn with a sterile syringe and stored at 2 °C until measurement.

Solution turbidity was visually assessed after thermal treatments (Section 3.2.2), showing progressive decrease from biotic (highest turbidity) to sterile (minimal turbidity) conditions, correlating with microbial load reduction. It cannot be excluded that thermal treatment caused partial precipitation of inorganic salts, which may have contributed to the residual turbidity.

3.3 Experimental methodologies for bioelectrochemical CO₂RR

3.3.1 Site selection and soil sampling

Soil samples were collected from the Monte Amiata geothermal area (southern Tuscany, Italy), a Quaternary volcanic complex characterized by extensive natural CO₂ degassing, active hydrothermal circulation, and well-documented geochemical gradients.^{173,174} The selected sampling site, "Acquapassante 1 ex SMI" (42.8942 °N, 11.6520 °E), represents a persistent CO₂-emitting vent (Fig. 3.2), providing an ideal natural system for investigating the influence of elevated CO₂ fluxes on soil microbial processes and assessing the potential of geothermal CO₂ as a carbon source for bioelectrochemical methane production.

Sampling was conducted along a natural CO₂ flux gradient, to capture contrasting geochemical conditions. Two distinct soil samples were collected: i) High-CO₂/SO₄²⁻ sample (inoculum HCS, point A, Figure 3.2) obtained immediately adjacent to the emission point, characterized by elevated gas flux and sulfate concentrations, and ii) a low-CO₂/SO₄²⁻ sample (inoculum LCS, point B, Figure 3.2) collected approximately 30 m from the vent, where both gas flux and sulfate levels were substantially reduced (detailed characterization provided in Section 10.3.1).

Gas composition at 10 cm soil depth was determined in situ using a portable Combimass[®] GA-m analyzer equipped with three non-dispersive infrared (NDIR) and three electrochemical (EC) sensors.

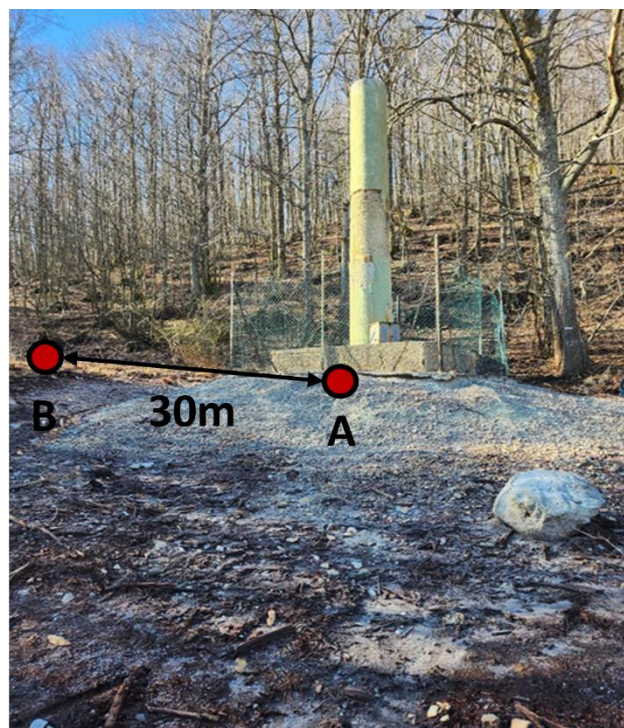


Figure 3.2. Soil sampling area "Acquapassante 1 ex SMI" (42.8942 °N, 11.6520 °E), Monte Amiata geothermal area (southern Tuscany, Italy). A schematic representation of the soil sampling area is presented in the picture with point A (inoculum HCS, high $\text{CO}_2/\text{SO}_4^{2-}$ soil) representing the soil sampled adjacent to the emission point and B (inoculum LCS, low $\text{CO}_2/\text{SO}_4^{2-}$ soil) collected approximately 30 m from the vent.

3.3.2 Experimental design and BES configuration

A two-phase experimental approach was adopted. The first phase consisted of a 90-day preliminary screening to evaluate the effects of three operational parameters on bioelectrochemical system performance: i) inoculum type (high-sulfate vs. low-sulfate soil), ii) reactor configuration (presence or absence of a cation exchange membrane), and iii) sulfate-reducing bacteria (SRB) inhibition. SRB inhibition was achieved through the addition of 2 mM sodium molybdate dihydrate, which acts as a sulfate analog and disrupts adenosine 5'-phosphosulfate (APS) formation, a critical intermediate in the dissimilatory sulfate reduction pathway.¹⁷⁵ To our knowledge, this represents the first application of molybdate-mediated SRB inhibition in electromethanogenesis systems.

Six dual-chamber H-type bioelectrochemical reactors (250 mL working volume) were constructed and operated according to the configurations summarized in Table 3.2 and Figure 3.3. Each cathode chamber was inoculated with approximately 40 g of soil suspended in 0.1 M potassium phosphate buffer (pH 7.0, final working volume 150 mL). In membrane-free configurations (R2,

R5), soil was added to both anode and cathode compartments to evaluate whether the soil matrix itself could function as an ion-permeable separator, eliminating the need for a synthetic membrane.

Table 3.2. Experimental conditions tested in the preliminary screening phase.

Soil type	Membrane	No membrane	Sulfate-reduction inhibitor
HCS (High CO ₂ /SO ₄ ²⁻)	R1	R2	R3
LCS (Low CO ₂ /SO ₄ ²⁻)	R4	R5	R6

The cathode electrode consisted of a 25 cm² carbon cloth (SAATI, Legnano, Italy), while the anode comprised an iridium-coated titanium mesh DSA[®] (60 cm² nominal area, 40 cm × 1.5 cm) connected via titanium wire. In membrane-equipped reactors (R1, R3, R4 and R6), the two chambers were separated by a Nafion[®] 117 (DuPont[™]) proton exchange membrane. A saturated KCl Ag/AgCl reference electrode ($E = +0.197$ vs SHE, AMEL Srl, mod. 373/12) was positioned in the anode compartment for electrochemical measurements. To maintain CO₂ saturation and ensure strict anoxic conditions, cathode chambers were purged with 100% CO₂ at 3 mL min⁻¹ for 30 minutes (44.5 mmol dosed) three times weekly. Tests were carried out in a thermostatic sand bath with temperature control (25 ± 2 °C).

All glassware and electrodes were sterilized prior to use to prevent contamination. The sterilization procedure consisted of multiple steps, including washing with bleach, rinsing with water, treatment with 70% (v/v) ethanol, and final sterilization in an oven at 121 °C for 60 min. Following the preliminary screening, a second 31-day experiment was conducted using the best-performing catholytes (based on methane production rates) as enriched inoculum (details provided in Section 10.3.3). Three replicate reactors (R7, R8, R9) were then assembled in the optimal configuration (identical to R4) identified during the preliminary phase, alongside an unpolarized control reactor (R_{control}) maintained under open-circuit conditions. During this validation experiment, cathode chambers were purged daily with 100% CO₂ using identical gas flow parameters (3 mL min⁻¹, 30 minutes).

Headspace gas samples (10 mL) were withdrawn periodically using gas-tight syringes and analyzed by micro-gas chromatography (Agilent MicroGC 990, Santa Clara, CA, USA) to

quantify CH_4 , H_2 , and CO_2 concentrations. Methane production rates were normalized to cathode geometric surface area (m^2) and time (days) to enable performance comparison across reactors.

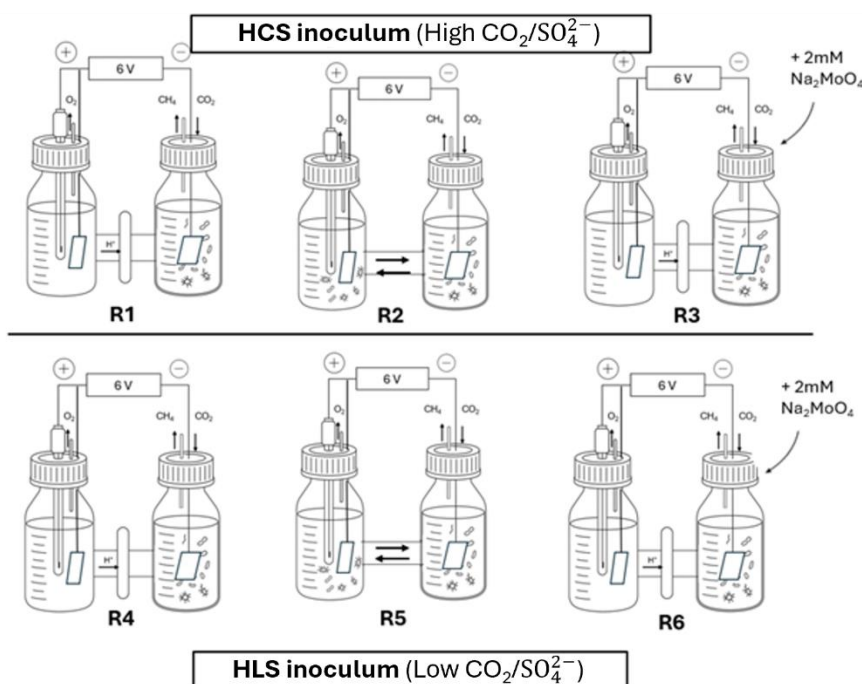


Figure 3.3. Schematic representation of the different tested experimental setups.

3.3.3 Electrochemical measurements and analysis

Initial attempts to polarize the cathode at a conventional potential of $-1.0 \text{ V vs. Ag/AgCl}$ resulted in negligible current generation. Consequently, all subsequent experiments were conducted under galvanostatic control by applying a fixed cell voltage (E_{cell}) of $-6.6 \pm 0.05 \text{ V}$ between anode and cathode. Current and cathode potential were continuously monitored throughout the experimental period using a multi-channel data logger (Graphtech Midi Logger GL820) recording voltage drop across a 10Ω external resistor at 10-minute intervals. Current generation (I) was calculated according to Ohm's law (Eq. 3.1):

$$I = E_{\text{cell}} R^{-1} \quad (3.1)$$

Cyclic voltammetry (CV) was performed on days 17, 24, and 31 for the three enriched reactors and the unpolarized control using a potentiostat (Gamry Interface 1000). CV scans were conducted in a potential window from -0.5 V to $+0.5 \text{ V}$ vs. open circuit potential (E_{OC}) at a scan rate of 10 mV s^{-1} with 10 mV step increments to identify redox-active species.

Electrochemically active surface area (ECSA) was determined through CV measurements conducted in a non-Faradaic potential window centered at 0 V vs. Ag/AgCl. An adaptive windowing protocol was employed: the default window spanned ± 0.10 V; if fewer than eight data points were acquired within this range, the window was expanded to ± 0.15 V. For each scan rate v (10-50 mV s⁻¹), anodic and cathodic current sweeps were segmented based on the sign of dV/dt . Within the selected potential window, mean anodic (i_a) and cathodic (i_c) currents were calculated, and the capacitive current (Δi) was determined following Equation 3.2.

$$\Delta i(A) = \frac{|i_a - i_c|}{2} \quad (3.2)$$

The capacitive current was then regressed against scan rate using the linear model presented in Equation 3.3, where C_{dl} represents the electrical double-layer capacitance (F), and b is the intercept. Goodness of fit was assessed using the coefficient of determination (R^2).

$$\Delta i (V \cdot s^{-1}) = C_{dl} \cdot v + b \quad (3.3)$$

ECSA was calculated from C_{dl} (Eq. 3.4) assuming a specific capacitance (C_s) of 20 $\mu F \text{ cm}^{-2}$.

$$ECSA (cm^2) = \frac{C_{dl}}{C_s} \quad (3.4)$$

The roughness factor (R_f), defined as the ratio of electrochemically active to geometric surface area, was calculated following Equation 3.5, where A_{geom} corresponds to 25 cm².

$$R_f = \frac{ECSA}{A_{geom}} \quad (3.5)$$

Coulombic efficiency (CE) was calculated separately for methane (Eq. 3.6) and acetate (Eq. 3.7) as the fraction of electrons supplied via applied current that were recovered in each product.

$$CE_{CH_4} = \frac{n_{CH_4}}{n_{CH_4, theoretical}} = \frac{n_{CH_4} \cdot F \cdot n_e}{\int_{t=0}^t I dt} \quad (3.6)$$

$$CE_{acetate} = \frac{n_{acetate}}{n_{acetate, theoretical}} = \frac{n_{acetate} \cdot F \cdot n_e}{\int_{t=0}^t I dt} \quad (3.7)$$

For methane Equation 3.6 was used, where n_{CH_4} represents the moles of methane determined from headspace volume and CH_4 concentration, F is Faraday's constant (96485 C mol^{-1}), n_e is the number of electrons required per mole of product (8 mol e^- per mol CH_4), and $\int Idt$ represents the total charge passed. An analogous equation was applied for acetate (Eq. 3.7), where $n_{acetate}$ was determined from measured concentration (mol L^{-1}) and reactor volume, and $n_e = 8\text{ mol e}^-$ per mol acetate. Total CE was calculated as the sum of individual product efficiencies.

3.3.4 Chemical characterization

Soil samples were air-dried, sieved through a 2 mm mesh, and analyzed for total carbon and nitrogen content using the Dumas dry combustion method.¹⁷⁶ Plant-available phosphorus was quantified by the Olsen extraction method,¹⁷⁷ and sulfate concentrations were determined by ion chromatography.

At experimental termination, catholytes and anolytes (from membrane-free reactors R2 and R5) were analyzed for inorganic and organic constituents. Volatile fatty acids (VFAs), including acetate, and other organic acids (e.g., carbonate, oxalate) were quantified by ion chromatography (IC, Dionex ICS-1000, Thermo Fisher Scientific, Waltham, MA, USA). Total sulfur content was determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES, PlasmaQuant[®] PQ9000, Analytik Jena, Jena, Germany), while sulfate was measured by ion chromatography (IC, Dionex Integration HPIC, Thermo Fisher Scientific). Sulfide concentration was calculated as the difference between total sulfur and sulfate. Additional analyses included quantification of major ions (nitrate, fluoride, chloride) and trace elements (Fe, Mn, Cu) by appropriate analytical methods.

3.3.5 Cathode morphology characterization

Cathode electrode morphology and biofilm formation were examined using field-emission scanning electron microscopy (FE-SEM, Tescan Mira II XMH, Brno, Czech Republic). Prior to imaging, carbon cloth samples were oven-dried to remove residual moisture. Imaging was performed at an accelerating voltage of 20 kV and 5000 $\times$ magnification.

3.3.6 Characterization of the bacterial community

Genomic DNA was extracted from catholytes, anolytes (membrane-free reactors), and cathode biofilms (1 cm^2 carbon cloth sections). Liquid samples were vacuum-filtered through $0.22\text{ }\mu\text{m}$ pore-size nitrocellulose membranes, and DNA was extracted from half of each filter using the FastDNA[®] Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) according to manufacturer

specifications. DNA quality and integrity were assessed by PCR amplification of serial dilutions (undiluted, 1:10, 1:100).

Prokaryotic community composition was characterized by amplification and sequencing of the V3-V4 hypervariable region of the 16S rRNA gene using primers Pro341F (5'-CCTACGGGNBGCASCAG-3') and Pro805R (5'-GACTACNVGGGTATCTAATCC-3') (Takahashi et al., 2014). For each sample, duplicate 50 μ L PCR reactions were prepared containing 25 μ L GoTaq G2 Green Master Mix (Promega Corporation, Madison, WI, USA), 12.5 μ L nuclease-free water, 2.5 μ L template DNA, and 1 μ M of each primer. Thermal cycling conditions consisted of initial denaturation at 94 °C for 5 min, followed by 30 cycles with a touchdown annealing protocol starting at 65 °C and decreasing by 1 °C per cycle to 55 °C (15 s annealing, 72 °C extension for 30 s), with the remaining cycles conducted at a constant 55 °C annealing temperature. Final extension was performed at 72 °C for 5 min.

Amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega Corporation) and quantified fluorometrically using a Qubit® 3.0 fluorometer (Life Technologies, Carlsbad, CA, USA). Libraries were prepared by pooling nine amplicons with unique dual-index barcode combinations. Paired-end sequencing (2 × 300 bp) was performed on the Illumina MiSeq® platform (Illumina, Inc., San Diego, CA, USA) at the University of Milano-Bicocca. Quality-filtered reads were processed into amplicon sequence variants (ASVs) using DADA2.

3.3.7 Quantitative PCR analysis

Absolute quantification of total bacteria and methanogenic archaea was performed by quantitative real-time PCR (qPCR) targeting the 16S rRNA gene and *mcrA* gene (encoding the α -subunit of methyl-coenzyme M reductase), respectively. Each reaction was conducted in triplicate in a final volume of 10 μ L using Luna Universal qPCR Master Mix 2× (New England Biolabs, Ipswich, MA, USA) with 0.3 μ M final primer concentration. Standard curves were generated using serial five-fold dilutions of appropriate standards, and reactions included no-template controls. All assays were performed on a QuantStudio Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

For bacterial 16S rRNA gene quantification, primers 331F and 797R were employed with the following thermal profile: initial denaturation at 95 °C for 4 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 15 s. Melt curve analysis was performed with transitions from 95 °C (15 s) to 55 °C (15 s) to 95 °C (15 s).

For methanogen quantification, mcrA-specific primers mcrA-F (5'-GTGGTGTMGDDTTCACMCARTA-3') and mcrA-R (5'-TTCATBGCRTAGTTVGGRTAGT-3') were used with the following conditions: initial denaturation at 95°C for 1 min, followed by 40 cycles of 95 °C for 45 s, 47 °C for 30 s, and 72 °C for 45 s. Melt curve analysis consisted of 95 °C for 15 s, 60 °C for 20 s, and 95 °C for 15 s.

Gene copy numbers were normalized to sample volume (mL^{-1}) for liquid samples and to electrode geometric surface area (cm^{-2}) for biofilm samples to enable quantitative comparison across matrices.

RESULTS

Chapter 4

4 Oxygen Evolution Reaction

Proton exchange membrane water electrolysis (PEMWE) is a promising technology for sustainable hydrogen production. Reducing the iridium oxide loading at the anode is critical for lowering costs and minimizing dependence on precious metals in large-scale systems. Supporting IrO₂ on non-precious substrates provides an effective strategy to decrease iridium usage while maintaining high catalytic performance. As discussed in the Introduction, optimizing the geometry of the support can further enhance catalyst efficiency. In this study, we present a core-shell architecture in which a thin, conformal layer of IrO₂ is grown on rutile TiO₂ spheres. Due to the structural compatibility between rutile IrO₂ and rutile TiO₂, a continuous IrO₂ shell is achieved, whereas anatase-rich TiO₂ supports yield only a discontinuous, decorated IrO₂ structure. The core-shell morphology and uniform IrO₂ distribution were confirmed using imaging, diffraction, and spectroscopy. Electrochemical testing demonstrates superior mass activity and pseudocapacitance for the rutile-based core-shell material compared to the anatase-based decorated catalyst. Remarkably, the core-shell catalyst shows performance comparable to that of a commercial unsupported IrO₂ reference. These results highlight the advantageous interaction between rutile IrO₂ and rutile TiO₂ in improving catalyst utilization. Overall, such core-shell structures represent a promising approach for low-loading PEMWE systems, where unsupported IrO₂ often suffers from limited in-plane conductivity.

4.1 Electrocatalysts: surface and morphology investigation

A sol-gel synthesis route was developed to produce TiO₂ spheres with identical morphology but distinct crystalline phases. The particle size and shape were controlled using tetradecylamine (TDA) as a surfactant in a mixed methanol-acetonitrile solvent system. A small amount of water was added to the mixture and preferentially located within the templating micelles formed by TDA. Upon addition of titanium(IV) isopropoxide (TTIP), rapid hydrolysis occurred inside the micelles. The system was then aged for 24 h prior to surfactant removal and subsequent freeze-drying¹⁷⁸. The resulting powder was subjected to thermal treatment under a reducing atmosphere

(5% H₂/Ar), where the calcination temperature dictated the crystalline phase obtained,¹⁷⁹ resulting in the formation of anatase and/or rutile TiO₂.

Two calcination temperatures were selected to produce either fully rutile particles (800 °C, TiO₂-R) or anatase-rich particles (550 °C, TiO₂-AR). Both resulting materials exhibit a black coloration, suggesting incomplete oxidation of the titanium lattice. This hypothesis is supported by thermogravimetric analysis (TGA), shown in Figure S.4.1 (Supplementary Information), which presents the thermogram of TiO₂-R. The measurement was performed in synthetic air from 50 to 800 °C at a heating rate of 3 °C min⁻¹. A mass increase reaching a maximum at 570 °C was observed, corresponding to a 0.7 wt.% gain relative to the initial mass. This behavior is attributed to the oxidation of titanium suboxides at the surface, consistent with previous reports.^{180–184} The oxidation is accompanied by a visible color transition of the powder from black to white after thermal treatment.

Assuming a homogeneous distribution of suboxide defects throughout the rutile lattice, the observed mass increase corresponds to approximately 1.6% oxygen vacancies. Alternatively, if the suboxide defects are localized exclusively at the surface and the full mass gain arises from the oxidation of *TiO*_(2-*x*) to stoichiometric TiO₂, the mass fraction of material in the suboxide state can be estimated using Equation 4.1.

$$\frac{m_{\text{sub}}}{m_{\text{ox}}} = \frac{1}{\frac{M_{\text{ox}}}{M_{\text{sub}} \cdot \text{wt. \%}_{\text{gain}}} - 1} \quad (4.1)$$

With m_{sub} and m_{ox} denoting the masses of the suboxide and oxide phases, respectively, M_{sub} and M_{ox} their corresponding molar masses, and wt. %_{gain} the mass gain measured by TGA at 570 °C, the mass fraction of material in the suboxide state can be estimated. Assuming the suboxide has the composition *TiO*_{1.75} (*Ti*₄*O*₇), a mass gain of 0.7% results in an $m_{\text{sub}}/m_{\text{ox}}$ ratio of 0.6%. For a particle diameter of approximately 430 nm, this corresponds to an external suboxide layer on the order of 1 nm. Such a thin surface layer is not expected to be detectable by X-ray diffraction (XRD), which predominantly probes the bulk crystalline structure.^{185,186}

XRD analysis of TiO₂-R (Fig. S.4.2, Supplementary Information) confirms the exclusive presence of the rutile phase, consistent with the ICSD-80843 reference pattern. In contrast, the material calcined at 550 °C (TiO₂-AR) exhibits a mixed phase composition, containing both anatase (ICSD-63711) and rutile. The characteristic reflections at $2\theta = 27.4^\circ$ (110, rutile) and $2\theta =$

25.5° (101, anatase) were used for semi-quantitative phase analysis, indicating a composition of 74% anatase and 26% rutile.

The mean coherent domain size (crystallite size, D) of each phase was estimated using the Scherrer equation (Eq. 4.2):¹⁸⁷

$$D = \frac{k\lambda}{\beta \cos\theta} \quad (4.2)$$

where β is the full width at half maximum (FWHM) corrected for instrumental broadening (0.18°), and k is the Scherrer constant, taken as 0.89 for all phases. The rutile crystallite sizes are estimated to be 40 nm in TiO₂-R and 18 nm in TiO₂-AR, while the anatase crystallites in TiO₂-AR measure approximately 17 nm.

Scanning transmission electron microscopy (STEM) images of the bare supports (Fig. 4.1a–b) confirm the spherical morphology of both materials. Only minimal agglomeration is observed, even for TiO₂-R calcined at 800 °C. TiO₂-AR particles appear noticeably smoother than TiO₂-R, consistent with the smaller crystallites determined from XRD. The nanoscale features visible in the magnified images align well with the crystallite dimensions obtained by Scherrer analysis. Particle size distributions for TiO₂-R and TiO₂-AR were obtained by manual counting of SEM images (Figure S.4.3, Supplementary Information), yielding mean diameters of 432 ± 89 nm and 403 ± 93 nm, respectively. These closely matched morphologies provide a robust platform for evaluating how crystal structure influences the formation of IrO₂@TiO₂ core-shell architectures.

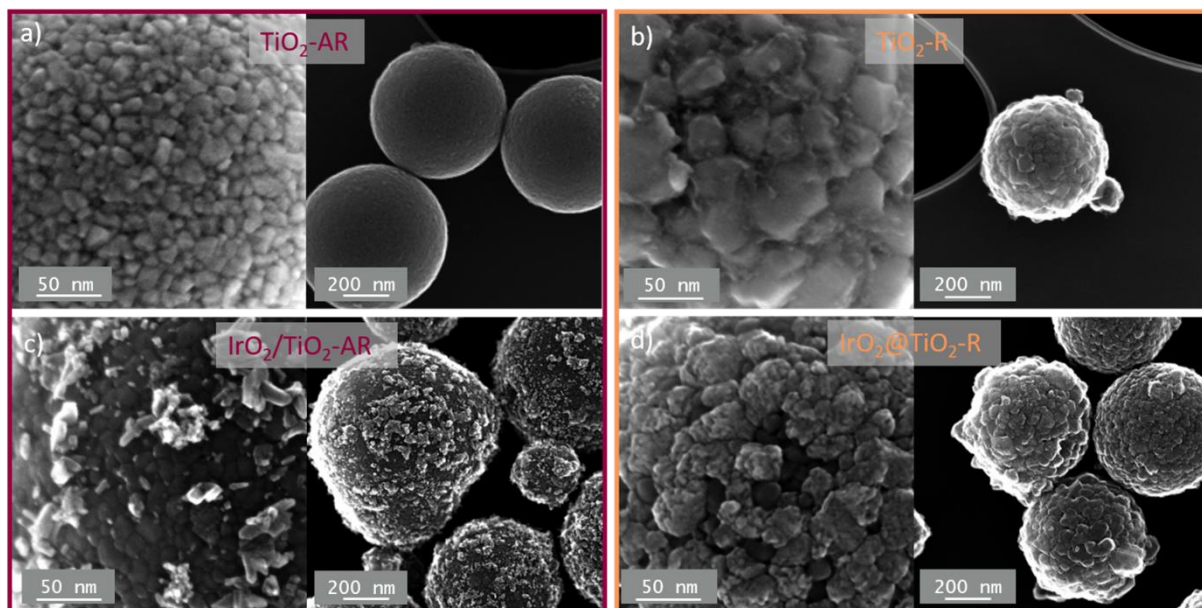


Figure 4.1. STEM images of (a) TiO_2 spheres calcined at 550 °C, (b) TiO_2 spheres calcined at 800 °C, (c) iridium oxide decorated spheres of anatase-rutile TiO_2 and (d) core-shell iridium oxide on TiO_2 rutile.

Iridium oxide was deposited onto each support by wet impregnation using a lab-prepared aqueous solution of IrCl_6^{2-} , followed by thermal treatment in air. The resulting catalyst materials are denoted $\text{IrO}_2/\text{TiO}_2\text{-AR}$ and $\text{IrO}_2@\text{TiO}_2\text{-R}$. STEM secondary electron images (SEI; Fig. 4.2c–d) reveal clear morphological differences between the two: on the anatase-rich $\text{TiO}_2\text{-AR}$ support, the deposition process yields distinct nanoparticles that increase surface roughness, whereas on the rutile $\text{TiO}_2\text{-R}$ support, the morphology remains largely unchanged relative to the bare material.

In the XRD patterns of the catalyst samples (Figure S.4.2, Supplementary Information), diffraction features associated with rutile IrO_2 are negligible compared to those of the TiO_2 support. The crystallographic nature of the iridium oxide phase is therefore further examined in the discussion that follows. It is worth noting that the heat treatments used to convert the iridium precursor into IrO_2 also induce modifications in the TiO_2 supports and specifically, an increase in crystallite size and partial transformation of anatase to rutile. For the anatase-rich $\text{TiO}_2\text{-AR}$ support, the synthesis involves an initial heat treatment at 550 °C in a reducing atmosphere (5% H_2/Ar), followed by a second calcination at 500 °C in air, a sequence that likely promotes conversion toward the thermodynamically favored rutile phase. The evolution of the TiO_2 crystalline phases for each support is summarized in Table 4.1.

Table 4.1. Rutile fraction (%) and rutile size (nm) of bare support and iridium.

	TiO₂-AR	IrO₂/TiO₂-AR	TiO₂-R	IrO₂@TiO₂-R
Rutile fraction [%]	26	43	100	100
Rutile size [nm]	18	21	40	58

The nominal iridium loading was estimated from the average support particle size (Figure S.4.3, Supplementary Information), where an 8 wt.% IrO₂ loading would theoretically correspond to a conformal shell thickness of 2-3 nm, assuming complete surface coverage. The actual iridium content was quantified by inductively coupled plasma mass spectrometry (ICP-MS) following complete digestion of the IrO₂/TiO₂-AR and IrO₂@TiO₂-R samples. The measured loadings are 4.8 ± 0.5 wt. % for IrO₂/TiO₂-AR and 2.6 ± 0.1 wt. % for IrO₂@TiO₂-R. For the latter, this corresponds to an estimated shell thickness of approximately 0.7 nm under the assumption of uniform coverage.

Both measured loadings fall below the targeted nominal value, likely due to inefficiencies inherent to the impregnation step. Qualitatively, a larger fraction of iridium precursor remained undeposited in the synthesis of IrO₂@TiO₂-R compared with IrO₂/TiO₂-AR, which plausibly accounts for the lower final loading measured on the rutile support.

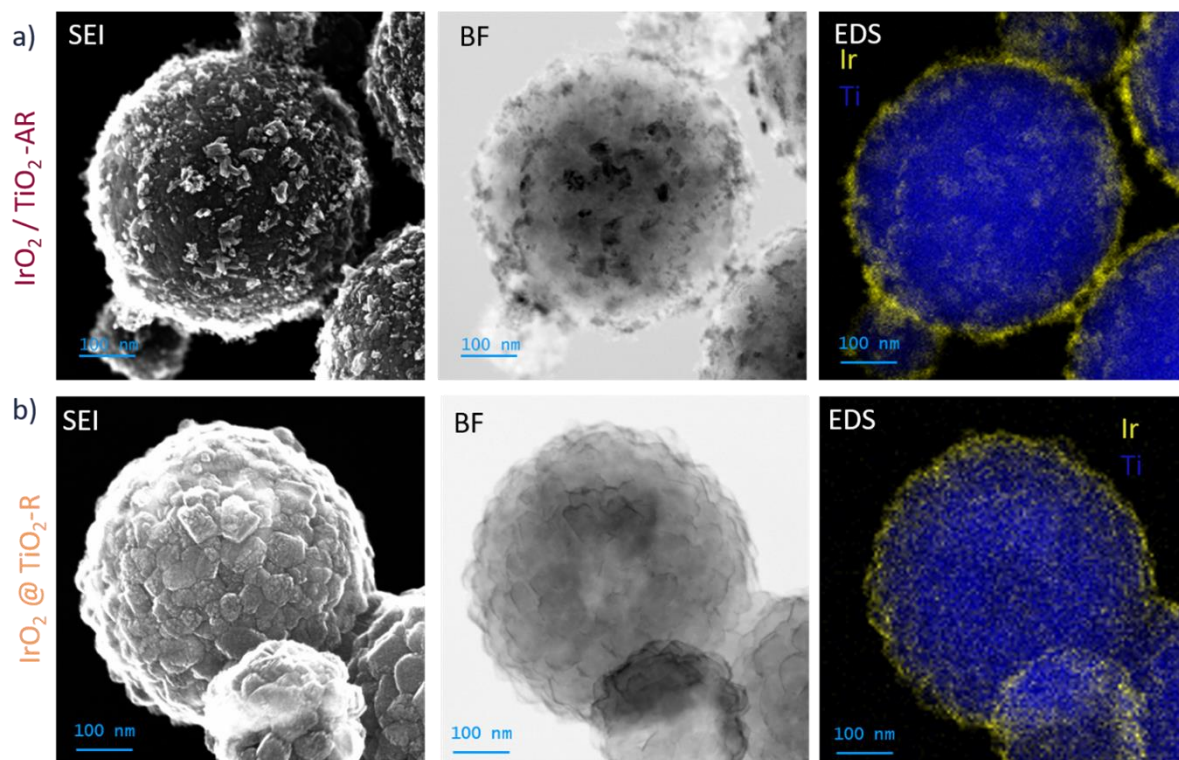


Figure 4.2. STEM imaging with secondary electron image (SEI) and bright field images (BF) and EDS mapping of Ir K edge and Ti M5 edge for (a) $\text{IrO}_2/\text{TiO}_2\text{-AR}$ and (b) $\text{IrO}_2@\text{TiO}_2\text{-R}$.

The morphology and architecture of the supported catalysts were examined by scanning transmission electron microscopy (STEM), as shown in Figure 4.3. Secondary electron images (SEI) provide a three-dimensional view of the particle surfaces, while bright-field images, generated from transmitted electrons, highlight atomic-weight contrast. For $\text{IrO}_2@\text{TiO}_2\text{-R}$, the particle surface exhibits a uniform contrast, whereas $\text{IrO}_2/\text{TiO}_2\text{-AR}$ displays pronounced surface roughness arising from nanoparticles composed of heavier atoms, presumably iridium. This interpretation is corroborated by energy-dispersive X-ray spectroscopy (EDS) elemental mapping of titanium and iridium. For $\text{IrO}_2/\text{TiO}_2\text{-AR}$, discrete nanoparticles at the surface are confirmed to be iridium oxide, while for $\text{IrO}_2@\text{TiO}_2\text{-R}$ the iridium signal is strong and continuous along the particle edges, consistent with the formation of a conformal IrO_2 shell. Together with the absence of visible IrO_2 nanoparticles on the rutile support, these observations indicate complete coating and the successful formation of $\text{IrO}_2@\text{TiO}_2\text{-R}$ core-shell structures. In contrast, $\text{IrO}_2/\text{TiO}_2\text{-AR}$ adopts a decorated morphology, with IrO_2 nanoparticles several nanometers in size clearly visible at the surface.

Since the only difference between the two supports lies in their crystalline structure, these results suggest a favorable interaction between rutile TiO_2 and rutile IrO_2 that promotes shell formation.

The observation is consistent with the likely formation of an epitaxial heterostructure, in which the rutile TiO_2 lattice serves as a template for the thermal growth of rutile IrO_2 . Direct atomic-scale confirmation would require thin-film model systems and lies beyond the scope of this work.

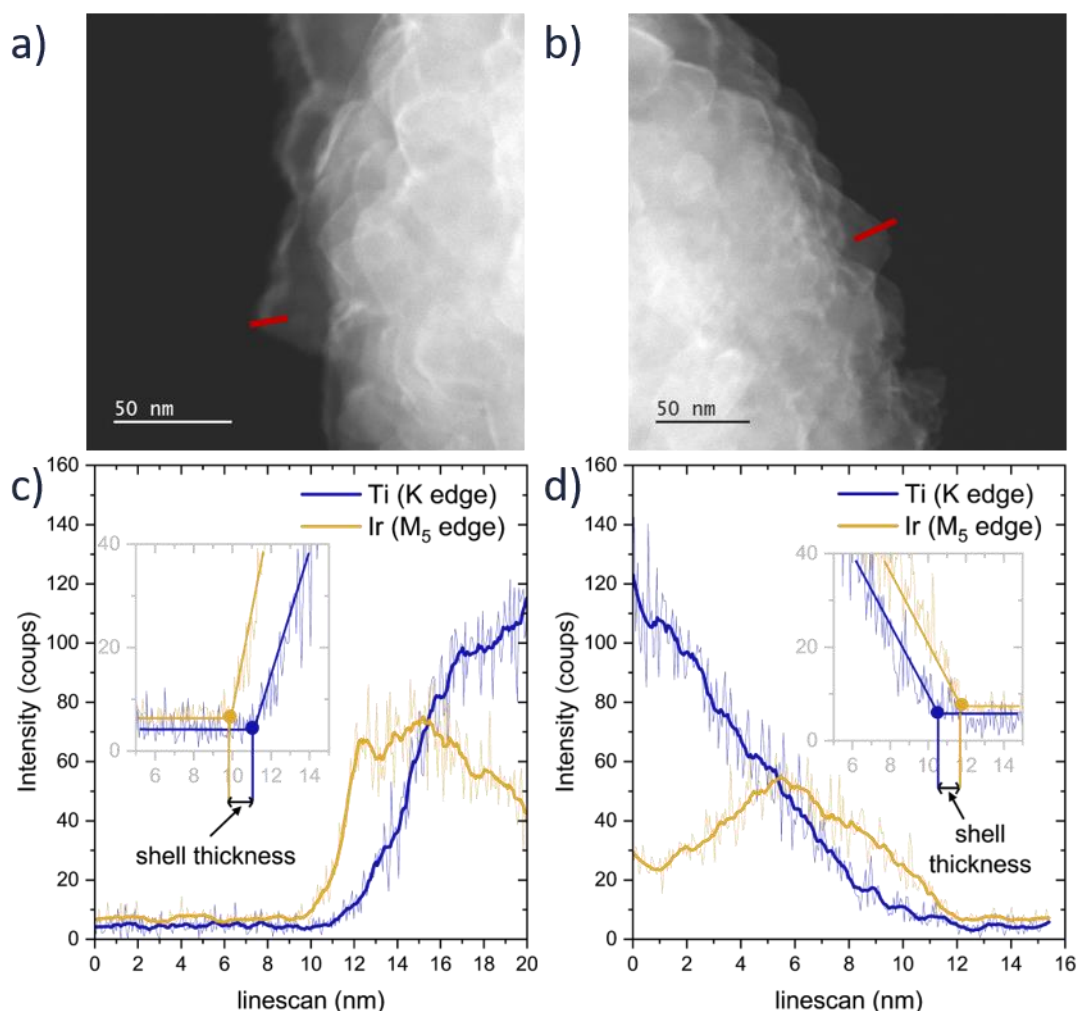


Figure 4.3. (a,b) STEM bright field image and (c,d) corresponding line scans from EDS for two zones of IrO_2 @ TiO_2 -R.

A comparison of bare and IrO_2 -coated supports is presented in Figure S.4.4 (Supplementary Information) using transmission electron microscopy (TEM). For rutile TiO_2 , the presence of a thin IrO_2 layer manifests as a darker edge at high magnification, absent in the uncoated support. In contrast, on anatase-rich TiO_2 , IrO_2 is clearly visible as discrete nanoparticles at the sphere surface. To quantify the shell thickness, EDS line scans were acquired across the edges of individual rutile particles (Fig. 4.3). The scanned region was restricted to ensure that only a single TiO_2 crystallite contributed to the signal. In all cases, the Ir signal rises prior to the Ti signal,

indicating that the outermost layer is composed exclusively of IrO₂. The distance between the onset of the Ir and Ti signals yields an estimated shell thickness of 1-2 nm, in good agreement with the calculated value of ~0.7 nm based on ICP-MS loading measurements.

The crystallinity of the IrO₂ phase was further examined using X-ray diffraction (XRD) and X-ray photoelectron spectroscopy (XPS). The XRD patterns of the catalysts are shown in Figure 4.4a, including a magnified view of the 2 θ region between 20° and 40° to highlight the IrO₂ reflections. A commercial rutile IrO₂ reference was used for comparison, and the IrO₂ peaks were deconvoluted using pseudo-Voigt line shapes. For IrO₂@TiO₂-R, the (110) reflection is barely discernible, whereas the (101) reflection appears more clearly. Semi-quantitative analysis based on relative intensity ratios (RIR = 7.9, 5.1, and 4.0 for rutile IrO₂, anatase TiO₂, and rutile TiO₂, respectively), following the methodology detailed in our previous work,¹⁸⁸ yields strongly divergent loading estimates from the (110) and (101) peaks. For IrO₂@TiO₂-R in particular, calculated loadings span 2-12%, indicating pronounced underrepresentation of the (110) reflection and suggesting preferential orientation of the IrO₂ shell. Although the (110) facet is expected to be the most exposed surface, it is scarcely detected, while the (101) reflection is intensified relative to isotropic crystallites. Whether such preferential exposure is already present in the rutile TiO₂ support cannot be determined from XRD due to its much larger crystallite size. Because of extensive peak convolution and overlapping contributions from TiO₂, crystallite-size analysis using the Scherrer equation is not appropriate and was therefore not performed.

The structure of the IrO₂ layer was further assessed by XPS using the O 1s fitting methodology previously developed by our group for unsupported IrO₂.¹⁸⁹ As the IrO₂ layer is thinner than the XPS probing depth, photoelectrons originating from the TiO₂ support are detected, as shown in the survey spectra (Figure S.4.5, Supplementary Information). Consequently, contributions from TiO₂ must be included in the O 1s fitting. Fine spectra of the bare supports (Figure S.4.6a and S.4.6c, Supplementary Information) reveal distinct O 1s signatures for anatase and rutile TiO₂, and these contributions were incorporated into the deconvolution of the O 1s spectra for IrO₂/TiO₂-AR and IrO₂@TiO₂-R (Figure 4.4b). Although the presence of TiO₂-related oxygen peaks reduces the robustness of the fitting compared to unsupported IrO₂, the extracted spectral features unambiguously correspond to a rutile-type IrO₂ structure in both materials.

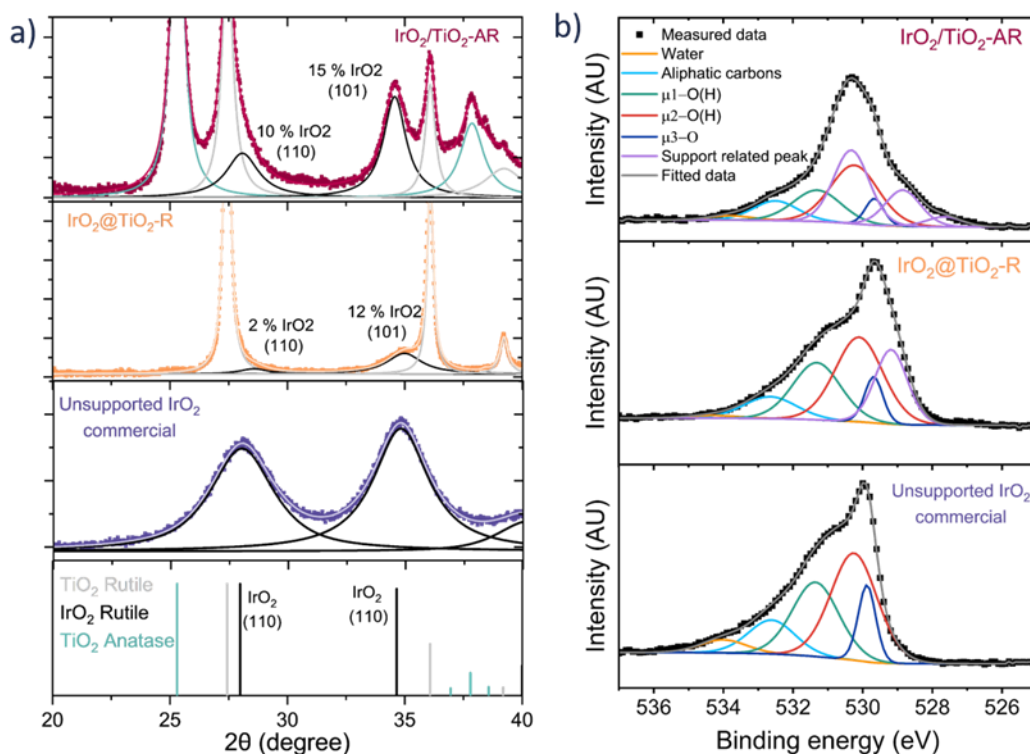


Figure 4.4. (a) X-Ray diffractograms of IrO₂/TiO₂-AR, IrO₂@TiO₂-R and commercial IrO₂ unsupported rutile, (b) O 1s spectra and corresponding fine analysis of IrO₂/TiO₂-AR, IrO₂@TiO₂-R and IrO₂ unsupported rutile TKK.

4.2 Electrochemical performances in OER

The electrochemical performance of the catalytic materials was evaluated using a rotating disk electrode (RDE) configuration, with a commercial unsupported rutile IrO₂ serving as the benchmark. Binder-free catalyst inks were deposited onto a gold electrode tip to ensure consistent contact. Before each measurement, the high-frequency resistance was determined by electrochemical impedance spectroscopy and dynamically compensated at 85%, with the remaining 15% corrected post-measurement. All currents were normalized by the calculated IrO₂ loading, and all potentials are reported versus the reversible hydrogen electrode (RHE).

Following electrode activation, cyclic voltammetry (CV) was conducted at various sweep rates between 1.0 and 1.3 V to evaluate the pseudocapacitive contribution. Representative CVs at 100 mV s⁻¹ are shown in Figure 4.5b. All samples exhibit minimal faradaic redox activity and display nearly rectangular voltammograms in the 1.0-1.2 V region. However, the current at 1.1 V primarily reflects pseudocapacitive behavior, acknowledging that small redox contributions cannot be entirely excluded. The IrO₂@TiO₂-R core-shell catalyst exhibits a significantly higher specific pseudocapacitance ($0.25 \pm 0.03 \text{ F g}^{-1}_{\text{IrO}_2}$) compared with the decorated IrO₂/TiO₂-AR

material ($0.025 \pm 0.002 \text{ F g}_{\text{IrO}_2}^{-1}$). Because the IrO_2 phase in both catalysts was formed under identical conditions, this large difference is unlikely to arise from variations in intrinsic double-layer capacitance. Instead, it indicates a substantially lower electrochemically active surface area (ECSA) for $\text{IrO}_2/\text{TiO}_2\text{-AR}$. This reduced ECSA is consistent with its morphology, which consists of discrete IrO_2 nanoparticles with low surface-to-volume ratio and limited electronic percolation. In contrast, the continuous IrO_2 shell in $\text{IrO}_2@\text{TiO}_2\text{-R}$, with a thickness below 2 nm, enables efficient utilization of the Ir mass.

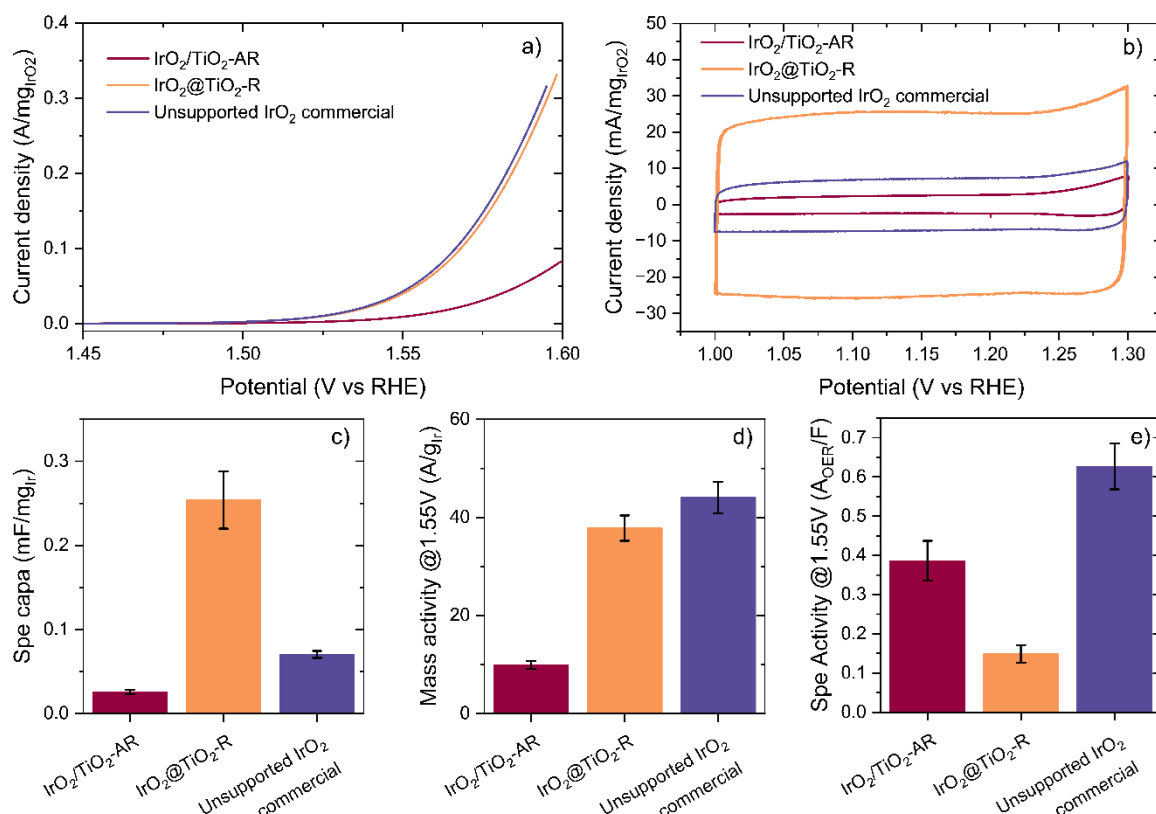


Figure 4.5. Electrochemical characterization of the iridium oxide catalysts. (a) Polarization curves obtained by linear sweep voltammetry at 2 mV s^{-1} . The potential is compensated at 85% for iR-drop, the current is normalized by the loading calculated from ICP-MS measurements. (b) Cyclic voltammetry at 100 mV s^{-1} in a range with minimal redox activity for assessment of the pseudo-capacitive current. (c) Specific capacitance obtained by normalization of the capacitance at 1.1 V by the iridium loading. (d) Mass activity obtained from normalization of the current measured at 1.55 V on the LSV by the iridium loading and (e) Specific activity obtained from normalizing the mass activity at 1.55 V by the specific capacitance.

Catalytic performance was probed by linear sweep voltammetry (LSV) at 2 mV s^{-1} from 1.3 to 1.6 V (Fig. 4.5a). Activities were compared at 1.55 V, a potential region free from mass-transport artifacts associated with O_2 bubble removal and are reported as mass activities in Figure 4.5d. The decorated $\text{IrO}_2/\text{TiO}_2\text{-AR}$ catalyst displays markedly lower activity than the $\text{IrO}_2@\text{TiO}_2\text{-R}$ core-shell material, consistent with its lower ECSA. Remarkably, the core-shell catalyst achieves a mass activity comparable to that of unsupported IrO_2 despite exhibiting a much smaller pseudocapacitive current. Consequently, the unsupported IrO_2 displays a much higher specific activity when the mass activity is normalized by the specific pseudocapacitance (Fig. 4.5e). This normalization is commonly employed to estimate the intrinsic activity of the iridium oxide surface, using pseudocapacitance as a proxy for ECSA. However, in the present case, the discrepancy between the two specific activities is so large that it implies fundamentally different surface states and/or reaction mechanisms. This disparity undermines the validity of using pseudocapacitance as a universal ECSA metric here, as it presumes identical double-layer properties and surface chemistries across all samples conditions that are not satisfied.

To further analyze the polarization curves, the methodology described in the Supporting Information was applied to reveal differences between the supported and unsupported IrO_2 catalysts. First, the Tafel parameters (i.e., Tafel slope and exchange current density) were determined by linear fitting over the optimal overpotential region. These parameters were then incorporated into a Butler-Volmer model to simulate the full polarization behavior. For all materials, the simulated curves deviated from the experimental data. In each case, the deviation could be corrected by adding an additional resistive term within a non-linear Butler-Volmer fit, while keeping both the exchange current density and the charge transfer coefficient fixed. Representative Tafel and Butler-Volmer fit for each catalyst are shown in Figure S.4.7 (Supplementary Information). The extracted exchange current densities, Tafel slopes, additional resistances, and charge transfer coefficients are summarized in Figure 4.6.

Interestingly, both supported catalysts exhibit higher exchange current densities than the commercial unsupported IrO_2 (Fig. 4.6a). For the core-shell material, this likely reflects improved utilization of the IrO_2 surface, yielding more accessible active sites per mass of Ir. However, for the decorated catalyst, where larger IrO_2 particles are present, greater utilization relative to unsupported IrO_2 is improbable. The higher exchange current density in this case, instead, suggests faster charge-transfer kinetics at equilibrium. The unsupported catalyst compensates for its slower near-equilibrium charge transfer by displaying a lower Tafel slope (Fig. 4.6c). This difference is not large enough to imply a mechanistic change (e.g., altered electron count in the rate-determining step); instead, it arises solely from variations in the charge transfer coefficient

(Fig. 4.6d). Consequently, while the unsupported material is less effective near equilibrium, it becomes more responsive to increases in overpotential.

A major divergence among the catalysts appears in the additional resistance extracted from the Butler-Volmer fits. The decorated material exhibits a resistance more than three times higher than those of the core-shell and unsupported samples (Fig. 4.6b). This excess resistance may stem from increased film resistance, mass-transport limitations, or poor electrical connectivity between the catalyst and the electrode. Because IrO_2 does not passivate and the catalyst layer is thin under 2000 rpm rotation, mass-transport and passivation-related effects are unlikely. The most plausible explanation is poor electrical contact: in the decorated sample, many IrO_2 nanoparticles are electrically connected only through the weakly conducting TiO_2 support, unlike the core-shell and unsupported catalysts where electronic pathways are continuous.

Potentiostatic electrochemical impedance spectroscopy recorded at 1.0 V further highlights this discrepancy. Modeling of the spectra yields characteristic frequencies of 263 ± 56 Hz for $\text{IrO}_2/\text{TiO}_2\text{-AR}$ and 26 ± 3 Hz for $\text{IrO}_2@\text{TiO}_2\text{-R}$ (Supplementary Information). This order-of-magnitude shift is evident in the imaginary capacitance plotted versus frequency (Fig. S.4.8). The substantially higher characteristic frequency for the decorated material indicates significant charge accumulation at the interface, demonstrating the consequences of poor electronic percolation. Achieving a continuous IrO_2 shell is therefore critical for enhancing catalyst performance.

Notably, the core-shell catalyst displays resistive behavior comparable to that of unsupported IrO_2 , further confirming the functional integrity of the shell structure. These results show that depositing a low IrO_2 loading onto rutile TiO_2 enables the formation of a core-shell architecture that retains the electrochemical characteristics of bulk IrO_2 while dramatically reducing the iridium loading. This approach is particularly attractive for low-loading PEMWE applications, where maximizing iridium utilization is essential.

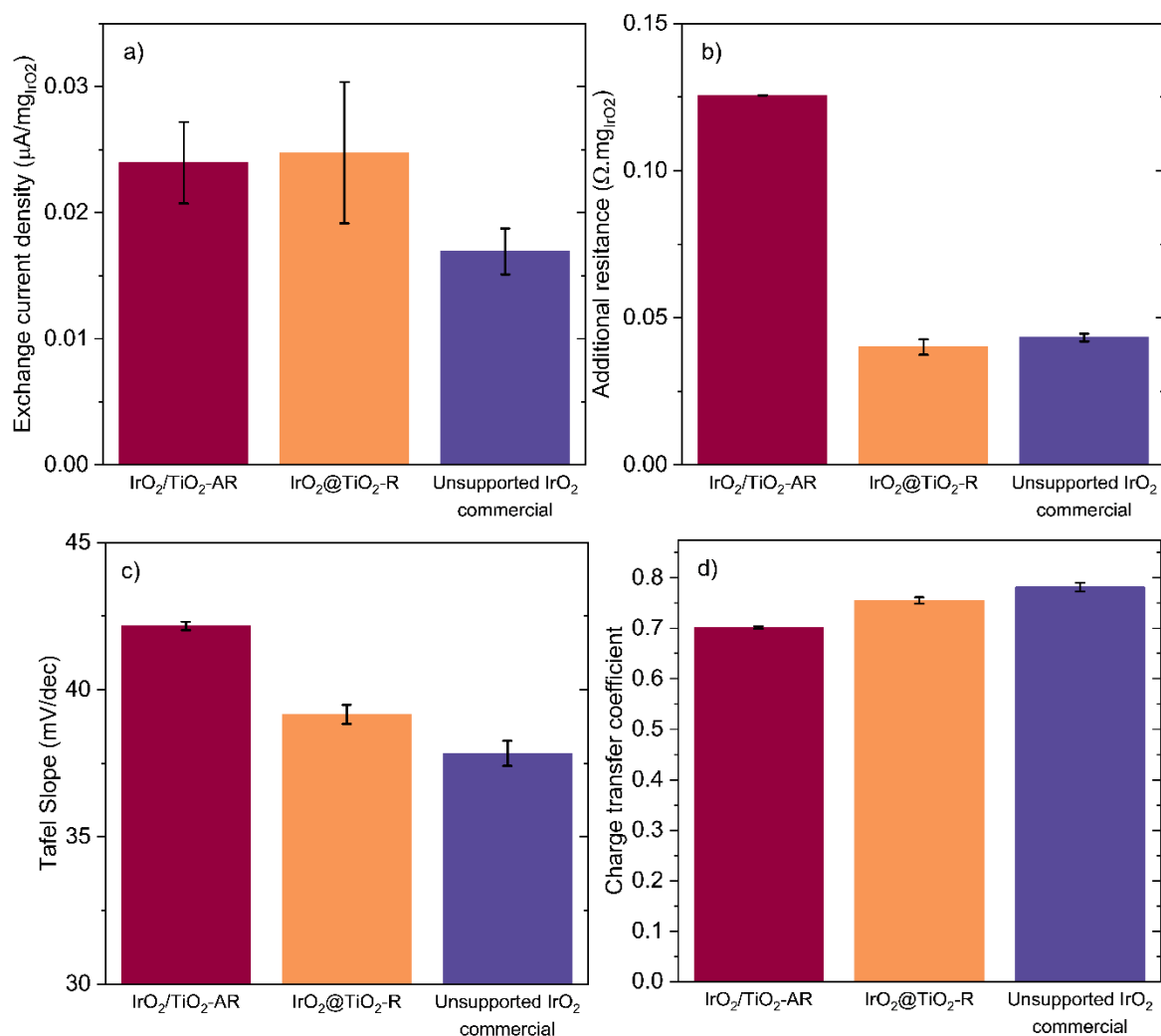


Figure 4.6. Parameters extracted from the Tafel analysis and non-linear Butler-Volmer fitting. (a) Mass normalized exchange current density, (b) Additional resistance calculated and (c) Tafel slope and (d) Charge transfer coefficient.

4.3 Concluding remarks

This study demonstrates the successful synthesis of core-shell IrO₂@TiO₂-R structures as highly efficient electrocatalysts for the oxygen evolution reaction (OER). By exploiting the lattice compatibility between rutile TiO₂ and rutile IrO₂, a continuous and uniform IrO₂ shell was achieved, in contrast to the discontinuous and agglomerated IrO₂ nanoparticles formed on anatase-rich TiO₂ supports. The thin IrO₂ shell in the core-shell architecture enabled excellent catalyst utilization, yielding electrochemical performance comparable to that of a commercial unsupported IrO₂ reference. For the decorated material, charge accumulation arising from the poor conductivity of the TiO₂ support limited the activity; however, this drawback was effectively mitigated in the core-shell system. Overall, this work highlights a promising strategy for reducing noble metal

loading in PEMWE catalysts by combining materials engineering with nanoscale structural control, thereby contributing to the advancement of sustainable hydrogen production technologies.

Chapter 5

5 How Do Thermal Treatments Shape Microbial Community?

Thermal treatment of microbial consortia is widely applied across biotechnology, environmental engineering, and materials science, traditionally as a sterilization strategy aimed at eliminating microbial activity.¹⁹⁰ Increasingly, however, it is recognized as a selective intervention that exploits differential thermotolerance among microorganisms to reshape community composition, favor specific metabolic functions, and influence material-microbe interactions.¹⁹¹ Understanding how treatment severity governs microbial survival and post-treatment activity is therefore critical for the rational design of bioprocesses, including bioelectrochemical systems and strategies for controlling microbiologically influenced corrosion (MIC).

An emerging and counterintuitive application of thermal treatment concerns corrosion mitigation. Conventional corrosion inhibition treatments, rely on sterilization and biocides to suppress MIC, yet growing evidence shows that selective enrichment of thermoresistant microorganisms can instead inhibit corrosion. Mild to intermediate thermal treatments preferentially suppress aggressive, non-spore-forming populations, such as sulfate-reducing bacteria, while enriching spore-forming and thermophilic taxa capable of promoting surface passivation through biomineralization and barrier film formation.^{192–194} Studies on marine, industrial, and bioelectrochemical systems have demonstrated that such communities can induce carbonate or oxide precipitation, reduce mass transport of corrosive species, and stabilize metal surfaces more effectively than sterile conditions.^{195–199} This protective effect is closely linked to solution chemistry rather than microbial metabolism alone. Thermal treatments modify the chemical environment by decomposing organic matter and releasing carbonate, phosphate, and other inorganic species that control passivation pathways. In systems containing copper alloys and ferrous materials, enrichment of alkaliphilic, carbonate-precipitating bacteria or methanogenic archaea has been shown to promote formation of compact, adherent layers such as calcite, siderite, or magnetite, whereas sterile or partially treated systems often favor porous, metastable phosphate-organic deposits prone to failure.²⁰⁰ As a result, corrosion outcomes depend primarily on the interplay between thermally selected microbial populations and the post-treatment chemical environment.

Complete thermal sterilization of complex and abundant microbial consortia embedded in organic matrices remains exceptionally difficult and often counterproductive.^{193,201,202} High initial

biomass concentrations, thermal buffering within aggregates, and the extraordinary resistance of bacterial endospores and thermophilic archaea mean that even rigorous autoclaving rarely achieves absolute sterility. Moreover, thermal inactivation of sensitive populations generates a substantial pool of readily bioavailable nutrients through cell lysis and hydrolysis, which fuels rapid regrowth of surviving thermoresistant organisms. This “death-feeds-the-survivors” dynamic accelerates selective enrichment, often leading to communities dominated by *Bacillus*, *Geobacillus*, or thermophilic methanogens that can rapidly recolonize treated systems. In organic-rich inocula such as those derived from biological methanation plants, this effect is particularly pronounced. Thermal treatments eliminate a large fraction of heat-sensitive microorganisms, but the resulting release of amino acids, nucleotides, and short-chain organics supports rapid proliferation of resistant populations during subsequent exposure periods. Consequently, thermal treatment does not simply reduce microbial abundance but fundamentally reshapes both community structure and solution chemistry, with direct implications for corrosion behavior.

Within this context, the present chapter examines how different thermal treatment regimes, ranging from boiling to autoclaving and extended sterilization, alter the microbial composition and chemical environment of a complex methanation inoculum. Additionally, Sections 6-9 report corrosion tests conducted using these differently enriched inocula, allowing assessment of how thermal treatment-induced changes affect corrosion behavior. By combining microbial community analysis with solution chemistry characterization, the study evaluates whether selective thermal enrichment can outperform sterilization as a corrosion mitigation strategy. The results challenge the traditional assumption that eliminating microorganisms is inherently beneficial, instead demonstrating that controlled thermal selection of beneficial microbial communities, coupled with favorable carbonate and phosphate chemistry, can provide more effective and durable protection through biomineralization-mediated passivation.

5.1 Characterization of the bacterial community

Figure 5.1 represents 16S rRNA gene sequencing of the bacterial and archaeal fractions of the methanation inoculum, revealing profound compositional shifts induced by thermal treatment. Results are presented as relative abundance of the dominant genera, with remaining taxa aggregated as “others”.

The untreated biotic inoculum exhibited high bacterial diversity characteristic of complex anaerobic digester communities (Figure 5.1a). The dominant genera included members of the Comamonadaceae family (approximately 15-20% relative abundance), *Pseudomonadaceae* (10-

15%), and *Lentilactobacillus* (5-10%), alongside a substantial fraction (>50%) of low-abundance taxa individually representing <5% of the community. *Comamonadaceae* are metabolically versatile facultative anaerobes capable of denitrification, fermentation, and organic acid metabolism, commonly found in wastewater treatment systems and anaerobic digesters.²⁰³ Their dominance in the untreated inoculum reflects the complex redox conditions and diverse carbon sources characteristic of methanation plant operation. *Pseudomonadaceae*, while typically considered aerobic or facultatively anaerobic, can persist in nominally anaerobic systems by occupying microoxic niches or utilizing alternative electron acceptors such as nitrate.^{204,205} *Lentilactobacillus* represents fermentative lactic acid bacteria that contribute to the acidogenic phase of anaerobic digestion, producing lactate, acetate, and other volatile fatty acids from complex carbohydrates.²⁰⁶

Boiling treatment (120 minutes at 101 °C) dramatically restructured the bacterial community, selecting for thermotolerant populations (Figure 5.1a). The most striking enrichment was observed for *Tepidiphilus*, which increased from negligible abundance (<1%) in the biotic inoculum to become the dominant genus, representing approximately 19% of the boiled community. *Tepidiphilus* is a thermophilic, facultatively anaerobic bacterium of the *Hydrogenophilaceae* family, capable of growth at temperatures up to 60-65 °C and possessing notable resistance to thermal stress through spore formation.^{207,208} Members of this genus are nitrate-reducing heterotrophs that degrade diverse organic compounds including amino acids, carbohydrates, and volatile fatty acids, producing acetate, formate, and hydrogen as metabolic end products. The substantial enrichment of *Tepidiphilus* following boiling indicates that this organism either survived the thermal treatment in spore form or persisted as vegetative cells in thermally protected microenvironments within the complex organic matrix of the digestate. The boiled inoculum also showed elevated proportions of *Chryseobacterium* (8-12%), *Alcaligenes* (5-8%), and various unclassified genera within the Bacteroidota and Pseudomonadota phyla. *Chryseobacterium* species are aerobic to facultatively anaerobic organisms commonly found in soil and aquatic environments, with some strains exhibiting surprising thermal tolerance and the capacity to form biofilms.²⁰⁹ *Alcaligenes*, already present in the biotic inoculum, maintained representation after boiling, consistent with the known thermal resistance of certain *Alcaligenes* strains that form protective biofilm matrices.

Critically, the relative abundance of typical mesophilic anaerobic digester taxa such as *Syntrophomonas*, *Clostridium*, and various Bacteroidetes declined dramatically or became undetectable following boiling, confirming that the 101 °C treatment effectively eliminated heat-

sensitive vegetative cells. However, the persistence of substantial bacterial diversity (>20 detected genera at >1% abundance) demonstrates that boiling did not achieve sterilization but rather imposed selective pressure favoring thermophilic, spore-forming, or biofilm-protected populations.

Autoclaving (double cycle at 121 °C, 20 minutes each) imposed even more stringent selection, further simplifying community structure and enriching ultra-thermoresistant taxa (Figure 5.1a). The autoclaved inoculum exhibited the highest proportion of unclassified bacteria (approximately 30%), reflecting either the survival of rare, taxonomically poorly characterized extremophiles not well represented in reference databases, partial DNA degradation during autoclaving that reduced sequence quality and taxonomic assignment confidence, or the presence of cultivable but not-yet-sequenced thermo-resistant strains.

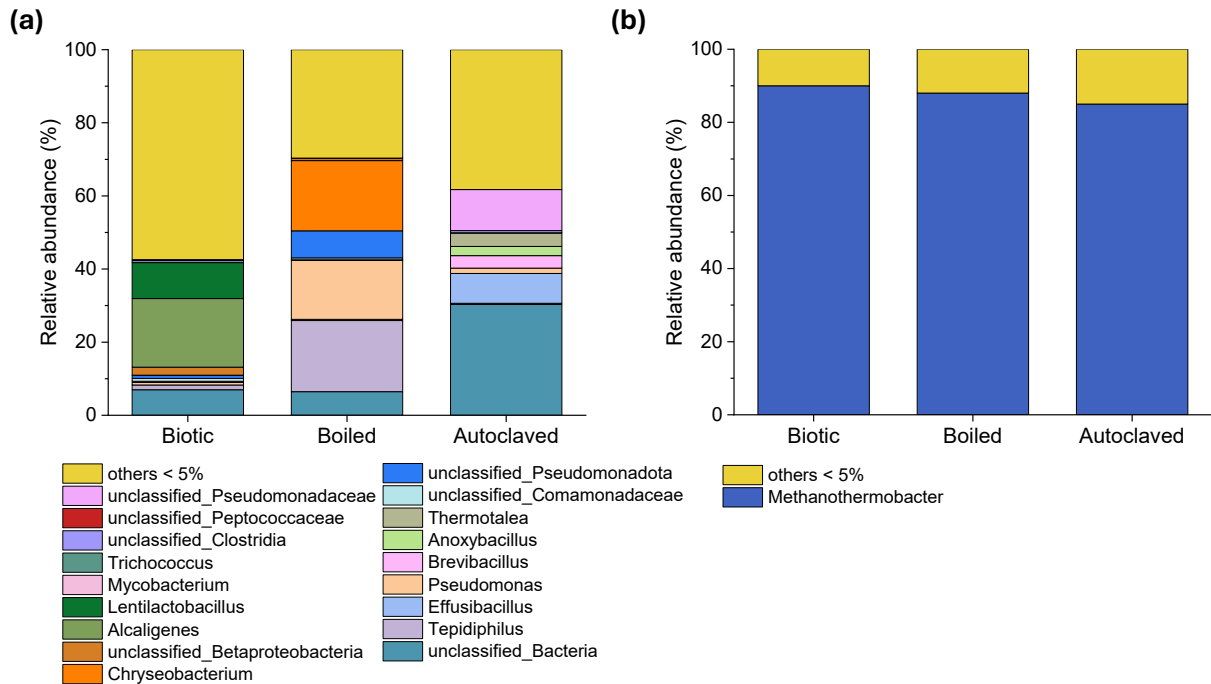


Figure 5.1. Genera relative abundances of the (a) bacterial and (b) Archaea communities present in the studied inocula (biotic, boiled and autoclaved).

Among classified taxa, the autoclaved community was dominated by spore-forming genera of the Bacillaceae family, particularly *Effusibacillus* (8%), *Caldalkalibacillus* (3-5%), and *Brevibacillus* (2-4%). *Effusibacillus* is a thermophilic, acidophilic, spore-forming bacterium originally isolated from acidic hot springs, capable of growth at pH 3-6 and temperatures up to 65 °C.²¹⁰⁻²¹³ Its high abundance in the autoclaved inoculum reflects exceptional spore resistance, as *Effusibacillus* endospores can withstand not only standard autoclaving but also extended exposure to elevated

temperatures, low pH, and desiccation. *Caldalkalibacillus* represents the opposite end of the pH spectrum, alkaliphilic, thermophilic spore-formers that thrive at pH 8-10 and temperatures up to 60 °C, commonly found in alkaline hot springs and geothermal environments.²¹⁴⁻²¹⁶ *Brevibacillus*, while less extreme in its pH preferences, produces highly resistant endospores that rank among the most thermotolerant biological structures known, capable of surviving not only autoclaving but also radiation and chemical disinfection.²¹⁷

The near-complete elimination of non-spore-forming bacteria (with the exception of small residual populations of *Pseudomonas* and related genera) confirms that double autoclaving approaches the theoretical limit of thermal sterilization for bacterial populations. However, the persistence of these ultra-resistant spore-formers demonstrates that even extreme thermal treatment (exceeding standard sterilization protocols) cannot guarantee complete sterility in complex organic matrices such as anaerobic digester sludge.

The effectiveness of the different thermal treatments in reducing bacterial genera was also confirmed by qPCR analysis (Fig. S.5.1, Supplementary Information), which showed that sterility was successfully achieved, while bacterial presence progressively decreased with more aggressive thermal treatments.

The archaeal fraction of the microbial community exhibited markedly different responses to thermal treatment compared to bacteria, reflecting the fundamental physiological differences between these domains (Figure 5.1b). In all three thermally treated inocula (biotic, boiled, autoclaved), the archaeal communities were dominated by *Methanothermobacter*, a strictly anaerobic, thermophilic, hydrogenotrophic methanogen.

In the biotic inoculum, *Methanothermobacter* represented approximately 85-90% of archaeal sequences, with minor contributions from *Methanoculleus* (3-5%), *Methanobacterium* (2-4%), and other unclassified methanogens. This dominance reflects both the thermophilic operational conditions of the source methanation plant (operated at 45-50 °C) and the inherent competitive advantage of *Methanothermobacter* in hydrogen-rich, CO₂-saturated environments. Members of this genus exhibit optimal growth at 50-65 °C, utilize H₂ and CO₂ exclusively as energy and carbon sources (strict hydrogenotrophy), and possess robust cell envelopes that confer resistance to environmental stresses.

Remarkably, both boiling and autoclaving had minimal impact on archaeal community composition at the genus level. *Methanothermobacter* maintained dominance at 85-95% relative abundance across all three conditions, indicating exceptional thermal tolerance. This resilience

likely arises from multiple mechanisms: the thermophilic growth temperature of *Methanothermobacter* (optimum 55-60 °C) implies intrinsic thermal stability of cellular components including heat-shock proteins, thermostable enzymes, and reinforced membrane lipids;^{218–220} the formation of protective microenvironments within aggregated archaeal clusters or biofilm matrices that buffer against thermal stress or the potential sporulation-like dormant states, though classical endospore formation has not been definitively demonstrated in archaea, cryptic survival mechanisms analogous to bacterial spores may exist.

From a functional perspective, the persistence of hydrogenotrophic methanogens across all thermal treatments has important implications for subsequent corrosion experiments. The capacity for methanogenesis was preserved even in heavily autoclaved inocula, indicating that thermal treatment does not inherently eliminate productive CO₂-reducing metabolism. However, as demonstrated in the MIC experiments (Chapters 6-9), the bacterial populations that co-occur with these methanogens, and the solution chemistry modifications induced by thermal treatment, exert dominant control over corrosion processes and overall system behavior.

5.2 Solution analysis

Chemical characterization of the thermally treated inocula via ion chromatography (IC, Table 5.1) and inductively coupled plasma atomic emission spectroscopy (ICP-AES, Table 5.2) revealed that thermal treatment not only reshapes microbial community composition but also fundamentally alters solution chemistry through microbial cell lysis, thermal decomposition of organic matter, and shifts in mineral solubility equilibria.

These chemical transformations have direct implications for both microbial metabolism and electrochemical corrosion processes during subsequent metal exposure experiments. Ion chromatography analysis quantified five anionic organic species (oxalate, formate, acetate, propionate, carbonate) that serve as indicators of microbial metabolic activity and thermal degradation processes (Table 5.1). Oxalate was present at high concentrations across all conditions, indicating it originates primarily from the methanation plant inoculum; its progressive decline with increasing thermal severity likely reflects thermal decomposition or oxidation. Formate was detectable only in biotic and boiled conditions, suggesting residual microbial activity, while its absence after autoclaving reflects inactivation of formate-producing microorganisms and potential disruption of electron transfer pathways.

Table 5.1. Concentrations of anions detected in the biotic, boiled, autoclaved and sterile inocula, determined by ion chromatography (IC). Results are reported in the ionic form detected by the instrument.

Analyte		Concentration ^a (mg L ⁻¹)			
		Experimental condition			
		Biotic	Boiled	Autoclaved	Sterile
Oxalate	$C_2O_4^{2-}$	2170 ± 4.8	2086 ± 6.3	1814 ± 9.6	1407 ± 7.3
Formate	$HCOO^-$	8.9 ± 0.63	8.2 ± 0.57	<LOD	n.d.
Acetate	CH_3COO^-	139 ± 0.97	178 ± 0.73	6.8 ± 0.17	n.d.
Propionate	$CH_3CH_2COO^-$	<LOD	<LOD	<LOD	n.d.
Carbonates	CO_3^{2-}	120 ± 0.26	126 ± 0.25	223 ± 0.29	n.d.

^a Values are expressed as mean ± standard deviation (n = 2). Concentrations below the limit of detection (LOD = 0.05 mg L⁻¹) are indicated as “< LOD”, while analytes not determined are indicated as “n.d.” in the Table.

Acetate showed the most pronounced dynamics: levels increased after boiling probably due to cell lysis and residual metabolism by thermotolerant populations, but nearly vanished after autoclaving, likely consumed by surviving methanogens or chemically transformed under high-temperature conditions. Propionate remained below detection limits in all cases, consistent with rapid syntrophic consumption or unfavorable conditions for its production. Carbonate concentrations increased markedly after autoclaving, arising probably from thermal degradation of organic matter.

ICP-AES analysis (Table 5.2) quantified seven elements (Cr, Fe, Ni, Cu, Zn, S, P) to assess metal dissolution, nutrient availability, and redox dynamics. Chromium, copper and zinc were undetectable in all conditions, confirming negligible contamination from medium components or glassware. Iron and nickel mainly originated from the inoculum and were partially solubilized or precipitated depending on thermal treatment. Sulfur concentrations decreased with thermal severity due to volatilization, oxidation, or precipitation, whereas phosphorus increased after boiling due to cell lysis and remained elevated after autoclaving, influencing phosphate availability for mineral layer formation.

Table 5.2. Total elemental concentration of inorganic elements detected in the biotic, boiled, autoclaved and sterile inocula, determined by inductively coupled plasma – atomic emission spectroscopy (ICP-AES).

Element		Concentration ^b (mg L ⁻¹)			
		Experimental condition			
		Biotic	Boiled	Autoclaved	Sterile
Chromium	Cr	n.d.	n.d.	n.d.	n.d.
Iron	Fe	0.06 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	n.d.
Nichel	Ni	0.25 ± 0.04	0.15 ± 0.01	0.13 ± 0.02	n.d.
Copper	Cu	<LOD	<LOD	<LOD	<LOD
Zinc	Zn	<LOD	<LOD	<LOD	<LOD
Sulfur	S	8.2 ± 0.14	3.4 ± 0.10	1.4 ± 0.05	4.20 ± 0.11
Phosphorous	P	308 ± 1.0	417 ± 1.3	318 ± 0.89	125 ± 0.70

^b Values are expressed as mean ± standard deviation (n = 2). Concentrations below the limit of detection (LOD = 0.05 mg L⁻¹) are indicated as “< LOD”, while analytes not determined are indicated as “n.d.” in the Table.

5.3 Discussion

The combined microbiological and chemical characterization clearly demonstrates coherent relationships between thermal treatment severity, surviving microbial populations, and the resulting solution chemistry. In the biotic condition, a highly diverse bacterial community coexists with dominant *Methanothermobacter* archaea, supporting active metabolism. This is reflected in moderate acetate concentrations (139 mg L⁻¹), maintained formate (8.9 mg L⁻¹), substantial oxalate (2170 mg L⁻¹), low carbonate (120 mg L⁻¹), and high phosphorus levels (308 mg L⁻¹) arising from both metabolic activity and nutrient supplementation.

Boiling significantly alters this environment. The community becomes dominated by *Tepidiphilus* alongside *Methanothermobacter*, with thermal lysis of sensitive cells and residual thermophilic metabolism shaping the chemistry. Consequently, acetate increases to 178 mg L⁻¹ due to release from lysed cells, formate remains at 8.2 mg L⁻¹, oxalate slightly decreases to 2086 mg L⁻¹, carbonate rises modestly to 126 mg L⁻¹, and phosphorus peaks at 417 mg L⁻¹, reflecting extensive release from cell lysis.

Autoclaving imposes the most severe thermal stress, leaving only spore-forming *Bacilli* and thermoresistant *Methanothermobacter*. Under these conditions, metabolic activity is minimal,

resulting in strongly depleted acetate (6.8 mg L^{-1}), complete loss of formate, reduced oxalate (1814 mg L^{-1}), and markedly elevated carbonate (223 mg L^{-1}) from thermal decarboxylation. Phosphorus is moderate (318 mg L^{-1}), as severe heat promotes partial precipitation despite release from lysed cells.

In the sterile condition, with no viable microorganisms, only the chemical baseline of the medium remains. Acetate and formate are absent, oxalate is lowest (1407 mg L^{-1}), carbonate is absent, and phosphorus is minimal (125 mg L^{-1}), representing only the supplemented salts.

These compositional differences define the distinct chemical environments in which corrosion processes occur in the following Chapters 6-9 and help explain the counterintuitive observation that sterile conditions often result in the worst corrosion outcomes.

5.4 Concluding remarks

Thermal treatment of methanation plant inocula strongly shapes both microbial community composition and solution chemistry, which in turn govern corrosion behavior (Chapters 6-9). Mild treatments, such as boiling, preserve thermophilic and spore-forming populations, release intracellular nutrients, and enhance acetate, formate, carbonate, and phosphate levels, creating chemical conditions that could favor formation of protective surface films in corrosion tests. Severe autoclaving retains only the most heat-resistant taxa, drastically reducing metabolic activity and key intermediates, while elevating carbonate through thermal decomposition. Achieving truly sterile conditions proved extremely challenging, requiring the combination of autoclaving, filtration, and UV exposure, highlighting the complexity of thermal interventions in real biomethanation plant inocula. These results highlight the complexity of sterilizing inocula characterized by an extremely high abundance and diversity of genera, such as those originating from a biomethanation plant. Furthermore, they emphasize that thermal treatments can lead to the enrichment of specific genera due to the presence of heat-resistant spores that cannot be eliminated by simple thermal processing.

Chapter 6

6 Microbiologically Influenced Corrosion on Copper

Sterilization in laboratory and industrial contexts is commonly achieved by autoclaving, i.e., exposure to saturated steam at 121 °C for 15-30 min under pressure (≈ 15 psi). This protocol is widely regarded as sufficient to inactivate most microorganisms, including bacteria, fungi, yeasts, viruses, and the majority of environmental spores.²²¹ Nevertheless, increasing evidence indicates that certain thermophilic and hyperthermophilic microorganisms, particularly archaea and spore-forming bacteria, may survive such thermal treatments under specific conditions.^{193,202,222,223} These resilient taxa are frequently associated with extreme natural environments, such as deep marine sediments, hydrothermal vents, and seafloor ecosystems, where elevated temperatures, low oxygen availability, and high metal concentrations prevail.

Microorganisms adapted to these environments not only tolerate thermal stress but can also colonize and persist on metallic substrates, including materials traditionally considered antimicrobial, such as copper and its alloys.^{224,225} Their ability to adhere to surfaces, form biofilms, and remain metabolically active following thermal or chemical stress challenges the assumption that standard sterilization protocols necessarily ensure microbially inert conditions. This issue is particularly relevant for high-temperature systems, where selective pressure may favor thermophilic and hyperthermophilic populations exhibiting enhanced tolerance to metal-induced stress and unusual metabolic capabilities.

Copper and copper-based alloys are extensively employed in industrial and marine infrastructures due to their high thermal conductivity, corrosion resistance, and well-documented antimicrobial properties.²²⁶ In marine environments, copper alloys are widely used in heat exchangers, piping, desalination units, and antifouling applications, where they are continuously exposed to saline waters, chloride ions, and complex microbial communities. Similarly, in industrial systems such as anaerobic digesters, biological methanation reactors, and heat recovery units, copper components often operate under thermophilic conditions (typically 50-65 °C), in chemically aggressive and anaerobic environments.^{227,228} While copper's antimicrobial activity is generally considered beneficial for limiting biofouling, prolonged exposure to complex microbial consortia, especially those adapted to thermal and redox extremes, may instead promote the selection of metal-tolerant microorganisms capable of interacting with and degrading copper surfaces.^{168,229}

From a mechanistic perspective, copper corrosion is a thermodynamically driven electrochemical process involving anodic metal dissolution coupled with cathodic reduction reactions.^{230,231} Copper oxidation produces cuprous (Cu^+) and/or cupric (Cu^{2+}) ions, which can react with environmental anions such as chlorides, carbonates, hydroxides, or sulfides to form a wide range of corrosion products, including cuprite (Cu_2O), tenorite (CuO), copper chlorides, sulfides, and mixed secondary phases.²³² Chloride ions, ubiquitous in marine and saline environments, are particularly aggressive toward copper, as they destabilize passive films and promote localized corrosion phenomena such as pitting.²³³ Pitting corrosion is characterized by highly localized anodic dissolution, which can cause severe structural damage despite limited overall mass loss.

The presence of microorganisms further complicates copper corrosion behavior. Numerous studies have demonstrated that microbial activity can either accelerate or inhibit corrosion processes, depending on the composition of the microbial community, its metabolic pathways, and the physicochemical conditions at the metal-biofilm interface.^{234–236} Many industrially relevant environments, such as natural waters, cooling systems, biogas digesters, and marine settings, support biofilm growth on metal surfaces, giving rise to microbially influenced corrosion (MIC). Microorganisms can accelerate copper corrosion through several mechanisms, including: i) production of corrosive species (e.g., sulfides from sulfate-reducing bacteria), ii) release of acidic metabolites such as organic acids, and iii) secretion of electroactive compounds or enzymes. Through these pathways, microbial activity can significantly modify local pH, redox potential, oxygen gradients, and metal ion speciation within biofilms, thereby altering corrosion kinetics and mechanisms.^{18,237–239} Conversely, microorganisms may also mitigate corrosion through processes collectively referred to as microbially influenced corrosion inhibition (MICI). Protective biofilms can act as diffusion barriers, consume oxidants, stabilize surface films, or promote the precipitation of passivating mineral layers via extracellular polymeric substances (EPS).^{230,239,240} For instance, *Shewanella* spp. have been reported to reduce corrosion in several alloy systems, including Al 2024, brass, and mild steel exposed to artificial seawater.^{241–244} These observations underscore that the role of microorganisms in corrosion is not unidirectional but highly dependent on microbial composition and environmental context. Biological methanation plants, for example, host dense consortia of hydrogenotrophic bacteria and methanogenic archaea and are frequently operated under thermophilic conditions, with periodic thermal treatments applied for performance optimization or contamination control.^{245,246}

In this context, the present study investigates copper corrosion in the presence of a microbial consortium that is ecologically and physiologically relevant to thermophilic industrial

environments, rather than relying on inocula from inaccessible extreme marine habitats such as hydrothermal vents. The inoculum, derived from the blowdown water of a biological methanation pilot plant, contains thermophilic and hyperthermophilic microorganisms sharing key metabolic traits with microbes inhabiting deep-sea and seafloor ecosystems. By subjecting this inoculum to different thermal treatments prior to copper exposure, this work explores how thermal-driven shifts in microbial community structure, rather than complete sterilization, affect corrosion behavior, biofilm development, and corrosion product evolution.

Overall, this study provides mechanistic insights into copper-microbe interactions under high-temperature, anaerobic, and chloride-containing conditions directly relevant to marine corrosion science and advanced industrial applications. Understanding how thermophilic microbial consortia influences copper corrosion contributes to a broader framework for predicting material performance in systems operating beyond mesophilic conditions, including deep-sea and extreme marine environments.

6.1 Surface and morphology investigation

Figure 6.1 shows the macroscopic changes on the copper surfaces after 15 days of immersion in biotic, boiled, autoclaved, and sterile inocula at 45 °C. Light-optical microscopy reveals that the corrosion products formed under each condition display distinct morphological features.

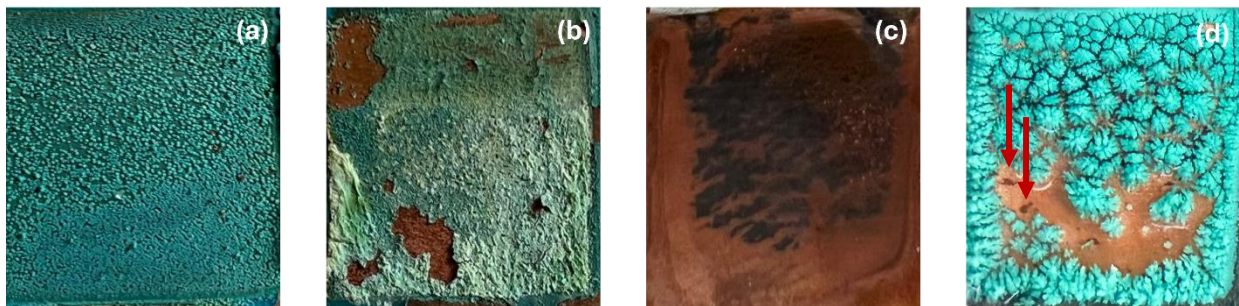


Figure 6.1. Optical microscopy images of Cu coupons after 15-day test in the (a) biotic, (b) boiled, (c) autoclaved and (d) sterile solutions at 45 °C.

Both biotic and boiled copper coupons exhibit a thick green patina of corrosion products that develop uniformly across the surface. The brown areas observed on the boiled coupon are attributed to patina detachment during post-exposure handling. At higher magnification, the patina shows a heterogeneous, multilayered structure, with globular aggregates atop a thinner underlying layer of fine precipitates, suggesting a two-stage growth mechanism (Figs. 6.2a,b). Energy-dispersive spectroscopy (EDS) analyses (Figs. 6.3 and 6.4) reveal that the globular aggregates are

primarily composed of Cu, O, and P, consistent with copper phosphate phases such as $\text{Cu}_2\text{PO}_4(\text{OH})$, while the underlying layer contains significant Cl, indicating chloride-containing phosphate products. Cross-sectional images confirm a thick, uniform phosphate layer on the coupon surface (Figs. 6.7 and 6.8). The sterile coupon exhibits a greenish to light-blue patina with markedly different morphology (Figure 6.1d). The corrosion layer is non-uniform, with localized corrosion sites (red arrows), and features acicular (needle-like) structures emerging from a very thin oxidized base layer (Figs. 6.1d and 6.2d). EDS shows Cl as a major constituent along with Cu and O, suggesting copper chloride and oxychloride phases (Fig. 6.6). Cross-sections reveal an inner copper oxide layer overlying a more complex chloride-rich layer (Fig. 6.10). In contrast, the autoclaved coupon shows a far less degraded surface. The patina is a thin, uniform brown layer with isolated black deposits (Fig. 6.1c). At higher magnification, it is compact and primarily composed of copper oxides, with only occasional chloride-containing precipitates (Figures 6.2c and 6.5). Cross-sectional images indicate a dense, continuous oxide layer at the metal interface (Figure 6.9), consistent with passive corrosion behavior and limited degradation.

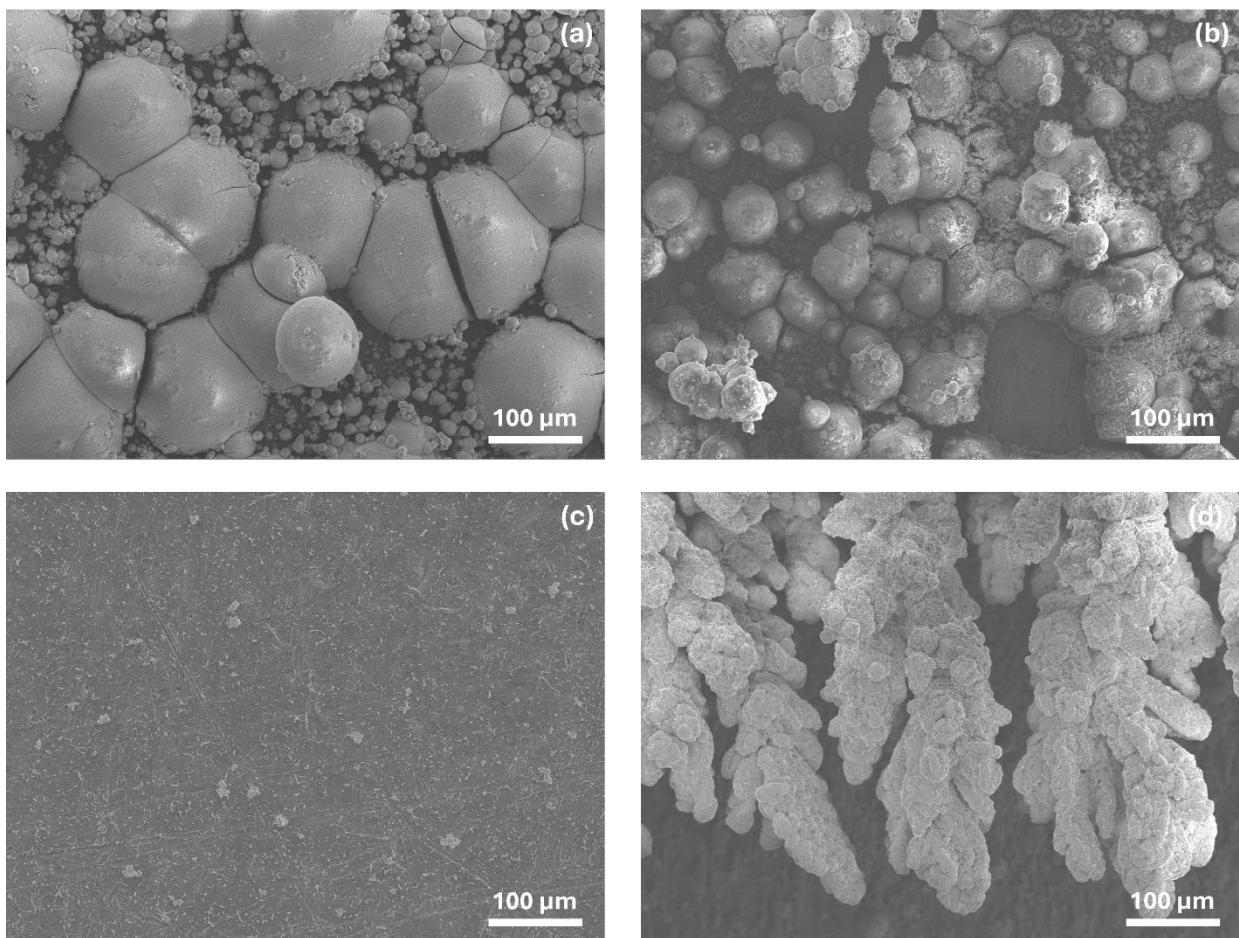


Figure 6.2. Scanning electron microscopy (SEM) images of Cu coupons after 15-day test in the (a) biotic, (b) boiled, (c) autoclaved and (d) sterile solutions at 45 °C.

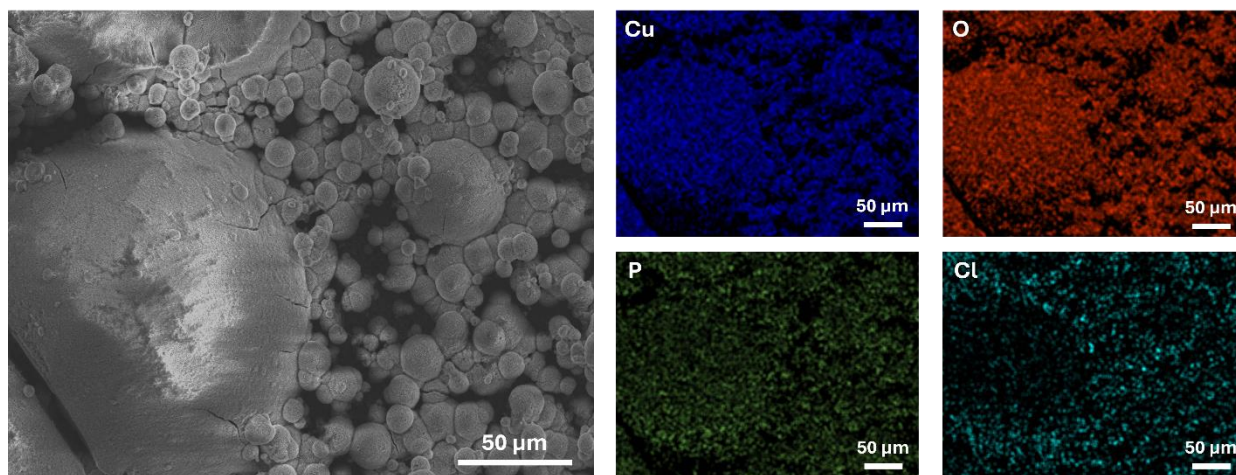


Figure 6.3. SEM-EDS images of Cu coupon after 15-day test in the biotic solution at 45 °C.

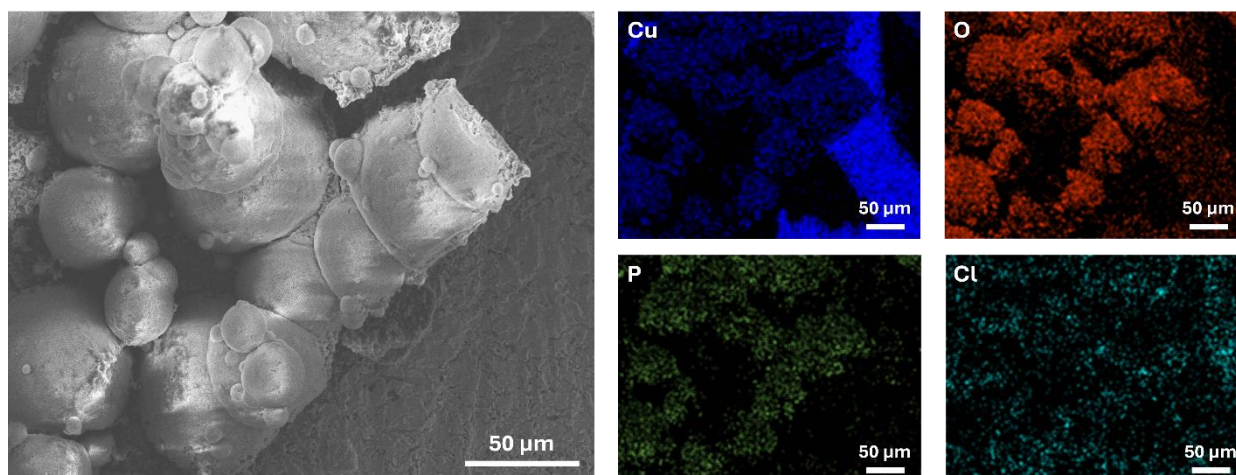


Figure 6.4. SEM-EDS images of Cu coupon after 15-day test in the boiled solution at 45 °C.

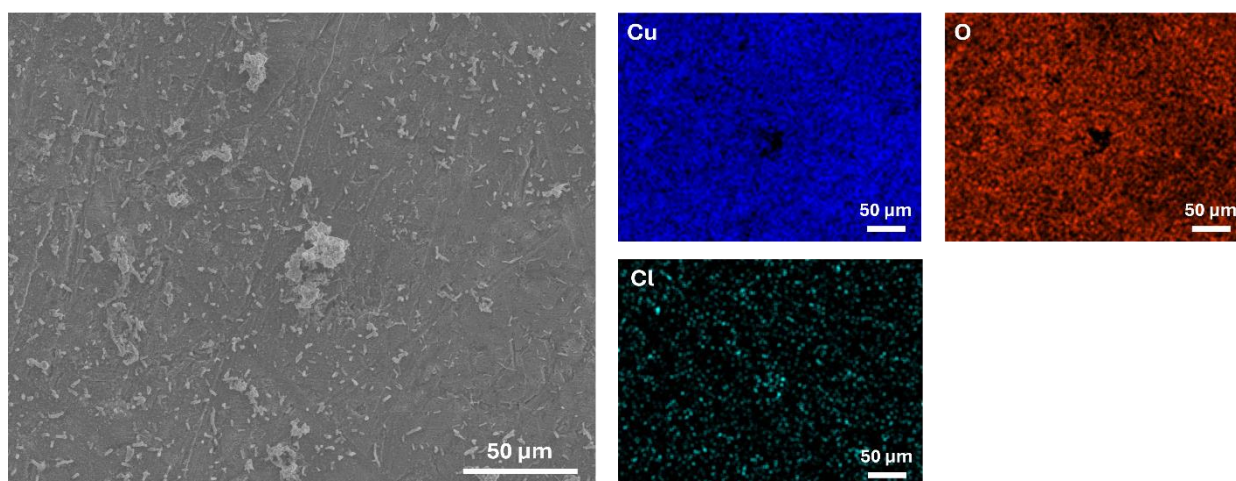


Figure 6.5. SEM-EDS images of Cu coupon after 15-day test in the autoclaved solution at 45 °C.

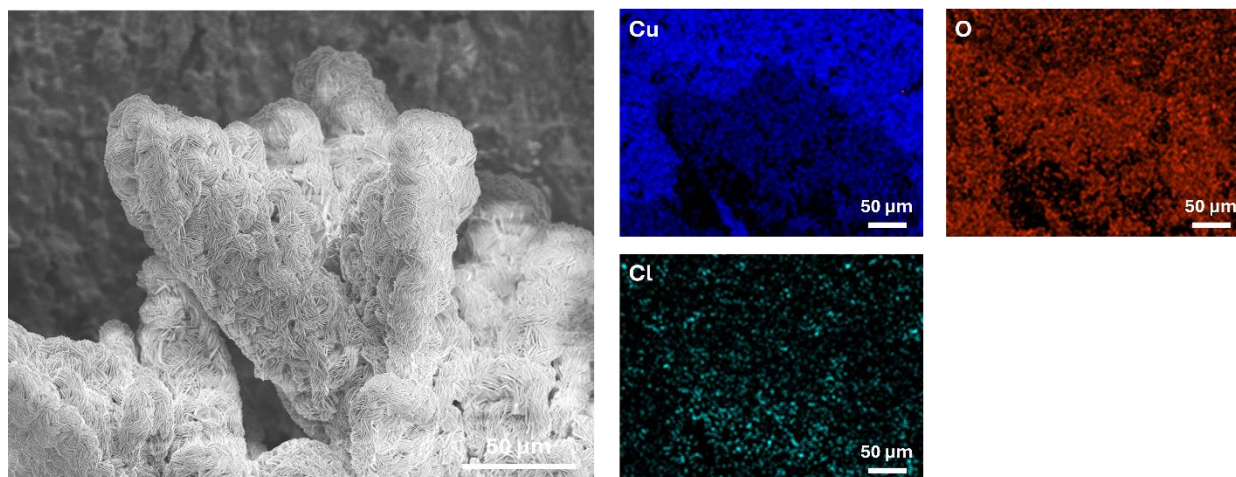


Figure 6.6. SEM-EDS images of Cu coupon after 15-day test in the sterile solution at 45 °C.

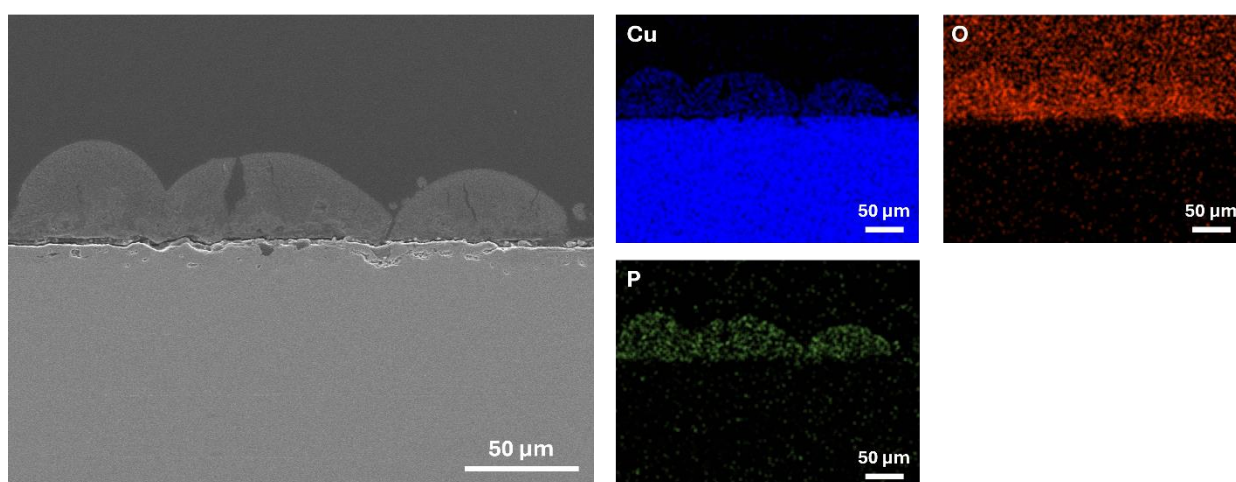


Figure 6.7. SEM-EDS images of Cu cross section after 15-day test in the biotic solution at 45 °C.

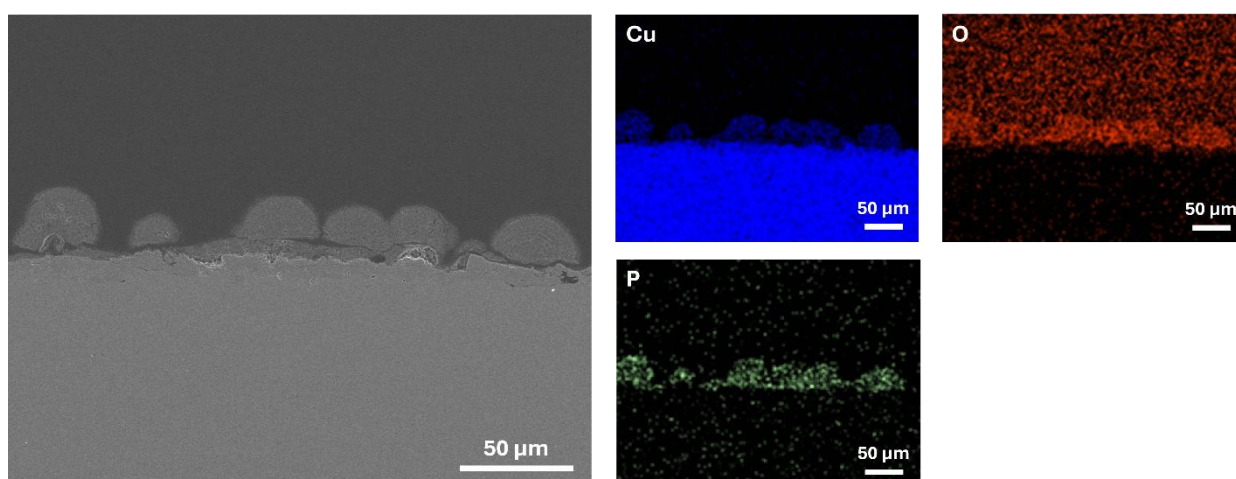


Figure 6.8. SEM-EDS images of Cu cross section after 15-day test in the boiled solution at 45 °C.

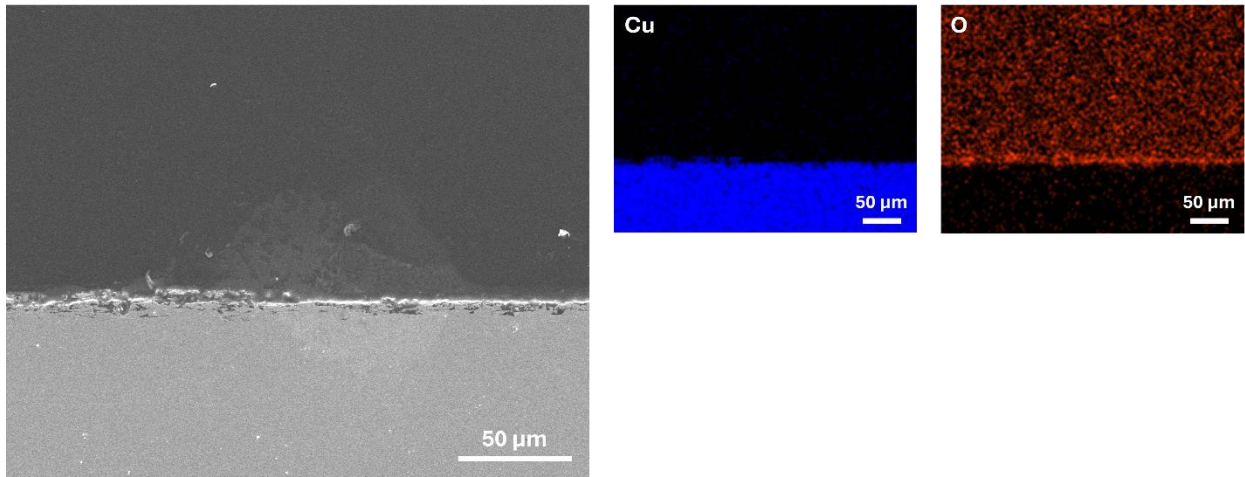


Figure 6.9. SEM-EDS images of Cu cross section after 15-day test in the autoclaved solution at 45 °C.

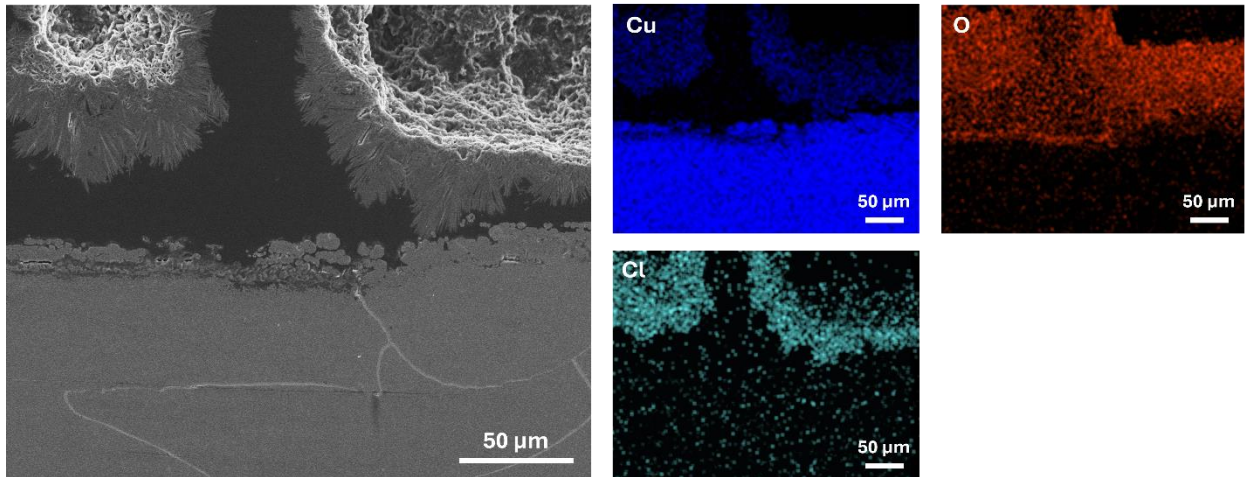


Figure 6.10. SEM-EDS images of Cu cross section after 15-day test in the sterile solution at 45 °C.

Raman spectroscopy analysis of the copper surfaces after 15 days of immersion (Fig. 6.11) revealed clear differences in the composition of corrosion products depending on the exposure conditions, consistent with the EDS observations. The distinct Raman signatures indicate that the corrosion mechanisms and resulting surface chemistries are strongly influenced by the immersion environment, leading to the formation of different corrosion phases under biotic, boiled, and autoclaved conditions. In the biotic sample (red curve), intense Raman bands at approximately 218, 410, and 520-530 cm^{-1} confirm the formation of cuprite (Cu_2O), while additional peaks in the 350-400 cm^{-1} and 950-1000 cm^{-1} regions indicate the presence of $\text{NaCaCu}_5(\text{PO}_4)_4\text{Cl}$ and $\text{Cu}_2\text{PO}_4(\text{OH})$. These results highlight a strong interaction between the copper surface, the phosphate-containing medium, and microbial activity, leading to the precipitation of mixed

copper-phosphate compounds. The boiled sample (blue curve) displays a similar spectral fingerprint, suggesting that boiling does not entirely suppress microbial activity and allows the formation of comparable corrosion products. In contrast, the autoclaved sample is dominated by features attributable to Cu_2O , with no detectable phosphate-rich phases, indicating a less aggressive corrosion process. The sterile coupon shows weak Raman peaks around 430 and 500 cm^{-1} , corresponding to Cu-Cl vibrations, characteristic of abiotic chloride-induced corrosion, consistent with the formation of CuCl_2 .^{247,248} Overall, these observations demonstrate that microbial activity and medium composition strongly influence corrosion, promoting phosphate-rich mixed layers under biotic conditions, whereas sterilization limits corrosion mainly to oxide and chloride species.

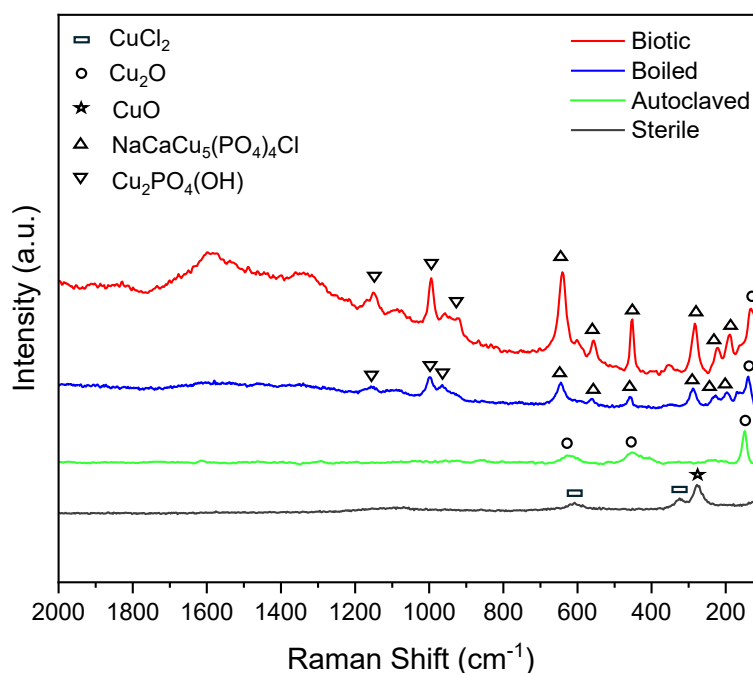


Figure 6.11. Raman spectra of Cu corrosion products on the surface of the coupons after 15-day test immersed in the biotic (red), boiled (blue), autoclaved (green) and sterile (gray) solutions at 45 °C.

6.2 Solution analysis

Post-experimental analysis of the solutions enabled the evaluation of potential microbial metabolic byproducts. The pH measurements revealed slight deviations from the initial value of 6.96: 6.90 for the biotic test, 6.79 for the boiled sample, 7.09 for the autoclaved sample, and 6.24

for the sterile control. Ion Chromatography (IC) analysis (Table 6.1) highlighted notable variations in organic acids and carbonates over the 15-day period.

In the biotic inoculum, acetate accumulated markedly, rising from below the detection limit (BDL) to 132.2 mg L^{-1} , reflecting active microbial metabolism consistent with known anaerobic microbial activity.²⁴⁹ Formate, initially present at 9.010 mg L^{-1} , was almost completely consumed by the end of the test (BDL), consistent with microbial utilization of formate as an electron donor.²⁵⁰

In contrast, the boiled and autoclaved inocula showed significant increases in acetate, particularly in the boiled condition (from 178.1 to 899.2 mg L^{-1}), likely due to abiotic chemical reactions or residual enzymatic activity surviving the thermal treatments.²⁵¹ Formate was largely absent or present at trace levels, supporting the hypothesis of limited microbial activity in these conditions. The sterile control exhibited no detectable organic acids, aside from oxalates, confirming the absence of microbial metabolism.

Oxalate concentrations increased moderately in all samples, with the highest final values in the boiled and autoclaved inocula, suggesting chemical transformations independent of microbial activity, as previously reported in abiotic aqueous corrosion systems. Carbonates remained relatively stable or slightly increased in the autoclaved samples, whereas they decreased in the boiled inoculum, likely reflecting variations in inorganic carbon equilibria influenced by pH and microbial presence.²⁵²

Table 6.1. Concentrations of anions detected in the biotic, boiled, autoclaved and sterile inocula after 15-day test, determined by ion chromatography (IC). Results are reported in the ionic form detected by the instrument.

Analyte		Concentration ^a (mg L^{-1})			
		Experimental condition			
		Biotic	Boiled	Autoclaved	Sterile
Oxalate	$\text{C}_2\text{O}_4^{2-}$	2257 ± 5.1	2637 ± 4.3	2157 ± 5.5	1207 ± 3.3
Formate	HCOO^-	n.d.	n.d.	1.0 ± 0.03	n.d.
Acetate	CH_3COO^-	132 ± 1.0	899 ± 0.96	30.8 ± 0.22	n.d.
Propionate	$\text{CH}_3\text{CH}_2\text{COO}^-$	<LOD	<LOD	<LOD	n.d.
Carbonates	CO_3^{2-}	114 ± 0.22	77.1 ± 0.12	421 ± 0.24	n.d.

^a Values are expressed as mean $\pm$ standard deviation ($n = 2$). Concentrations below the limit of detection ($\text{LOD} = 0.05 \text{ mg L}^{-1}$) are indicated as “< LOD”, while analytes not determined are indicated as “n.d.” in the Table.

ICP-AES analysis (Table 6.2) revealed a marked increase in dissolved copper concentrations across all inocula after 15 days. Initial trace levels (e.g., 0.008 mg L⁻¹ in the biotic inoculum) rose to over 3 mg L⁻¹ in the biotic, boiled, and autoclaved samples, indicating active copper corrosion and solubilization consistent with the known susceptibility of copper to aqueous environments.^{253,254} Notably, the sterile control, which initially contained no copper, reached 7.1 mg L⁻¹ by the end of the test, suggesting that copper release can occur to a greater extent via abiotic chemical corrosion in the absence of microbial activity.²⁵⁵

Phosphorus concentrations exhibited distinct trends depending on microbial activity: decreases in the biotic and boiled inocula likely reflect the precipitation of phosphate-based corrosion products, whereas the autoclaved inoculum showed an increase, possibly due to the absence of microbial-driven precipitation or altered chemical equilibria. Sulfur levels decreased in the biotic and boiled samples, which may indicate microbial sulfate reduction or incorporation into biomass, whereas the autoclaved and sterile conditions remained largely stable, consistent with minimal biological activity.²⁵⁶

Table 6.2. Total elemental concentration of inorganic elements detected in the biotic, boiled, autoclaved and sterile inocula after 15-day test, determined by inductively coupled plasma – atomic emission spectroscopy (ICP-AES).

Element		Concentration ^b (mg L ⁻¹)			
		Experimental condition			
		Biotic	Boiled	Autoclaved	Sterile
Chromium	Cr	n.d.	n.d.	n.d.	n.d.
Iron	Fe	<LOD	0.10 ± 0.01	0.11 ± 0.01	n.d.
Nichel	Ni	0.18 ± 0.02	0.14 ± 0.01	0.14 ± 0.02	n.d.
Copper	Cu	3.1 ± 0.03	3.7 ± 0.05	3.1 ± 0.03	7.1 ± 0.07
Zinc	Zn	n.d.	<LOD	<LOD	n.d.
Sulfur	S	2.7 ± 0.04	2.2 ± 0.03	4.4 ± 0.04	5.0 ± 0.04
Phosphorous	P	208 ± 0.98	277 ± 1.0	493 ± 0.89	100 ± 0.98

^b Values are expressed as mean ± standard deviation (n = 2). Concentrations below the limit of detection (LOD = 0.05 mg L⁻¹) are indicated as “< LOD”, while analytes not determined are indicated as “n.d.” in the Table.

Overall, these findings underscore the dual influence of biological and chemical processes on elemental dynamics. Microbial activity drives the transformation of organic acids (notably acetate accumulation and formate depletion) and modulates phosphorus and sulfur behavior, while copper

release occurs under both biotic and abiotic conditions. The elevated copper concentrations observed in the sterile control highlight the need to account for purely chemical corrosion when interpreting solution chemistry.

6.3 Characterization of the bacterial community

Microbial community profiling was performed using 16S rRNA gene next-generation sequencing (Illumina) on both the biofilms and inocula from the four experimental conditions (biotic, boiled, autoclaved, and sterile control). After 15 days, samples were collected from the inoculum, electrode biofilm, and surrounding solution for analysis. Sterility of the control was confirmed by the absence of growth on LD agar (Fig. S.6.1, Supplementary Information), whereas microbial presence was detected in the biotic, boiled, and autoclaved systems.

Quantitative PCR (qPCR) analysis (Figure S.6.2, Supplementary Information) demonstrated that thermal treatments substantially decreased the 16S rRNA gene copy numbers in the inocula. The autoclaved sample exhibited the lowest bacterial load (4.6×10^5 gene copies mL^{-1}), compared to the boiled inoculum (5.6×10^{10} gene copies mL^{-1}) and the untreated biotic control (5.5×10^{13} gene copies mL^{-1}).

Figure 6.12 shows the relative abundance at the genus level of bacterial communities in the inocula prior to electrochemical tests (Inocula), and at the end of the exposure period in both the biofilm formed on the metal coupon (Biofilm) and the surrounding solution (Solution) for each tested condition (biotic, boiled, and autoclaved). Notably, in all three conditions, a substantial fraction of the bacterial communities (30-60%) consists of genera with relative abundances below 5%. The initial inocula display marked differences in composition. In the biotic sample, *Alcaligenes* and *Lentilactobacillus* are the most abundant genera, reflecting active roles in nitrogen cycling and fermentation.^{257,258} Boiling shifts the community toward heat-tolerant taxa such as *Tepidiphilus* (19%), a nitrate-reducing bacterium of the *Hydrogenophylaceae* family,²⁵⁹ commonly detected in thermophilic aerobic digesters and hot springs.^{260,261} The autoclaved inoculum shows an increased proportion of unclassified bacteria (30%) and spore-formers such as *Effusibacillus* (8%), indicating that only the most resilient microorganisms survive this treatment. Analysis of the biofilm communities reveals enrichment in taxa capable of tolerating or metabolizing heavy metals.²⁶² In particular, *Anoxybacillus* and *Brevibacillus* dominate the autoclaved biofilm, with relative abundances of 34% and 23%, respectively, compared to their low abundance in the bulk solution. *Effusibacillus* is also exclusively found in the autoclaved

biofilm (18%). Conversely, in the bulk solution, *Tepidiphilus* is predominant in the biotic and boiled conditions (42% and 85%, respectively), whereas *Anoxybacillus* dominates the autoclaved solution (47%), demonstrating high resilience under thermal stress. These results highlight the differential enrichment of bacterial taxa in biofilm versus solution environments and the selective impact of thermal treatments on community composition. Species richness, assessed via amplicon sequence variants (ASVs), further reflects these differences. After rarefaction at 5685 sequences for the inocula, the number of ASVs was 431 for the biotic, 244 for the boiled, and 210 for the autoclaved conditions. For the biofilm samples, rarefaction at 364 sequences yielded 188 ASVs in the biotic, 149 in the boiled, and 15 in the autoclaved conditions. For the solution samples, rarefaction at 8564 sequences produced 328 ASVs in the biotic, 202 in the boiled, and 72 in the autoclaved samples.

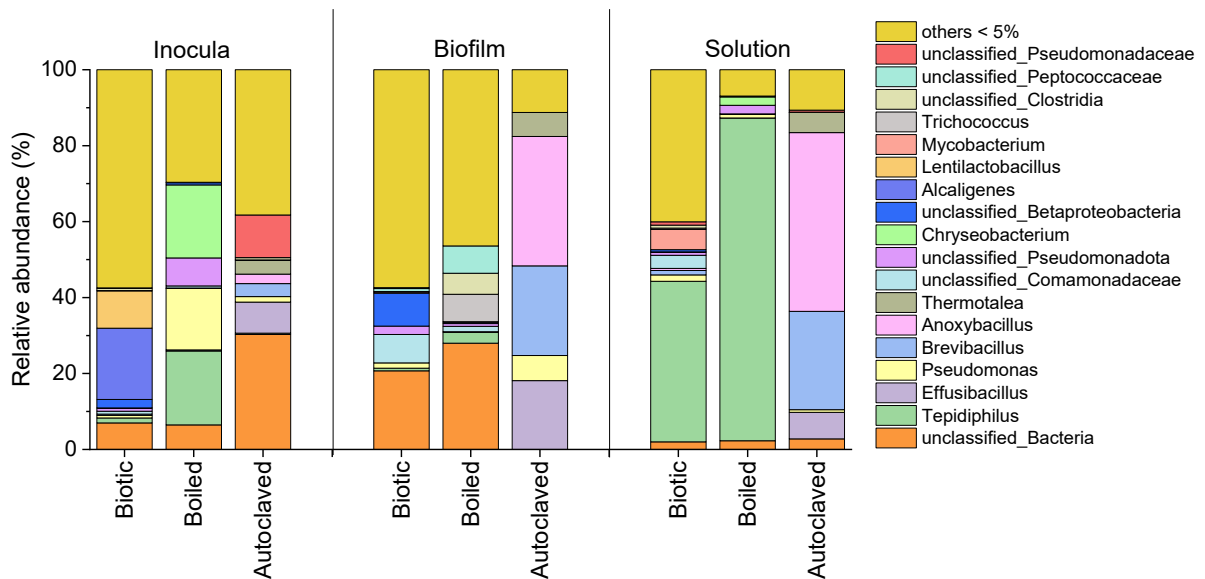


Figure 6.12. Bar plot showing the relative abundances of bacterial genera in the inocula prior the test, and in the biofilm and in solution after the test. The chart presents results for the untreated (biotic), boiled, and autoclaved conditions.

Cluster analysis based on Hellinger distance using ASV data (Fig. 6.13) shows that autoclaved samples cluster separately from biotic and boiled samples, confirming that autoclaving leads to substantially different bacterial communities compared to the other two conditions.

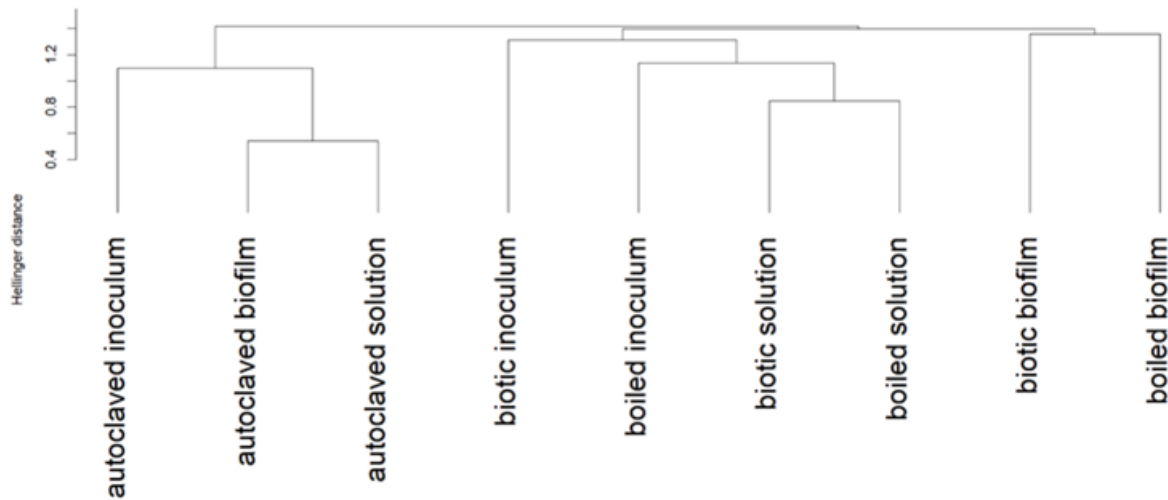


Figure 6.13. Cluster analysis of bacterial communities based on Hellinger transformed data.

Figure 6.14 shows the relative abundance at the genus level of archaeal communities before and after the exposure tests for each experimental condition. In all cases, both prior to and following the exposure period (i.e., inocula and bulk solution), the communities are dominated by *Methanothermobacter*, a group of thermophilic hydrogenotrophic methanogens.²⁶³ This observation indicates a high resilience of this genus to the different experimental treatments and suggests that the archaeal composition in solution largely reflects that of the initial inoculum.

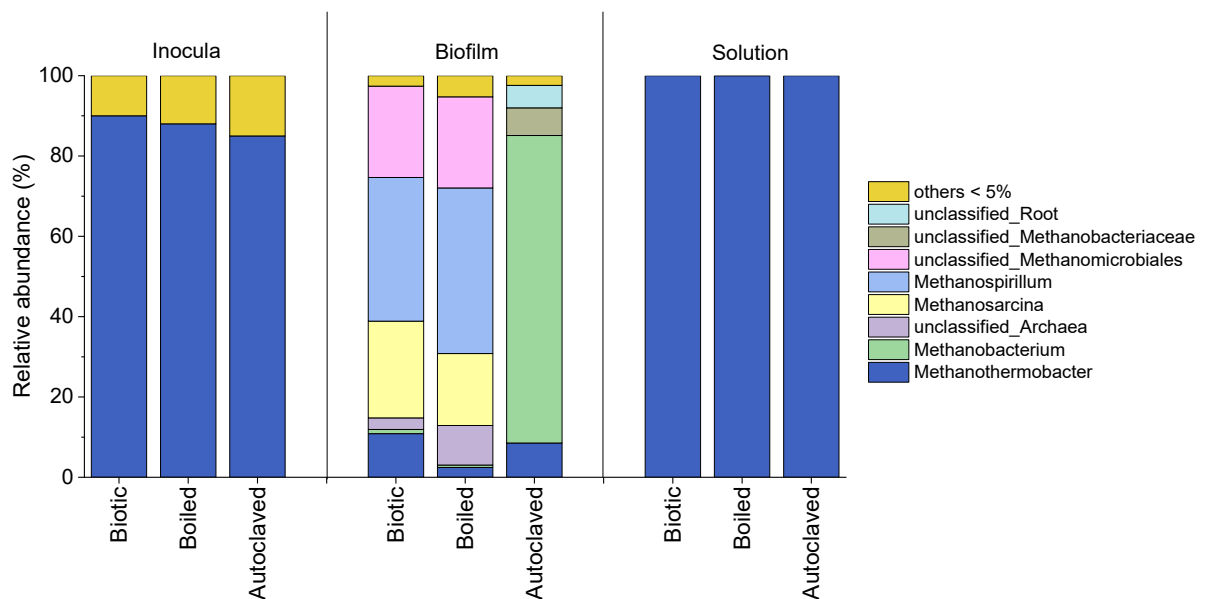


Figure 6.14. Bar plot showing the relative abundances of Archaea genera in the inocula prior the test, and in the biofilm and in solution after the test. The chart presents results for the untreated (biotic), boiled, and autoclaved conditions.

In contrast, pronounced shifts are observed in the archaeal communities within the biofilms formed on the copper coupons. The biotic and boiled conditions exhibit similar biofilm compositions, dominated by *Methanospirillum* (35-40%), *Methanosarcina* (17-24%), and members of the order *Methanomicrobiales* ($\approx 22\%$). This similarity suggests a stable and reproducible colonization of the copper surface under both conditions, despite the thermal treatment applied to the boiled inoculum. Notably, the biofilm formed under autoclaved conditions displays a markedly different archaeal community structure, overwhelmingly dominated by *Methanobacterium* (76%). This shift indicates that autoclaving selectively favors archaeal taxa with higher resistance or better adaptability to the altered physicochemical conditions, resulting in a distinct biofilm community compared to the biotic and boiled systems.

Overall, these results demonstrate that while archaeal populations in the bulk solution remain largely unaffected by the experimental treatments, the copper surface acts as a strong selective niche, promoting distinct archaeal assemblages depending on the applied sterilization strategy. For the archaeal communities, species richness was assessed by determining the number of ASVs in each sample following rarefaction. The dataset was rarefied at 110885 sequences for the inoculum samples, yielding 4 ASVs in both the biotic and boiled conditions and 3 ASVs in the autoclaved condition. For the biofilm samples, rarefaction was performed at 1498 sequences, resulting in 67 ASVs in the biotic sample, 68 in the boiled sample, and 17 in the autoclaved sample. The solution samples were rarefied at 67043 sequences, with 4 ASVs detected in the biotic condition, 5 in the boiled condition, and 4 in the autoclaved condition.

Cluster analysis based on ASV composition (Fig. 6.15) reveals that the biofilm samples group closely together, indicating a higher degree of similarity among biofilm-associated archaeal communities compared to those in the inocula and bulk solutions. In addition, the autoclaved inoculum and the corresponding solution form a distinct cluster, reflecting the impact of autoclaving on community structure. Consistent with the relative abundance data, both the biotic and boiled inocula and solutions cluster together, suggesting that boiling does not substantially alter the archaeal community composition and that these communities remain more similar to each other than to those subjected to autoclaving.

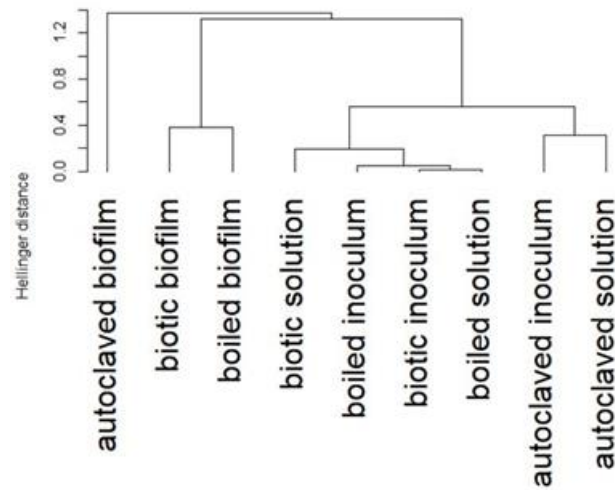


Figure 6.15. Cluster analysis of archaeal community based on Hellinger transformed data.

6.4 Electrochemical characterization

Figure 6.16 shows the evolution of the average open circuit potential (OCP, E vs. Ag/AgCl sat.) of the Cu samples over a 15-day immersion period under four experimental conditions: untreated biomethanation solution (biotic), boiled, autoclaved, and sterile control. The reported values represent the mean of two independent replicates, with error bars indicating the corresponding standard deviation. All OCP measurements were conducted at an initial solution pH of 6.96.

The evolution of the open-circuit potential (OCP) exhibited distinct trends depending on the thermal treatment applied to the microbial inoculum. Both the autoclaved and control (untreated) samples showed a rapid initial increase in potential, stabilizing around -0.01 V vs Ag/AgCl. In the control condition, however, a notable decrease in potential occurred after day 7, likely reflecting the breakdown or destabilization of protective surface films and a transition toward more active corrosion.

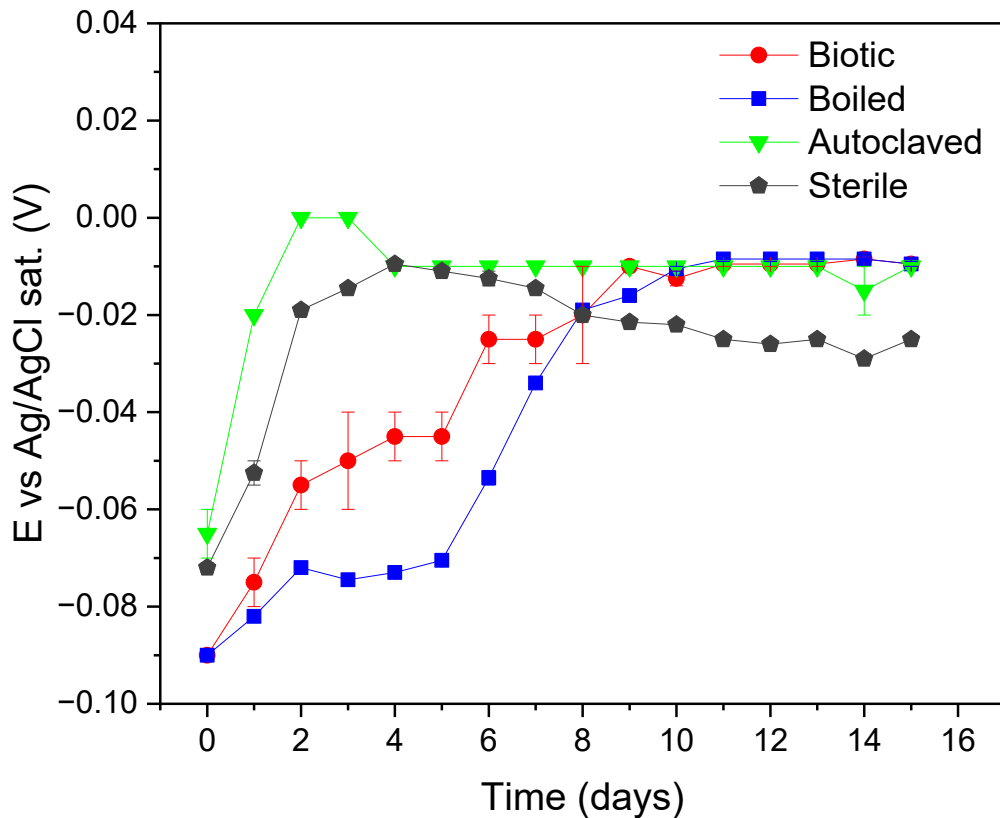


Figure 6.16. Open circuit potential (average and standard deviation, E vs Ag/AgCl sat.) of Cu coupons over the entire test period (15 days). Biotic (red solid line), boiled (blue solid line), autoclaved (green solid line) and sterile condition (gray solid line).

In contrast, the biotic and boiled samples displayed a more gradual rise in potential, reaching a stable value near -0.01 V vs Ag/AgCl only after approximately 9-10 days. This slower evolution suggests a delayed establishment of surface equilibrium, potentially influenced by ongoing microbial activity or slower formation of corrosion products.

The rapid ennoblement observed in the autoclaved and control conditions is indicative of reduced corrosion activity and the development of a relatively stable passive film on the copper surface. Conversely, the initially low and progressively increasing potentials recorded in the biotic and boiled systems (starting at approximately -0.10 V vs Ag/AgCl) point to ongoing anodic dissolution of copper, likely driven by microbial metabolism and associated localized chemical changes at the metal–solution interface.

Overall, these results demonstrate that active microbial communities strongly influence interfacial electrochemistry, promoting more negative potentials and accelerating anodic processes, whereas

thermal treatment (autoclaving) attenuates microbial activity and favors the formation of protective surface films.

To complement the OCP measurements, polarization curves were recorded in duplicates (Figs. 6.17 and S.6.3, Supplementary Information) on days 1 and 15 to assess the corrosion kinetics while minimizing disturbance of the interface. Linear polarization resistance (LPR) was determined by performing a linear fit of the current (I) versus potential (E) curve within ± 20 mV of the corrosion potential (Fig. 6.17), following established protocols.²⁶⁴ The LPR values provide an indirect measure of the corrosion rate through the Stern-Geary relationship, though limitations exist in cases of localized corrosion or pitting.²³⁸ The summarized results of the LPR measurements (Table 6.3) confirm the distinct corrosion behavior among the four inoculum conditions, with the autoclaved system exhibiting the highest polarization resistance and the sterile and biotic systems showing comparatively lower resistance, consistent with more aggressive metal dissolution.

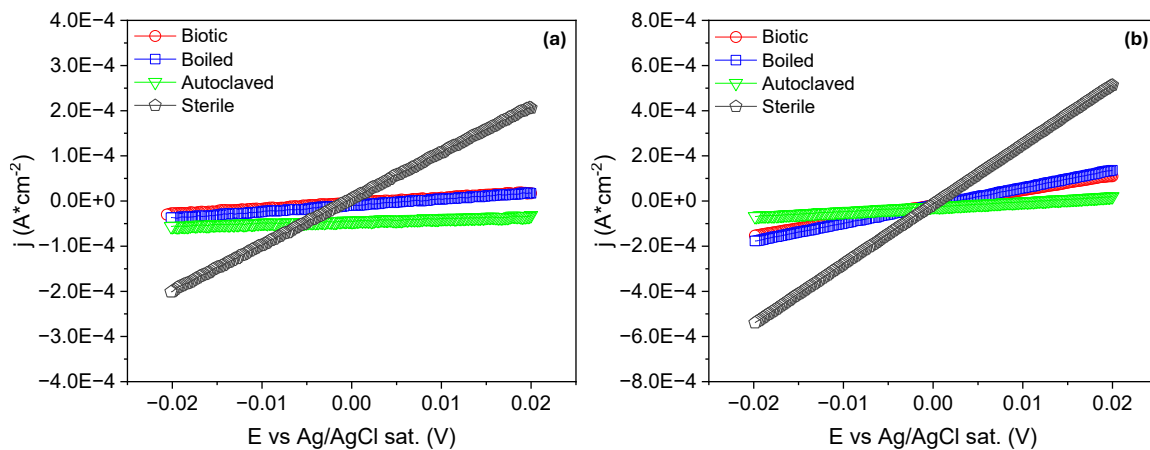


Figure 6.17. Linear polarization resistances of Cu coupons and replicates tested in the biotic (red), boiled (blue) and autoclaved (gray) inocula after (a) 1 day and (b) 15 days.

As the data show, all experimental conditions exhibited a decrease in polarization resistance (R_p) over time. Lower R_p values were observed in the biotic and boiled conditions, indicating a significant role of microorganisms in destabilizing the copper surface. The autoclaved condition consistently displayed the highest R_p values (1.20 and 0.35 $\text{k}\Omega\cdot\text{cm}^2$ at days 1 and 15, respectively), reflecting higher corrosion resistance compared to the sterile condition. Interestingly, the sterile condition did not exhibit high resistance values, suggesting that microbially influenced corrosion (MIC) is not the only corrosion mechanism affecting the copper

surface. Initial R_p values in the sterile solution were comparable to those measured in the boiled condition, with a marked decline by the end of the experiment.

It is important to note that the LPR technique provides an average assessment of the electrochemical behavior normalized over the entire exposed surface. As such, it does not capture localized phenomena, such as the initiation of pitting corrosion, which is characteristic of pure copper.²⁶⁵ Therefore, R_p values should be interpreted with caution in light of this limitation.

Table 6.3. R_p values obtained from LPR measurements at the beginning and end of the test (1 and 15 days, respectively) for each experimental condition (biotic, boiled, autoclaved and sterile). Values are reported as mean $\pm$ standard deviation.

Time days	Biotic $k\Omega \cdot \text{cm}^2$	Boiled $k\Omega \cdot \text{cm}^2$	Autoclaved $k\Omega \cdot \text{cm}^2$	Sterile $k\Omega \cdot \text{cm}^2$
1	0.28 ± 0.073	0.73 ± 0.21	1.2 ± 0.47	0.38 ± 0.26
15	0.20 ± 0.057	0.13 ± 0.034	0.35 ± 0.067	0.11 ± 0.036

Electrochemical Impedance Spectroscopy (EIS) was employed to investigate interfacial processes at the electrode-solution interface, including capacitive effects, charge transfer, and mass transport, which strongly influence the kinetics of electrochemical reactions. EIS measurements were performed with 10 points per decade over a frequency range of 100 kHz to 0.01 Hz at different exposure times (1, 7, and 15 days). The impedance data are presented as both Bode and Nyquist plots (Fig. 6.18), showing the real (Z') and imaginary (Z'') components, as well as the impedance modulus ($\log |Z|$) and phase angle as a function of frequency ($\log f$).

The shape of the impedance curves varies notably among the different conditions, with each system displaying a distinct and progressively evolving profile. During the first week of exposure, nearly all conditions exhibit a primary semicircle in the Nyquist plot, slightly depressed toward the x-axis, indicative of non-ideal capacitive behavior and interfacial dispersion at the metal-solution boundary.²⁶⁶ A secondary semicircle is also discernible, suggesting heterogeneity in surface reactivity, likely arising from variations in surface film properties and interfacial processes. At 7 days, the sterile condition shows a broader secondary semicircle at very low frequencies, implying the presence of at least two distinct electrochemical processes, possibly related to separate resistive or capacitive contributions from surface films and charge transfer.

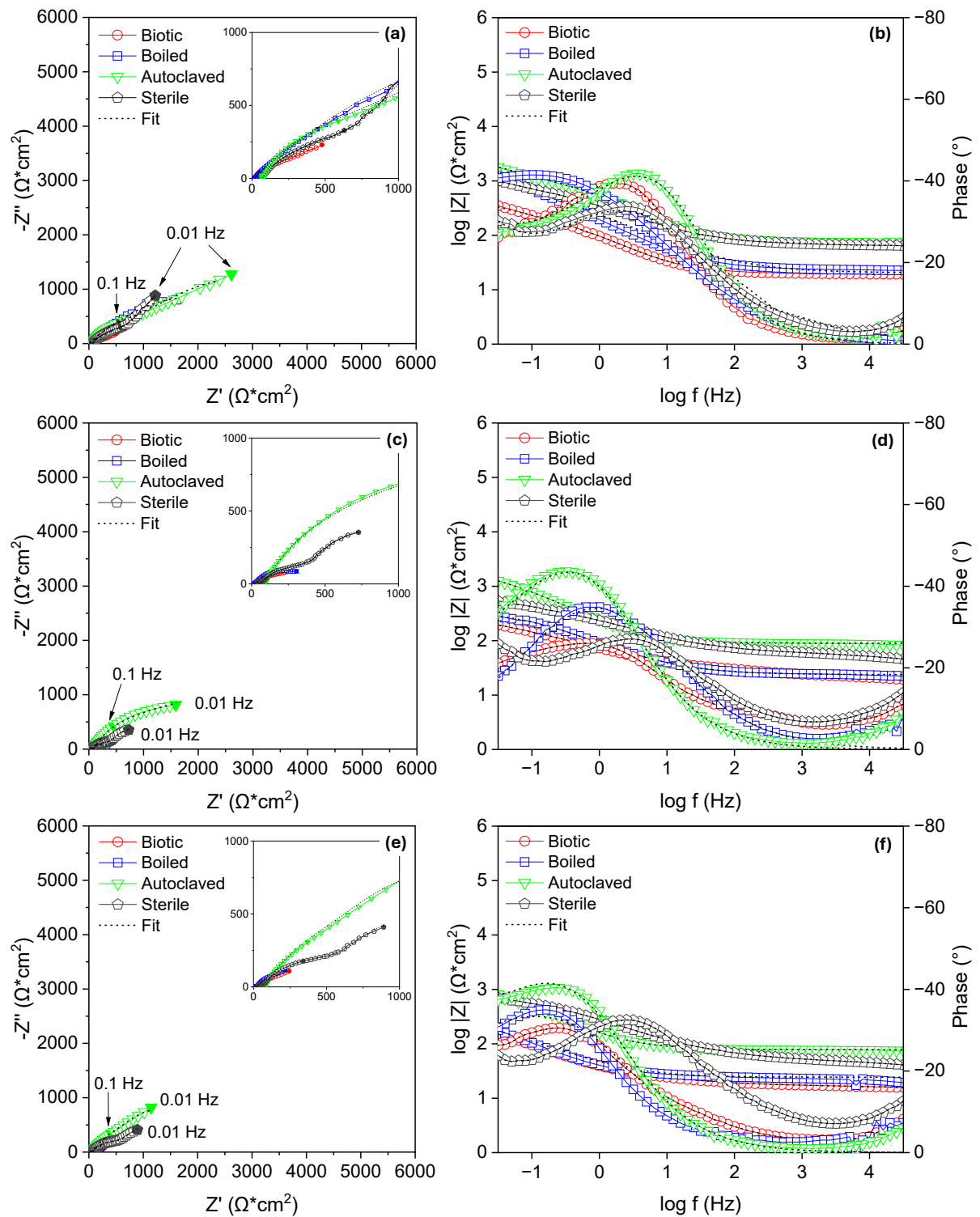


Figure 6.18. Nyquist and bode plots of Cu coupons tested in the biotic (red), boiled (blue), autoclaved (green) and sterile (gray) inocula after (a-b) 1 day, (c-d) 7 days and (e-f) 15 days.

After 15 days, the low-frequency region of the impedance spectra for all conditions (except the control) begins to approach a linear response with a slope near 45° , characteristic of Warburg

impedance. This indicates that diffusion-controlled processes, such as ion transport through corrosion products or biofilms, increasingly dominate over time.

In terms of magnitude, the autoclaved system consistently exhibits the highest overall impedance across all time points, exceeding those of the biotic, boiled, and sterile conditions. This suggests that autoclaving promotes the formation of a more resistive interfacial layer, likely due to the absence of aggressive microbial activity. It is also important to note that in cases of significant corrosion, the capacitive contribution of the biofilm formed during the test is generally negligible, as it is largely overshadowed by the dominant impedance of the corrosion products.^{238,267}

Impedance data were systematically post-processed by fitting the experimental results using the equivalent electrical circuit (EEC) models (I–III) shown in Figure 6.19.

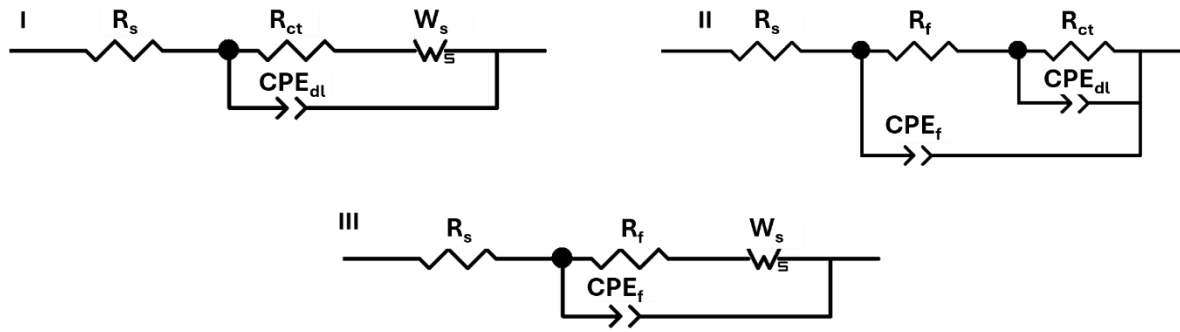


Figure 6.19. Equivalent electrical circuit (EECs) I–III used to model impedance data.

Circuit elements were assigned to specific physical or interfacial processes, including solution resistance (R_s), charge transfer resistance (R_{ct}), double-layer capacitance (CPE_{dl}), film-related electrical properties (R_f and CPE_f), and Warburg impedance (W), with a coefficient of determination $\chi = 10^{-4}$. Ideal capacitive behavior was not observed, as the experimental data in all systems deviated from that of a perfect capacitor. Therefore, the capacitive contributions were modeled using Constant Phase Elements (CPE_s), which account for the non-ideal, frequency-dependent behavior typically observed at electrochemical interfaces. The impedance of a CPE (Z_{CPE}) is described by the following expression (Eq. 6.1):

$$Z_{CPE} = \frac{1}{Y_0(j\omega)^n} \quad (6.1)$$

In the CPE model, Y_0 represents the capacitance, ω is the angular frequency ($2\pi\nu$), and n is a dimensionless exponent that characterizes the deviation from ideal capacitive behavior. The exponent ranges from 0 to 1, where $n = 1$ corresponds to an ideal capacitor and $n = 0$ corresponds to an ideal resistor. Values between 0.5 and 1 indicate a distributed or non-ideal system, typically arising from surface roughness, porosity, or other forms of interfacial heterogeneity.

Fitting results are summarized in Tables 6.4-6.7, along with a detailed attribution of the corresponding electrochemical parameters. The biotic condition exhibited the most complex corrosion behavior among all tested environments, necessitating the use of three distinct equivalent electrical circuits (Fig. 6.19, Table 6.4) to accurately model its evolving electrochemical response.

On day 1, the system is best described by EEC I, which captures dominant diffusion-controlled behavior. This model consists of a single R//CPE (resistor in parallel with a constant phase element) mesh followed by a Warburg element (W). The first time constant corresponds to the charge transfer resistance (R_{ct}) and the double-layer capacitance (C_{dl}), with the effective double-layer capacitance (C_{eff}) calculated using the CPE parameters (magnitude and exponent n) via Brug's equation.²⁶⁸ Valid C_{eff} values, falling within 10^{-4} to 10^{-5} F·cm⁻², confirm the physical consistency of the fit. The Warburg element represents diffusion-limited transport of electroactive species at the electrode–electrolyte interface, highlighting the role of ion migration in early-stage biotic corrosion.

After 7 days, the corrosion mechanism evolves and is best represented by EEC II, which incorporates two R//CPE meshes arranged in a ladder configuration. The first mesh captures the electrochemical processes at the metal–electrolyte interface (R_{ct} and C_{dl}), while the second accounts for the electrical properties of the surface film (R_f and CPE_f) that has formed. Surface analyses suggest this film primarily consists of copper phosphates. Despite its formation, the film is likely non-protective (porous or unstable) and insufficient to significantly inhibit ongoing corrosion processes.

At 15 days, the system once again exhibits a diffusion-controlled response, modeled by EEC III. This circuit consists of an R//CPE mesh in series with a Warburg element, reflecting mass transport limitations through the developed patina. At this stage, the charge transfer and double-layer contributions are partially obscured by the patina's presence. The resistance (R_f) and pseudocapacitance (CPE_f) are therefore attributed to the electrical properties of the patina itself,

which, according to surface analyses, likely contains complex copper phosphates. The Warburg element indicates continued diffusion-limited transport of Cu ions through the patina, suggesting that while the surface film is more developed and uniform, it remains porous and does not fully hinder ion mobility.

The boiled coupon exhibits a corrosion mechanism similar to that observed under biotic conditions, particularly at 7 and 15 days, and can be modeled using EEC II and EEC III, respectively (Fig. 6.19, Table 6.5). On day 1, the electrochemical behavior is less dominated by diffusion, reflecting the early formation of a surface patina composed of corrosion products. At this stage, the system is best described by EEC II, which consists of two R//CPE meshes: the first representing the charge transfer resistance (R_{ct}) and double-layer capacitance (C_{dl}) at the metal–electrolyte interface, and the second capturing the film resistance (R_f) and non-ideal film capacitance (CPE_f), likely associated with the initial development of copper oxide layers. By day 15, the surface patina has evolved further, and the corrosion products resemble those observed on the biotic coupon, justifying the use of EEC III to account for diffusion-controlled processes through the now more developed film.

Table 6.4. Fit results using the EECs from Figure 6.19 for the biotic condition.

Biotic ^c											
EEC	Time	R _s	R _{ct}	CPE _{dl} Q	CPE _{dl-n}	R _f	CPE _f Q	CPE _{f-n}	W	C _{eff}	R _{tot}
	days	$\Omega \cdot \text{cm}^2$	$\text{k}\Omega \cdot \text{cm}^2$	$\text{k}\Omega^{-1} \cdot \text{cm}^{-2} \cdot \text{s}^n$		$\text{k}\Omega \cdot \text{cm}^2$	$\text{k}\Omega^{-1} \cdot \text{cm}^{-2} \cdot \text{s}^n$		$\text{k}\Omega \cdot \text{cm}^2$	$\mu\text{F} \cdot \text{cm}^{-2}$	$\text{k}\Omega \cdot \text{cm}^2$
I	1	20±2	17±0.3	2.4±0.19	0.73± 0.05	n.d.	n.d.	n.d.	2.7±0.12	740±28	2.9±0.12
II	7	18±2	850±20	7.3±2.0	0.55±0.09	49±3.0	0.48±0.014	0.98± 0.01	n.d.	130±19	58±3.6
III	15	18±5	n.d.	n.d.	n.d.	39±6.2	0.015±0.0001	0.52 ±0.08	900±72	n.a.	48±6.2

^c Values are expressed as mean ± standard deviation. Depending on the equivalent electrical circuit (EEC) used, parameters that are not detected are reported as 'n.d.', whereas parameters that are not applicable are indicated as 'n.a.'.

Table 6.5. Fit results using the EECs from Figure 6.19 for the boiled condition.

Boiled ^d											
EEC	Time	R _s	R _{ct}	CPE _{dl} Q	CPE _{dl-n}	R _f	CPE _f Q	CPE _{f-n}	W	C _{eff}	R _{tot}
	days	$\Omega \cdot \text{cm}^2$	$\text{k}\Omega \cdot \text{cm}^2$	$\text{k}\Omega^{-1} \cdot \text{cm}^{-2} \cdot \text{s}^n$		$\text{k}\Omega \cdot \text{cm}^2$	$\text{k}\Omega^{-1} \cdot \text{cm}^{-2} \cdot \text{s}^n$		$\text{k}\Omega \cdot \text{cm}^2$	$\mu\text{F} \cdot \text{cm}^{-2}$	$\text{k}\Omega \cdot \text{cm}^2$
II	1	20±1	90±4.3	1.3±0.01	0.59±0.01	2.7±0.27	1.6±0.06	0.59±0.03	n.a.	98±0.01	3.6±0.27
II	7	24±6	36±7.0	3.9±0.19	0.61±0.01	0.044±0.01	0.074±0.00	0.95±0.02	n.a.	800±69	0.41±0.07
III	15	24±1	n.d.	n.d.	n.d.	0.052±0.005	8.5±0.18	0.67±0.01	0.41±0.01	n.a.	0.46±0.01

^d Values are expressed as mean ± standard deviation. Depending on the equivalent electrical circuit (EEC) used, parameters that are not detected are reported as 'n.d.', whereas parameters that are not applicable are indicated as 'n.a.'.

The autoclaved condition exhibits a distinct corrosion behavior, dominated by diffusion-controlled processes throughout the experiment (Fig. 6.19, Table 6.6). At all time points, the system is best described by an equivalent electrical circuit consisting of an R//CPE mesh in series with a Warburg element (W), indicative of ion transport limitations at the interface. Up to day 7, the electrochemical response is accurately modeled by EEC II, where the first time constant corresponds to the charge transfer resistance (R_{ct}) and double-layer capacitance (C_{dl}), reflecting the formation of a non-uniform patina, primarily composed of copper oxides and hydroxides. By day 15, the system is better represented by EEC III, as the patina becomes more compact and uniformly distributed, effectively masking contributions from R_{ct} and C_{dl} . At this stage, the R//CPE mesh predominantly reflects the electrical properties of the protective film itself (R_f and CPE_f), rather than the charge transfer processes at the metal–solution interface. The sterile coupon exhibits a distinctive corrosion behavior. On day 1, its response closely resembles that of the biotic and autoclaved systems and is best modeled by EEC I (Fig. 6.19, Table 6.7), reflecting a diffusion-controlled mechanism. The first time constant corresponds to charge transfer resistance (R_{ct}) and double-layer capacitance (C_{dl}) in series with a Warburg element (W), indicative of ion transport limitations at the metal-electrolyte interface. Between days 7 and 15, the corrosion mechanism evolves and is better described by EEC II, consisting of two R//CPE meshes. The first time constant represents interfacial electrochemical reactions (R_{ct}/C_{dl}), while the second reflects the formation of a surface film (R_f/CPE_f). Post-experimental analyses reveal a heterogeneous, stratified film: an inner layer of copper oxides overlaid by an outer layer likely composed of copper chlorides and oxychlorides. This layered structure illustrates the progressive accumulation and transformation of corrosion products, leading to increased heterogeneity in both surface chemistry and electrochemical behavior.

Table 6.6. Fit results using the EECs from Figure 6.19 for the autoclaved condition.

Autoclaved ^e											
EEC	Time	R _s	R _{ct}	CPE _{dl} Q	CPE _{dl-n}	R _f	CPE _f Q	CPE _{f-n}	W	C _{eff}	R _{tot}
	days	$\Omega \cdot \text{cm}^2$	$\text{k}\Omega \cdot \text{cm}^2$	$\text{k}\Omega^{-1} \cdot \text{cm}^{-2} \cdot \text{s}^n$		$\text{k}\Omega \cdot \text{cm}^2$	$\text{k}\Omega^{-1} \cdot \text{cm}^{-2} \cdot \text{s}^n$		$\text{k}\Omega \cdot \text{cm}^2$	$\mu\text{F} \cdot \text{cm}^{-2}$	$\text{k}\Omega \cdot \text{cm}^2$
I	1	76±1	0.46±0.04	30±1.7	0.77±0.01	n.a.	n.a.	n.a.	4.3±0.08	92±4.5	4.8±0.09
I	7	90±1	1.2±0.16	1.3±0.01	0.72±0.01	n.a.	n.a.	n.a.	5.2±0.48	590±32	6.4±0.48
III	15	78±1	n.d.	n.d.	n.d.	0.90±0.01	2.4±0.03	0.71±0.06	1.5±0.12	n.a.	2.4±0.14

^e Values are expressed as mean ± standard deviation. Depending on the equivalent electrical circuit (EEC) used, parameters that are not detected are reported as 'n.d.', whereas parameters that are not applicable are indicated as 'n.a.'.

Table 6.7. Fit results using the EECs from Figure 6.19 for the sterile condition.

Sterile ^f											
EEC	Time	R _s	R _{ct}	CPE _{dl} Q	CPE _{dl-n}	R _f	CPE _f Q	CPE _{f-n}	W	C _{eff}	R _{tot}
	days	$\text{k}\Omega \cdot \text{cm}^2$	$\text{k}\Omega \cdot \text{cm}^2$	$\text{k}\Omega^{-1} \cdot \text{cm}^{-2} \cdot \text{s}^n$		$\text{k}\Omega \cdot \text{cm}^2$	$\text{k}\Omega^{-1} \cdot \text{cm}^{-2} \cdot \text{s}^n$		$\text{k}\Omega \cdot \text{cm}^2$	$\mu\text{F} \cdot \text{cm}^{-2}$	$\text{k}\Omega \cdot \text{cm}^2$
I	1	66 ± 1	0.68±0.02	0.8 ± 0.02	0.61 ± 0.01	n.a	n.a	n.a	3.1±0.29	110±19	3.8±0.29
II	7	56 ± 1	0.55±0.02	1.5±0.03	0.53±0.01	0.85±0.07	0.17±0.12	0.9± 0.03	n.a.	150±14	1.4±0.07
II	15	47 ± 1	0.89±0.03	1.3±0.02	0.52 ± 0.01	0.89±0.15	0.22±0.04	0.97 ±0.06	n.a.	97±0.3	1.8±0.15

^f Values are expressed as mean ± standard deviation. Depending on the equivalent electrical circuit (EEC) used, parameters that are not detected are reported as 'n.d.', whereas parameters that are not applicable are indicated as 'n.a.'.

The temporal evolution of the resistive contributions for each condition is shown in Figure 6.20. The total resistance (R_{tot}) values obtained from electrochemical impedance spectroscopy (EIS) corroborate the corrosion trends observed via linear polarization resistance (LPR). Throughout the experiment, the biotic and boiled conditions exhibit the lowest corrosion resistances, indicative of more aggressive corrosion. By the end of the test, their R_{tot} values are approximately one order of magnitude lower than those measured in the autoclaved and sterile conditions ($\sim 10^2$ vs. $\sim 10^3 \Omega \cdot \text{cm}^2$), reflecting the persistent influence of microbial activity. This divergence becomes particularly pronounced from day 7 onward, highlighting the increasing role of biological factors over time. The film resistance (R_f) is the dominant contributor to R_{tot} and aligns with the trends observed previously. In contrast, the charge transfer resistance (R_{ct}) exhibits opposing behavior depending on the condition: it increases over time in the sterile and autoclaved environments, whereas it sharply decreases in the biotic and boiled conditions, suggesting that microbial activity enhances electron transfer, likely via redox-active metabolites that accelerate electrochemical corrosion.

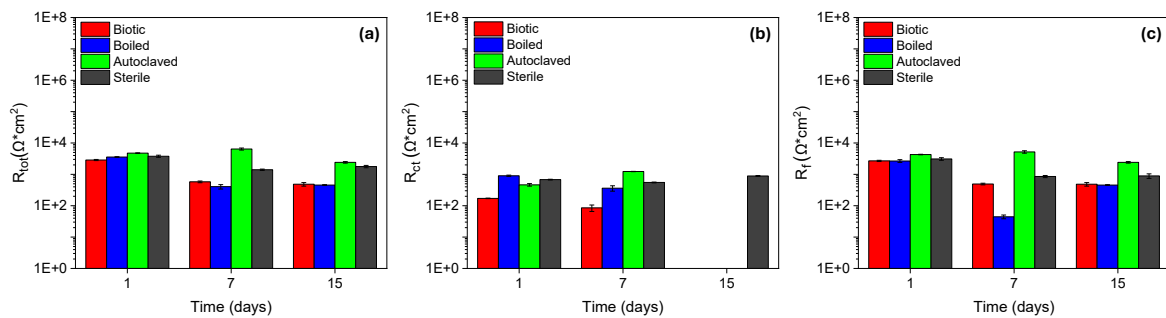


Figure 6.20. Evolution of the resistive contributions for each condition and for each time point: a) $R_{\text{tot}} = R_{\text{ct}} + R_f$; b) R_{ct} ; c) R_f .

6.5 Corrosion mechanisms

Data obtained through a multifaceted experimental approach, encompassing electrochemical monitoring, surface characterization, solution chemistry analysis, and microbial community profiling, provide clear evidence that bacterial and archaeal activity exerts a pronounced yet distinctive influence on copper corrosion across the different experimental conditions examined. The results suggest that the abundant bacterial populations detected in both biotic and boiled systems likely contribute to the initial acceleration of the corrosion process while simultaneously delaying the onset of mineral-mediated stabilization. This behavior partially counteracts the archaeal-mediated corrosion inhibition mechanisms observed under autoclaved conditions.

Collectively, the findings reveal a dynamic interplay between microbiologically influenced corrosion (MIC) and microbiologically influenced corrosion inhibition (MICI). Active microbial populations and their metabolic byproducts initially promote copper dissolution through anodic reactions coupled with cathodic anaerobic processes, including proton reduction and nitrate or sulfate reduction. Over time, biofilm-mediated phosphate precipitation leads to the formation of mineral-rich surface layers that progressively reduce the corrosion rate. Conversely, abiotic systems lack this self-regulating biological mechanism, resulting in the development of less protective and inherently unstable passive films.

6.5.1 Chloride-driven pitting versus biofilm-assisted phosphate mineralization

The distinct electrochemical trends observed for each coupon throughout the 15-day exposure period provide valuable mechanistic insight into the different corrosion pathways associated with each test condition. Under biotic conditions, the free corrosion potential initially exhibited negative values (approximately -0.10 V vs. Ag/AgCl sat.) and progressively shifted toward more noble potentials after 7-8 days of exposure. This electrochemical evolution reflects an early phase dominated by active anodic copper dissolution, driven by microbially mediated interfacial reactions and the production of acidic metabolic byproducts, followed by the gradual formation of a protective layer composed of complex phosphate and chloride compounds that partially stabilizes the electrochemical response (Eqs. 6.2-6.5). The boiled condition exhibited a similarly delayed ennoblement trend comparable to that of the biotic condition, indicating that thermal treatment did not completely suppress corrosion-relevant microbial activity. Instead, heat treatment appears to have selected for thermotolerant microbial populations capable of sustaining analogous interfacial processes. In contrast, both autoclaved and sterile systems displayed early potential ennoblement, consistent with the rapid chemical formation of oxide layers on the copper surface. The sterile condition, unlike the others, subsequently experienced potential drops indicative of early film destabilization and a transition toward localized corrosion that was more pronounced than in the microbially active systems.

Electrochemical impedance spectroscopy (EIS) measurements further elucidate these trends. Biotic and boiled samples initially displayed low impedance responses that progressively evolved toward diffusion- and film-controlled electrochemical behavior, consistent with the development of thick, porous corrosion product layers. Autoclaved samples exhibited comparatively higher and more stable impedance values throughout the exposure period, reflecting the formation of more

compact, oxide-dominated patinas. The sterile condition, while initially resembling the autoclaved system, evolved toward a heterogeneous electrochemical response characterized by multiple time constants. This behavior is consistent with the sequential formation and localized breakdown of stratified corrosion products and the progression of localized corrosion attack.

Collectively, these electrochemical results demonstrate that microbial presence does not merely increase or decrease overall corrosion rates but fundamentally alters the mechanistic pathway through which corrosion evolves. This interpretation was further confirmed through complementary surface analysis techniques, including optical microscopy, scanning electron microscopy (SEM), and Raman spectroscopy.

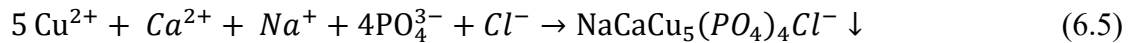
The corrosion patinas formed on biotic and boiled samples were compositionally similar, consisting primarily of mixed copper-calcium-sodium phosphates, including $\text{NaCaCu}_5(\text{PO}_4)_4\text{Cl}$ and $\text{Cu}_2\text{PO}_4(\text{OH})$. The formation of these crystalline phases can be rationalized through the progression of the anodic half-reaction (Eq. 6.2)



In the absence of insoluble and uniform Cu^+ precipitates, this reaction is followed by the disproportionation of cuprous ions (Eq. 6.3):



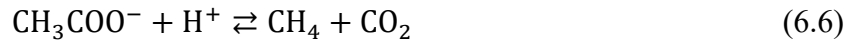
Subsequently, the generated cupric ions participate in the precipitation of phosphate-based compounds (Eqs. 6.4-6.5):



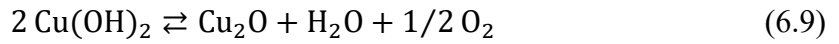
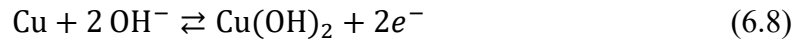
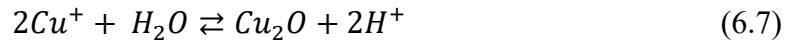
The substantial phosphorus depletion observed in both biotic and boiled conditions (ranging from 100 to 140 mg L⁻¹) provides quantitative confirmation of the formation of these phosphate-based corrosion products.

Microbial community analysis revealed that biotic and boiled samples exhibited no major compositional differences in the microbial communities present in either the biofilm or the

solution phase following the exposure period. These findings suggest that the presence of a rich and compositionally comparable bacterial population, including a notable abundance of the *Tepidiphilus* genus, likely sustained similar metabolic activities that favored the formation of analogous corrosion products under both conditions. In these systems, microbial metabolism appears to play a dual functional role: initial metabolic activity, including acetogenesis and formate oxidation, accelerates copper dissolution during early exposure stages, while subsequent biofilm-mediated phosphate precipitation contributes to a progressive decrease in corrosion rates. The accumulation of acetate (132.2 mg L^{-1}) and the concurrent consumption of formate in the biotic system provide evidence for active syntrophic and methanogenic metabolic pathways (Eq. 6.6).



In contrast to the biotic and boiled coupons, the copper surface exposed under autoclaved conditions appears to have been dominated by Cu_2O formation proceeding through purely abiotic chemical pathways Eqs. 6.2, 6.3, and Eqs. 6.7-6.9.



Notably, the Cu_2O layer forms a dense diffusion barrier that effectively restricts oxygen transport to the underlying metallic substrate, thereby preventing secondary oxidation to CuO . The presence of thermoresistant microorganisms and the notable abundance of *Methanobacterium* archaea in the autoclaved system highlights the preservation of strict anaerobic conditions at the metal-biofilm interface, which were better maintained than in the sterile control. Indeed, surface examination of the sterile coupon revealed a distinctive layer of green-blue deposits composed of hydrated CuO . Surface inspection of the sterile samples revealed clear evidence of pitting corrosion (Figs. 6.10 and 6.1d), indicating that the passive oxide layer formed under abiotic conditions is inherently unstable in the chloride-rich inoculum environment.

The high concentration of Cl^- ions likely promote localized film breakdown and subsequent transformation to CuCl_2 (Eq. 6.10), which undermines the protective character of the CuO layer and enhances pit propagation:



These conditions are consistent with surface acidification occurring in conjunction with CuO formation (Eq. 6.11). ICP-AES analysis revealed that the highest copper release occurred in the sterile control system (7.1 mg L^{-1}), underscoring the limited protective capacity of purely chemical $\text{Cu}(\text{OH})_2/\text{CuO}$ films in the absence of biofilm-mediated mineralization.

6.5.2 Bacterial-driven corrosion enhancement versus archaeal-mediated corrosion stabilization

Microbial community analysis revealed a strong correlation between community composition and the observed copper corrosion behavior. Comparative analysis of bacterial and archaeal abundances across the different experimental conditions suggests that copper corrosion in these systems results from a dynamic interplay between bacterial-driven corrosion enhancement and archaeal-mediated surface stabilization. During the early stages of exposure, abundant and metabolically active bacterial populations likely dominate the corrosion response by sustaining low open circuit potential (OCP) values, reduced polarization resistance, and enhanced rates of copper dissolution. This interpretation is supported by the observed acetate accumulation and formate depletion, which reflect active bacterial and syntrophic metabolism capable of modifying local pH, redox conditions, and copper speciation at the metal-biofilm interface. Such metabolic processes are expected to delay the immediate precipitation of protective copper oxide minerals by maintaining dissolved copper species in solution and shifting interfacial chemical equilibria away from mineral supersaturation conditions.

As the exposure period progresses, however, archaeal populations-particularly methanogenic archaea enriched within the biofilm matrix-appear to assume a progressively more significant role in controlling the corrosion pathway. Archaeal-dominated biofilms are characteristically associated with the stabilization of interfacial redox conditions, consumption of intermediate metabolites (such as formate and molecular hydrogen), and the development of structurally

persistent extracellular polymeric matrices possessing high metal-binding capacity. These features collectively favor the local accumulation and immobilization of Cu^{2+} ions, thereby promoting the precipitation of phosphate-rich corrosion products and facilitating the transition toward the diffusion-controlled electrochemical behavior observed in EIS measurements. Thus, while bacterial metabolic activity enhances corrosion kinetics during initial exposure, it does not preclude the subsequent establishment of archaeal-driven patina stabilization.

This mechanistic interpretation is further corroborated by microbial community profiling data. Despite different initial thermal treatments, biotic and boiled systems converged toward remarkably similar bacterial and archaeal biofilm compositions, consistent with their similar corrosion product morphologies and electrochemical signatures. In contrast, autoclaving resulted in a substantially simplified microbial community dominated by resilient thermophilic archaeal taxa, correlating with a distinct corrosion phenotype characterized by oxide-dominated, comparatively more protective surface films. Notably, the sterile system, which lacked both bacterial and archaeal biofilms, exhibited the most severe copper release and the most extensive localized corrosion damage. This observation underscores that abiotic chloride-driven corrosion can exceed the severity of biotic corrosion when biologically mediated copper immobilization pathways are absent.

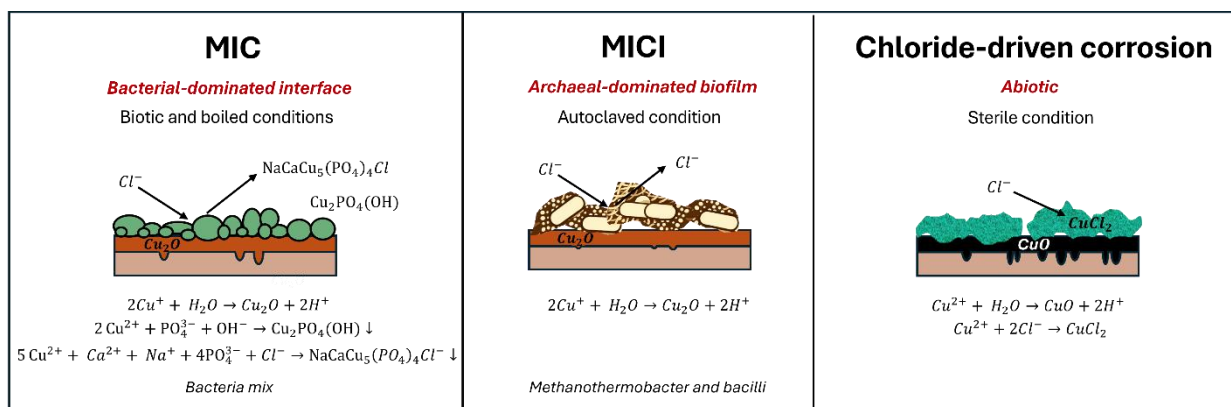


Figure 6.21. Mechanistic schematic of thermophilic bacteria and archaea impact on copper: bacterial activity is primarily associated with electrochemical MIC and acid production, whereas archaeal-dominated biofilms promote chemically mediated mineralization that leads to MIC inhibition (MICI). The sterile condition, dominated by a stronger impact of chlorides, is also reported.

Overall, these results demonstrate that copper corrosion in the analyzed systems is governed by a thermally dependent transition controlled by microbial functional balance, which determines

whether the corrosion process proceeds via microbiologically influenced corrosion (MIC) or microbiologically influenced corrosion inhibition (MICI). A metabolically active bacterial population sustains early-stage copper dissolution, whereas archaeal-dominated biofilms subsequently redirect the corrosion pathway toward mineralized, diffusion-limited mechanisms that mitigate localized attack, as illustrated schematically in Figure 6.21. This nuanced microbial interaction highlights the critical importance of considering both bacterial and archaeal contributions to biofilm temporal evolution when assessing copper material durability in warm, anaerobic, chloride-containing environments.

6.6 Concluding remarks

This study demonstrates that standard autoclaving procedures (121 °C, 20 min, 15 psi/1 bar) do not achieve complete sterilization of methanation-derived inocula, as substantial microbial activity persists even under hyperthermophilic conditions. Rather than functioning as true sterilization protocols, thermal treatments primarily serve as community-selective procedures that preferentially enrich thermotolerant bacterial and archaeal populations capable of forming biofilms and interacting distinctively with copper surfaces.

The abundant bacterial populations characterizing both biotic and boiled systems sustained an electrochemically active copper interface throughout the exposure period. The development of a thick yet inhomogeneous phosphate-rich surface layer, evidenced by phosphorus depletion in solution and copper immobilization via mineral precipitation, drove the progressive evolution of corrosion kinetics toward diffusion- and film-controlled mechanisms.

Thermotolerant methanogenic archaea demonstrated the ability to survive autoclaving conditions, subsequently promoting the formation of a more stable, oxide-dominated patina that conferred enhanced corrosion resistance. Conversely, sterile abiotic conditions facilitated the formation of stratified copper oxide/chloride corrosion products, exhibited pronounced localized corrosion attack, and resulted in the highest measured copper release into solution. These findings indicate that purely abiotic, chloride-driven corrosion processes can be substantially more aggressive than biotic corrosion when biofilm-mediated mineralization pathways are absent.

Collectively, these results reveal a thermally dependent transition from microbiologically influenced corrosion (MIC) toward mineralization-mediated corrosion stabilization (exhibiting MICI-like behavior) in systems hosting metabolically active microbial communities. Within this conceptual framework, bacterial metabolic activity primarily governs early-stage corrosion

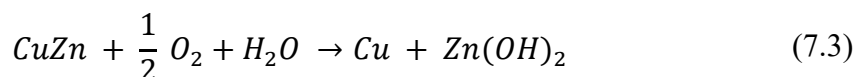
kinetics, whereas archaeal-dominated biofilms exert control over corrosion morphology and long-term surface stability by redirecting dissolved copper species toward precipitation as low-solubility mineral phases. Although the inoculum employed in this investigation originated from a methanation plant and was therefore primarily enriched in methanogenic microorganisms, the mechanistic insights obtained are broadly relevant to any warm, anoxic, chloride-containing environment. Such environments include deep marine hydrothermal settings, geothermal systems, and various engineered infrastructures operating under comparable physico-chemical conditions. Understanding these dynamic MIC-MICI transitions is essential for accurately predicting copper material durability and for developing effective corrosion mitigation strategies in microbially active environments.

Chapter 7

7 Microbiologically Influenced Corrosion on Brass

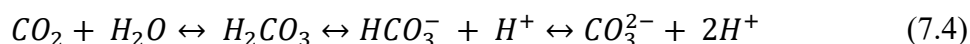
Brasses (Cu-Zn alloys) are widely employed in biogas plant components due to their presumed corrosion resistance and antimicrobial properties.^{269–273} Among copper-based alloys, duplex brasses ($\alpha+\beta$ phase) containing approximately 40 wt. % Zn, such as OT60, are widely employed in biogas and methanation plant components due to their excellent combination of mechanical strength, machinability, and corrosion resistance under normal operating conditions.²⁷⁴ These alloys are commonly used in piping, fittings, and valves, particularly in biogas upgrading and gas-cleaning sections, where they are exposed to aggressive compounds such as hydrogen sulfide and organic acids generated during anaerobic digestion.^{275,276} Despite their antimicrobial behavior, brasses can undergo severe forms of microbially influenced corrosion (MIC), as extensively reported in seawater condenser bundles and other industrial systems.^{277–279} Anaerobic digesters represent a cornerstone technology for organic waste management and biogas production, providing a sustainable route for energy recovery.^{280–282} These systems rely on the metabolic activity of complex microbial consortia, mainly belonging to the Bacteria and Archaea domains, which thrive under oxygen-depleted conditions, nutrient-rich substrates, and controlled temperatures. Digestate and sludge from anaerobic reactors are characterized by high organic content and elevated microbial abundance and diversity.^{273,283–287} Depending on the metabolic pathways activated within biofilms and on the nature of the metabolic by-products released into solution, brass corrosion may be either accelerated or mitigated under both aerobic and anaerobic conditions.^{239,267} Recent studies have demonstrated that nutrient availability, particularly carbon sources, critically affects microbial corrosion behavior and can shift microbial activity between aggressive and protective modes.²⁸⁸ In methanation systems, microbial communities typically include both mesophilic and thermophilic microorganisms, whose metabolic versatility enables survival under extreme environmental conditions.^{18,289} Variations in pH, redox potential, and availability of electron donors and acceptors selectively promote different functional groups, including acetogens, acidogens, syntrophic bacteria, and spore-forming Bacilli, which can either enhance or inhibit corrosion processes.^{193,290} Through localized acidification or alkalization, as well as through the release of organic ligands capable of complexing or reprecipitating Cu and Zn species, these microorganisms significantly modulate the electrochemical and chemical conditions at the metal/solution interface. Under purely abiotic conditions, brass corrosion is

generally controlled by the redox behavior of copper and zinc, coupled with the formation of hydroxides, oxides, or basic salts (Eqs. 7.1-7.2).



Dezincification represents the most common degradation mechanism, leading to the formation of porous, Cu-rich layers (Eq. 7.3). In sulfide-containing environments, mixed copper and zinc sulfides may precipitate, whereas in phosphate-rich solutions the formation of Cu- and Zn-phosphate phases can result in partially protective surface layers.^{291,292}

In microbiologically active environments, however, metabolite-mediated MIC often prevails over purely electrochemical corrosion pathways. Fermentative microorganisms can produce organic acids such as acetate and formate, which destabilize passive films on brass surfaces. Conversely, some bacterial taxa, particularly spore-forming Bacilli, are capable of inducing localized alkalization, thereby promoting carbonate precipitation (Eq. 7.4) and inhibiting corrosion.¹⁹⁶



The resulting corrosion behavior therefore reflects a dynamic balance between acidifying and alkalizing metabolic pathways, as well as the ability of specific microbial subgroups to survive environmental stresses such as thermal treatments.²⁹³ In addition to microbial metabolism, the structure and diversity of biofilms play a key role in determining corrosion severity. Biofilms dominated by a single microbial species typically induce corrosion mechanisms that directly mirror the metabolic activity of that organism. In contrast, multispecies biofilms may exhibit synergistic interactions that enhance corrosion processes in more complex and less predictable ways. Consequently, the composition and diversity of microbial communities developing on metal surfaces are closely correlated with biofilm corrosiveness, as extensively demonstrated in marine environments.^{281,294} Moreover, highly diverse biofilms tend to be more resilient and adaptable to fluctuating environmental conditions, rendering them more difficult to control and often more aggressive toward metallic substrates.^{18,294} Reducing microbial load and diversity in aqueous systems is therefore a common strategy for mitigating MIC risk.^{288,295} Among the available approaches, targeted thermal treatments represent a simple method to control microbial

populations and selectively enrich specific functional groups.²⁹⁶ However, this strategy may be ineffective against thermophilic and hyperthermophilic microorganisms, such as spore-forming Bacilli, which are frequently encountered in biogas plants and methanation processes and are known to withstand temperatures exceeding 100 °C.^{193,290,297,298} A detailed understanding of how thermal treatments influence microbe-material interactions is thus essential for the development of effective corrosion-mitigation strategies and for ensuring the long-term durability of components used in biotechnological plants.²⁹⁵

In this context, the present study investigates the microbial corrosion behavior of OT60 duplex brass (40 wt.% Zn) exposed to a microbial community derived from the aqueous phase of a methanation pilot plant. The inoculum was thermally modified to generate four experimental conditions: i) untreated inoculum (biotic control); ii) boiled inoculum (120 min); iii) inoculum autoclaved twice at 121 °C; and iv) a sterile reference obtained by extended autoclaving followed by filtration and UV irradiation. A multi-analytical approach combining electrochemical techniques (OCP, LPR, EIS), chemical analyses (ICP-AES, IC), Raman spectroscopy, SEM–EDS surface and cross-sectional characterization, and 16S rRNA gene sequencing was employed to evaluate the influence of these thermally reshaped microbial consortia on corrosion-product formation and protectiveness, dezincification severity, film evolution, and overall brass susceptibility to MIC. Notably, the sterile reference exhibited the most severe corrosion, highlighting the unexpected yet significant protective role of thermally selected microbial communities in phosphate-rich methanation environments.

7.1 Surface and morphology investigation

Figure 7.1 presents macroscopic observations of brass surfaces after 15 days of immersion in biotic, boiled, autoclaved, and sterile inocula at 45 °C. Light optical microscopy (LOM) reveals distinct morphologies of the corrosion products formed under the four conditions. Coupons exposed to biotic and boiled media exhibit similar surface characteristics, characterized by thick, heterogeneous layers of green corrosion products. In contrast, the coupon immersed in the autoclaved solution shows a less severely corroded surface, with black deposits distributed across the exposed area. A markedly different morphology is observed for the coupon exposed to the sterile solution, which displays an inhomogeneous corrosion layer.

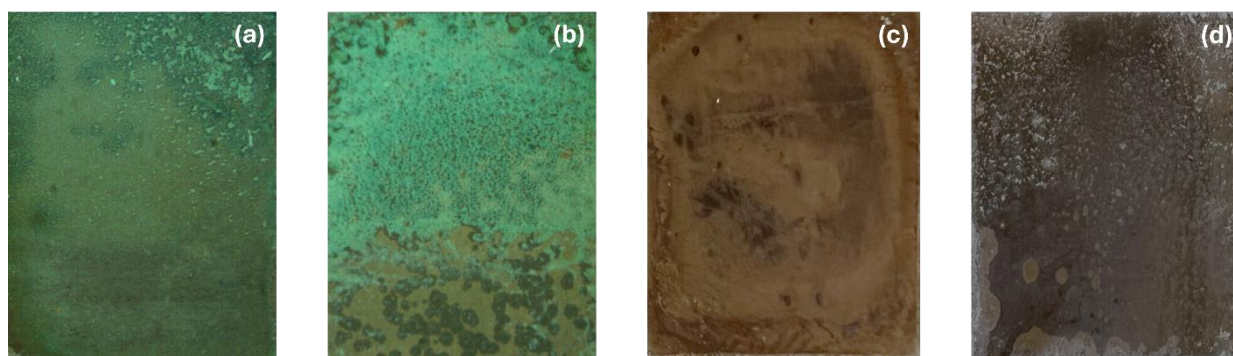


Figure 7.1. Light optical microscopy images of Cu-Zn alloy coupons after 15-day test in the (a) biotic, (b) boiled, (c) autoclaved and (d) sterile solutions at 45 °C.

Scanning electron microscopy (SEM) was employed on different surface regions to gain detailed insight into the morphology of the corrosion products formed under each experimental condition at the end of the exposure period (Fig. 7.2). Due to the heterogeneous nature of biofilm distribution and corrosion product formation typical of microbially influenced corrosion, different surface regions were examined for different analytical purposes. Figure 7.2a-d focus on documenting microbial presence and surface heterogeneity, while Figures 7.4-7.6 (EDS mapping) was acquired from regions optimized for elemental characterization of the corrosion products. Surfaces exposed to biotic and boiled inocula are characterized by an outer layer of globular secondary corrosion products, differing primarily in their size and distribution (Figs. 7.2a and 7.2b). This outer globular layer is absent on coupons exposed to autoclaved and sterile conditions (Figs. 7.2c and 7.2d).

For the biotic condition, the corrosion product matrix appears highly compact and consists of very fine particles with a low degree of crystallinity (Fig. 7.2a). In contrast, the boiled condition exhibits small precipitates decorating the globular features, resulting in a more complex surface morphology (Fig. 7.2b). In this case, both a dense underlying layer and acicular, needle-like crystalline structures are observed. Conversely, coupons immersed in the autoclaved inoculum display smoother corrosion regions that develop heterogeneously across the surface (Fig. 7.2c). Notably, the presence of residual biofilm on these coupons highlights the limited effectiveness of autoclaving alone in fully sterilizing the inocula used in this study. Finally, the sterile condition is distinguished by the formation of well-defined crystalline corrosion products interspersed with porous deposits (Fig. 7.2d).

In the biotic inoculum, bacterial activity strongly influenced the nature and evolution of corrosion products. After 15 days of exposure, SEM observations revealed a heterogeneous surface partially covered by clusters of compact, complex crystalline structures characteristic of mixed phosphate

phases (Figs. 7.2a and 7.3). Energy-dispersive spectroscopy (EDS) analyses confirmed an increased phosphorus content and revealed the coexistence of Zn-rich and Cu-rich regions, indicating chemical stratification within the corrosion layer (Fig. 7.3).

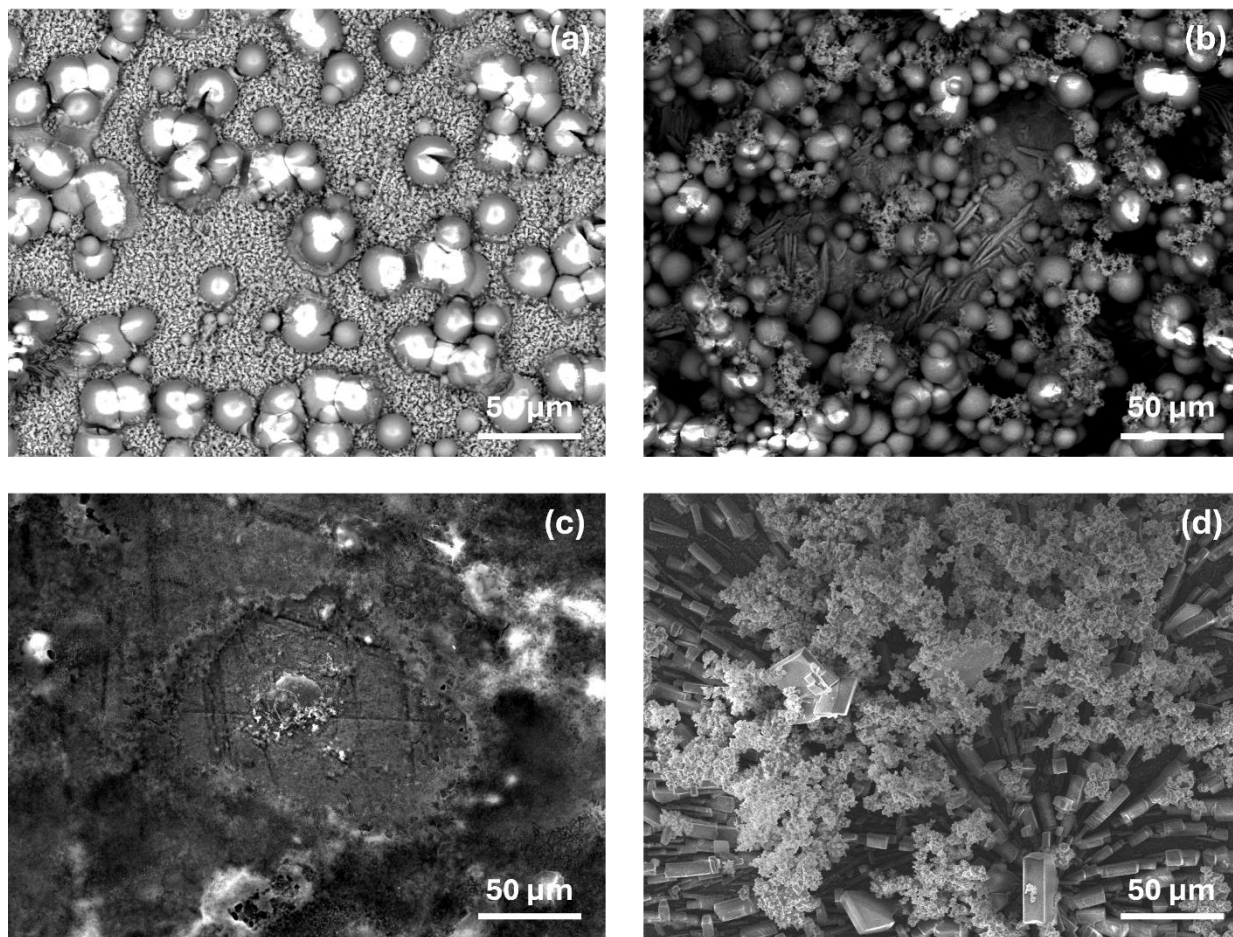


Figure 7.2. Scanning electron microscopy (SEM) images of Cu-Zn alloys coupons after 15-day test, showing the heterogeneous surface morphology in the (a) biotic, (b) boiled, (c) autoclaved and (d) sterile solutions at 45 °C.

Raman spectroscopy further identified the presence of both zinc- and copper-containing phosphate species, evidenced by the characteristic PO_4^{3-} stretching band near 945 cm^{-1} (Fig. 7.7). These results are consistent with a bilayered corrosion structure, where the larger globular features correspond to sampleite ($\text{NaCaCu}_5(\text{PO}_4)_4\text{Cl}\cdot 5\text{H}_2\text{O}$), while the smaller crystalline domains are attributed to zincolibethenite (CuZnPO_4OH). Cross-sectional analyses (Fig. 7.8) confirmed the presence of a dense inner zinc-phosphate layer overlaid by a more fragmented copper-phosphate, chlorine-containing outer layer, thereby validating the proposed stratified architecture. Notably, microstructural defects and cracks were observed within the outer layer, reflecting its mechanical instability. Such degradation is consistent with biofilm-induced acidification, where acidic

metabolic by-products likely promote partial dissolution and disruption of the outer phosphate film.

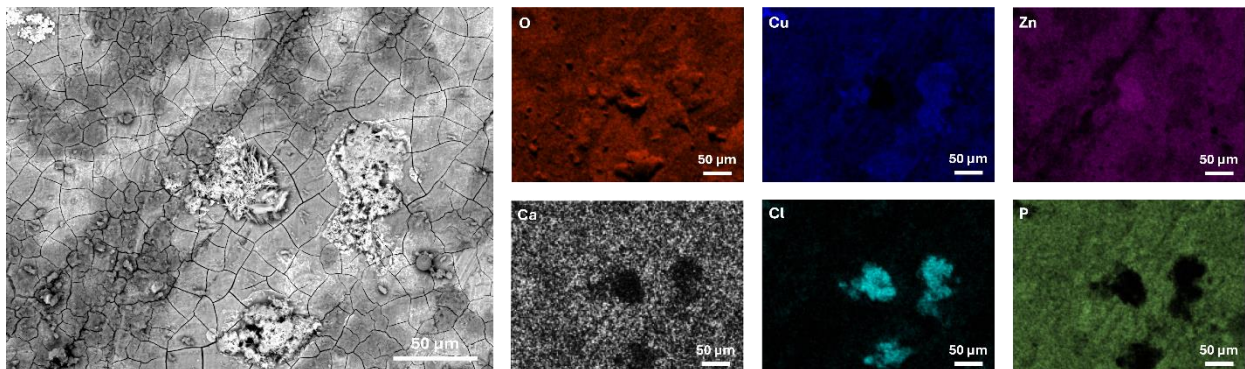


Figure 7.3. SEM-EDS images of brass coupon after 15-day test in the biotic solution at 45 °C.

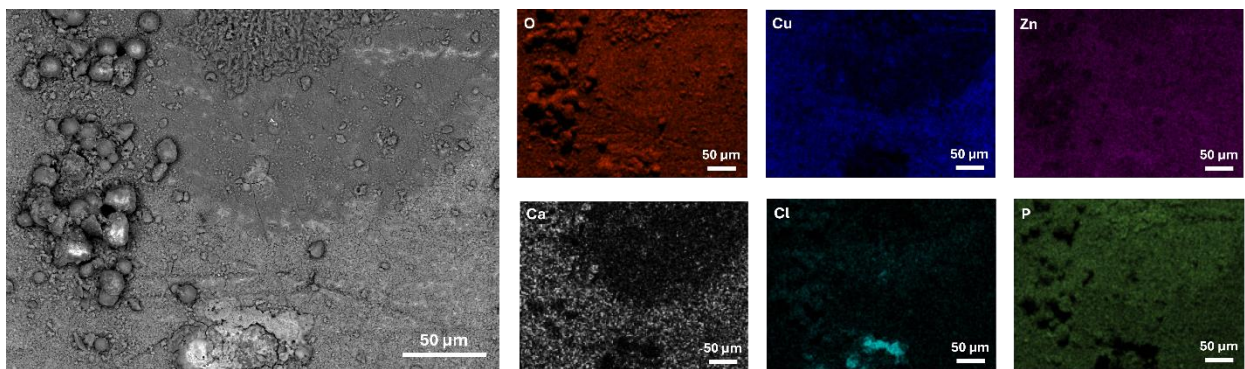


Figure 7.4. SEM-EDS images of brass coupon after 15-day test in the boiled solution at 45 °C.

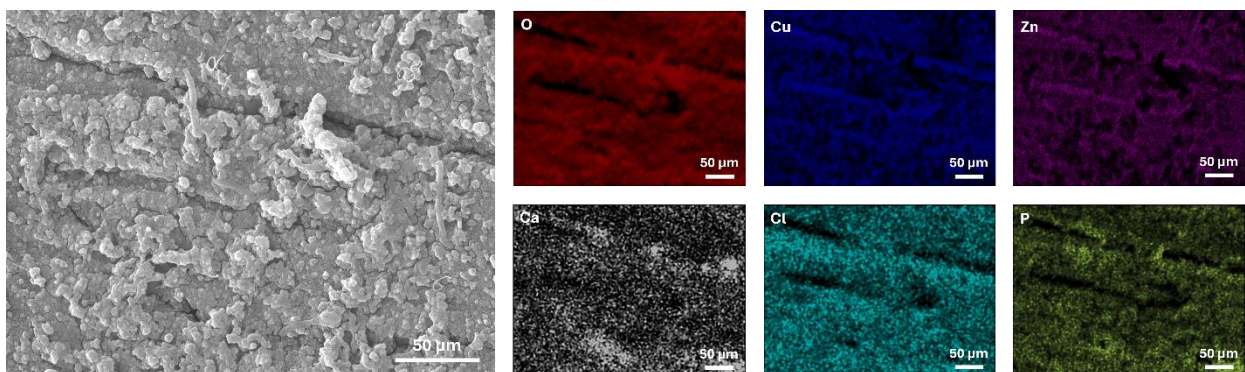


Figure 7.5. SEM-EDS images of brass coupon after 15-day test in the autoclaved solution at 45 °C.

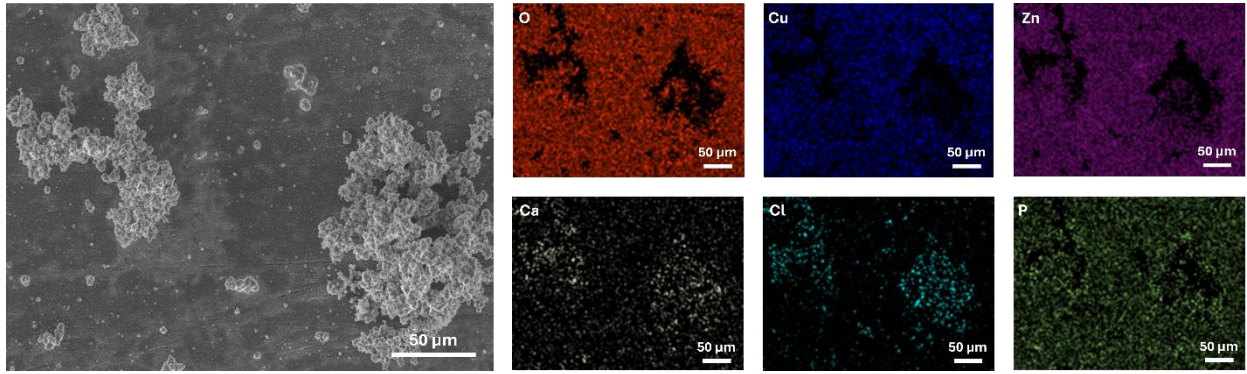


Figure 7.6. SEM-EDS images of brass coupon after 15-day test in the sterile solution at 45 °C.

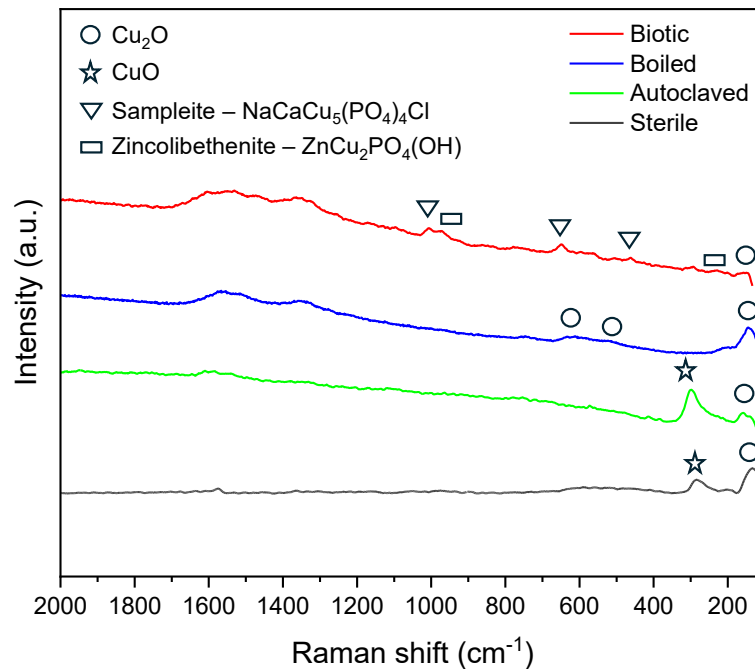


Figure 7.7. Raman spectra of brass corrosion products on the surface of the coupons after 15-day test immersed in the biotic (red), boiled (blue), autoclaved (green) and sterile (gray) solutions at 45 °C.

The boiled inoculum resulted in the formation of surface films with intermediate density and surface coverage. SEM observations revealed smoother and more coherent deposits than those formed under sterile conditions, although they remained less compact than the films observed for the biotic and autoclaved systems (Fig. 7.2b). EDS mapping (Fig. 7.4) confirmed phosphorus enrichment and a moderately increased zinc content within the corrosion layer. While both sampleite and zincolibethenite were detected on the biotic coupon, the boiled condition

predominantly exhibited plate-like, zinc-enriched crystals (Fig. 7.4), tentatively assigned to $\text{Zn}_2(\text{PO}_4)(\text{OH})$. These results suggest that microbial selection, likely associated with reduced acetogenic activity following boiling, favored the precipitation of zinc phosphate phases rather than mixed Cu-Zn phosphates. Raman spectra (Fig. 7.7) displayed predominantly Cu_2O -related bands, indicative of partially oxygenated conditions. Cross-sectional analyses (Fig. 7.9) revealed a thin but continuous phosphate layer, providing moderate corrosion protection. The absence of severe acidification combined with residual microbial activity enabled controlled film growth; however, the reduced microbial diversity hindered the development of complex, multilayered corrosion structures.

In contrast, the autoclaved inoculum produced the most protective surface film. SEM images (Fig. 7.2c) showed a dense and compact deposit exhibiting granular and globular morphologies characteristic of CaCO_3 biomineralization. EDS maps (Fig. 7.5) revealed strong calcium enrichment at the surface, accompanied by oxygen signals consistent with carbonate formation. Raman spectra (Fig. 7.7) exhibited distinct Cu_2O and CuO features. Cross-sectional observations (Fig. 7.10) revealed a well-defined, thick bilayer structure, consisting of an inner compact $\text{Cu}_2\text{O}/\text{CuO}$ layer adherent to the brass substrate and an outer calcium carbonate layer forming a continuous barrier to ionic transport.

Under sterile conditions, the brass surface was covered by a discontinuous layer of fine crystalline particles interspersed with large regions of exposed alloy. SEM images (Fig. 7.2d) revealed a non-compact, heterogeneous film composed of scattered agglomerates consistent with copper oxides and a phosphorus-rich phase tentatively attributed to sampleite. EDS analyses (Fig. 7.6) supported this interpretation, showing elevated phosphorus content along with significant zinc depletion from the alloy surface. Raman spectra (Fig. 7.7) displayed only Cu_2O and CuO signals. Cross-sectional analysis (Fig. 7.11) revealed uniform corrosion penetration to a depth of approximately 1 μm , with no evidence of multilayered structures or dense protective deposits. The sterile condition therefore represents a purely chemical corrosion regime dominated by direct alloy dissolution and the formation of fragile, non-protective surface films.

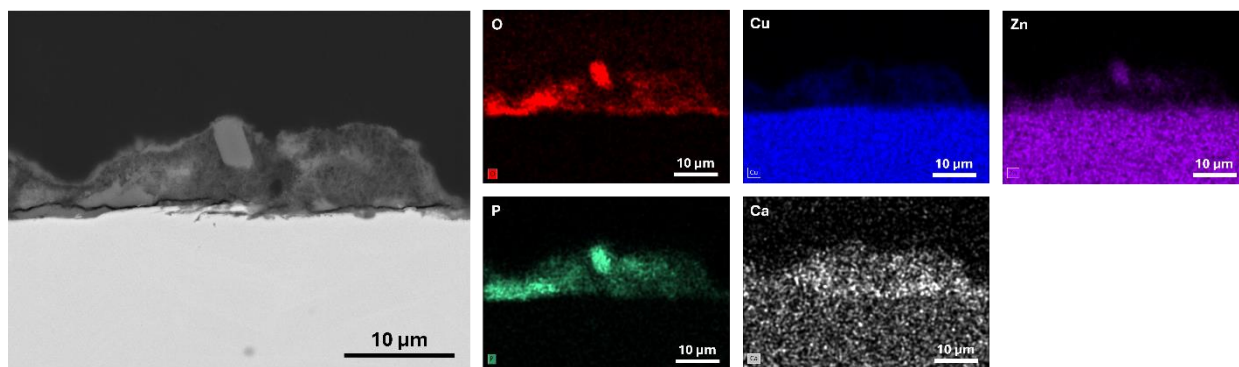


Figure 7.8. SEM-EDS images of brass cross section after 15-day test in the biotic solution at 45 °C.

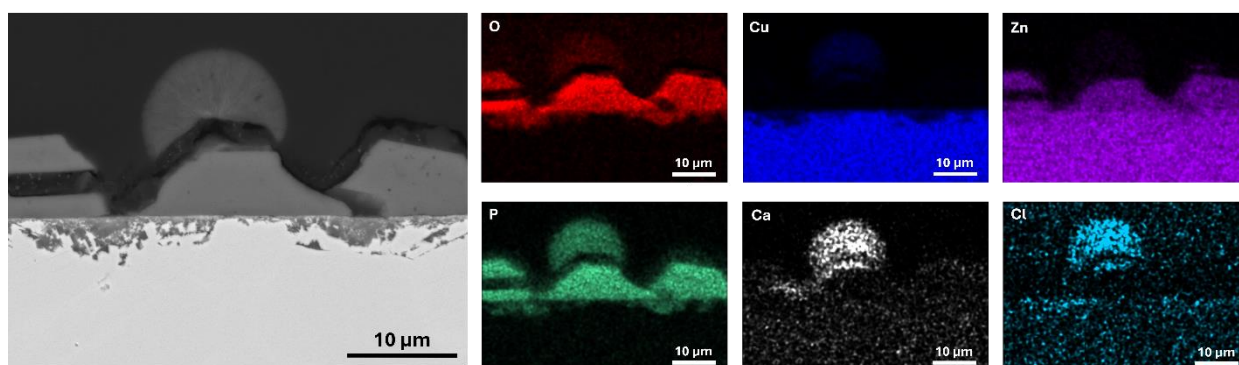


Figure 7.9. SEM-EDS images of brass cross section after 15-day test in the boiled solution at 45 °C.

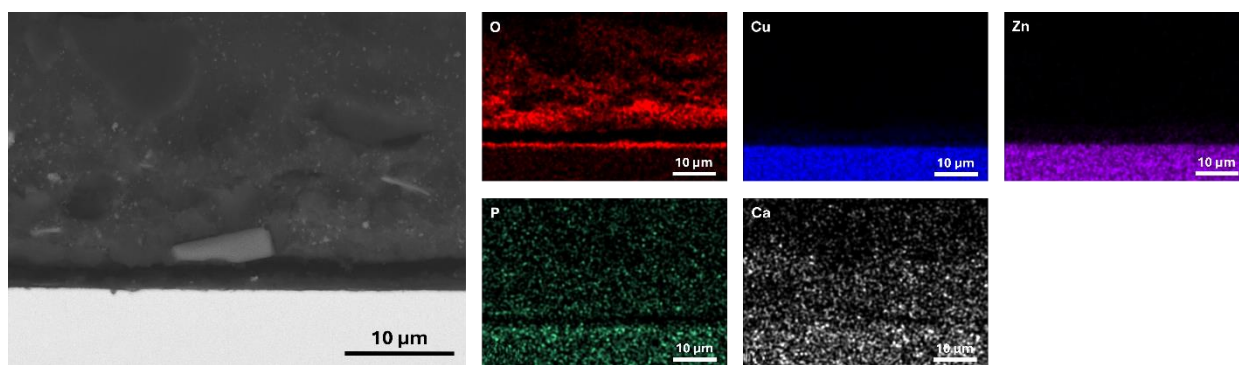


Figure 7.10. SEM-EDS images of brass cross section after 15-day test in the autoclaved solution at 45 °C.

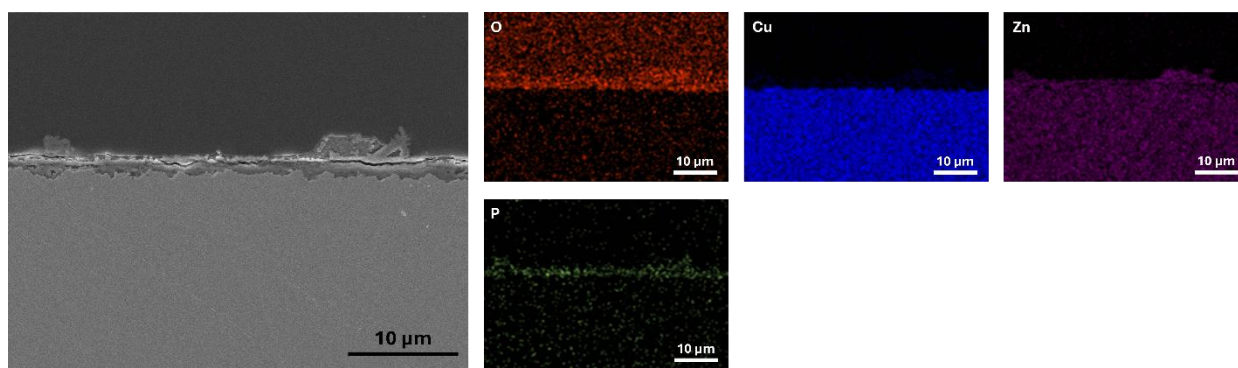


Figure 7.11. SEM-EDS images of brass cross section after 15-day test in the sterile solution at 45 °C.

7.2 Solution analysis

Solution chemistry was analyzed after the tests, revealing only minor pH variations relative to the initial value (pH 6.96). As the medium was buffered, significant pH changes were not expected. At the end of the experiment, the pH values were 7.11 for the biotic condition, 6.89 for the boiled condition, 7.23 for the autoclaved condition, and 6.80 for the sterile condition.

Ion chromatography (IC) and inductively coupled plasma-atomic emission spectroscopy (ICP-AES) were employed to assess the enrichment of potential microbial metabolites in solution and to investigate the nature of precipitates formed on the metal surfaces. Concentrations of acetates, formates, oxalates, and carbonates were measured both before and after exposure (Table 7.1). The results indicate that acetate production occurred exclusively in the biotic system, whereas acetate was completely consumed in the boiled system. Both the biotic and boiled conditions exhibited consumption of formates, while oxalates remained at high concentrations across all conditions. In contrast, the sterile system showed a decrease in oxalate concentration by the end of the test, which may be attributed to chemical consumption through complexation or precipitation with corrosion products, or to metal-catalyzed oxidation reactions.

The autoclaved solution exhibited a significantly higher carbonate concentration, which decreased by approximately 47% over the course of the experiment. Acetate was only partially consumed (c.a., 20%) under this condition, suggesting possible carbonate deposition on the metal surface induced by local pH shifts at the metal-solution interface. Overall, the IC data demonstrated that each inoculum treatment resulted in a distinct evolution of solution chemistry, with the sterile system exhibiting the fewest active transformations and the autoclaved condition showing the most pronounced changes, particularly in carbonate speciation.

Table 7.1. Concentrations of anions detected in the biotic, boiled, autoclaved and sterile inocula after 15-day test, determined by ion chromatography (IC). Results are reported in the ionic form detected by the instrument.

Analyte		Concentration ^a (mg L ⁻¹)			
		Experimental condition			
		Biotic	Boiled	Autoclaved	Sterile
Oxalate	$C_2O_4^{2-}$	2490 ± 3.5	2225 ± 4.3	2722 ± 6.2	896 ± 3.6
Formate	$HCOO^-$	<LOD	<LOD	<LOD	n.d.
Acetate	CH_3COO^-	5.1 ± 0.76	<LOD	<LOD	n.d.
Propionate	$CH_3CH_2COO^-$	<LOD	<LOD	<LOD	n.d.
Carbonates	CO_3^{2-}	196 ± 0.21	112 ± 0.13	506 ± 0.19	n.d.

^a Values are expressed as mean ± standard deviation (n = 2). Concentrations below the limit of detection (LOD = 0.05 mg L⁻¹) are indicated as “< LOD”, while analytes not determined are indicated as “n.d.” in the Table.

The concentrations of dissolved metals after 15 days (Table 7.2) further highlight the differences among the four systems. The sterile solution exhibited some of the highest concentrations of dissolved Cu and Zn, resulting in the highest Cu/Zn ratio (7.25) among all conditions. This indicates a pronounced preferential release of copper from the alloy into solution.

The biotic and boiled inocula displayed intermediate concentrations of dissolved Cu and Zn. In both cases, copper was released to a greater extent than zinc; however, the Cu/Zn ratios differed markedly between the two treatments (1.43 for the biotic condition and 3.84 for the boiled condition), reflecting differences in corrosion and precipitation mechanisms.

The autoclaved condition showed the lowest concentrations of both metals in solution. Its Cu/Zn ratio (0.79), the lowest among all systems, indicates minimal overall metal release, with a slight predominance of zinc dissolution over copper.

Phosphorus concentrations were higher in the biotic and boiled solutions, while significantly lower values were measured in the autoclaved and sterile media. Sulfur consumption was observed exclusively in the biotic system, with no detectable changes in the other three inocula.

Taken together, the ICP results indicate that the sterile and biotic conditions led to the greatest extent of metal release into solution, characterized by the highest copper release under sterile conditions and the highest zinc release under biotic conditions, whereas the autoclaved treatment resulted in the lowest degree of alloy dissolution.

Table 7.2. Total elemental concentration of inorganic elements detected in the biotic, boiled, autoclaved and sterile inocula after 15-day test, determined by inductively coupled plasma - atomic emission spectroscopy (ICP-AES).

Element		Concentration ^b (mg L ⁻¹)			
		Experimental condition			
		Biotic	Boiled	Autoclaved	Sterile
Chromium	Cr	n.d.	n.d.	n.d.	n.d.
Iron	Fe	<LOD	<LOD	0.16 ± 0.03	n.d.
Nichel	Ni	0.10 ± 0.03	0.12 ± 0.01	0.18 ± 0.02	n.d.
Copper	Cu	1.5 ± 0.13	2.1 ± 0.15	0.50 ± 0.03	5.8 ± 0.11
Zinc	Zn	1.1 ± 0.11	0.53 ± 0.08	0.18 ± 0.04	0.8 ± 0.03
Sulfur	S	3.5 ± 0.10	3.8 ± 0.11	4.6 ± 0.15	6.2 ± 0.15
Phosphorous	P	419 ± 1.1	355 ± 0.99	619 ± 1.7	120 ± 0.78

^b Values are expressed as mean ± standard deviation (n = 2). Concentrations below the limit of detection (LOD = 0.05 mg L⁻¹) are indicated as “< LOD”, while analytes not determined are indicated as “n.d.” in the Table.

7.3 Characterization of the bacterial community

16S rRNA gene sequencing was performed using the Illumina platform to identify the bacterial genera present in the inoculum solutions before and after the electrochemical tests, as well as to quantify the 16S rRNA gene copy numbers per unit volume in the biofilm formed after the experiments. Thermal treatments applied to the inocula (i.e., boiling and autoclaving) significantly reduced microbial abundance but were not sufficient to achieve complete sterilization. Complete sterility of the inoculum was achieved only by combining prolonged autoclaving with filtration and UV treatment. Sterility was confirmed by the absence of bacterial growth on LD agar plates (Figure S.7.1, Supplementary Information).

Quantitative PCR (qPCR) analysis (Figure S.7.2, Supplementary Information) demonstrated that thermal treatments substantially decreased the 16S rRNA gene copy numbers in the inocula. The autoclaved sample exhibited the lowest bacterial load (4.1×10^4 gene copies mL⁻¹), compared to the boiled inoculum (8.6×10^{10} gene copies mL⁻¹) and the untreated biotic control (7.1×10^{12} gene copies mL⁻¹).

Figure 7.12 presents the relative abundance (%) of bacterial genera detected in the inocula prior to the corrosion tests for the untreated (biotic), boiled, and autoclaved conditions. The biotic inoculum was characterized by bacterial genera associated with acetogenic and formate-consuming metabolic pathways, including *Lentilactobacillus* and *Alcaligenes*, the latter being known to oxidize formate to CO₂.^{299,300} The boiled inoculum showed a marked enrichment in *Chryseobacterium* (22.6%), a genus previously reported as an eco-friendly corrosion inhibitor in acidic environments.²⁰⁹ In contrast, the autoclaved inoculum exhibited a higher relative abundance of spore-forming and thermotolerant *Bacilli*, including *Effusibacillus*, *Caldalkalibacillus*, and *Brevibacillus*.

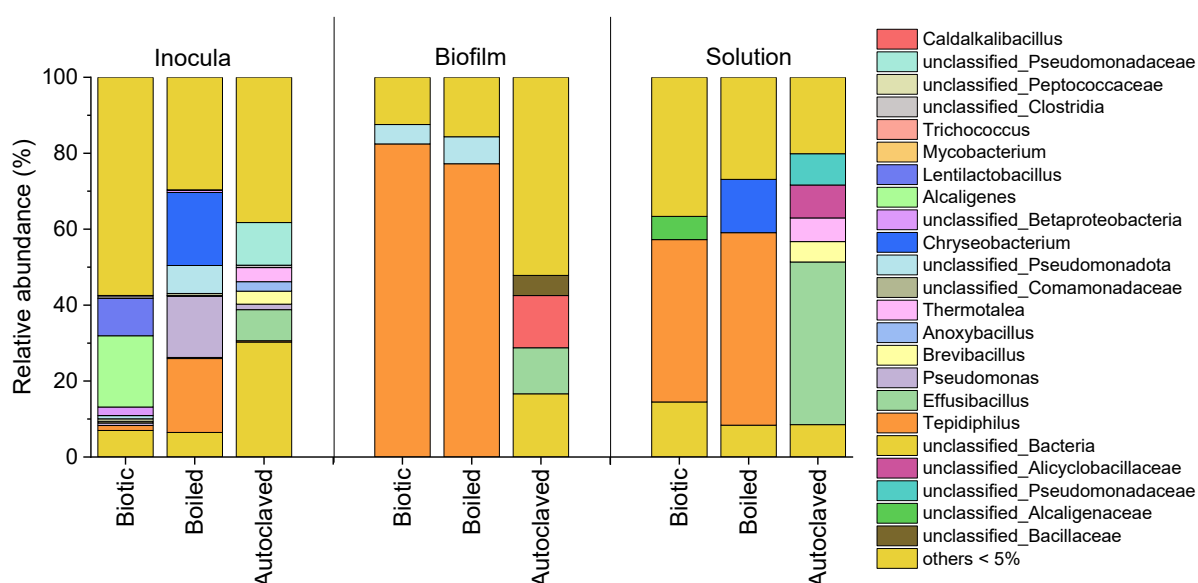


Figure 7.12. Bar plot showing the relative abundances of bacterial genera in the inocula prior the test, and in the biofilm and in solution after the test. The chart presents results for the untreated (biotic), boiled, and autoclaved conditions.

The microbial communities detected in the biofilms formed on the metal coupons and in the corresponding solutions after the 15-day exposure are presented in Figure 7.12. In both the biotic and boiled conditions, the biofilms were dominated by the genus *Tepidiphilus*, whereas the autoclaved condition exhibited a more diverse microbial consortium, with a predominance of *Bacilli*, mainly *Effusibacillus* and *Caldalkalibacillus*. A similar trend was observed in the bulk solutions at the end of the test: *Tepidiphilus* was the most abundant genus in the biotic and boiled solutions, while *Effusibacillus*, together with other *Bacilli* such as *Brevibacillus*, dominated the autoclaved solution.

Tepidiphilus is a Gram-negative, non-sporulating, moderately thermophilic genus capable of growth under anaerobic conditions in the presence of nitrate. Members of this genus can degrade a wide range of organic compounds, producing volatile fatty acids as metabolic byproducts.³⁰¹ *Effusibacillus* is a spore-forming, thermophilic, and acidophilic bacterium.²¹⁰ *Brevibacillus* is an endospore-forming genus that includes aerobic and facultative anaerobic thermophilic strains.³⁰² *Caldalkalibacillus* is an alkaliphilic and thermophilic genus capable of forming single terminal endospores.³⁰³ Notably, several Bacilli are known to possess oxalate-degrading enzymes, enabling them to convert oxalate into CO₂, formate, or other intermediate products. This metabolic capability may contribute to the distinct chemical environments and corrosion behaviors observed under the autoclaved condition.

To assess microbial diversity, the amplicon sequence variant (ASV) table for inoculum samples was rarefied to 3238 sequences. This resulted in 86 ASVs for the autoclaved condition, 77 for the boiled condition, and 87 for the biotic condition. For biofilm samples, rarefaction was performed at 800 sequences, yielding 53 ASVs in the autoclaved condition and 21 ASVs in both the boiled and biotic conditions. For solution samples, rarefaction at 686 sequences resulted in 27 ASVs for the autoclaved condition, 68 for the boiled condition, and 62 for the biotic condition. The reduced microbial load induced by the sterilization treatments led to lower DNA extraction yields. As a consequence, the number of ASVs in the rarefied datasets does not fully reflect the actual bacterial diversity, and the rarefaction curves do not reach a plateau, particularly for samples with total sequence counts below 1000.

Cluster analysis performed at the ASV level showed that samples from the autoclaved condition clustered distinctly from those of the boiled and biotic conditions (Fig. 7.13), whereas no clear separation was observed between the boiled and biotic samples. This indicates that autoclaving significantly altered the bacterial community structure, consistent with the enrichment of spore-forming taxa such as members of the family *Bacillaceae*. In addition, the analysis revealed that both the biotic solution and the biofilm formed during the experiment differed markedly from the initial biotic inoculum. In contrast, the bacterial community composition of the biofilms formed under biotic and boiled conditions was highly similar.

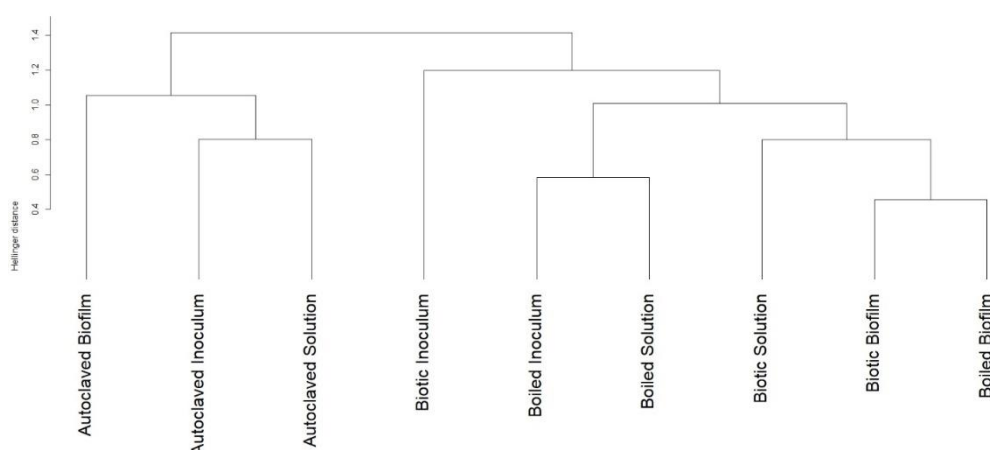


Figure 7.13. Cluster analysis of the bacterial genera on the Hellinger transformed ASV table showing all samples.

Representative ASVs affiliated with the genera *Tepidiphilus*, *Caldalkalibacillus*, *Brevibacillus*, and *Effusibacillus* were subjected to BLAST analysis against the NCBI database to better characterize their taxonomic identity. *Tepidiphilus* was represented by ten ASVs, nine of which contained fewer than 50 sequences, while one dominant ASV comprised 13639 sequences. BLAST analysis of this dominant ASV identified *Tepidiphilus baoligensis* strain B18-69 as the closest match, with 99% sequence identity. *Caldalkalibacillus*, detected in the autoclaved biofilm at the end of the test, was represented by five ASVs; the most abundant contained 841 sequences, while the remaining ASVs had fewer than 411 sequences. The dominant ASV showed 99% identity with *Caldalkalibacillus thermarum* strain HA6. *Brevibacillus*, which was primarily enriched in the autoclaved solution at the end of the experiment, was represented by three ASVs. The most abundant ASV contained 1443 sequences, while the others had fewer than 86 sequences. BLAST analysis of the dominant ASV revealed 100% identity and an E-value of 9×10^{-132} for the first 19 matches, all affiliated with the genus *Brevibacillus*, with *Brevibacillus reuszeri* strain NBRC 15719 as the top hit. *Effusibacillus* was represented by a single ASV comprising 4,295 sequences; BLAST analysis yielded a maximum identity of 96.05% with an E-value of 9×10^{-111} . The top eight matches corresponded to uncultured bacterial clones, while the ninth match was *Alicyclobacillus pohliae*. Finally, BLAST analysis was performed on the two most abundant ASVs assigned to the family *Bacillaceae* but not classified at the genus level. One of these ASVs showed 100% identity and an E-value of 1×10^{-128} across 96 matches, with the top hits corresponding to *Bacillus halodurans* isolate LB 35 and *Bacillus* sp. AP2.

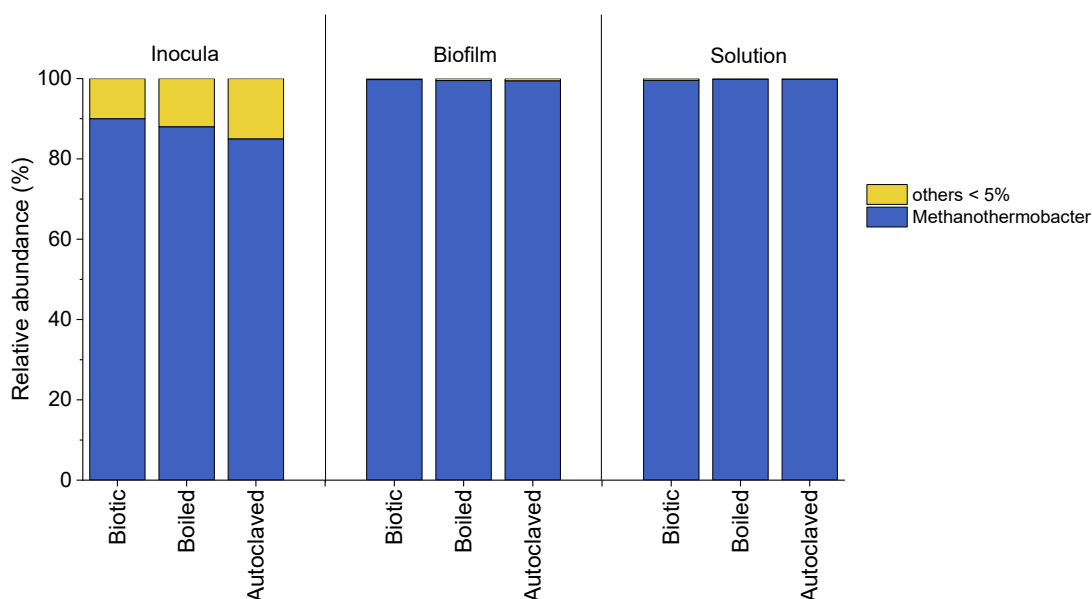


Figure 7.14. Bar plot showing the relative abundances of Archaea genera in the inocula prior the test, and in the biofilm and in solution after the test. The chart presents results for the untreated (biotic), boiled, and autoclaved conditions.

Figure 7.14 shows the relative abundance at the genus level of archaeal communities before and after the exposure tests for each experimental condition. In all cases, both prior to and following the exposure period (i.e., inocula and bulk solution), the communities are dominated by *Methanothermobacter*, a group of thermophilic hydrogenotrophic methanogens.²⁶³ This observation indicates a high resilience of this genus to the different experimental treatments and suggests that the archaeal composition in solution largely reflects that of the initial inoculum. The same results are observed in the biofilm in all the experimental conditions tested, with *Methanothermobacter* dominating.

7.4 Electrochemical characterization

Figure 7.15 shows the evolution of the average open-circuit potential (OCP, E vs Ag/AgCl sat.) of brass coupons during 15 days of immersion under four conditions: untreated biomethanation solution (biotic), boiled, autoclaved, and sterile. Each value represents the mean of two independent experiments, and error bars correspond to the standard deviation, demonstrating good reproducibility. Although the coupons were exposed to four distinct environments, the OCP profiles exhibit clear and systematic differences that can be correlated with variations in the early formation and long-term stability of surface films. In all systems, a slight ennoblement was

observed within the first 24-48 h, consistent with the initial precipitation of corrosion products on the brass surface. Beyond this initial stage, however, the OCP behavior diverged markedly depending on the treatment applied.

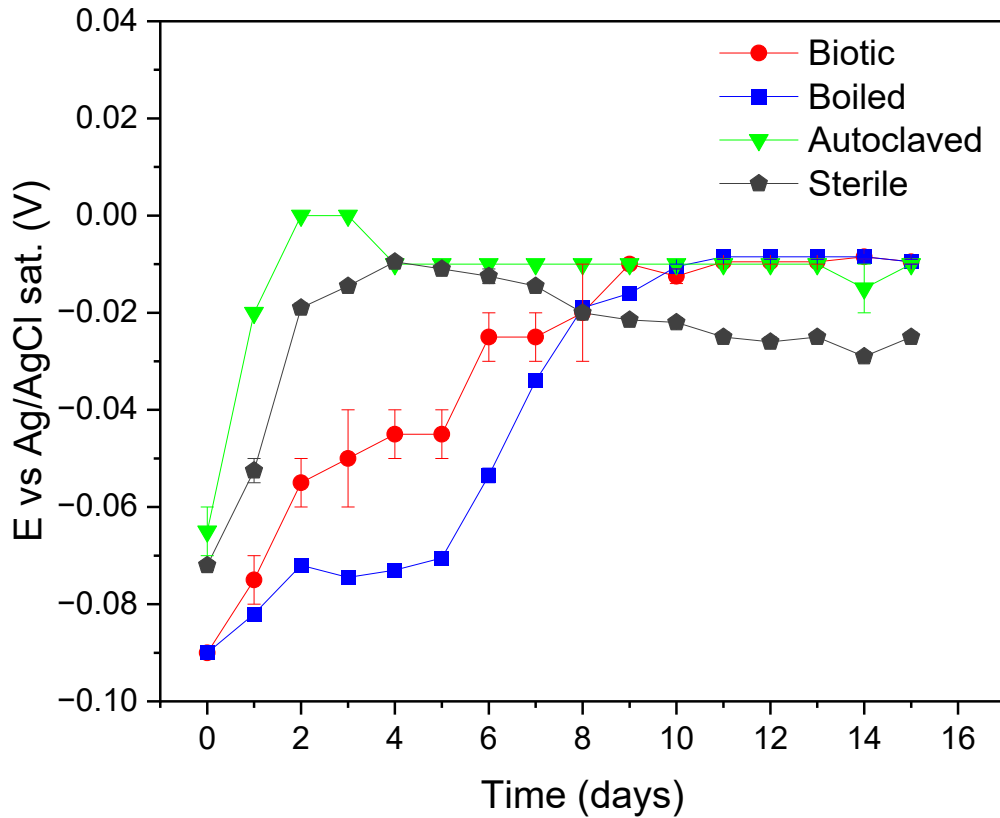


Figure 7.15. Open circuit potential (average and standard deviation, E vs Ag/AgCl sat.) of brass coupons over the entire test period (15 days). Biotic (red solid line), boiled (blue solid line), autoclaved (green solid line) and sterile condition (gray solid line).

In the biotic, boiled, and autoclaved solutions, the OCP progressively increased and stabilized between -0.075 V and -0.050 V vs Ag/AgCl (sat.), indicating the development of increasingly protective surface layers. In the biotic system, the relatively lower OCP values recorded during the first 6-7 days are consistent with an initial selective dezincification process driven by acidogenic microbial activity. The subsequent shift toward more positive and stable potentials is associated with the redeposition and maturation of corrosion products, a process that is strongly influenced by biofilm formation and metabolic activity.

A faster ennoblement was observed in both boiled and autoclaved inocula. This behavior can be attributed to the rapid precipitation of Zn-rich phosphate phases and the preferential formation of protective copper oxides and/or hydroxides on the brass surface. The slight differences between the boiled and autoclaved conditions may reflect the presence of distinct microbial populations surviving the boiling treatment, which can locally affect phosphate precipitation and film compactness. In contrast, the sterile solution exhibited a markedly different trend. After a rapid ennoblement during the first two days, the OCP gradually decreased throughout the remainder of the experiment, reaching the most negative values among all conditions at day 15. This monotonic decline suggests that, in the absence of biofilm-mediated processes and under oxygen-depleted conditions, the surface film remains poorly protective and increasingly porous, thereby allowing sustained anodic dissolution of the brass alloy.

Linear polarization resistance (LPR) measurements were performed after 1 and 15 days of exposure (Figs. 7.16 and S.7.3, Supplementary Information) by polarizing the electrodes in a narrow potential range around the open-circuit potential (OCP). The polarization resistance (R_p) was determined from the slope of the potential (E) versus current density (I) response. According to the Stern–Geary relationship,³⁰⁴ R_p is inversely proportional to the corrosion current density (J_{corr}) under activation-controlled conditions, where charge-transfer kinetics govern the corrosion rate.²³⁸

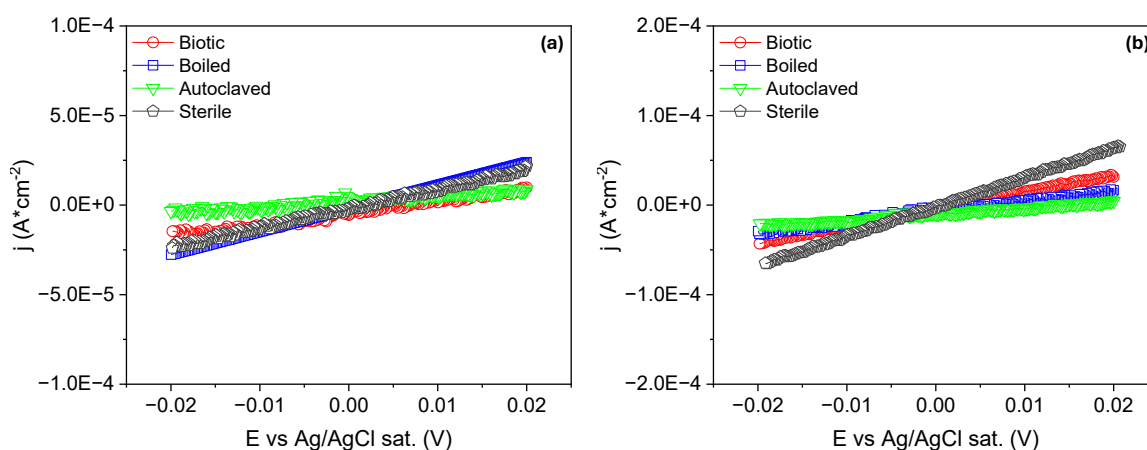


Figure 7.16. Linear polarization resistances of Cu-Zn alloy coupons and replicates tested in the biotic (red), boiled (blue) and autoclaved (gray) inocula after (a) 1 day and (b) 15 days.

In the present system, however, corrosion kinetics are likely influenced not only by charge transfer but also by diffusion-related processes associated with biofilm development and the formation of

thick corrosion product layers. As a result, conventional Tafel extrapolation cannot be reliably used to quantitatively estimate J_{corr} . Nevertheless, the R_p values provide a meaningful qualitative comparison of the corrosion behavior under the different experimental conditions. As summarized in Table 7.3, the distinct R_p trends highlight the markedly different electrochemical responses of the four systems.

Table 7.3. R_p values obtained from LPR measurements at the beginning and end of the test (1 and 15 days, respectively) for each experimental condition (biotic, boiled, autoclaved and sterile). Values are reported as mean $\pm$ standard deviation.

Time days	Biotic $\text{k}\Omega\cdot\text{cm}^2$	Boiled $\text{k}\Omega\cdot\text{cm}^2$	Autoclaved $\text{k}\Omega\cdot\text{cm}^2$	Sterile $\text{k}\Omega\cdot\text{cm}^2$
1	2.0 ± 0.50	1.2 ± 0.25	2.8 ± 0.67	1.2 ± 0.42
15	0.79 ± 0.27	1.2 ± 0.16	1.2 ± 0.37	0.52 ± 0.067

At the early stage of exposure (day 1), all samples exhibited comparable polarization resistance values of approximately $10^3 \Omega\cdot\text{cm}^2$, with the boiled and sterile conditions showing the lowest R_p , indicative of limited initial surface protection. After 15 days, the electrochemical behavior diverged markedly among the systems. The sterile and biotic coupons displayed the lowest R_p values, suggesting enhanced corrosion activity. Conversely, coupons exposed to the boiled and autoclaved conditions showed a significant increase in R_p , consistent with the formation of more protective surface layers.

The decrease in R_p observed in the sterile system, in agreement with the OCP evolution, indicates that in the absence of microbial activity and under oxygen-limited conditions, corrosion proceeds through the formation of a thin and unstable corrosion product layer, likely composed of oxides, phosphates, and chlorides. In contrast, the increase in R_p in the autoclaved system reflects the development of a thick and compact carbonate-rich deposit. The boiled condition exhibits an intermediate behavior, attributed to the formation of Zn-rich phosphate layers that provide partial surface protection.

Electrochemical impedance spectroscopy (EIS) measurements were performed after 1, 7, and 15 days of exposure. The results are presented as Bode and Nyquist plots (Fig. 7.17). At day 1, the biotic and autoclaved conditions exhibited the highest impedance values, indicating a more resistive initial electrochemical interface. In the biotic system, the impedance magnitude increased further and reached a maximum after 7 days, suggesting the temporary formation of a protective

layer or the influence of microbial metabolic activity, such as the adsorption of metabolites on the metal surface. This behavior was followed by a pronounced decrease at day 15, indicative of layer destabilization and/or enhanced corrosion processes at longer exposure times.

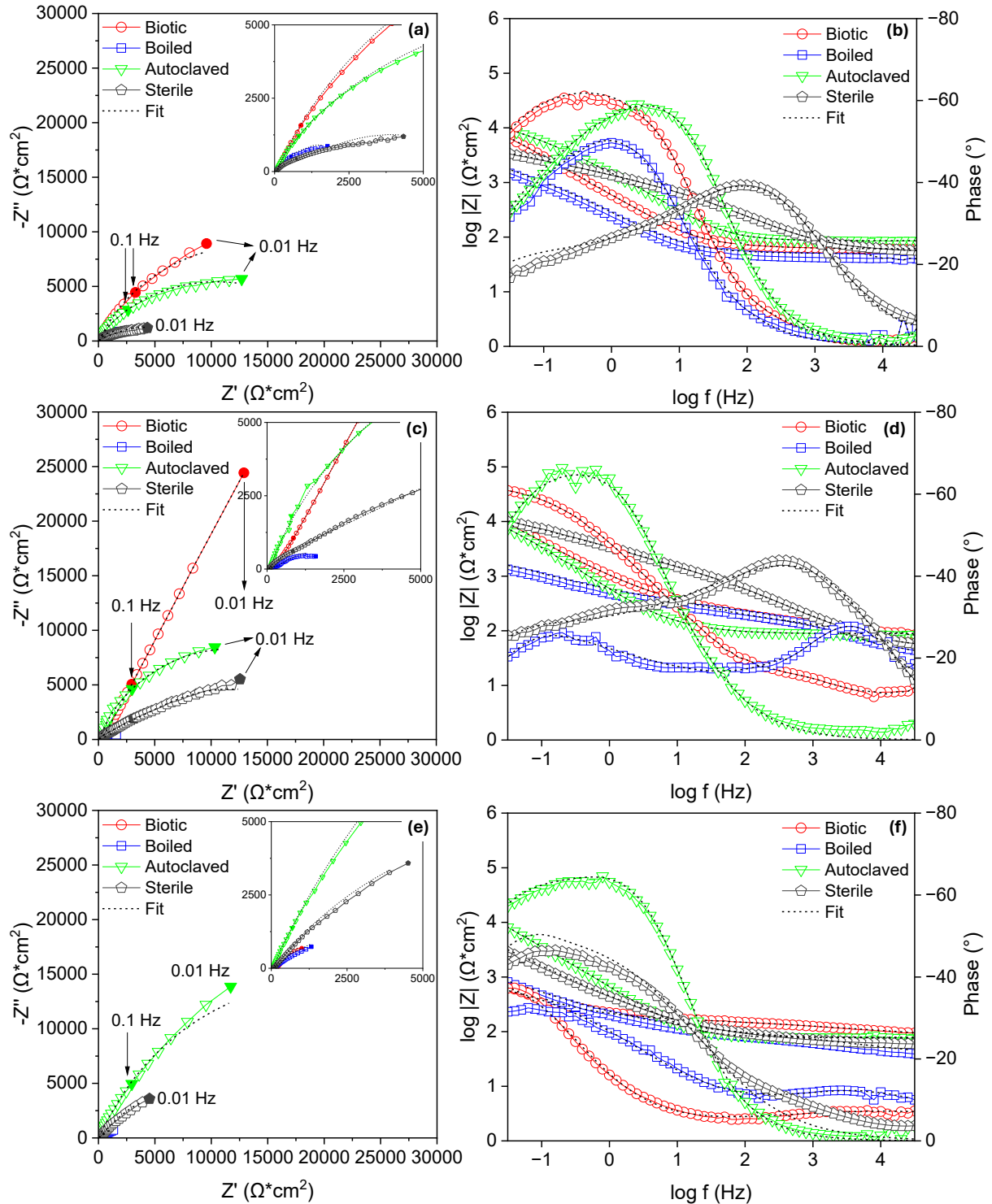


Figure 7.17. Nyquist and bode plots of brass coupons tested in the biotic (red), boiled (blue), autoclaved (green) and sterile (gray) inocula after (a-b) 1 day, (c-d) 7 days and (e-f) 15 days.

In contrast, the autoclaved and boiled conditions showed a progressive increase in impedance throughout the entire immersion period. Notably, the autoclaved samples exhibited the highest impedance at the end of the test (day 15), consistent with the development of a stable and protective surface film. The sterile control displayed a similar trend, maintaining relatively high impedance values at the final time point when compared to the biotic and boiled systems.

Different equivalent electrical circuit (EEC I-IV) models were employed to fit the EIS experimental data (Fig. 7.18). The selection of appropriate equivalent electrical circuits (EECs) for each condition was based on the physical processes occurring at the brass/solution interface, following established principles of impedance interpretation.^{305,306} Different EECs were required because thermal treatment-induced microbial selection produced fundamentally different corrosion mechanisms and surface film structures, as confirmed by cross-sectional SEM-EDS analysis (Figs. 7.8-7.11). Each circuit element was assigned based on: i) characteristic frequency ranges and impedance magnitudes; ii) validation of CPE assignments using Brug's equation (Eq. 7.6) to ensure C_{eff} values consistent with double-layer or film capacitance; and iii) correlation with surface analysis showing the presence or absence of protective layers. The evolution of EECs over time (e.g., biotic condition using three different circuits at days 1, 7, and 15) reflects genuine mechanistic changes in the corrosion process, not arbitrary model selection. All fitting procedures yielded satisfactory agreement between experimental and simulated spectra, with a chi-square (χ^2) value on the order of 10^{-4} for each model. In all cases, the solution resistance (R_s) was included to account for the ohmic contribution of the electrolyte.

The biotic condition was fitted using three different equivalent electrical circuit (EEC) models (EEC I, II, and III), as shown in Figure 7.18 and summarized in Table 7.4, highlighting a clear evolution of the corrosion mechanism over the exposure period. At day 1 (EEC I), the electrochemical response is adequately described by a modified Randles circuit consisting of a parallel combination of a resistance and a constant phase element ($R//CPE$), coupled with a Warburg (W) diffusion element. In this configuration, the ideal capacitance is replaced by a constant phase element (CPE) to account for non-ideal interfacial behavior, whose impedance is defined as:

$$Z_{CPE} = \frac{1}{Y_0(j\omega)^n} \quad (7.5)$$

where Y_0 is the CPE magnitude, ω is the angular frequency ($2\pi\nu$), and n is a dimensionless exponent describing the deviation from ideal capacitive behavior. The value of n ranges between 0 and 1, where 0 corresponds to an ideal resistor and 1 to an ideal capacitor. Values between 0.5 and 1 are commonly associated with interfacial inhomogeneities such as surface roughness, porosity, or heterogeneity of the corrosion product layer.³⁰⁷

The assignment of the charge transfer resistance (R_{ct}) and the double-layer capacitance (C_{dl}) was performed using mathematical relations derived from Brug's equation:²⁶⁸

$$C_{dl} = C_{eff} = Q^{\frac{1}{\alpha}} \left(\frac{1}{R_s} + \frac{1}{R_{ct}} \right)^{\left(\frac{\alpha-1}{\alpha} \right)} \quad (7.6)$$

where Q and α represent the magnitude and exponent of the CPE, respectively. R_s denotes the solution resistance, while R_{ct} corresponds to the charge transfer resistance. The resulting effective capacitance values (C_{eff}) fall within the commonly accepted range for double-layer capacitance (10–100 μF).³⁰⁸

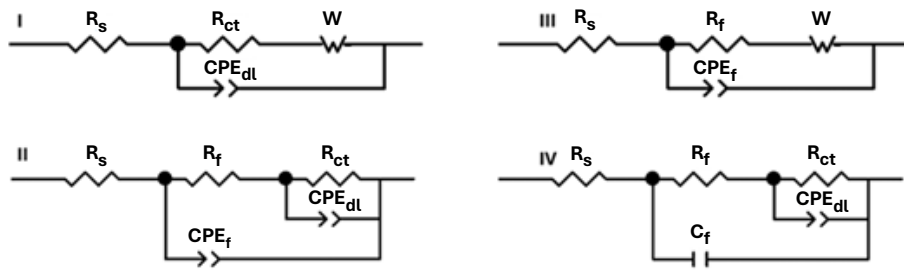


Figure 7.18. Equivalent electrical circuit (EECs) I–IV used to model impedance data.

Accordingly, in EEC I the high-frequency time constant is attributed to the $R_{ct} - C_{dl}$ pair, while the low-frequency process, represented by the Warburg element, is associated with the diffusion of ionic species (primarily Cu and Zn) toward the metal/electrolyte interface, leading to the formation of a patina of secondary corrosion products.³⁰⁶

After 7 days of exposure, the impedance spectra are better described by EEC II, characterized by two R//CPE elements. The high-frequency time constant remains associated with charge transfer and double-layer capacitance, whereas the low-frequency process reflects the electrical properties of a surface film, represented by a film resistance (R_f) and film capacitance (CPE_f). This transition indicates the formation of a dense and protective corrosion product layer, likely composed of mixed copper and zinc phosphates, as supported by surface characterization analyses.^{267,309}

At day 15, the corrosion mechanism of the biotic system is described by EEC III. In this case, the high-frequency process can no longer be attributed to R_{ct} and C_{dl} , as the effective capacitance calculated using Brug's equation yields unrealistically low values ($C_{eff} = 0.4 \mu F$). Instead, this time constant is associated with the resistive and capacitive properties of a surface-covering patina (R_f and CPE_f). The presence of a thick but porous oxidation layer partially impedes direct access of the electrolyte to the underlying metal, and the corrosion process becomes predominantly diffusion-controlled, governed by the transport of Cu and Zn ions through the corrosion product layer toward the metal/solution interface, as represented by the Warburg element (W).

The electrochemical behavior of the boiled condition was modeled using two different equivalent electrical circuits (EEC III and EEC IV), as shown in Figure 7.18 and summarized in Table 7.5. At day 1, the impedance response was best described by EEC IV, consisting of two parallel meshes ($R//CPE-R//C$). The high-frequency time constant was attributed to the charge transfer resistance (R_{ct}) and the double-layer capacitance (C_{dl}), while the low-frequency process, represented by a film resistance (R_f) and a capacitance (C_f), indicated the formation of a corrosion product layer exhibiting ideal capacitive behavior ($n = 1$).

Based on surface characterization analyses and considering the possible presence of residual oxygen in the boiled inoculum, potentially at higher levels than in the biotic system despite CO_2 purging, the corrosion products formed at early exposure times are likely attributable to copper oxides.

The impedance spectra recorded after 7 and 15 days of exposure were instead fitted using EEC III, comprising a parallel $R//CPE$ element coupled with a Warburg (W) diffusion component. In this configuration, the high-frequency process (R_f , CPE_f) is associated with the presence of a corrosion product film, identified by surface analyses as copper- and zinc-based phosphates, exhibiting a highly distributed capacitive behavior ($n = 0.54$). The Warburg element accounts for diffusion limitations of Cu and Zn ions at the metal/solution interface imposed by the phosphate-rich layer.

Table 7.4. Fit results using the EECs from Figure 7.18 for the biotic condition.

EEC	Time days	Biotic ^c									
		R_s $\Omega \cdot \text{cm}^2$	R_{ct} $\text{k}\Omega \cdot \text{cm}^2$	$\text{CPE}_{dl} Q$ $(\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$	CPE_{dl-n}	R_f $\text{k}\Omega \cdot \text{cm}^2$	$\text{CPE}_f Q$ $(\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$	CPE_{f-n}	W $\text{k}\Omega \cdot \text{cm}^2$	C_{eff} $\mu\text{F} \cdot \text{cm}^{-2}$	R_{tot} $\text{k}\Omega \cdot \text{cm}^2$
I	1	65±1	4.0±0.38	0.23±0.01	0.75±0.01	n.d.	n.d.	n.d.	8.6± 0.82	55±0.1	13±0.90
II	7	70±7	1.6±0.06	0.12±0.03	0.75±0.03	400±52	0.11±0.04	0.71±0.08	n.a.	37±5.0	410±52
III	15	69±5	n.d.	n.d.	n.d.	0.23±0.01	0.01±0.001	0.72±0.02	3.6 ±0.06	n.a.	3.8±0.06

^c Values are expressed as mean ± standard deviation. Depending on the equivalent electrical circuit (EEC) used, parameters that are not detected are reported as 'n.d.', whereas parameters that are not applicable are indicated as 'n.a.'.

Table 7.5. Fit results using the EECs from Figure 7.18 for the boiled condition.

EEC	Time days	Boiled ^d									
		R_s $\Omega \cdot \text{cm}^2$	R_{ct} $\text{k}\Omega \cdot \text{cm}^2$	$\text{CPE}_{dl} Q$ $(\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$	CPE_{dl-n}	R_f $\text{k}\Omega \cdot \text{cm}^2$	$\text{CPE}_f Q$ $(\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$	CPE_{f-n}	W $\text{k}\Omega \cdot \text{cm}^2$	C_{eff} $\mu\text{F} \cdot \text{cm}^{-2}$	R_{tot} $\text{k}\Omega \cdot \text{cm}^2$
IV	1	50±1	1.7±0.2	0.93±0.01	0.74±0.01	0.98±0.08	0.03±0.001	1.0± 0.01 ^e	n.a.	110±5	2.7±0.2
III	7	15±1	n.d	n.d	n.d	0.20±0.03	0.04±0.004	0.54±0.01	2.1±0.2	n.a	2.3±0.2
III	15	33±6	n.d	n.d	n.d	0.07±0.01	0.22±0.043	0.54±0.01	6.5±0.1	n.a	6.5±0.1

^d Values are expressed as mean ± standard deviation. Depending on the equivalent electrical circuit (EEC) used, parameters that are not detected are reported as 'n.d.', whereas parameters that are not applicable are indicated as 'n.a.'.

^epure capacitance (C).

The impedance response of the autoclaved condition was successfully fitted using a single equivalent electrical circuit (EEC II) throughout the entire exposure period. As shown in Figure 7.18 and summarized in Table 7.6, this circuit consists of two parallel R//CPE elements. The high-frequency time constant is attributed to interfacial processes, where the resistive component (R_{ct}) represents the charge transfer resistance and the capacitive element (CPE_{dl}) accounts for the non-ideal double-layer capacitance. The second R//CPE element, characterized by a film resistance (R_f) and a film constant phase element (CPE_f), is associated with the formation of a resistive surface layer during exposure.

Based on surface characterization analyses, this surface film is likely composed of copper oxides and calcium carbonates, which contribute to the enhanced corrosion resistance observed in the autoclaved system.

The impedance spectra of the sterile condition were fitted using a single equivalent electrical circuit (EEC I) throughout the entire exposure period. As illustrated in Figure 7.18 and detailed in Table 7.7, this circuit comprises a single parallel R//CPE element coupled with a Warburg (W) diffusion component. The resistive and capacitive elements are attributed to the charge transfer resistance (R_{ct}) and the non-ideal double-layer capacitance (CPE_{dl}), respectively. The Warburg element accounts for diffusion-controlled mass transport of ionic species through a corrosion product layer, likely composed of copper oxides and mixed phosphates, as evidenced by surface characterization analyses. This layer partially hinders ionic transport toward the metal/electrolyte.

Table 7.6. Fit results using the EECs from Figure 7.18 for the autoclaved condition.

Autoclaved ^e										
EEC	Time days	R _s	R _{ct}	CPE _{dl} Q	CPE _{dl-n}	R _f	CPE _f Q	CPE _{f-n}	C _{eff}	R _{tot}
		$\Omega \cdot \text{cm}^2$	$\text{k}\Omega \cdot \text{cm}^2$	$(\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$		$\text{k}\Omega \cdot \text{cm}^2$	$(\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$		$\mu\text{F} \cdot \text{cm}^{-2}$	$\text{k}\Omega \cdot \text{cm}^2$
II	1	86±1	2.2±0.35	0.12±0.003	0.83±0.01	20±1.0	1.5±0.06	0.54±0.02	44±0.1	22±1.1
II	7	89±1	0.47±0.01	0.39±0.006	0.77±0.01	23±0.3	0.31±0.06	0.95±0.04	130±2.0	24±0.40
II	15	79±1	5.0±0.15	0.31±0.03	0.84±0.01	37±2.0	1.5±0.08	0.69±0.02	150±2.0	42±2.0

^e Values are expressed as mean ± standard deviation. Depending on the equivalent electrical circuit (EEC) used, parameters that are not detected are reported as 'n.d.', whereas parameters that are not applicable are indicated as 'n.a.'.

Table 7.7. Fit results using the EECs from Figure 7.18 for the sterile condition.

Sterile ^f								
EEC	Time days	R _s	R _{ct}	CPE _{dl} Q	CPE _{dl-n}	W	C _{eff}	R _{tot}
		$\Omega \cdot \text{cm}^2$	$\text{k}\Omega \cdot \text{cm}^2$	$(\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$		$\text{k}\Omega \cdot \text{cm}^2$	$\mu\text{F} \cdot \text{cm}^{-2}$	$\text{k}\Omega \cdot \text{cm}^2$
I	1	54±1	0.37±0.11	0.05±0.005	0.68±0.01	6.4±0.36	8.0±1.0	6.8±0.37
I	7	48±1	0.45±0.18	0.02±0.003	0.67±0.01	20±7.5	10±0.2	21±7.7
I	15	76±1	0.88±0.22	0.68±0.018	0.62±0.03	13±3.2	110±52	14±3.2

^f Values are expressed as mean ± standard deviation.

Figure 7.19 illustrates the evolution of the charge transfer resistance (R_{ct}), the film resistance (R_f , which also includes the resistive contribution associated with diffusion limitations), and the total resistance (R_{tot}) for each immersion condition at the different exposure times. The total resistance obtained from EEC fitting (R_{tot}) is defined as the sum of the individual resistive contributions associated with each electrochemical process included in the model. The magnitude of the overall resistive response varies markedly among the different systems.

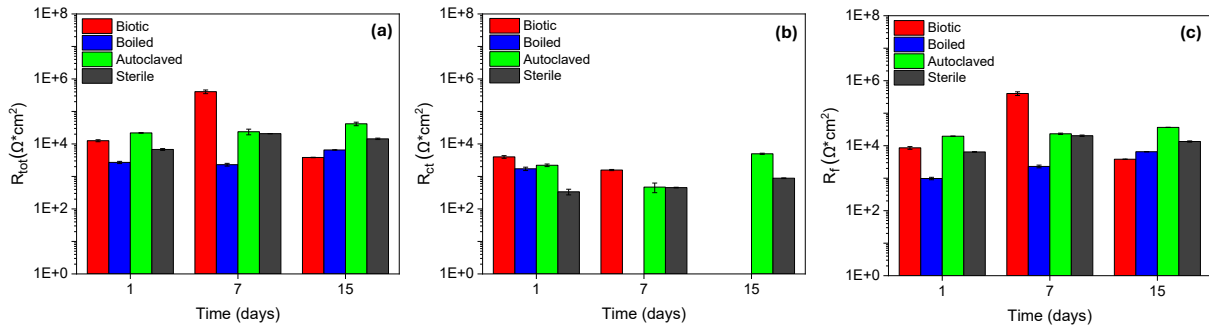


Figure 7.19. Evolution of the resistive contributions for each condition and for each time point: (a) $R_{tot} = R_{ct} + R_f$; (b) R_{ct} ; (c) R_f .

The biotic samples exhibited the highest corrosion resistance during the first seven days of exposure, with total resistance values ranging from 10⁴ to 10⁵ $\Omega \cdot \text{cm}^2$, indicating the formation of an initially thick and highly resistive layer of reprecipitated corrosion products. This protective layer is likely composed of mixed zinc and copper phosphates, which effectively inhibit corrosion processes. However, after 15 days, a pronounced decrease in resistance to approximately 10³ $\Omega \cdot \text{cm}^2$ was observed, suggesting destabilization of the protective layer. This behavior can be attributed to microbial activity at the metal surface, likely associated with acidogenic metabolic processes that compromise the integrity of the corrosion product layer.

In contrast, the boiled, autoclaved, and sterile conditions exhibited a gradual increase in corrosion resistance during the first seven days, followed by stabilization or a slight decrease on day 15. Among these, the autoclaved samples displayed the highest corrosion resistance at the end of the experiment (approximately 10⁴ $\Omega \cdot \text{cm}^2$), whereas the boiled and sterile samples showed lower values on the order of 10³ $\Omega \cdot \text{cm}^2$. The enhanced performance of the autoclaved system is likely associated with the formation of a heterogeneous but compact layer of corrosion products, predominantly carbonates, which provides effective protection to the underlying metal substrate.

The charge transfer resistance (R_{ct}) increased progressively throughout the exposure period in both the sterile and autoclaved systems, whereas a decreasing trend was observed under biotic

conditions during the first seven days. This behavior suggests that microbial activity in the biotic environment promotes electron transfer at the metal/solution interface, in agreement with previous studies.^{272,273} Across all systems, the film resistance (R_f) represents the dominant contribution to the total corrosion resistance, highlighting the critical role of the composition and electrical properties of the corrosion product patina in governing the overall corrosion behavior.

7.5 Corrosion mechanisms

The results demonstrate that thermal treatments alone, including boiling and autoclaving at 121 °C, were insufficient to achieve complete sterilization of the mixed microbial consortium. Although these treatments significantly reduced the 16S rRNA gene copy numbers and induced marked shifts in the relative abundance of specific microbial genera, they did not fully suppress microbial activity. Although 16S rRNA gene detection alone does not definitively prove bacterial viability, multiple complementary lines of evidence strongly support the presence of viable, metabolically active bacteria in the boiled and autoclaved conditions: i) selective enrichment of specific thermoresistant genera (Figs. 7.12-7.14); ii) distinct corrosion behaviors and electrochemical responses for each thermal treatment (Figs. 7.15-7.19, Tables 7.3-7.7); iii) formation of structured biofilms and intact bacterial cells on coupon surfaces (Figs. 7.2c, 7.5, 7.20a-c); iv) active metabolite production and consumption (Table 7.1); and v) distinct corrosion product chemistry driven by microbial metabolism (Figs. 7.3-7.6). The persistence of viable microorganisms under both boiling and autoclaving conditions (Fig. 7.20a–c), together with their continued interaction with the metallic substrate (Fig. 7.20c), highlights the resilience of the microbial community and its sustained potential to influence corrosion processes. These findings emphasize the necessity of applying extended autoclave cycles in combination with filtration and UV irradiation to ensure complete sterility of the biomethanation inoculum employed in this study.

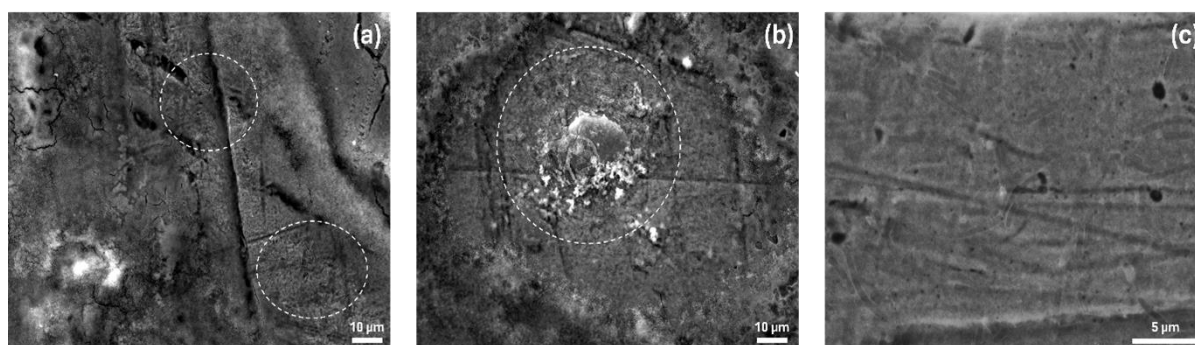


Figure 7.20. SEM micrographs showing the presence of microorganisms and biofilm on the surface of the Cu–Zn coupon after exposure to (a) boiled and (b) autoclaved inocula. (c) Microbial footprints observed on the coupon surface after biofilm removal under autoclaved conditions.

The multi-technique approach adopted herein further reveals that the corrosion behavior of OT60 brass in methanogenic media is governed by a complex interplay between electrochemical processes, solution chemistry, and the structure and activity of the microbial community shaped by thermal treatments. The four inoculum conditions (biotic, boiled, autoclaved, and sterile) resulted in distinct electrochemical responses and corrosion product assemblages, which can be rationalized by integrating experimental observations with the underlying corrosion, precipitation, and microbially mediated reaction pathways. Moreover, thermal treatments applied to the methanation inoculum are expected to induce partial microbial cell lysis, releasing intracellular compounds and, in some cases, leaving residual enzymatic activity. These lysis products can influence brass corrosion by complexing Cu and Zn ions, buffering local pH at the metal/solution interface, and affecting the nucleation and growth of corrosion-product films. In the boiled and autoclaved systems, such effects likely contributed to the formation of phosphate- and carbonate-rich layers and to the transition from charge-transfer-controlled to diffusion-limited corrosion observed by EIS. Conversely, the sterile condition, in which lysis products were largely removed, exhibited a predominantly abiotic corrosion mechanism characterized by higher metal release and poorly protective surface films.

Electrochemical measurements provide an integrated overview of corrosion behavior under the different experimental conditions. Open circuit potential (OCP) trends (Fig. 7.15) indicate that coupons exposed to biotic, boiled, and autoclaved inocula progressively stabilize within a relatively ennobled potential range (-0.075 to -0.050 V vs. Ag/AgCl). In contrast, the sterile system exhibits a continuous shift toward more negative potentials, reaching the most active value on day 15.

This behavior is consistent with linear polarization resistance (LPR) results (Table 7.3), which show that at the end of the exposure period the highest R_p values are measured for the autoclaved and boiled conditions, whereas the lowest values are observed for the biotic and sterile systems, with the sterile condition exhibiting the minimum resistance ($\sim 0.5 \times 10^3 \Omega \cdot \text{cm}^2$). These trends suggest that, although some degree of surface film formation occurs in all systems, only the autoclaved and boiled inocula promote the development of sufficiently resistive layers, while in the sterile medium the metal/electrolyte interface remains highly electrochemically active.

Electrochemical impedance spectroscopy (EIS; Fig. 7.17) provides deeper insight into these differences. Nyquist plots reveal that autoclaved samples consistently exhibit the largest impedance semicircles at all exposure times, with total resistance values (R_{tot} ; Fig. 7.19a) reaching approximately $10^4 \Omega \cdot \text{cm}^2$ at day 15. Boiled and biotic systems display intermediate behavior: in

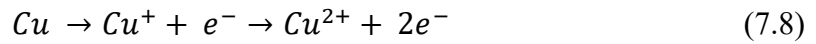
the biotic inoculum, impedance increases up to day 7 and then decreases sharply by day 15, whereas in the boiled condition R_{tot} increases more gradually and stabilizes at intermediate values. In contrast, the sterile system is characterized by the lowest impedance moduli throughout the experiment and by a persistent low-frequency diffusion tail, indicative of limited surface protection.

Equivalent electrical circuit (EEC) fitting (Tables 7.4-7.7) further corroborates these distinctions. The sterile condition is adequately described at all exposure times by a single R_{ct} - CPE_{dl} element coupled with a Warburg diffusion component (EEC I), indicating corrosion controlled by charge transfer and ionic diffusion through a thin, poorly protective layer. Conversely, the biotic, boiled, and autoclaved systems require additional film-related elements (R_{f} , CPE_{f}), reflecting the growth and evolution of surface layers with significant resistive and capacitive contributions.

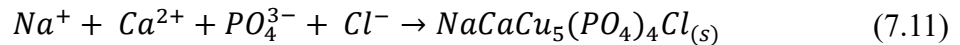
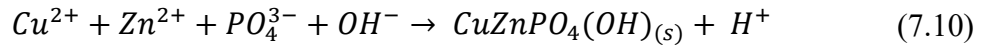
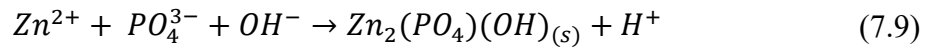
Surface and cross-sectional analyses clarify the nature of these layers (Figs. 7.3-7.6 and 7.8-7.11). Optical and SEM observations show that both biotic and boiled coupons are covered by thick, green corrosion products, characterized by an outer layer of globular deposits. In the biotic system, the underlying matrix is compact and composed of very fine, poorly crystalline products, whereas the boiled condition exhibits a more heterogeneous matrix with acicular crystals and secondary precipitates decorating the globules. The autoclaved coupon presents smoother regions and a relatively thin but dense corrosion layer with scattered dark deposits. In contrast, the sterile coupon displays well-defined crystalline features interspersed with porous areas and lacks the globular outer layer characteristic of the biotic and boiled systems.

Cross-sectional observations reveal that the biotic coupon develops a heterogeneous corrosion layer up to $\sim 10 \mu\text{m}$ thick, with localized penetration reaching $\sim 1 \mu\text{m}$. The boiled sample shows a corrosion depth of 1-5 μm and a stratified phosphate-rich layer. In the biotic system, acidic metabolites likely promote partial dissolution and destabilization of the outer phosphate film, accounting for the decrease in film resistance (R_{f}) observed at day 15 and the moderate corrosion resistance measured electrochemically. In the autoclaved condition, no visible pits are observed and a uniform, compact layer rich in Ca and O is present. Conversely, the sterile coupon undergoes relatively uniform corrosion to a depth of $\sim 1 \mu\text{m}$ and exhibits a thin, discontinuous surface layer mainly composed of O, Zn, and P. This morphology is fully consistent with the electrochemical response, including the persistently low impedance, the negative OCP shift (Fig. 7.15), and the minimal R_{p} values (Table 7.3).

Raman spectroscopy (Fig. 7.7) directly identifies the corrosion products responsible for these behaviors. In the biotic condition, the compact matrix consists predominantly of mixed Cu–Zn phosphates, such as $\text{CuZnPO}_4(\text{OH})$, while the globular outer deposits are attributed to a complex secondary phase, $\text{NaCaCu}_5(\text{PO}_4)_4\text{Cl}$. These phases suggest an initial anodic dissolution of Zn and Cu (Eqs. 7.7-7.8), followed by reprecipitation as phosphate-based corrosion products (Eqs. 7.9-7.11).

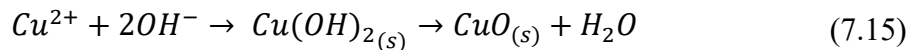
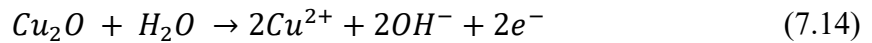
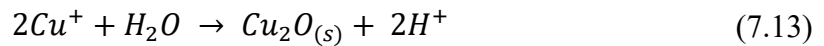


and then precipitate as phosphates (Eq. 7.9-7.11) according to reactions such as:



In the boiled inoculum, Raman spectra reveal the presence of $\text{Zn}_2(\text{PO}_4)(\text{OH})$ both as a compact inner layer and as needle-like crystals, together with globular Cu-phosphate phases similar to those observed in the biotic system. This stratified structure is clearly visible in cross sections, where an inner lamellar Zn-phosphate layer is overlain by globular Cu-phosphate deposits.

The autoclaved condition exhibits a markedly different chemistry. Raman analysis identifies Cu_2O and CuO as the dominant phases, with CuO preferentially localized within the dark surface spots. These oxides form through a sequence of copper oxidation and hydrolysis reactions (Eqs. 7.12–7.15).



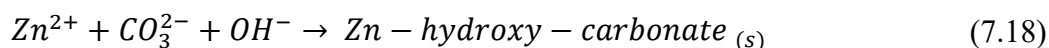
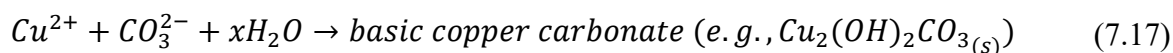
SEM-EDS further indicates that this inner copper oxide layer is covered by a Ca- and O-rich overlayer, consistent with calcium carbonate precipitation. Ion chromatography data support this interpretation, showing a high initial carbonate concentration in the autoclaved solution and an

approximately 47% decrease by the end of the experiment, consistent with CaCO_3 formation (Eq. 7.16).



This bilayer structure accounts for the high R_f and R_{tot} values measured by EIS (Fig. 7.19), as well as for the stable OCP ennoblement and the highest impedance moduli observed at day 15 (Fig. 7.17f).

The sterile condition provides a critical reference. Raman spectra show no phosphate-related bands and instead reveal Cu_2O , basic copper carbonates, and zinc hydroxycarbonates, indicating that only basic corrosion pathways are active (Eqs. 7.17-7.18).



EDS confirms the absence of P-rich compact layers, with only sparse Ca- and Mn-containing deposits detected. The corresponding electrochemical response, low R_{ct} , low R_{tot} , persistent Warburg diffusion, and progressively negative OCP, reflects the formation of a thin, porous, and non-protective film that does not significantly hinder corrosion.

Solution chemistry further supports this interpretation. The varying initial concentrations of organic acids (Table 7.1) reflect both the metabolic state of the inoculum at collection and the effects of thermal treatments on cellular lysis and metabolite stability. The high initial acetate in the boiled condition (178.1 ppm) likely results from thermal degradation of cellular components, while the low organic acid content in the sterile condition confirms extensive removal through the combined sterilization protocol. These residual organic compounds, along with CO_2 , provided limited carbon sources for microbial metabolism during the 15-day exposure period. The limited organic carbon availability in our system is consistent with recent findings showing that carbon source starvation fundamentally alters microbial metabolism and corrosion behavior.²⁸⁸ In our carbon-limited conditions, the autoclaved consortium shifted toward carbonate precipitation rather than aggressive metabolite production, demonstrating how nutrient limitation can redirect microbial activity toward protective rather than corrosive pathway. Ion chromatography shows acetate production exclusively in the biotic system and complete consumption in the boiled condition, whereas in the autoclaved system acetate is only partially consumed and remains

essentially unchanged in the sterile medium. Formate is consumed in both biotic and boiled systems but remains below detection or unchanged in the autoclaved and sterile conditions. Oxalates persist at high concentrations in all systems except the sterile one, where their decrease suggests chemical consumption through complexation with dissolved Cu^{2+} and Zn^{2+} or metal-catalyzed oxidation.

ICP analyses emphasize the contrasting roles of microbial activity and sterility. The sterile solution exhibits the highest Cu/Zn ratio (~ 7.25) and elevated concentrations of both metals, indicative of intense dezincification and copper dissolution. In the biotic and boiled systems, metal release is lower and Cu/Zn ratios are more moderate, consistent with partial reprecipitation of both cations as phosphates and oxychlorides. In the autoclaved condition, the lowest Cu and Zn concentrations and the lowest Cu/Zn ratio (~ 0.79) reflect strongly limited dissolution, in agreement with the formation of a compact Cu-oxide/Ca-carbonate bilayer.

The paradoxical observation that complete sterilization resulted in the most severe corrosion while selective microbial enrichment provided protection can be explained through fundamental differences in surface film formation kinetics and thermodynamics under biotic versus abiotic conditions.

In the sterile system, brass corrosion proceeds through classical electrochemical pathways (Eqs. 7.7-7.8, 7.12-7.15) governed by the local electrochemical potential and solution chemistry. Despite high phosphate concentration (125 ppm initial, Table 7.2), the formation of protective metal-phosphate films was kinetically limited. The pH (6.80 final) was insufficiently alkaline to drive rapid $\text{Cu}(\text{OH})_2/\text{Zn}(\text{OH})_2$ precipitation and subsequent conversion to stable oxides or phosphates. Moreover, in the absence of nucleation sites provided by bacterial cells and extracellular polymeric substances (EPS), homogeneous precipitation of protective phases in bulk solution was thermodynamically unfavorable compared to heterogeneous nucleation on the metal surface, which occurred slowly and produced only thin, discontinuous deposits.

In contrast, bacterial metabolism in the autoclaved system fundamentally altered the thermochemical environment at the metal/biofilm interface through multiple synergistic mechanisms:

i) Spore-forming genera (*Caldalkalibacillus*, *Brevibacillus*, *Effusibacillus*) are known to elevate local pH through various metabolic pathways including urea hydrolysis, nitrate reduction, and deamination reactions.^{196,197,200,310} This localized alkalinization (evidenced by final pH 7.23, highest among all conditions) shifts the carbonate equilibrium (Eq. 7.16) toward CO_3^{2-} ,

dramatically increasing the saturation index for CaCO_3 precipitation. The decrease in solution carbonate concentration (from 2762 to 1476 ppm, Table 7.1) indicates massive CaCO_3 precipitation specifically in this condition, forming the dense outer protective layer observed in cross-sections (Fig. 7.10).

ii) Bacterial cells and their EPS matrix provide high-energy heterogeneous nucleation sites for mineral precipitation. The negatively charged functional groups in EPS (carboxyl, phosphate, hydroxyl) bind Ca^{2+} and Cu^{2+} cations, locally concentrating them and reducing the activation energy barrier for crystal nucleation.¹⁹⁶ This explains the superior compactness and adhesion of the carbonate layer in the autoclaved condition compared to the poorly adherent oxides in the sterile system.

iii) The dual-layer structure (inner $\text{Cu}_2\text{O}/\text{CuO}$ + outer CaCO_3) creates a superior diffusion barrier compared to single-phase oxide films. The inner oxide layer blocks electron transfer while the outer carbonate layer impedes ionic transport, resulting in the highest total resistance ($R_{\text{tot}} = 42 \text{ k}\Omega \cdot \text{cm}^2$, Fig. 7.19a) among all conditions.

In the biotic and boiled conditions, *Tepidiphilus* and other genera promoted Cu-Zn phosphate precipitation (sampleite, zincolibethenite) through a different mechanism. Phosphate precipitation does not require alkalization but depends on direct complexation (Eq. 7.9-7.11). However, these phosphate phases proved less protective than carbonates for several reasons, such as CaCO_3 superior thermodynamical stability at neutral pH compared to mixed Cu-Zn phosphates, which are susceptible to reductive dissolution. Additionally, carbonate layers are more compact and adherent whereas phosphate layers exhibited visible cracks (Fig. 7.8). Lastly, in the biotic condition, fermentative bacteria produced organic acids (acetate production, Table 7.1) that locally acidified the interface, destabilizing phosphate films and explaining the sharp impedance decline at day 15 (Fig. 7.17).

The boiled condition, with intermediate *Tepidiphilus* dominance and reduced acidogenic activity, achieved moderate protection through Zn-phosphate layers but lacked the carbonate-precipitating Bacilli to form the most protective bilayer.

In summary, these results challenge the conventional view that microorganisms universally accelerate corrosion. In phosphate-rich, buffered methanation environments, the complete absence of bacteria (sterile condition) led to the most severe corrosion because it eliminated the biomineralization pathways responsible for protective layer formation. Specifically, the survival of spore-forming Bacilli in the autoclaved condition enabled formation of a dense CaCO_3

overlayer (Eq. 7.16) that effectively suppressed brass corrosion, achieving three times higher electrochemical resistance ($R_{\text{tot}} = 42 \text{ k}\Omega \cdot \text{cm}^2$) compared to the sterile system ($R_{\text{tot}} = 14 \text{ k}\Omega \cdot \text{cm}^2$). This demonstrates that in certain chemical environments, selective microbial enrichment can provide superior corrosion protection compared to complete sterilization, with significant implications for corrosion management strategies in methanation facilities. Thus, attempting complete sterilization may be counterproductive in phosphate-rich, buffered environments, whereas controlled thermal treatment (standard autoclaving) that selectively enriches carbonate-precipitating *Bacilli*, while eliminating acidogenic competitors, provides superior protection. The protective effect of *Bacilli* depends critically on high Ca^{2+} and carbonate availability. In methanation plants with different water chemistry, alternative microbial communities may provide protection through different biomineralization pathways. The superior performance of the autoclaved system under carbon-limited conditions aligns with observations that nutrient limitation can suppress aggressive microbial metabolism while favoring biomineralization processes.²⁸⁸ The selective enrichment of *Bacilli* through autoclaving, combined with limited organic carbon availability, created conditions favoring protective carbonate precipitation over acidogenic attack.

Rather than attempting biofilm elimination, operators should consider biofilm engineering, promoting beneficial biofilms dominated by mineralizing bacteria while suppressing aggressive acidogenic populations. The proposed reactions (Eqs. 7.7-7.18) provide a coherent framework to link electrochemical parameters, surface and cross-section features, Raman/EDS signatures, and solution chemistry, highlighting the central role of bio-driven mineralization pathways in controlling brass corrosion under these conditions.

7.6 Concluding remarks

The four inoculum conditions investigated in this study generated clearly distinct corrosion scenarios for OT60 brass. The sterile condition led to the most severe degradation, driven by the formation of thin and weakly protective oxide films. The untreated inoculum promoted the development of mixed Cu-Zn phosphate layers that initially conferred corrosion protection but progressively destabilized over time. Boiling resulted in simpler and more homogeneous Zn-phosphate deposits, providing an intermediate level of protection. In contrast, the autoclaved inoculum yielded the most protective system, characterized by the formation of a compact $\text{Cu}_2\text{O}/\text{CuO}$ inner layer overlain by a dense CaCO_3 deposit, arising from the survival of spore-forming *Bacilli* following heat treatment.

These findings demonstrate that the corrosion behavior of OT60 brass in methanogenic media is strongly governed by the structure and metabolic activity of the microbial consortium, which can be substantially reshaped by thermal treatments. Rather than eliminating microbial activity, boiling and autoclaving selectively enriched distinct microbial groups: autoclaving favored heat-tolerant, sporulating members of the *Bacilli* class, which promoted sustained carbonate precipitation and effectively mitigated corrosion, whereas both the boiled and untreated inocula were enriched in the thermophilic genus *Tepidiphilus*, whose metabolic activity contributed to the formation of phosphate-based corrosion products with differing degrees of compactness and protective efficacy. Contrary to the common assumption that sterilization reduces corrosion risk, our results demonstrate that complete elimination of microbial activity (achieved only after filtration and UV treatment) resulted in the most aggressive brass degradation, while thermally-selected microbial communities, particularly spore-forming *Bacilli*, provided effective corrosion inhibition through biogenic carbonate precipitation. These results underscore the protective role of specific microbial communities in modulating brass degradation.

Overall, microbial enrichment controls not only the extent of corrosion but also the composition, structure, and long-term stability of the passivating films formed on brass surfaces. Microorganisms selectively enriched by thermal treatments can redirect corrosion pathways away from aggressive abiotic dissolution and toward the precipitation of protective phosphate- or carbonate-based layers. These results highlight the intrinsic difficulty of achieving complete sterility in methanogenic environments and emphasize the central role of microbial community structure in determining corrosion outcomes.

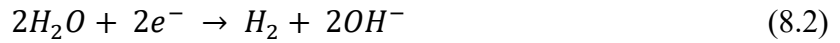
Chapter 8

8 Microbiologically Influenced Corrosion on ARMCO Iron

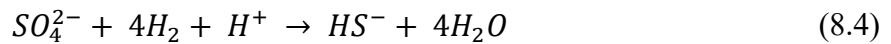
Corrosion of ferrous materials in anaerobic digesters for biogas production represents a complex interplay between abiotic electrochemical processes and biologically mediated mechanisms, collectively known as microbially influenced corrosion (MIC), which poses significant challenges for the structural integrity and longevity of digester infrastructure.^{311–313} Under abiotic anaerobic conditions, iron oxidation occurs through the anodic reaction following Eq. 8.1:^{314–316}



with the cathodic reaction typically involving water reduction (Eq. 8.2) or proton reduction (Eq. 8.3):



leading to the formation of iron hydroxides, oxides, and carbonates depending on solution chemistry. However, in the presence of microbial communities characteristic of anaerobic digesters, these fundamental electrochemical processes can be dramatically altered through multiple biologically mediated pathways. Sulfate-reducing bacteria (SRB), historically considered the primary causes of anaerobic MIC, accelerate corrosion through consumption of cathodic hydrogen via the reaction:^{317,318}

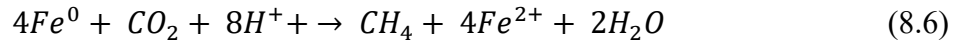


which removes the protective hydrogen layer from the metal surface and promotes continued iron oxidation, while simultaneously producing hydrogen sulfide (HS^-), a highly corrosive agent that reacts with ferrous ions to form iron sulfides:³¹⁹



More recently, the understanding of anaerobic MIC mechanisms has expanded beyond the classical cathodic depolarization theory to encompass direct extracellular electron transfer (EET),

wherein microorganisms directly extract electrons from metallic iron surfaces to support their metabolism in carbon-limited environments, a phenomenon observed not only in SRB but also in methanogenic archaea such as *Methanobacterium*, which can utilize elemental iron as an electron donor for methanogenesis:^{319,320}



forming corrosion crusts composed of siderite ($FeCO_3$), calcite, and iron sulfides^{321,322}. Additionally, acid-producing bacteria within digester biofilms generate volatile fatty acids including acetic, formic, propionic, and oxalic acids through fermentation of complex organic substrates, which locally decrease pH and enhance iron dissolution through direct chemical attack and complexation of ferrous ions, while iron-reducing bacteria such as *Geobacter* and *Shewanella* can enzymatically reduce Fe^{3+} in corrosion products back to Fe^{2+} , maintaining a continuous corrosion cycle.^{323,324} The microbial community composition in anaerobic digesters is strongly influenced by operational parameters, with thermophilic conditions (45-60 °C) selecting for distinct populations including thermophilic methanogens (*Methanothermobacter*, *Methanoculleus*) and thermophilic bacteria (*Tepidiphilus*, *Bacillus*), while the presence of organic acids, phosphates, sulfides, and carbonates in the complex digestate matrix creates microenvironments with varying electrochemical characteristics that govern both abiotic passivation and biotic acceleration of corrosion. Thermal treatments such as boiling and autoclaving are commonly employed in research and industrial practice for sterilization or selective enrichment of microbial communities,³²⁵ yet the effectiveness of these treatments in eliminating corrosive microorganisms remains incompletely characterized, particularly given that certain bacterial genera produce endospores capable of surviving extended exposure to temperatures exceeding 100 °C,^{194,325,326} and recent evidence suggests that even standard autoclave conditions (121 °C, 15-60 minutes) may be insufficient to eliminate ultra-resistant spore-forming bacteria such as *Bacillus*, *Paenibacillus*, and *Effusibacillus*, as well as dormant states of non-spore-forming organisms like *Pseudomonas* when protected within organic matrices.^{194,298,302} The present study investigates the corrosion behavior of ARMCO iron, a high-purity ferritic material commonly used as a model substrate for mechanistic corrosion studies, when exposed to inoculum derived from an operating anaerobic digester subjected to different thermal treatments - i.e., biotic (untreated), boiled (101°C), autoclaved (121°C, 60 minutes), and fully sterilized (autoclave, filtration and UV treatment) - with the objective of elucidating how thermal sterilization efficacy influences microbial community recolonization patterns and how

these distinct microbial assemblages, in turn, govern the morphology, composition, and kinetics of corrosion product formation through differential activation of electrochemical, metabolic, and biofilm-mediated corrosion pathways, ultimately demonstrating that thermal treatments alone are insufficient for complete sterilization and that the selective survival and subsequent proliferation of thermoresistant microorganisms fundamentally alters corrosion mechanisms from localized pitting attack in sterile conditions to more uniform layer-forming corrosion in biotic and thermally treated conditions, with significant implications for understanding MIC prevention strategies and materials selection in biogas production facilities.

8.1 Surface and morphology characterization

Light optical microscopy of ARMCO iron coupons (Fig. 8.1) after 15 days of immersion at 45 °C revealed marked differences in surface appearance among the four experimental conditions, with biotic and boiled samples exhibiting heterogeneous reddish-brown corrosion layers distributed irregularly across the surface, while iron coupon exposed to the autoclaved inoculum displayed comparatively lighter and more uniform coloration. On the contrary, the coupon exposed to the sterile inoculum, exhibited a completely different surface with an heterogeneous dispersion of acicular-like brownish corrosion products.

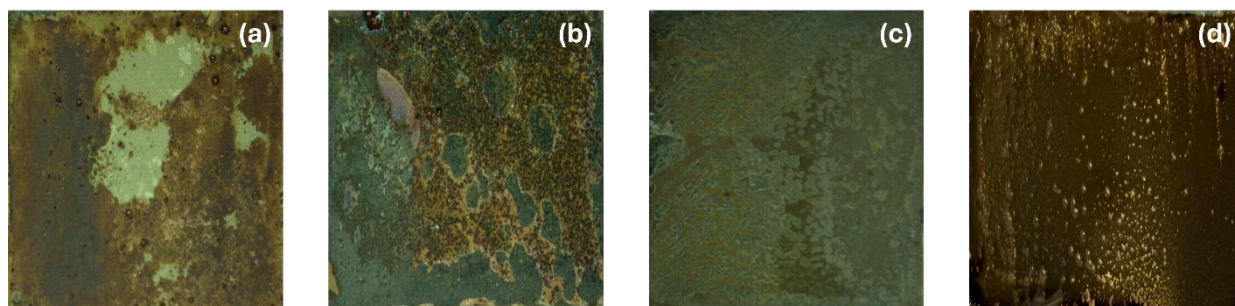


Figure 8.1. Optical microscopy images of ARMCO iron coupons after 15-day test in the (a) biotic, (b) boiled, (c) autoclaved and (d) sterile solutions at 45 °C.

Scanning electron microscopy (Fig. 8.2) provided further insight into the microstructural characteristics of the corrosion products, revealing that despite macroscopic similarities between biotic and boiled conditions, substantial morphological differences exist at the microscale. The boiled sample was notably distinguished by the presence of well-defined elongated parallelepiped-shaped crystals, a morphology not observed on other coupons, which instead displayed more irregular and less geometrically ordered corrosion structures. Energy-dispersive X-ray spectroscopy (Figs. 8.3-8.6) detected oxygen and phosphorus in the corrosion layers formed under biotic, boiled, and autoclaved conditions, whereas the sterile coupon showed no detectable

phosphorus signal, suggesting either complete absence or concentrations below the detection limit. In the biotic condition (Fig. 8.3), SEM imaging clearly revealed the presence of a biofilm covering portions of the metal surface and embedding the corrosion products, consistent with active microbial colonization and metabolic activity. The autoclaved coupon (Figs. 8.2d and 8.5) presented a relatively smooth and continuous surface layer with minimal textural heterogeneity, indicative of limited corrosion propagation.

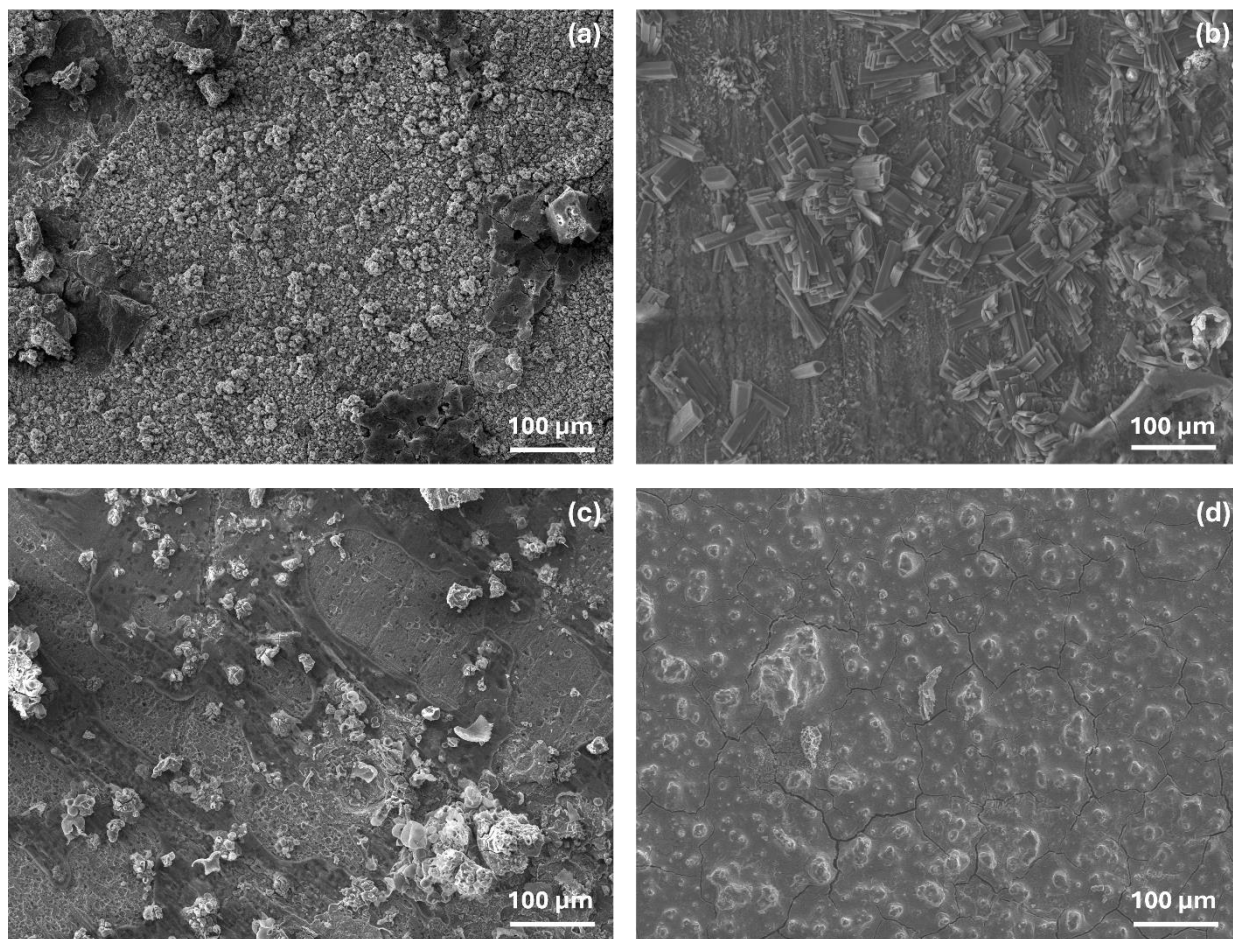


Figure 8.2. Scanning electron microscopy (SEM) images of ARMCO iron coupons after 15-day test in the (a) biotic, (b) boiled, (c) autoclaved and (d) sterile solutions at 45 °C.

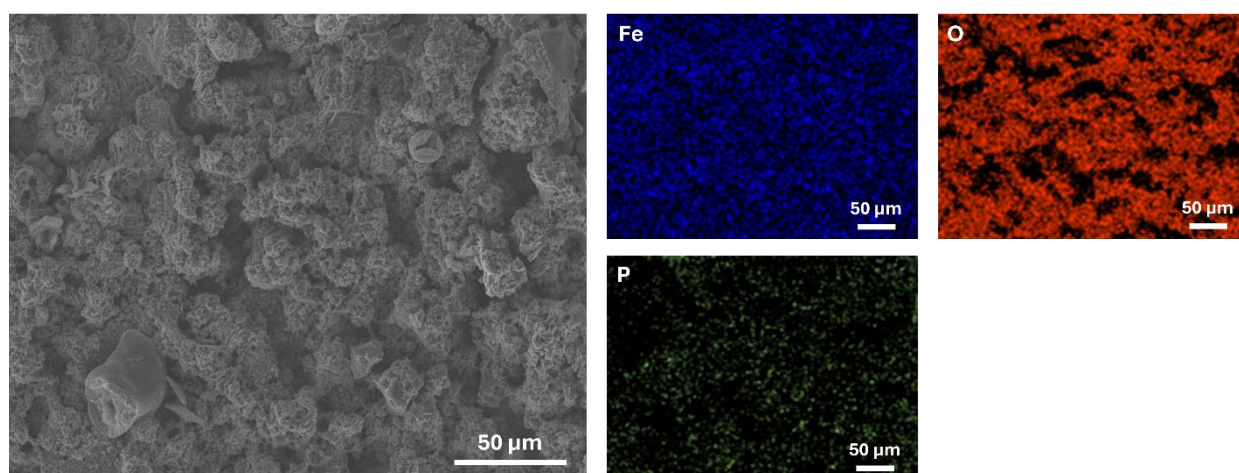


Figure 8.3. SEM-EDS images of ARMCO iron coupon after 15-day test in the biotic solution at 45 °C.

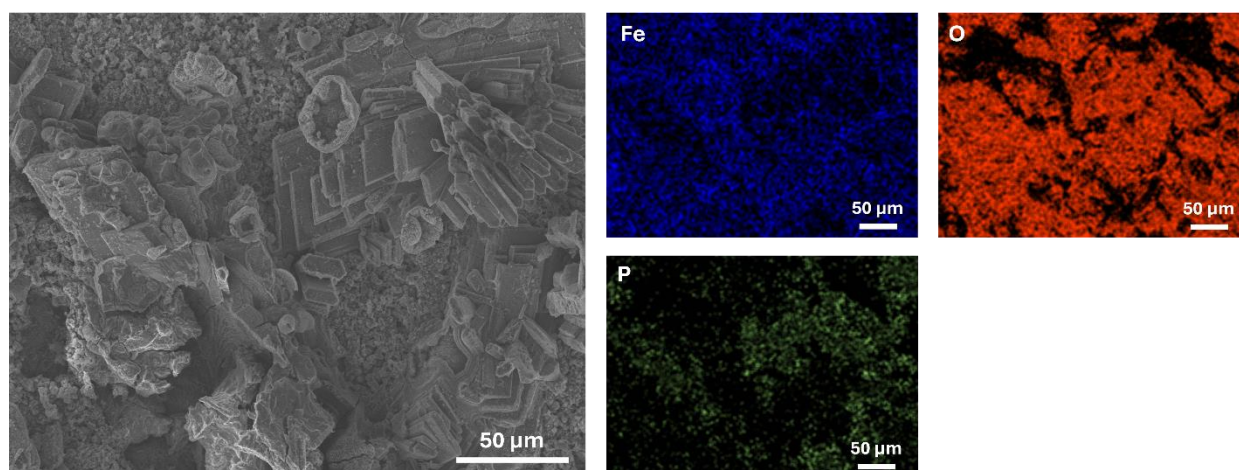


Figure 8.4. SEM-EDS images of ARMCO iron coupon after 15-day test in the boiled solution at 45 °C.

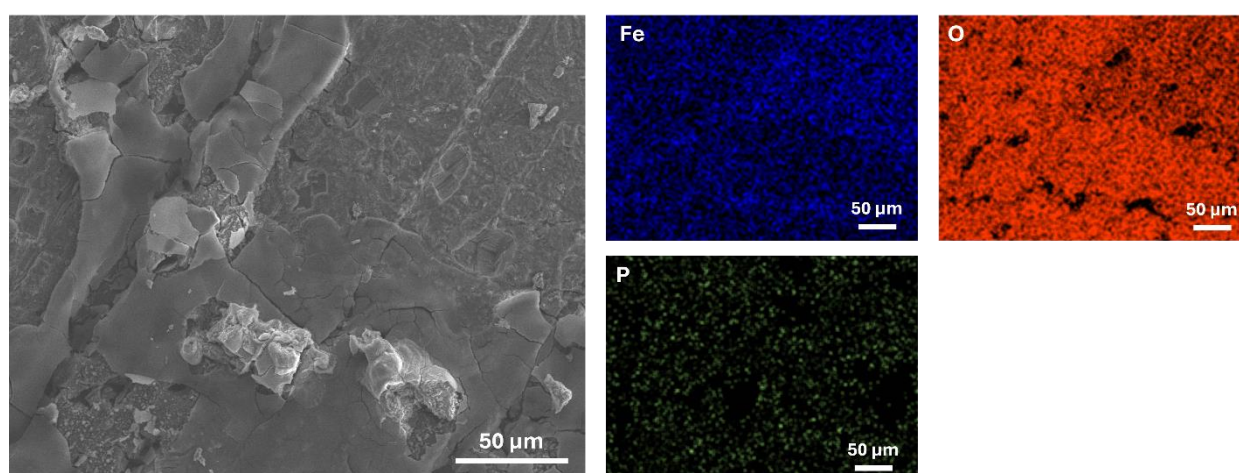


Figure 8.5. SEM-EDS images of ARMCO iron coupon after 15-day test in the autoclaved solution at 45 °C.

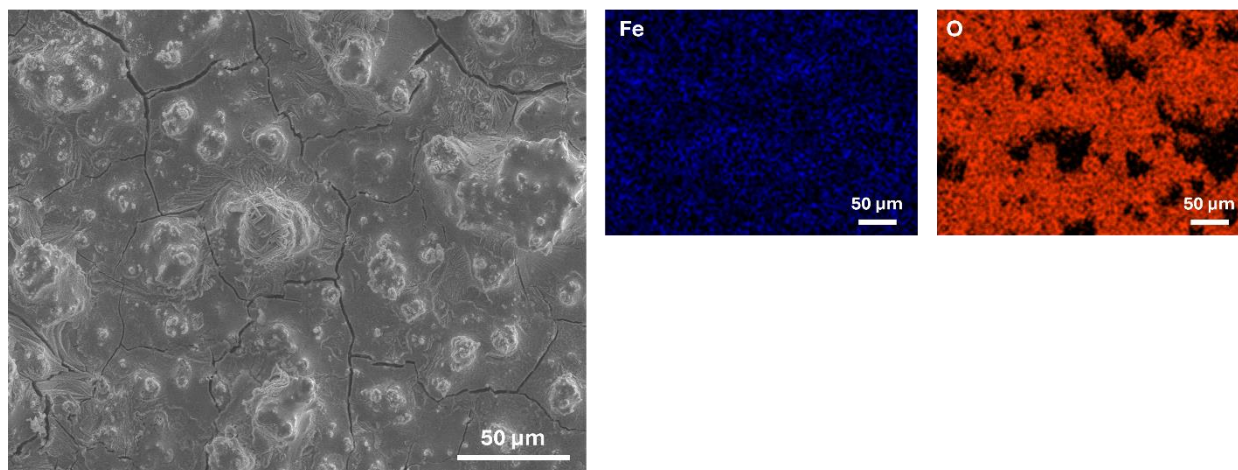


Figure 8.6. SEM-EDS images of ARMCO iron coupon after 15-day test in the sterile solution at 45 °C.

Cross-sectional analysis (Figs. 8.7-8.10) quantified the extent of material degradation, revealing striking differences in corrosion layer thickness: the boiled sample (Fig. 8.8) exhibited the thickest corrosion product layer at approximately 20 μm, followed by biotic (Fig. 8.7) and sterile (Fig. 8.10) samples at approximately 10 μm each, while the autoclaved (Fig. 8.9) sample retained a largely intact metallic substrate with only a thin approximately 1 μm oxide layer, indicating markedly reduced corrosion.

Notably, the corrosion layers formed under biotic and boiled conditions appeared compact and well-adhered to the substrate, whereas the sterile coupon displayed extensive localized attack with pitting penetration reaching depths of approximately 10 μm, demonstrating severe non-uniform corrosion despite an average layer thickness comparable to the biotic condition. This observation suggests that in the absence of microbial activity or thermally modified solution chemistry, corrosion proceeds through a localized mechanism rather than forming protective surface layers.

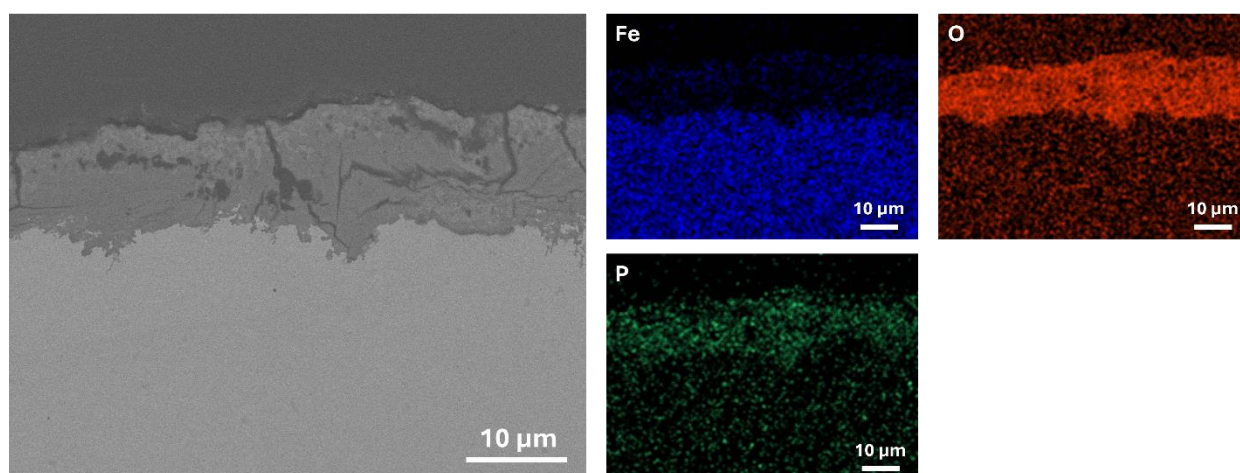


Figure 8.7. SEM-EDS images of ARMCO iron cross section after 15-day test in the biotic solution at 45 °C.

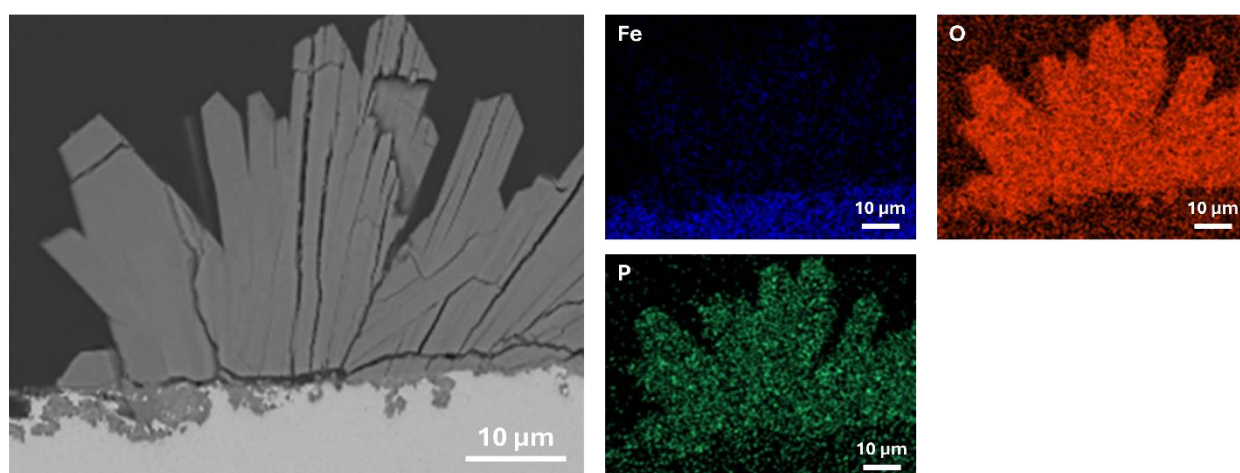


Figure 8.8. SEM-EDS images of ARMCO iron cross section after 15-day test in the boiled solution at 45 °C.

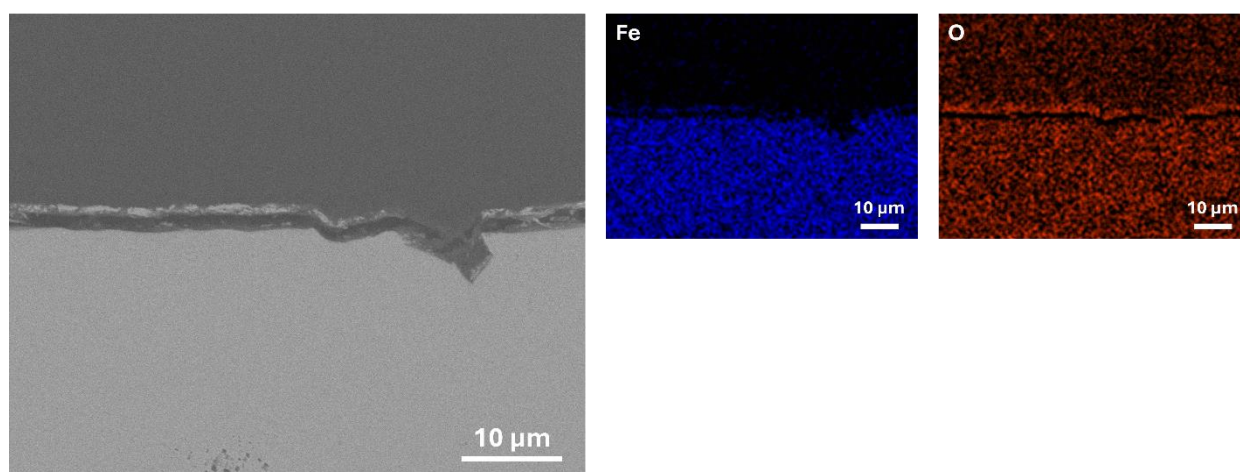


Figure 8.9. SEM-EDS images of ARMCO iron cross section after 15-day test in the autoclaved solution at 45 °C.

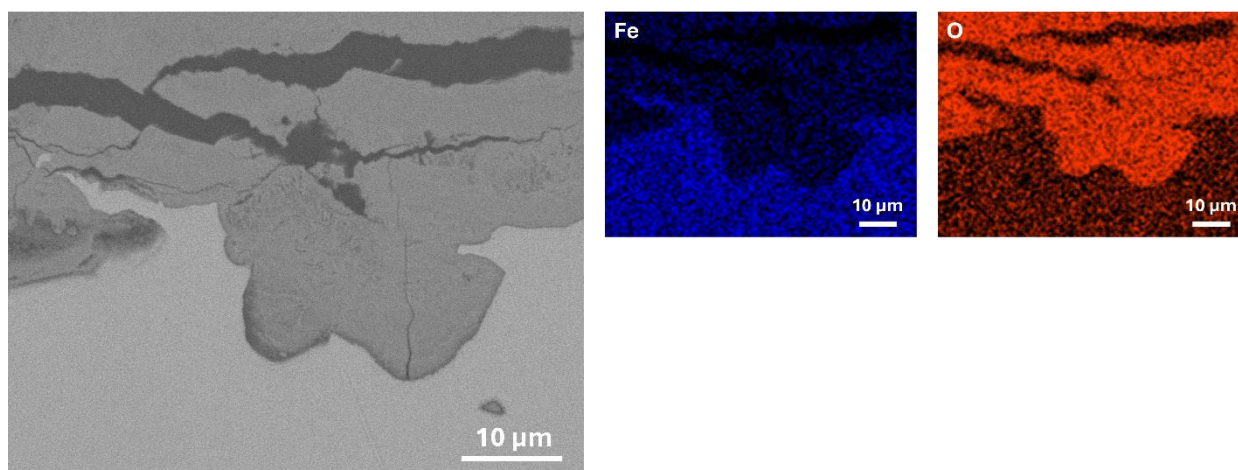


Figure 8.10. SEM-EDS images of ARMCO iron cross section after 15-day test in the sterile solution at 45 °C.

Raman spectroscopy analysis (Fig. 8.11) of the corrosion products revealed distinct phase compositions across the four experimental conditions, providing critical insight into the mineralogical pathways governing corrosion layer formation. The biotic condition exhibited the most complex spectral signature, with prominent peaks at approximately 670 cm^{-1} , 540 cm^{-1} , and in the $200\text{--}300\text{ cm}^{-1}$ region characteristic of magnetite (Fe_3O_4), alongside peaks at approximately 660 cm^{-1} , 410 cm^{-1} , 290 cm^{-1} , and 220 cm^{-1} consistent with hematite ($\alpha\text{-Fe}_2\text{O}_3$). The strong intensity of these oxide peaks indicates that the corrosion products in the biotic system are dominated by mixed-valence iron oxides, with magnetite likely forming through biologically mediated reduction of initially precipitated ferric phases or direct microbial Fe(III) reduction at the biofilm-metal interface. A weak broad feature around 1300 cm^{-1} suggests trace amounts of iron carbonate, though siderite is not a major phase in this condition. The boiled condition displayed a spectral pattern qualitatively similar to the biotic sample, with clear magnetite and hematite signatures evidenced by peaks at approximately 670 cm^{-1} , 540 cm^{-1} for magnetite and the characteristic hematite multiplet in the $200\text{--}700\text{ cm}^{-1}$ range. However, the relative intensity distribution differs subtly from the biotic condition, with a more pronounced peak at approximately 1320 cm^{-1} indicative of increased iron carbonate content, and the overall spectral intensity appears somewhat lower, consistent with a corrosion layer that, while thick, may contain more disordered or poorly crystalline phases. The presence of both magnetite and hematite in the boiled system, suggests that iron oxide formation can proceed through chemical pathways under the altered solution conditions created by thermal treatment, though the distinct crystalline morphology observed by SEM (Figs. 8.4 and 8.8) may correspond to mixed oxide-oxyhydroxide assemblages not fully resolved in the Raman spectrum.

The autoclaved condition presented a markedly different spectral profile characterized by lower overall intensity and a more balanced distribution of oxide and carbonate phases. Peaks assignable to magnetite and hematite are present but significantly less intense than in the biotic and boiled samples, while a clear peak at approximately 1080 cm^{-1} with an accompanying feature around 730 cm^{-1} confirms the presence of siderite (FeCO_3) as a major corrosion product. Additional weak peaks at approximately $280\text{--}300\text{ cm}^{-1}$ may arise from both iron oxide and carbonate lattice modes. This phase assemblage indicates that corrosion in this system proceeds predominantly through carbonate-mediated pathways, with rapid precipitation of protective iron carbonate films that limit further oxidation and oxide accumulation. The relatively low Raman signal intensity correlates well with the thin ($\sim 1\text{ }\mu\text{m}$) corrosion layer observed in cross-section (Fig. 8.9) and suggests a dense and compact structure.

The sterile condition exhibited the most distinctive Raman signature, dominated by a very strong, sharp peak at approximately 1320 cm^{-1} characteristic of the symmetric stretching mode (ν_1) of carbonate in siderite, accompanied by clear features at approximately 730 cm^{-1} (bending mode ν_4) and around 280 cm^{-1} . Iron oxide phases are also present, as evidenced by peaks in the $200\text{--}700\text{ cm}^{-1}$ region corresponding to both magnetite and hematite, though these are substantially weaker relative to the dominant carbonate signal compared to the biotic and boiled conditions. The prominence of siderite in the sterile system is paradoxical given the severe localized corrosion observed and suggests that while iron carbonate does precipitate from the carbonate-free sterile solution, the resulting deposits are either non-adherent, porous, or preferentially form away from active corrosion sites, failing to establish effective surface passivation. The coexistence of intense carbonate peaks with evidence of active corrosion indicates that siderite formation in the sterile condition occurs via precipitation from dissolved ferrous ions in bulk solution or within corrosion pits rather than as a protective surface film, fundamentally differing from the passivating carbonate layer formed in the autoclaved system.

Significantly, no Raman peaks characteristic of crystalline iron phosphate phases were detected in any sample, despite EDS confirmation of phosphorus in biotic, boiled and autoclaved corrosion layers (Figs. 8.3, 8.4, 8.7 and 8.8). The absence of peaks in the diagnostic $950\text{--}1050\text{ cm}^{-1}$ region for phosphate symmetric stretching modes indicates that phosphorus is either present in amorphous or poorly crystalline forms, incorporated into the oxide/carbonate matrix through substitution or surface adsorption, or in the case of the biotic sample, primarily associated with organic components of the biofilm such as phospholipids, nucleic acids, and extracellular polymeric substances rather than forming discrete phosphate mineral phases. This finding revises

the initial hypothesis that the distinctive crystalline morphology observed in the boiled sample corresponds to iron phosphate minerals; instead, these crystals are more likely iron oxyhydroxide phases (goethite, lepidocrocite, or akaganeite) whose Raman signatures overlap substantially with those of magnetite and hematite in the measured spectral range, or represent unusual crystalline habits of the oxide phases formed under the specific chemical conditions of the thermally treated medium.

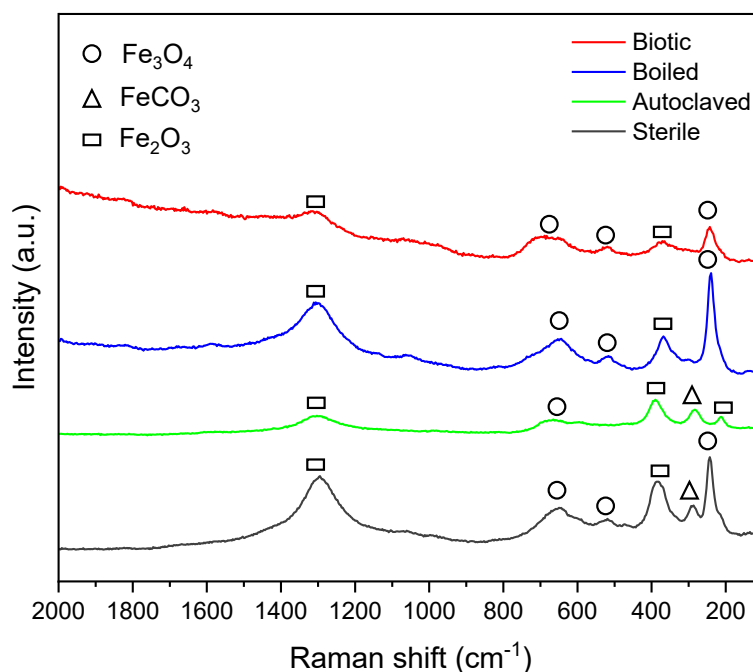


Figure 8.11. Raman spectra of ARMCO iron corrosion products on the surface of the coupons after 15-day test immersed in the biotic (red), boiled (blue), autoclaved (green) and sterile (gray) solutions at 45 °C.

8.2 Solution analysis

Ion chromatography and inductively coupled plasma optical emission spectroscopy of the test solutions after 15 days of immersion provided complementary insights into the chemical processes governing corrosion under the different experimental conditions. ICP-AES analysis (Table 8.1) revealed striking differences in dissolved iron concentrations, with the sterile condition exhibiting the highest Fe concentration at $4.5 \pm 0.15 \text{ mg L}^{-1}$, approximately five times greater than the boiled sample at $1.7 \pm 0.10 \text{ mg L}^{-1}$, twice that of the biotic condition at $0.83 \pm 0.04 \text{ mg L}^{-1}$, and nearly twenty times higher than the autoclaved sample at $0.24 \pm 0.02 \text{ mg L}^{-1}$. This trend in dissolved iron

concentration does not correlate directly with corrosion layer thickness measured by cross-sectional analysis (Figs. 8.7-8.10), indicating that iron released during corrosion is being precipitated back onto the surface with different efficiencies depending on the solution chemistry and microbial activity. The exceptionally low dissolved iron in the autoclaved condition, coupled with the minimal 1 μm corrosion layer observed in cross-section (Fig. 8.9), confirms that this treatment resulted in the most effective corrosion inhibition, whereas the high dissolved iron in the sterile condition, despite a corrosion layer thickness of only approximately 10 μm comparable to the biotic sample, suggests that corrosion products are either more soluble or less adherent in the absence of microbial influence and organic complexing agents.

Table 8.1. Total elemental concentration of inorganic elements detected in the biotic, boiled, autoclaved and sterile inocula after 15-day test, determined by inductively coupled plasma – atomic emission spectroscopy (ICP-AES).

Element		Concentration ^a (mg L ⁻¹)			
		Experimental condition			
		Biotic	Boiled	Autoclaved	Sterile
Chromium	Cr	n.d.	n.d.	n.d.	n.d.
Iron	Fe	0.83 $\pm$ 0.04	1.7 $\pm$ 0.10	0.24 $\pm$ 0.02	4.5 $\pm$ 0.15
Nichel	Ni	0.14 $\pm$ 0.11	0.12 $\pm$ 0.09	0.20 $\pm$ 0.01	n.d.
Copper	Cu	<LOD	0.2 $\pm$ 0.02	0.06 $\pm$ 0.01	n.d.
Zinc	Zn	n.d.	n.d.	n.d.	<LOD
Sulfur	S	6.0 $\pm$ 0.11	4.3 $\pm$ 0.14	4.8 $\pm$ 0.09	4.4 $\pm$ 0.10
Phosphorous	P	236 $\pm$ 1.3	403 $\pm$ 1.4	290 $\pm$ 1.1	80 $\pm$ 0.99

^a Values are expressed as mean $\pm$ standard deviation (n = 2). Concentrations below the limit of detection (LOD = 0.05 mg L⁻¹) are indicated as “< LOD”, while analytes not determined are indicated as “n.d.” in the Table.

Phosphorus concentrations determined by ICP-AES showed substantial variation among treatments, with the boiled condition exhibiting the highest concentration at 403 $\pm$ 1.4 mg L⁻¹, followed by autoclaved at 290 $\pm$ 1.1 mg L⁻¹, biotic at 236 $\pm$ 1.3 mg L⁻¹, and sterile at 80 $\pm$ 0.99 mg L⁻¹, confirming that thermal treatments significantly affect phosphorus solubility and speciation in the test solutions. These elevated phosphorus concentrations in solution are consistent with EDS detection of phosphorus in the corrosion layers of biotic, boiled and autoclaved samples (Figs. 8.3-8.5), suggesting an equilibrium between dissolved and surface-

associated phosphorus species, though the absence of crystalline phosphate phases in Raman spectroscopy indicates that phosphorus incorporation occurs primarily through adsorption or formation of amorphous compounds rather than precipitation of discrete phosphate minerals. The presence of sulfur at comparable concentrations across all conditions, ranging from 4.3 to 6.0 mg L⁻¹, suggests relatively uniform sulfur speciation regardless of thermal treatment, though no sulfide phases such as mackinawite were detected by Raman spectroscopy, indicating that sulfur is likely present as sulfate or organosulfur compounds rather than participating directly in corrosion product formation.

Ion chromatography (Table 8.2) revealed distinct organic acid profiles among the experimental conditions, with oxalate being the dominant anion detected in all samples but showing marked concentration differences: biotic 3019 ± 3.1 mg L⁻¹, boiled 2240 ± 2.3 mg L⁻¹, autoclaved 2358 ± 3.3 mg L⁻¹, and sterile 1161 ± 2.4 mg L⁻¹.

Table 8.2. Concentrations of anions detected in the biotic, boiled, autoclaved and sterile inocula after 15-day test, determined by ion chromatography (IC). Results are reported in the ionic form detected by the instrument.

Analyte		Concentration ^b (mg L ⁻¹)			
		Experimental condition			
		Biotic	Boiled	Autoclaved	Sterile
Oxalate	$C_2O_4^{2-}$	3019 ± 3.1	2240 ± 2.3	2358 ± 3.3	1161 ± 2.4
Formate	$HCOO^-$	<LOD	<LOD	14.4 ± 2.6	n.d.
Acetate	CH_3COO^-	2.4 ± 0.16	38 ± 0.12	28.4 ± 0.11	n.d.
Propionate	$CH_3CH_2COO^-$	<LOD	<LOD	<LOD	n.d.
Carbonates	CO_3^{2-}	213 ± 1.0	253 ± 0.36	525 ± 0.21	n.d.

^b Values are expressed as mean ± standard deviation (n = 2). Concentrations below the limit of detection (LOD = 0.05 mg L⁻¹) are indicated as “< LOD”, while analytes not determined are indicated as “n.d.” in the Table.

The substantially higher oxalate concentration in the biotic condition compared to sterile suggests active microbial production or release of oxalate during the experiment, while the intermediate concentrations in thermally treated samples indicate that oxalate survives heat treatment to varying degrees. Acetate was detected only in biotic, boiled, and autoclaved conditions at low concentrations ranging from 2.4 to 38 mg L⁻¹, with the boiled sample showing the highest acetate

content, while formate was detected exclusively in the autoclaved condition at $14.4 \pm 2.6 \text{ mg L}^{-1}$, suggesting that specific thermal treatment conditions promote the formation or preservation of certain low-molecular-weight organic acids through thermal degradation of more complex organic matter. Carbonate concentrations showed a progressive increase from biotic at $213 \pm 1.0 \text{ mg L}^{-1}$ to boiled at $253 \pm 0.36 \text{ mg L}^{-1}$ and autoclaved at $525 \pm 0.21 \text{ mg L}^{-1}$, while being absent in the sterile condition, correlating with the Raman spectroscopy (Fig. 8.11) identification of siderite in autoclaved and sterile samples and its absence in biotic and boiled samples. The elevated carbonate concentration in the autoclaved solution provides a plausible explanation for the minimal corrosion observed in this condition, as carbonate ions can promote the formation of protective iron carbonate layers, consistent with the thin $1 \text{ }\mu\text{m}$ surface film observed in cross-section, though the paradoxical detection of siderite by Raman in both autoclaved and sterile samples despite their vastly different corrosion behaviors suggests that carbonate-based passivation is effective only under specific solution chemistry conditions present in the autoclaved environment.

8.3 Characterization of the bacterial community

16S rRNA gene sequencing of bacterial communities (Fig. 8.12) and archaeal-specific primer amplification (Fig. 8.13) revealed marked differences in microbial composition among the experimental conditions, providing insight into the relationship between microbial community structure and corrosion behavior. In the biotic condition, both biofilm and solution were dominated by *Tepidiphilus*, representing over 74% of the total bacterial population, while the archaeal community in the biofilm exhibited greater diversity with the presence of *Methanospirillum*, *Methanosarcina*, and other *Methanomicrobiales*, whereas the solution contained primarily *Methanothermobacter*, indicating spatial stratification of methanogenic activity. The predominance of *Tepidiphilus*, a thermophilic genus known to thrive at elevated temperatures and capable of diverse metabolic activities, suggests that the experimental temperature of $45 \text{ }^{\circ}\text{C}$ selected for thermophilic microorganisms that likely contributed to the observed corrosion through metabolic production of organic acids and creation of localized electrochemical heterogeneities beneath the biofilm.

The boiled condition exhibited a surprisingly high abundance of *Tepidiphilus*, constituting over 83% of the biofilm community and approximately 75% of the solution, a finding that initially appears paradoxical given that boiling at $101 \text{ }^{\circ}\text{C}$ for an extended period should eliminate vegetative cells. This observation is consistent with previous reports that certain bacterial species

can survive autoclaving through spore formation, with genera such as *Bacillus* and *Paenibacillus* being particularly notable for producing heat-resistant spores, and standard autoclave conditions at 121 °C for 15 minutes are designed to inactivate even highly resistant bacterial spores including those of *Geobacillus stearothermophilus*.^{327–330} The persistence and dominance of *Tepidiphilus* in the boiled sample indicates that this thermophilic genus likely produces spores capable of surviving the boiling treatment, subsequently germinating and rapidly colonizing the available ecological niche during the 15-day incubation period.

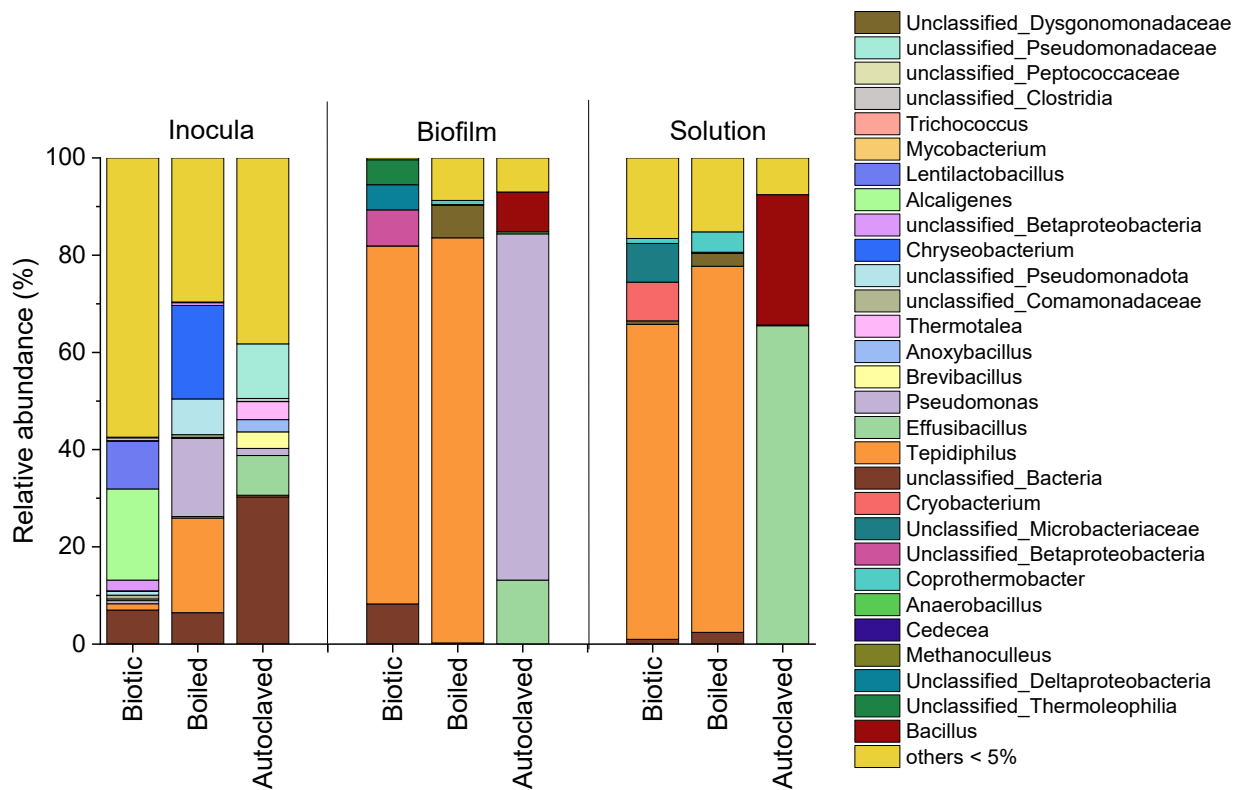


Figure 8.12. Bar plot showing the relative abundances of bacterial genera in the inocula prior the test, and in the biofilm and in solution after the test. The chart presents results for the untreated (biotic), boiled, and autoclaved conditions.

The higher abundance of *Tepidiphilus* in the boiled condition compared to the biotic condition suggests reduced microbial competition following thermal elimination of less heat-resistant species, allowing spore-forming thermophiles to proliferate without constraint. The archaeal community in the boiled condition was exclusively composed of *Methanothermobacter*, indicating that only the most thermotolerant methanogenic archaea survived the heat treatment, consistent with the known thermophilic character of this genus.

The autoclaved condition presented a markedly different microbial composition despite the more severe thermal treatment (i.e., double autoclaving at 121 °C for 20 minutes), which substantially exceeds standard sterilization protocols. The biofilm community was dominated by *Pseudomonas* at approximately 71%, with *Effusibacillus* representing 13%, while the solution showed an inverted pattern with *Effusibacillus* at 65% and the remainder attributed to *Bacillus* species. The presence of these organisms following autoclaving can be attributed to ultra-resistant spore formers, as certain *Bacillus* species possess mobile genetic elements that confer exceptionally high-level heat resistance to their spores, and non-spore-forming bacteria such as *Pseudomonas* have been documented to survive autoclaving in specific environmental matrices, likely through protection within organic matrices or through formation of highly resistant dormant states.^{331–333}

The archaeal community (Fig. 8.13) in the autoclaved condition consisted solely of *Methanothermobacter*, mirroring the pattern observed in the boiled sample. The extremely limited corrosion observed in the autoclaved condition, evidenced by the thin 1 µm oxide layer and minimal metal loss, suggests that despite microbial survival and recolonization, the altered solution chemistry resulting from autoclave treatment, particularly the elevated carbonate concentration documented by ion chromatography (Table 8.2), created conditions unfavorable for aggressive microbial corrosion, potentially through formation of protective iron carbonate surface films that inhibited microbial metabolic access to the metal substrate.

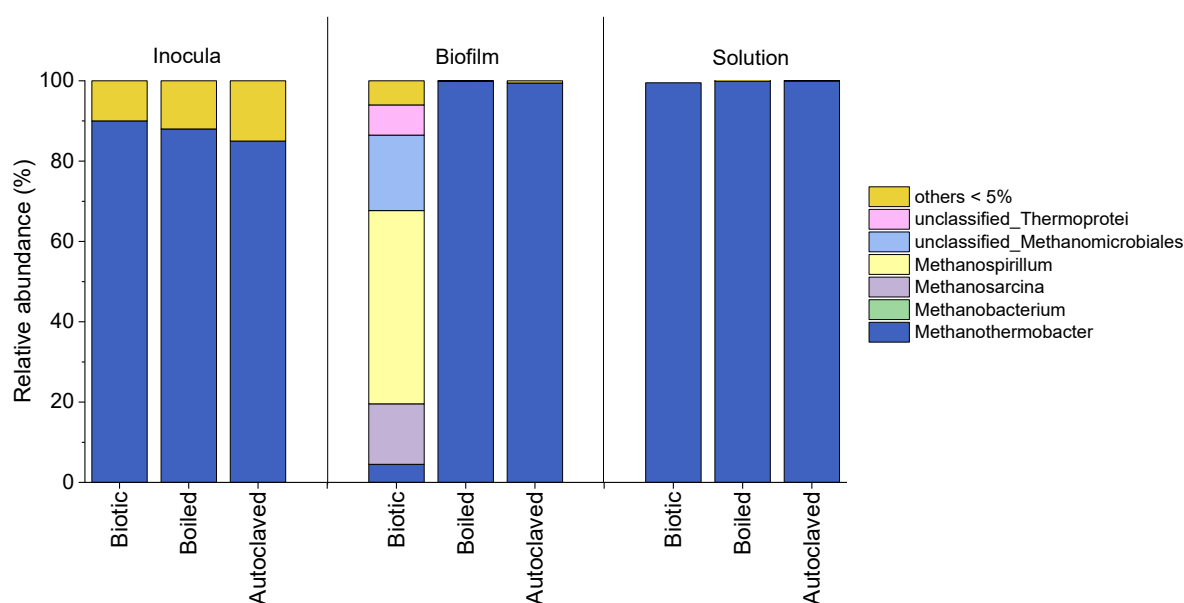


Figure 8.13. Bar plot showing the relative abundances of Archaea genera in the inocula prior the test, and in the biofilm and in solution after the test. The chart presents results for the untreated (biotic), boiled, and autoclaved conditions.

The sterile condition, which underwent both autoclave sterilization at 121 °C for 60 minutes and subsequent UV treatment, showed no detectable bacterial or archaeal presence by 16S rRNA sequencing (Fig. S.8.1, Supplementary Information), confirming the effectiveness of the combined sterilization protocol. The severe localized corrosion observed in this condition, characterized by pitting penetration reaching approximately 10 µm depth despite an average corrosion layer thickness comparable to the biotic condition, demonstrates that in the complete absence of microbial activity, corrosion proceeds through purely abiotic electrochemical mechanisms driven by solution chemistry rather than biologically mediated processes. The absence of phosphorus in the corrosion products of the sterile sample, as detected by EDS analysis (Fig. 8.6), further supports the conclusion that the phosphorus identified in biotic, boiled, and autoclaved conditions originates primarily from microbial biomass, extracellular polymeric substances, or microbially mediated phosphate precipitation, rather than from the sterile inoculum itself. The comparison between sterile and biotic conditions reveals that while microbial activity does not necessarily accelerate overall metal loss, it fundamentally alters the corrosion mechanism from localized pitting to more uniform layer formation, likely through production of organic acids that promote general dissolution and precipitation of iron salts, and through biofilm-mediated modification of the local electrochemical environment that homogenizes corrosion across the metal surface rather than concentrating attack at discrete sites.

Quantitative PCR (qPCR) analysis (Fig. S.8.2, Supplementary Information) highlighted that thermal treatments substantially decreased the 16S rRNA gene copy numbers in the biofilm and confirmed the absence of viable microorganisms in the sterile condition. The autoclaved sample exhibited the lowest bacterial load (3.4×10^8 gene copies mL⁻¹), compared to the boiled inoculum (1.7×10^{10} gene copies mL⁻¹) and the untreated biotic control (1.2×10^{15} gene copies mL⁻¹).

8.4 Electrochemical characterization

Open circuit potential measurements were conducted continuously over 15 days at 45 °C to monitor the electrochemical evolution of ARMCO iron surfaces in biotic, boiled, autoclaved, and sterile inocula (Fig. 8.14).

At the beginning of the immersion period, all samples exhibited similar initial potentials ranging from approximately -0.62 to -0.65 V vs Ag/AgCl sat. KCl, indicating comparable initial surface conditions. The temporal evolution of the OCP, however, diverged markedly among the four experimental conditions as corrosion processes progressed. The sterile condition maintained the most electronegative potential throughout the test period, remaining relatively stable between -

0.62 and -0.67 V, with a slight drift toward more negative values reaching approximately -0.67 V by day 15.

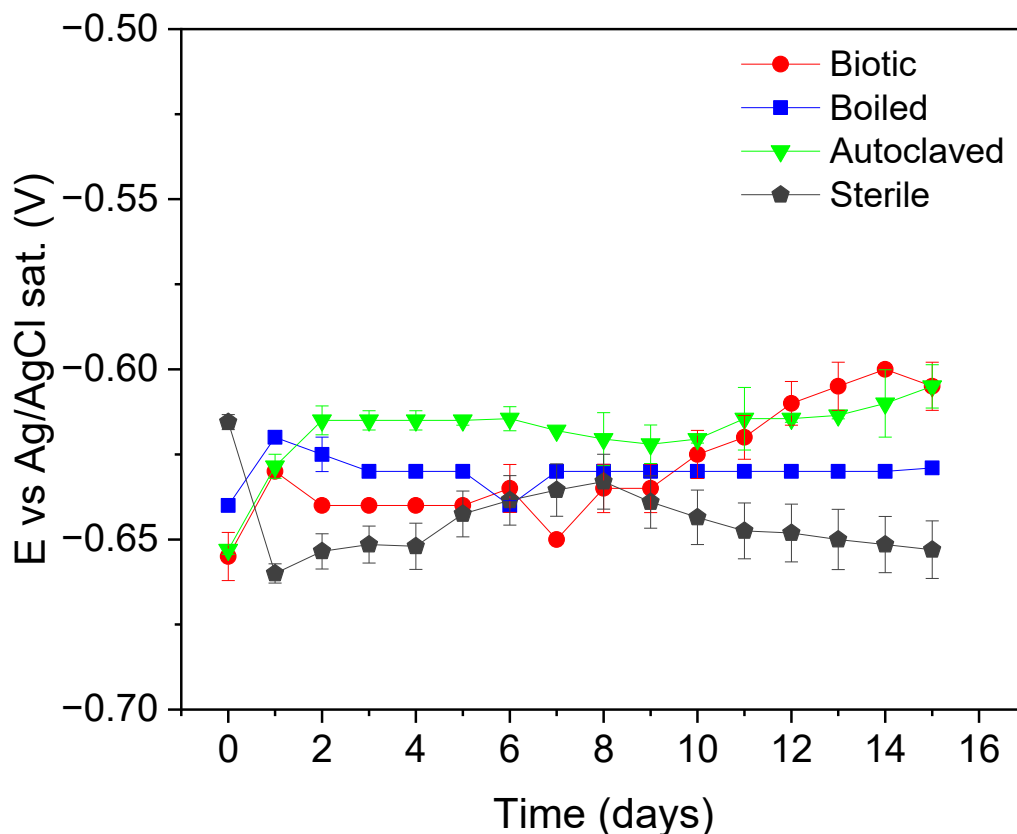


Figure 8.14. Open circuit potential (average and standard deviation, E vs Ag/AgCl sat.) of ARMCO iron coupons over the entire test period (15 days). Biotic (red solid line), boiled (blue solid line), autoclaved (green solid line) and sterile condition (gray solid line).

The biotic condition exhibited a more complex evolution characterized by an initial period around -0.64 V for approximately 7 days, followed by a transient shift toward more negative values reaching approximately -0.65 V at day 7, and subsequent ennoblement toward approximately -0.60 V by day 15. The boiled condition displayed the most stable OCP profile throughout the experiment, maintaining values between -0.62 and -0.64 V with minimal temporal variation and standard deviations comparable to the other treatments. The autoclaved condition exhibited progressive ennoblement from an initial value of approximately -0.65 V to approximately -0.60 V by day 15, representing the most pronounced positive potential shift of approximately 50 mV among all treatments. Standard deviations derived from replicate measurements ranged from approximately 10 to 30 mV across all conditions, indicating reasonable experimental

reproducibility, with the largest variations observed during transition periods, particularly in the biotic condition between days 7 and 12. By the end of the 15-day exposure period, the final OCP values ranked in order from most noble to most active as follows: biotic and autoclaved (both approximately -0.60 V), then boiled (approximately -0.63 V) and lastly sterile (approximately -0.65 V), corresponding to a total potential spread of approximately 50 mV among the different treatments.

Polarization resistance measurements were conducted at 1 and 15 days of immersion by applying a small potential perturbation within a restricted range centered on the open-circuit potential (Figs. 8.15 and S.8.3, Supplementary Information).

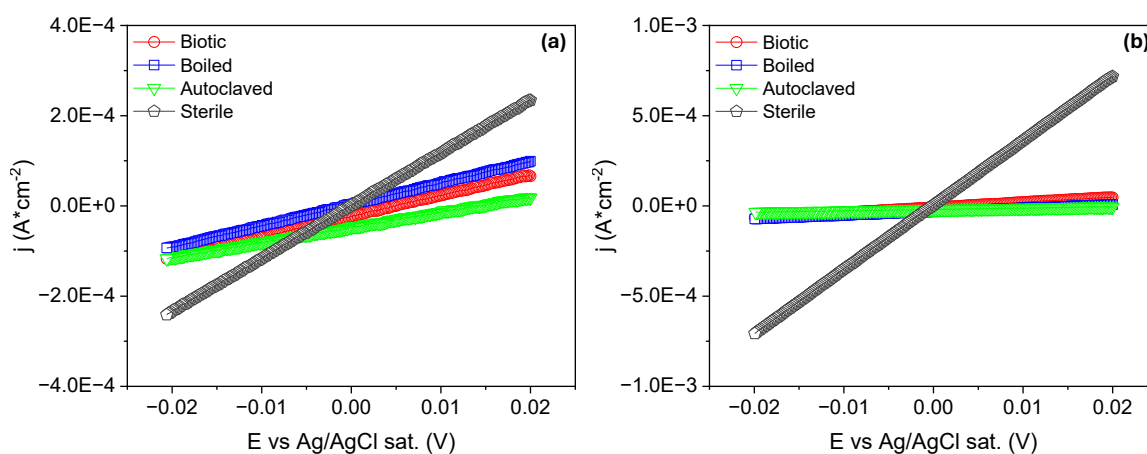


Figure 8.15. Linear polarization resistances of ARMCO iron coupons and replicates tested in the biotic (red), boiled (blue) and autoclaved (gray) inocula after (a) 1 day and (b) 15 days.

Polarization resistance values (R_p) were extracted from the linear relationship between applied potential and measured current density. Under purely activation-controlled corrosion, the Stern-Geary equation establishes an inverse relationship between R_p and the corrosion current density (J_{corr}), with charge-transfer reactions at the metal-electrolyte interface dictating the overall corrosion rate. However, in the experimental conditions examined here, the corrosion process is expected to involve not only interfacial charge-transfer kinetics but also mass-transport limitations arising from biofilm accumulation and the progressive buildup of corrosion product deposits. Consequently, direct quantitative determination of J_{corr} through standard Tafel analysis is not appropriate for this system. Nonetheless, the measured R_p values serve as a reliable metric for qualitative assessment and comparative evaluation of corrosion resistance across the different thermal treatment conditions, with the observed temporal trends revealing markedly divergent electrochemical behavior among the four experimental systems.

Table 8.3. Rp values obtained from LPR measurements at the beginning and end of the test (1 and 15 days, respectively) for each experimental condition (biotic, boiled, autoclaved and sterile). Values are reported as mean $\pm$ standard deviation.

Time days	Biotic $\text{k}\Omega\cdot\text{cm}^2$	Boiled $\text{k}\Omega\cdot\text{cm}^2$	Autoclaved $\text{k}\Omega\cdot\text{cm}^2$	Sterile $\text{k}\Omega\cdot\text{cm}^2$
1	0.67 ± 0.22	0.50 ± 0.20	0.56 ± 0.10	1.30 ± 1.13
15	0.56 ± 0.09	0.66 ± 0.04	2.01 ± 0.004	0.17 ± 0.14

At day 1, the polarization resistance values ranged from $0.50 \pm 0.20 \text{ k}\Omega\cdot\text{cm}^2$ for the boiled condition to $1.30 \pm 1.13 \text{ k}\Omega\cdot\text{cm}^2$ for the sterile condition, with intermediate values of $0.67 \pm 0.22 \text{ k}\Omega\cdot\text{cm}^2$ and $0.56 \pm 0.10 \text{ k}\Omega\cdot\text{cm}^2$ observed for the biotic and autoclaved conditions, respectively. The relatively large standard deviation observed for the sterile condition at day 1 indicates substantial variability among replicate measurements, suggesting heterogeneous initial surface conditions or the early stages of localized corrosion initiation.

By day 15, the polarization resistance values exhibited markedly different temporal evolutions depending on the experimental condition. The autoclaved sample showed a pronounced increase in Rp from $0.56 \pm 0.10 \text{ k}\Omega\cdot\text{cm}^2$ at day 1 to $2.01 \pm 0.004 \text{ k}\Omega\cdot\text{cm}^2$ at day 15, which indicates progressive decline in corrosion rate consistent with the formation and maturation of a protective surface layer that increasingly restricts charge-transfer processes at the metal-solution interface. The boiled condition exhibited a modest increase from $0.50 \pm 0.20 \text{ k}\Omega\cdot\text{cm}^2$ to $0.66 \pm 0.04 \text{ k}\Omega\cdot\text{cm}^2$, suggesting a moderate reduction in corrosion kinetics over time, though the protective effect appears less pronounced than in the autoclaved system and indicates development of surface layers with only partial barrier properties. In contrast, the biotic condition displayed a slight decrease in Rp from $0.67 \pm 0.22 \text{ k}\Omega\cdot\text{cm}^2$ at day 1 to $0.56 \pm 0.09 \text{ k}\Omega\cdot\text{cm}^2$ at day 15, representing a modest increase in corrosion rate or maintenance of sustained corrosion activity throughout the exposure period, possibly reflecting continuous metabolic activity within the biofilm that prevents establishment of fully passivating conditions. The sterile condition exhibited the most dramatic change, with Rp decreasing substantially from $1.30 \pm 1.13 \text{ k}\Omega\cdot\text{cm}^2$ at day 1 to $0.17 \pm 0.14 \text{ k}\Omega\cdot\text{cm}^2$ at day 15, yielding the lowest final polarization resistance value observed across all experimental conditions, indicating severe acceleration of corrosion kinetics and suggesting that in the absence of microbial colonization and associated surface modification processes, the system undergoes progressive degradation without development of protective corrosion product layers. The final

ranking of R_p values at day 15 followed the order: autoclaved ($2.01 \text{ k}\Omega\cdot\text{cm}^2$) $\gg$ boiled ($0.66 \text{ k}\Omega\cdot\text{cm}^2$) $\approx$ biotic ($0.56 \text{ k}\Omega\cdot\text{cm}^2$) $>$ sterile ($0.17 \text{ k}\Omega\cdot\text{cm}^2$), representing a ten times difference between the most and least resistant conditions. Given the inverse relationship between R_p and corrosion rate, the autoclaved condition exhibits a corrosion rate approximately 12 times lower than the sterile condition at day 15, while the biotic and boiled conditions show intermediate corrosion rates approximately 3 to 4 times higher than the autoclaved system but still significantly lower than the actively corroding sterile sample. These divergent temporal trajectories reveal three distinct corrosion behaviors: progressive passivation in the autoclaved system, accelerating degradation in the sterile system, and quasi-steady-state corrosion with persistent but moderate rates in the biotic and boiled systems.

Electrochemical impedance spectroscopy measurements were performed at 1, 7, and 15 days of immersion to characterize the interfacial electrochemical processes and evaluate the evolution of surface layer properties under the different experimental conditions. Nyquist plots (Figs. 8.16 a,c,e) revealed distinct impedance responses that evolved significantly over time, with the autoclaved condition consistently exhibiting the largest semicircle diameters, indicating the highest overall impedance, while the sterile condition displayed the smallest semicircles throughout the exposure period. Bode magnitude plots (Figs. 8.16b,d,f) corroborated these observations, showing that the autoclaved system maintained the highest impedance modulus across the entire frequency range, particularly at low frequencies where values approached $10^4 \text{ }\Omega\cdot\text{cm}^2$ by day 15, whereas the sterile condition exhibited substantially lower impedance values around $10^2 \text{ }\Omega\cdot\text{cm}^2$ at comparable frequencies. Bode phase angle plots revealed the presence of one or two time constants depending on the condition and exposure time, with maxima shifting toward lower frequencies as corrosion products accumulated, and phase angles approaching -80° in some systems, indicative of capacitive behavior associated with the formation of surface layers.

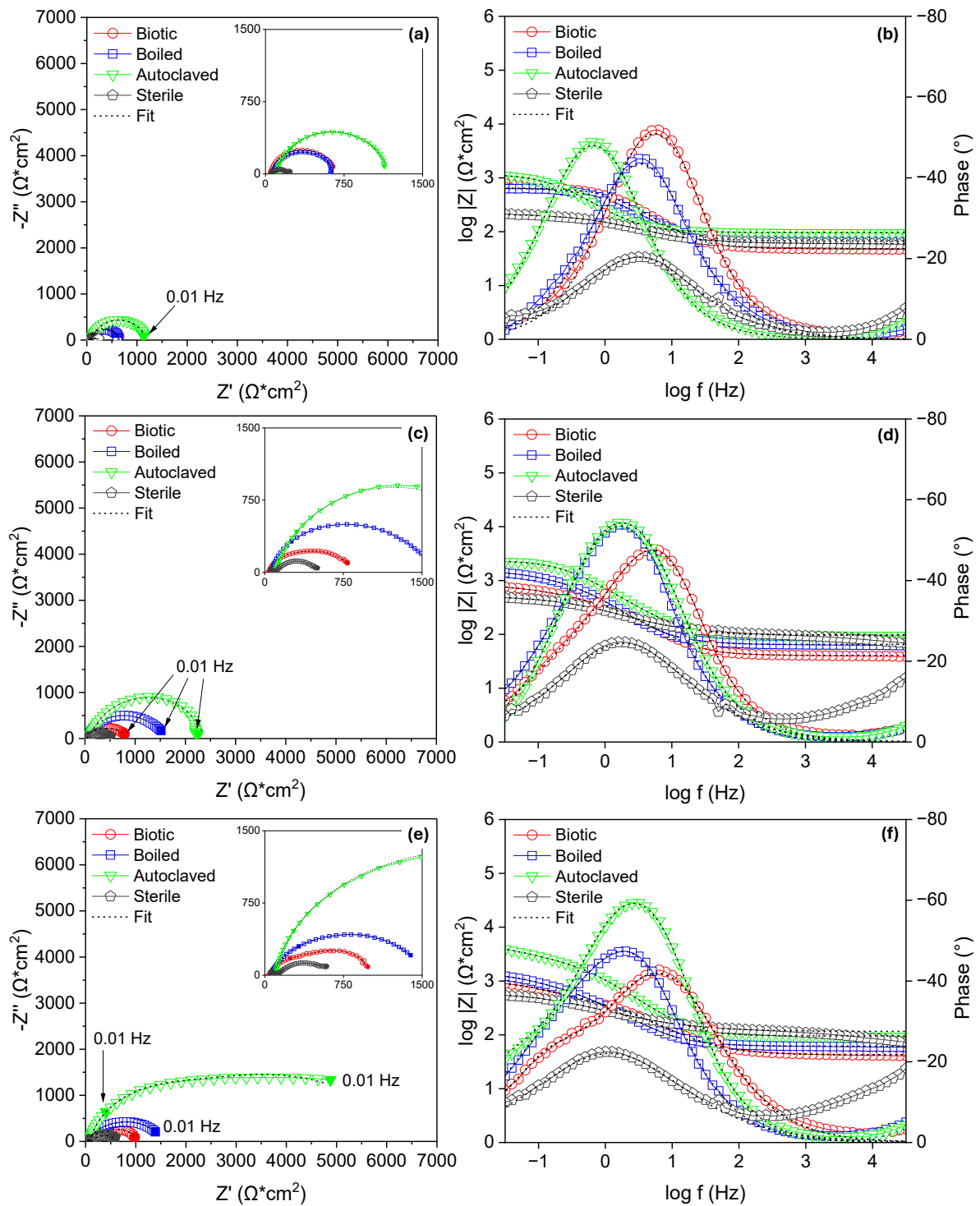


Figure 8.16. Nyquist and bode plots of ARMCO iron coupons tested in the biotic (red), boiled (blue), autoclaved (green) and sterile (gray) inocula after (a-b) 1 day, (c-d) 7 days and (e-f) 15 days.

The impedance spectra were fitted using two different equivalent electrical circuits depending on the complexity of the electrochemical response observed (Fig. 8.17).

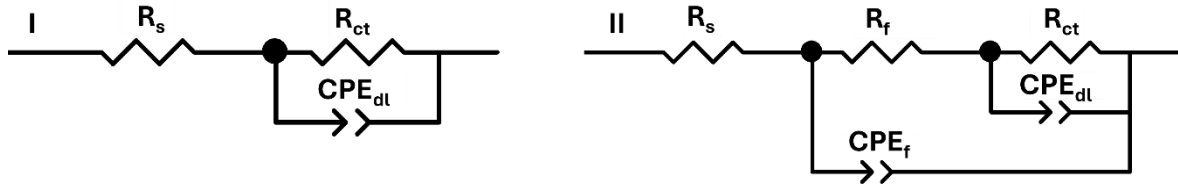


Figure 8.17. Equivalent electrical circuit (ECCs) I-II used to model impedance data.

Circuit I, consisting of a solution resistance (R_s) in series with a parallel combination of charge-transfer resistance (R_{ct}) and a constant phase element representing the electrical double layer (CPE_{dl}), was employed when a single well-defined time constant was evident in the impedance response. Circuit II, comprising R_s in series with two nested parallel R-CPE combinations; one representing the corrosion product film (R_f , CPE_f) and the other representing the charge-transfer process at the metal-electrolyte interface (R_{ct} , CPE_{dl}), was used when two distinct time constants were resolved, indicating the presence of a differentiated surface layer. All fittings yielded χ^2 values on the order of 10^{-4} , confirming excellent agreement between experimental data and the proposed models. Effective capacitance values (C_{eff}) were calculated from the CPE parameters using the Brug formula²⁶⁸ to facilitate comparison of dielectric properties across different conditions and to account for the non-ideal capacitive behavior associated with surface roughness and heterogeneity.

For the biotic condition, the impedance response at day 1 was adequately described by Circuit I, with $R_s = 48.2 \pm 0.18 \, \Omega \cdot \text{cm}^2$, $R_{ct} = 0.60 \pm 0.003 \, \text{k}\Omega \cdot \text{cm}^2$, $CPE_{dl} = 0.27 \pm 0.003 \, (\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$, $CPE_{dl-n} = 0.88 \pm 0.00$, and $C_{eff} = 149 \pm 1.65 \, \mu\text{F} \cdot \text{cm}^2$, yielding a total resistance (R_{tot}) of $0.60 \, \text{k}\Omega \cdot \text{cm}^2$. By day 7, the system transitioned to Circuit II, indicating the development of a distinct biofilm-corrosion product layer, with $R_s = 40.6 \pm 0.11 \, \Omega \cdot \text{cm}^2$, $R_{ct} = 0.37 \pm 0.02 \, \text{k}\Omega \cdot \text{cm}^2$, $R_f = 0.44 \pm 0.02 \, \text{k}\Omega \cdot \text{cm}^2$, and $R_{tot} = 0.81 \pm 0.026 \, \text{k}\Omega \cdot \text{cm}^2$, while at day 15, R_{tot} increased to $0.98 \pm 0.018 \, \text{k}\Omega \cdot \text{cm}^2$ ($R_{ct} = 0.53 \pm 0.01 \, \text{k}\Omega \cdot \text{cm}^2$, $R_f = 0.46 \pm 0.01 \, \text{k}\Omega \cdot \text{cm}^2$), representing a modest 63% increase from the initial value. The effective capacitance decreased from $149 \, \mu\text{F} \cdot \text{cm}^2$ at day 1 to $115 \, \mu\text{F} \cdot \text{cm}^2$ at day 15, suggesting thickening or densification of the interfacial region, while the CPE exponent associated with the double layer decreased from 0.88 to 0.75, indicating increasing surface heterogeneity as the biofilm matured.

The boiled condition exhibited a similar temporal evolution, with Circuit I describing the day 1 response ($R_s = 66.9 \pm 0.29 \, \Omega \cdot \text{cm}^2$, $R_{ct} = 0.58 \pm 0.003 \, \text{k}\Omega \cdot \text{cm}^2$, $C_{eff} = 204 \pm 2.57 \, \mu\text{F} \cdot \text{cm}^2$), followed by transition to Circuit II at days 7 and 15. At day 7, the system showed a substantial increase in

total resistance to $1.53 \pm 0.061 \text{ k}\Omega\cdot\text{cm}^2$ ($R_{\text{ct}} = 1.1 \pm 0.03 \text{ k}\Omega\cdot\text{cm}^2$, $R_{\text{f}} = 0.40 \pm 0.05 \text{ k}\Omega\cdot\text{cm}^2$), representing the highest impedance observed for the boiled condition during the experiment. However, by day 15, R_{tot} decreased to $1.45 \pm 0.043 \text{ k}\Omega\cdot\text{cm}^2$ ($R_{\text{ct}} = 0.80 \pm 0.03 \text{ k}\Omega\cdot\text{cm}^2$, $R_{\text{f}} = 0.65 \pm 0.03 \text{ k}\Omega\cdot\text{cm}^2$), indicating a slight reduction in overall corrosion resistance despite continued accumulation of corrosion products. The effective capacitance remained relatively stable at approximately $278 \pm 1 \text{ }\mu\text{F}\cdot\text{cm}^2$ for both days 7 and 15, while the CPE exponents ($\text{CPE}_{\text{dl-n}}$ and $\text{CPE}_{\text{f-n}}$) remained in the range of 0.72-0.86, suggesting a moderately heterogeneous surface with both conducting and insulating regions.

In stark contrast, the sterile condition was best described by Circuit I throughout the entire 15-day exposure period, indicating absence of a well-differentiated corrosion product layer despite ongoing metal dissolution. At day 1, $R_{\text{s}} = 60.2 \pm 0.10 \text{ }\Omega\cdot\text{cm}^2$, $R_{\text{ct}} = 0.15 \pm 0.001 \text{ k}\Omega\cdot\text{cm}^2$, and $C_{\text{eff}} = 469 \pm 4.10 \text{ }\mu\text{F}\cdot\text{cm}^2$, representing the lowest charge-transfer resistance among all conditions at this time point. The total resistance increased to $0.42 \pm 0.025 \text{ k}\Omega\cdot\text{cm}^2$ at day 7 ($R_{\text{s}} = 97.0 \pm 0.49 \text{ }\Omega\cdot\text{cm}^2$, $R_{\text{ct}} = 0.42 \pm 0.002 \text{ k}\Omega\cdot\text{cm}^2$) and further to $0.52 \pm 0.057 \text{ k}\Omega\cdot\text{cm}^2$ at day 15 ($R_{\text{s}} = 115.4 \pm 0.88 \text{ }\Omega\cdot\text{cm}^2$, $R_{\text{ct}} = 0.52 \pm 0.006 \text{ k}\Omega\cdot\text{cm}^2$). However, this final resistance remained the lowest among all experimental conditions, and the continued applicability of Circuit I suggest that any corrosion products formed remained poorly adherent or electrically conducting, failing to establish a distinct impedance signature separate from the charge-transfer process. The CPE exponent decreased progressively from 0.68 at day 1 to 0.57 at day 15, indicating increasing surface roughness and heterogeneity associated with localized corrosion attack, while the effective capacitance decreased from 469 to $329 \text{ }\mu\text{F}\cdot\text{cm}^2$, consistent with development of a rough, porous surface with altered dielectric properties.

The autoclaved condition displayed the most favorable impedance evolution, with Circuit I adequately describing the response at days 1 and 7, and Circuit II becoming necessary at day 15 to account for the well-developed protective layer. At day 1, $R_{\text{s}} = 96.9 \pm 0.29 \text{ }\Omega\cdot\text{cm}^2$, $R_{\text{ct}} = 1.08 \pm 0.004 \text{ k}\Omega\cdot\text{cm}^2$, and $C_{\text{eff}} = 623 \pm 3.23 \text{ }\mu\text{F}\cdot\text{cm}^2$, already representing higher initial resistance than the biotic and boiled conditions. By day 7, R_{ct} more than doubled to $2.29 \pm 0.011 \text{ k}\Omega\cdot\text{cm}^2$, with a concomitant decrease in C_{eff} to $141 \pm 0.81 \text{ }\mu\text{F}\cdot\text{cm}^2$, indicating rapid formation of a protective surface layer. At day 15, the system was best described by Circuit II, with $R_{\text{tot}} = 6.26 \pm 0.19 \text{ k}\Omega\cdot\text{cm}^2$ ($R_{\text{ct}} = 2.45 \pm 0.60 \text{ k}\Omega\cdot\text{cm}^2$, $R_{\text{f}} = 3.8 \pm 0.18 \text{ k}\Omega\cdot\text{cm}^2$), representing a rapid increase from day 1 and the highest total resistance observed across all conditions and time points. The film resistance contribution ($R_{\text{f}} = 3.8 \text{ k}\Omega\cdot\text{cm}^2$) exceeded the charge-transfer resistance ($R_{\text{ct}} = 2.45 \text{ k}\Omega\cdot\text{cm}^2$), indicating that mass transport through the protective layer became the dominant rate-

limiting process for corrosion. The effective capacitance decreased to $96 \pm 0.94 \mu\text{F}\cdot\text{cm}^2$ by day 15, the lowest value observed in the entire study, consistent with formation of a thick, dense, and highly insulating surface film. The CPE exponents remained high (0.84-0.88 for $\text{CPE}_{\text{dl-n}}$, 0.64 for $\text{CPE}_{\text{f-n}}$), suggesting relatively uniform and compact layer formation compared to the other conditions. Comparison of the final impedance parameters at day 15 reveals substantial differences in electrochemical behavior: the autoclaved condition exhibited a total resistance more than 12 times higher than the sterile condition (6.26 vs $0.52 \text{ k}\Omega\cdot\text{cm}^2$), approximately 6-7 times higher than the biotic and boiled conditions (0.98 and $1.45 \text{ k}\Omega\cdot\text{cm}^2$, respectively), and was the only condition at day 15 for which the film resistance component exceeded the charge-transfer resistance, demonstrating dominant diffusion control. The sterile condition maintained the highest effective capacitance ($329 \mu\text{F}\cdot\text{cm}^2$) and lowest CPE exponent (0.57), consistent with a rough, heterogeneous surface undergoing active localized corrosion, while the autoclaved condition showed the lowest effective capacitance ($96 \mu\text{F}\cdot\text{cm}^2$) and highest CPE exponents, indicative of a smooth, dense, and protective surface layer.

Table 8.4. Fit results using the EECs from Figure 8.17 for the biotic condition.

Biotic ^c										
EEC	Time	R _s	R _{ct}	CPE _{dl} Q	CPE _{dl-n}	R _f	CPE _f Q	CPE _{f-n}	C _{eff}	R _{tot}
	days	$\Omega \cdot \text{cm}^2$	$\text{k}\Omega \cdot \text{cm}^2$	$(\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$		$\text{k}\Omega \cdot \text{cm}^2$	$(\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$		$\mu\text{F} \cdot \text{cm}^{-2}$	$\text{k}\Omega \cdot \text{cm}^2$
I	1	48.2±0.18	0.60±0.003	0.27±0.003	0.88±0.00	n.a.	n.a.	n.a.	149±1.65	0.60±0.003
II	7	40.6±0.11	0.37±0.02	0.37±0.009	0.87±0.00	0.44±0.02	2.26±0.15	0.65±0.02	198 ±4.73	0.81±0.026
II	15	42.5±0.13	0.53±0.01	0.45±0.0007	0.75±0.00	0.46±0.01	3.02±0.14	0.82±0.02	115±1.95	0.98±0.018

^c Values are expressed as mean ± standard deviation. Depending on the equivalent electrical circuit (EEC) used, parameters that are not detected are reported as 'n.d.', whereas parameters that are not applicable are indicated as 'n.a.'.

Table 8.5. Fit results using the EECs from Figure 8.17 for the boiled condition.

Boiled ^d										
EEC	Time	R _s	R _{ct}	CPE _{dl} Q	CPE _{dl-n}	R _f	CPE _f Q	CPE _{f-n}	C _{eff}	R _{tot}
	days	$\Omega \cdot \text{cm}^2$	$\text{k}\Omega \cdot \text{cm}^2$	$(\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$		$\text{k}\Omega \cdot \text{cm}^2$	$(\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$		$\mu\text{F} \cdot \text{cm}^{-2}$	$\text{k}\Omega \cdot \text{cm}^2$
I	1	66.9±0.29	0.58±3.5	0.41±0.01	0.84±0.00	n.a.	n.a.	n.a.	204±2.57	0.58±0.00
II	7	65.0±0.20	1.1±0.03	0.48±0.01	0.86±0.00	0.40±0.05	5.02±0.12	0.75±0.07	278±3.60	1.53±0.06
II	15	61.5±0.13	0.80±0.03	0.59±0.01	0.82±0.00	0.65±0.03	2.44±0.22	0.72±0.02	277±3.81	1.45±0.04

^d Values are expressed as mean ± standard deviation. Depending on the equivalent electrical circuit (EEC) used, parameters that are not detected are reported as 'n.d.', whereas parameters that are not applicable are indicated as 'n.a.'.

Table 8.6. Fit results using the EECs from Figure 8.17 for the autoclaved condition.

Autoclaved ^e										
EEC	Time days	R _s Ω·cm ²	R _{ct} kΩ·cm ²	CPE _{dl} Q (kΩ) ⁻¹ ·s ⁿ ·cm ⁻²	CPE _{dl-n}	R _f kΩ cm ²	CPE _f Q (kΩ) ⁻¹ ·s ⁿ ·cm ⁻²	CPE _{f-n}	C _{eff} μF·cm ⁻²	R _{tot} kΩ·cm ²
I	1	96.9±0.29	1.08±0.004	0.93±0.005	0.86±0.00	n.a.	n.a.	n.a.	623±3.23	1.08±0.005
I	7	96.5±0.28	2.29±0.011	0.29±0.002	0.84±0.00	n.a.	n.a.	n.a.	141±0.81	2.29±0.011
II	15	95.3±0.20	2.45±0.60	0.17±0.002	0.88±0.00	3.8±0.18	0.97±0.06	0.64±0.02	96.0±0.94	6.26±0.19

^e Values are expressed as mean ± standard deviation. Depending on the equivalent electrical circuit (EEC) used, parameters that are not detected are reported as 'n.d.', whereas parameters that are not applicable are indicated as 'n.a.'.

Table 8.7. Fit results using the EECs from Figure 8.17 for the sterile condition.

Sterile ^f							
EEC	Time days	R _s Ω·cm ²	R _{ct} kΩ·cm ²	CPE _{dl} Q (kΩ) ⁻¹ ·s ⁿ ·cm ⁻²	CPE _{dl-n}	C _{eff} μF·cm ⁻²	R _{tot} kΩ·cm ²
I	1	60.2±0.10	0.15±0.001	1.66±0.01	0.68±0.00	469±4.10	0.15±0.001
I	7	97.0±0.49	0.42±0.002	1.13±0.01	0.64±0.00	285±2.84	0.42±0.025
I	15	115.4±0.88	0.52±0.006	1.45±0.02	0.57±0.01	329±4.00	0.52±0.057

^f Values are expressed as mean ± standard deviation. Depending on the equivalent electrical circuit (EEC) used, parameters that are not detected are reported as 'n.d.', whereas parameters that are not applicable are indicated as 'n.a.'.

The temporal evolution of the individual resistive contributions provides further insight into the divergent corrosion mechanisms operating under each condition. Analysis of total resistance ($R_{\text{tot}} = R_{\text{ct}} + R_{\text{f}}$) as a function of exposure time (Fig. 8.18a) reveals that the autoclaved system exhibited exponential growth, increasing from approximately $10^3 \Omega \cdot \text{cm}^2$ at days 1 and 7 to nearly $10^4 \Omega \cdot \text{cm}^2$ by day 15, representing more than an order of magnitude increase and the highest resistance achieved in this study. In contrast, the biotic and boiled conditions displayed relatively modest changes in R_{tot} over the 15-day period, maintaining values in the range of 10^2 to $10^3 \Omega \cdot \text{cm}^2$ with only gradual increases, while the sterile condition remained consistently around $10^2 \Omega \cdot \text{cm}^2$ throughout the experiment, showing minimal resistance development despite ongoing corrosion. Examination of the charge-transfer resistance (R_{ct}) component reveals similar trends (Fig. 8.18b), with the autoclaved condition showing progressive increases from 10^3 to approaching $10^4 \Omega \cdot \text{cm}^2$, while biotic, boiled, and sterile conditions exhibited more modest values that remained relatively stable or increased only slightly over time. The film resistance (R_{f}) component (Fig. 8.18c), which is only present in systems described by Circuit II (Fig. 8.17), provides particularly revealing information about surface layer development. At day 1, R_{f} values were either absent (Circuit I) or negligible for all conditions, indicating minimal formation of differentiated corrosion product layers during the initial exposure period. By day 7, both biotic and boiled conditions developed measurable R_{f} values on the order of $10^2 \Omega \cdot \text{cm}^2$, indicating emergence of distinct surface films, though these remained significantly lower than R_{ct} in both cases. At day 15, the autoclaved condition exhibited the most dramatic R_{f} development, reaching values approaching $10^4 \Omega \cdot \text{cm}^2$ and exceeding its corresponding R_{ct} value, demonstrating that the protective film had become the dominant barrier to charge transfer and indicating transition from activation-controlled to diffusion-limited corrosion kinetics. The biotic and boiled conditions showed moderate R_{f} values at day 15, remaining in the 10^2 to $10^3 \Omega \cdot \text{cm}^2$ range and contributing substantially but not dominantly to the total resistance. Notably, the sterile condition showed no detectable R_{f} contribution at any time point, confirming the absence of a well-developed protective surface layer throughout the experiment and supporting the interpretation that corrosion in this system proceeds through direct charge-transfer processes at an active, poorly passivated interface. The contrasting evolution of R_{f} across the different conditions, from negligible in the sterile system to dominant in the autoclaved system, underscores the critical role of surface film formation in determining long-term corrosion resistance and demonstrates that thermal treatment history profoundly influences not only the final resistance magnitude but also the relative contributions of interfacial charge transfer versus mass transport through surface layers. These impedance characteristics are fully consistent with the trends observed in polarization resistance

measurements (Table 8.3) and provide quantitative evidence for the formation of increasingly protective surface layers in the order: autoclaved >> boiled $\approx$ biotic >> sterile.

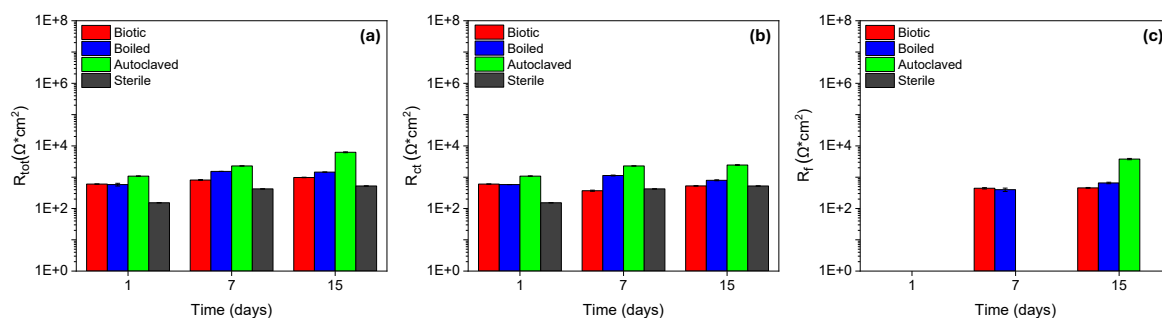


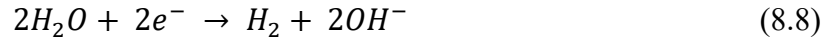
Figure 8.18. Evolution of the resistive contributions for each condition and for each time point: (a) $R_{\text{tot}} = R_{\text{ct}} + R_{\text{f}}$; (b) R_{ct} ; (c) R_{f} .

8.5 Corrosion mechanisms

At the onset of immersion, all four experimental conditions exhibited relatively similar open circuit potentials (-0.62 to -0.65 V vs Ag/AgCl sat., Fig. 8.14) and modest polarization resistances (0.15 to 1.30 $\text{k}\Omega \cdot \text{cm}^2$), indicating comparable initial surface states prior to significant microbial colonization or corrosion product accumulation. However, critical differences in solution chemistry resulting from the thermal treatments established the foundation for the dramatically divergent corrosion trajectories observed over the subsequent 15 days. The sterile condition, which underwent autoclave sterilization, filtration and UV treatment, exhibited complete elimination of microbial life as confirmed by 16S rRNA sequencing (Section 8.3), yet paradoxically displayed the lowest initial charge-transfer resistance ($R_{\text{ct}} = 0.15$ $\text{k}\Omega \cdot \text{cm}^2$) and notably contained no detectable carbonate by ion chromatography (Table 8.2). In contrast, the autoclaved condition, despite surviving colonization by ultra-resistant spore formers including *Pseudomonas* and *Effusibacillus* (Fig. 8.12), showed elevated carbonate concentration (525 mg L^{-1}) and correspondingly exhibited higher initial R_{ct} (1.08 $\text{k}\Omega \cdot \text{cm}^2$). The biotic and boiled conditions displayed intermediate initial resistances and carbonate levels (213 and 253 mg L^{-1} , respectively), with intact or partially viable microbial communities dominated by the thermophilic bacterium *Tepidiphilus*, which survived boiling through endospore formation³³⁴ and rapidly recolonized the system upon return to favorable growth conditions at 45 °C.

The fundamental electrochemical processes governing iron corrosion in all four systems involve the anodic dissolution reaction (Eq. 8.7), coupled with cathodic reactions that, in the anaerobic or

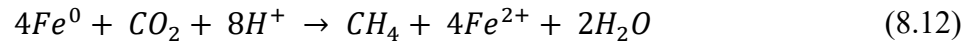
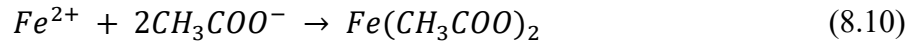
microaerobic conditions of the digester inoculum, primarily consist of water reduction (Eq. 8.8) and proton reduction (Eq. 8.9).



However, the rate-determining steps, the spatial distribution of anodic and cathodic sites, and the nature of secondary precipitation reactions differ profoundly depending on microbial activity and solution chemistry. In the sterile system, corrosion proceeds through purely abiotic electrochemical mechanisms, with initial uniform dissolution rapidly transitioning to localized attack as surface heterogeneities and incipient oxide formation create galvanic cells that concentrate anodic activity at discrete sites. The absence of carbonate in the sterile solution precludes formation of protective siderite films, and while Raman spectroscopy (Fig. 8.11) detected iron carbonate in the corrosion products at day 15, this phase likely precipitates within corrosion pits or as loose deposits from locally generated carbonate via organic acid degradation, rather than as adherent passivating layers. The progressively decreasing polarization resistance from $1.30 \text{ k}\Omega \cdot \text{cm}^2$ at day 1 to $0.17 \text{ k}\Omega \cdot \text{cm}^2$ at day 15, coupled with the persistent applicability of the single time-constant equivalent circuit (circuit I, Fig. 8.17) throughout the experiment, confirms that no protective surface layer developed, and that corrosion remained under activation control with increasingly facile charge transfer as pit propagation exposed fresh metal surfaces.

By day 7, the four systems had evolved along distinctly different mechanistic pathways, as evidenced by the emergence of differentiated impedance responses and marked changes in open circuit potential behavior (Fig. 8.14). The biotic condition exhibited a transient negative shift in OCP to approximately -0.65 V at day 7, coinciding with the transition from Circuit I to Circuit II in the impedance response, indicating establishment of a mature biofilm with metabolically active microorganisms. The 16S rRNA sequencing data revealing $>70\%$ *Tepidiphilus* in both biofilm and solution phases (Fig. 8.12), along with diverse methanogenic archaea including *Methanospirillum*, *Methanosarcina*, and *Methanomicrobiales* specifically enriched in the biofilm (Fig. 8.13), provides direct evidence for microbially influenced corrosion processes. These organisms can participate in corrosion through multiple pathways: i) production of organic acids (acetic, oxalic, formic) that locally acidify the metal-biofilm interface and enhance iron dissolution through complexation (Eq. 8.10); ii) cathodic depolarization via consumption of surface-adsorbed hydrogen by hydrogenotrophic methanogens (Eq. 8.11), which removes the

protective hydrogen layer and sustains continued anodic dissolution; and iii) direct extracellular electron transfer from metallic iron to methanogenic archaea according to Equation 8.12, a mechanism that has been increasingly recognized in recent literature for both pure cultures and mixed anaerobic communities.^{146,320}



The moderate oxalate concentration (3019 mg L⁻¹, Table 8.2) detected by ion chromatography in the biotic condition, substantially higher than in thermally treated samples, supports active microbial metabolism and organic acid production. The formation of both magnetite and hematite as dominant Raman-active phases (Fig. 8.11), coupled with detection of phosphorus by EDS (Fig. 8.3) but absence of crystalline phosphate minerals, indicates that corrosion products precipitate as mixed-valence iron oxides incorporating phosphorus from microbial biomass (phospholipids, nucleic acids, extracellular polymeric substances) in amorphous or surface-adsorbed forms. Critically, these biologically mediated corrosion products, while thick (~10 µm in cross-section, Fig. 8.7), provide only modest impedance ($R_{tot} = 0.81 \text{ k}\Omega \cdot \text{cm}^2$ at day 7), suggesting that the biofilm-corrosion product composite layer is porous and electrically conductive, allowing continued but spatially homogeneous corrosion rather than localized pitting.

The boiled condition at day 7 exhibited the highest total impedance among biotic and boiled systems (1.53 kΩ·cm²), correlating with the thickest corrosion product layer observed by SEM cross-section (Fig. 8.8). The survival and proliferation of *Tepidiphilus* (>80% relative abundance) following boiling treatment demonstrates that endospore formation renders certain thermophilic bacteria resistant to temperatures exceeding 101 °C, enabling rapid recolonization and establishment of metabolically active populations. However, the microbial community structure differs from the untreated biotic condition in two critical aspects: i) elimination of microbial diversity, with *Tepidiphilus* representing a near-monoculture, and ii) simplification of the archaeal community to exclusively *Methanothermobacter*, indicating that only the most thermotolerant methanogens survived boiling. This altered community composition, combined with elevated carbonate (253 mg L⁻¹) and the highest phosphorus concentration (403 mg L⁻¹) detected by ICP-AES (Table 8.1), creates conditions favoring rapid precipitation of corrosion products with distinctive crystalline morphology. The elongated parallelepiped-shaped crystals observed by

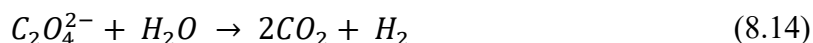
SEM (Fig. 8.2b), initially hypothesized to be iron phosphates, are now attributed to phosphate oxyhydroxide iron phases (goethite α -FeOOH, lepidocrocite γ -FeOOH, or akaganeite β -FeOOH) that form under the specific pH and redox conditions of the boiled inoculum. Literature reports that these species of iron oxyhydroxide strongly bind phosphate ions, acting as regulator of phosphorous in soils and water.^{335–337} Indeed, these materials capture phosphate through high-affinity surface complexation, influencing bacterial adhesion. The coexistence of magnetite, hematite, and likely oxyhydroxide phases, coupled with high dissolved iron (1.7 mg L^{-1} , Table 8.1), indicates that while corrosion products accumulate rapidly, they do not establish effective diffusion barriers, and corrosion proceeds through mixed charge-transfer and mass-transport control with continued metal dissolution balanced by extensive but non-protective precipitation.

The autoclaved condition at day 7 had already established clear protective characteristics, with R_{ct} increasing to $2.29 \text{ k}\Omega \cdot \text{cm}^2$ while maintaining Circuit I response, indicating enhanced charge-transfer resistance without yet forming a distinct film impedance signature. The dramatic difference between autoclaved and boiled conditions, despite both undergoing thermal treatment and both supporting microbial recolonization by spore-forming bacteria, underscores that the severity and duration of thermal treatment fundamentally alter solution chemistry in ways that override microbial effects. The exceptionally high carbonate concentration (525 mg L^{-1}) in the autoclaved condition, more than double that of the boiled system, creates a chemical environment strongly favoring siderite (FeCO_3) precipitation according to Equation 8.13:



The thermodynamic stability and solubility product of siderite ($K_{sp} \approx 10^{-10.7}$) dictate that at elevated carbonate activities, even modest ferrous iron concentrations rapidly exceed supersaturation, promoting nucleation and growth of adherent iron carbonate surface films.³³⁸ The critical distinction between the autoclaved and sterile conditions lies precisely in this carbonate-mediated passivation: both systems ultimately form siderite as detected by Raman spectroscopy (strong peak at 1080 cm^{-1}), but in the autoclaved case, the high bulk carbonate concentration drives rapid, spatially uniform precipitation directly on the metal surface, forming a dense protective layer, whereas in the sterile system, siderite forms only through local carbonate generation within corrosion pits or via precipitation from solution, yielding non-adherent deposits that fail to passivate the surface.

By day 15, the four experimental conditions had established fundamentally different corrosion regimes characterized by distinct electrochemical signatures, surface morphologies, and corrosion product assemblages that reflect the interplay between microbial activity, solution chemistry, and surface film formation. The sterile condition continued along its trajectory of progressive degradation, with polarization resistance decreasing to $0.17 \text{ k}\Omega\cdot\text{cm}^2$ and total impedance remaining below $1 \text{ k}\Omega\cdot\text{cm}^2$ throughout the exposure period, coupled with the highest dissolved iron concentration (4.5 mg L^{-1} , Table 8.1) indicating sustained metal loss. Cross-sectional analysis (Fig. 8.10) revealed severe localized corrosion with pitting penetration exceeding $10 \text{ }\mu\text{m}$, while SEM surface imaging (Fig. 8.6) showed irregular attack morphology devoid of protective deposits. The Raman spectrum (Fig. 8.11) dominated by intense siderite peaks appears paradoxical given this active corrosion, but can be reconciled by recognizing that in the absence of bulk carbonate, siderite forms only through secondary reactions involving locally produced carbonate from organic acid decomposition (e.g., oxalate degradation, Equation 8.14), which precipitates preferentially within pits or as loose surface accumulations rather than as dense adherent films.



The absence of phosphorus (Table 8.1) in the sterile corrosion products, contrasting with its presence in all microbially influenced conditions, further confirms that the observed phases result purely from abiotic electrochemical processes and chemical precipitation, without biogenic contribution. The impedance response remaining describable by Circuit I even after 15 days demonstrates that no differentiated protective layer forms, and that the loose siderite deposits detected by Raman (Figure 8.11) do not establish a distinct electrochemical impedance separate from the charge-transfer process at the corroding metal interface. This purely abiotic corrosion mechanism, characterized by localized attack and high metal dissolution, establishes the baseline against which microbially influenced processes can be evaluated.

The biotic condition at day 15 exhibited modest improvement in corrosion resistance relative to day 7, with total impedance increasing to $0.98 \text{ k}\Omega\cdot\text{cm}^2$ ($R_{\text{ct}} = 0.53 \text{ k}\Omega\cdot\text{cm}^2$, $R_{\text{f}} = 0.46 \text{ k}\Omega\cdot\text{cm}^2$) and OCP ennobling (Fig. 8.14) to approximately -0.60 V , indicating partial passivation through biofilm maturation and corrosion product consolidation. The comparable contributions of charge-transfer and film resistances suggest that while a distinct biofilm-mineral layer has formed, it remains only modestly protective, allowing continued metabolic activity and corrosion. The dissolved iron concentration (0.83 mg L^{-1} , Table 8.1) lies intermediate between the highly

protective autoclaved condition (0.24 mg L^{-1}) and the actively corroding sterile system (4.5 mg L^{-1}), indicating that microbial activity simultaneously promotes corrosion through cathodic depolarization and organic acid production, while also facilitating precipitation of corrosion products that partially inhibit further attack. The compact $10 \text{ }\mu\text{m}$ thick corrosion layer observed in cross-section (Fig. 8.7), composed of magnetite and hematite with incorporated phosphorus from biofilm components, demonstrates that microbially influenced corrosion in this thermophilic anaerobic system does not exhibit the severe acceleration sometimes associated with sulfate-reducing bacteria in marine environments, but rather establishes a quasi-steady-state condition where biofilm-mediated corrosion and biofilm-induced mineralization reach approximate balance. The moderate carbonate concentration (213 mg L^{-1} , Table 8.2) and high oxalate content (3019 mg L^{-1} , Table 8.2) create conditions where siderite formation is thermodynamically favorable but kinetically slow compared to oxide precipitation, explaining why Raman spectroscopy (Fig. 8.11) shows predominantly magnetite and hematite with only minor carbonate signatures despite the presence of bulk carbonate. The decreasing CPE exponent ($\text{CPE}_{\text{dl-n}} = 0.75$ at day 15) and effective capacitance ($115 \text{ }\mu\text{F}\cdot\text{cm}^2$) indicate progressive surface heterogeneity and layer densification, consistent with ongoing biofilm maturation and consolidation of the mineral-organic composite that characterizes mature microbially influenced corrosion layers.

The boiled condition at day 15 maintained its intermediate character, with total impedance ($1.45 \text{ k}\Omega\cdot\text{cm}^2$) and polarization resistance ($0.66 \text{ k}\Omega\cdot\text{cm}^2$) suggesting persistent corrosion despite the thickest corrosion product layer ($\sim 20 \text{ }\mu\text{m}$, Fig. 8.8) observed across all conditions. This apparent contradiction indicates that the crystalline phases forming in the thermally altered, *Tepidiphilus*-dominated system, while abundant, do not establish effective diffusion barriers. The elevated dissolved iron (1.7 mg L^{-1} , Table 8.1), highest phosphorus concentration (403 mg L^{-1} , Table 8.1), and presence of distinctive iron oxyhydroxide crystals suggest rapid cycling of iron dissolution and precipitation without formation of dense, adherent passivating layers. The Raman spectrum showing mixed magnetite, hematite, and enhanced carbonate relative to the biotic condition reflects the altered solution chemistry (elevated carbonate from boiling) and simplified microbial community (*Tepidiphilus* monoculture, *Methanothermobacter* exclusively), but the film resistance component ($R_f = 0.65 \text{ k}\Omega\cdot\text{cm}^2$) remaining lower than charge-transfer resistance ($R_{\text{ct}} = 0.80 \text{ k}\Omega\cdot\text{cm}^2$) indicates that mass transport through the thick corrosion layer is not the rate-limiting step. This suggests a porous, heterogeneous layer structure that allows continued access of corrosive species to the metal surface despite its substantial thickness, highlighting that layer

thickness alone does not determine protective efficacy-density, adherence, and ionic conductivity are equally critical factors governing long-term corrosion resistance.

The autoclaved condition at day 15 demonstrated unequivocally superior corrosion protection, with total impedance reaching $6.26 \text{ k}\Omega\cdot\text{cm}^2$ (more than 12 times higher than sterile and 6 times higher than biotic/boiled), open circuit potential ennobling to -0.60 V , and the lowest dissolved iron concentration (0.24 mg L^{-1} , Table 8.1) confirming minimal ongoing metal loss. The transition to Circuit II with film resistance ($R_f = 3.8 \text{ k}\Omega\cdot\text{cm}^2$) exceeding charge-transfer resistance ($R_{ct} = 2.45 \text{ k}\Omega\cdot\text{cm}^2$) provides definitive evidence that corrosion has transitioned from activation control to diffusion-limited kinetics, with mass transport through the protective surface layer becoming the rate-determining process. Cross-sectional analysis (Fig. 8.9) revealing only a thin ($\sim 1 \text{ }\mu\text{m}$) corrosion layer demonstrates that effective passivation does not require thick deposits but rather depends on formation of dense, adherent, low-porosity films that block ionic transport. The Raman spectrum (Fig. 8.11) showing both iron oxides and prominent siderite (1080 cm^{-1}), coupled with the exceptionally high carbonate concentration (525 mg L^{-1} , Table 8.2) and lowest effective capacitance ($96 \text{ }\mu\text{F}\cdot\text{cm}^2$), indicates formation of a compact mixed oxide-carbonate passivating layer. The mechanism of protection can be understood through considering that at high carbonate activities, ferrous ions released through anodic dissolution immediately encounter supersaturated conditions with respect to siderite, precipitating directly on the metal surface according to Eqs. 8.15-8.16:



This rapid, spatially uniform precipitation prevents the development of localized corrosion cells and establishes a continuous barrier that limits further anodic dissolution. Importantly, this carbonate-mediated passivation occurs in the presence of microorganisms, such *Pseudomonas*, *Effusibacillus*, *Bacillus*, *Methanothermobacter*, detected by 16S rRNA sequencing, demonstrating that this microorganisms can participate in the reaction, as already confirmed by the literature.^{196,198,199,339} The contrast with the sterile condition, which also contains carbonate-rich corrosion products but exhibits severe localized attack, highlights that the kinetics and spatial distribution of carbonate precipitation, determined by bulk carbonate availability, are critical: high bulk carbonate drives rapid, uniform passivation (autoclaved), while locally generated carbonate produces non-protective deposits (sterile).

8.6 Concluding remarks

The comparative analysis of these four conditions reveals that corrosion of ARMCO iron in anaerobic digester environments is governed by competition between carbonate-mediated passivation and various microbially influenced corrosion mechanisms, with the dominant process determined by solution chemistry rather than merely the presence or absence of microorganisms. The sterile condition establishes that purely abiotic corrosion in carbonate-depleted solutions proceeds through localized attack without an uniform passivation, accumulating corrosion products that do not protect the surface. The biotic condition demonstrates that thermophilic anaerobic microbial communities, while promoting corrosion through cathodic depolarization and organic acid production, simultaneously induce precipitation of mixed-valence iron oxides that provide modest protection, resulting in more uniform, layer-forming corrosion rather than severe localized pitting. Critically, the boiled and autoclaved conditions reveal that thermal treatment severity controls corrosion behavior not primarily through sterilization efficacy (both support microbial recolonization by resistant spore formers) but through modifying solution carbonate concentrations via thermal decomposition of organic matter. Boiling produces moderate carbonate elevation insufficient for effective passivation, while autoclave treatment generates carbonate levels that enable formation of dense, protective siderite-based films that render microbial corrosion mechanisms irrelevant. This finding has profound implications for understanding and controlling corrosion in biogas digesters and similar environments: effective corrosion mitigation may be achieved not through biocide addition or thermal sterilization aimed at eliminating microbial activity, but rather through manipulation of solution chemistry and microbiology, particularly carbonate alkalinity and *Bacilli* enrichment, to promote rapid formation of protective iron carbonate surface films that provide robust, long-term passivation regardless of microbial colonization.

Chapter 9

9 Microbiologically Influenced Corrosion on Carbon Steel

Anaerobic digestion represents a critical technology for sustainable waste management and renewable energy production, converting organic waste streams into biogas (primarily methane and carbon dioxide) while reducing waste volume and stabilizing organic matter.²⁸⁰ Industrial-scale anaerobic digesters operate under strictly oxygen-free conditions at mesophilic (35-40 °C) or thermophilic (50-60 °C) temperatures, hosting complex microbial consortia including fermentative bacteria, syntrophic acetogens, and methanogenic archaea that cooperatively degrade organic substrates.^{106,108,157,263,286,340,341} These systems process diverse feedstocks ranging from municipal sewage sludge and agricultural waste to industrial organic effluents, generating environments characterized by neutral to slightly alkaline pH (6.5-8.0), high organic acid concentrations, elevated ionic strength, and rich microbial populations (10^8 - 10^{10} cells mL⁻¹). Carbon steel remains the predominant structural material for anaerobic digester construction due to its favorable combination of mechanical strength, weldability, availability, and cost-effectiveness.^{342,343} However, the unique physicochemical and biological conditions within digesters create aggressive corrosive environments that significantly limit equipment lifespan and compromise operational safety.

Microbiologically influenced corrosion (MIC) in anaerobic digesters represents a multifaceted degradation process distinct from purely abiotic electrochemical corrosion, involving direct and indirect interactions between steel surfaces and microbial communities.^{152,152,318,320} The corrosion mechanism includes multiple coupled processes: electrochemical reactions at the metal/electrolyte interface, biofilm formation creating localized chemical microenvironments, microbial metabolic activities modifying pH and redox conditions, and precipitation of biologically-influenced corrosion products. Methanogenic archaea, particularly hydrogenotrophic methanogens of the genera *Methanothermobacter*, *Methanobacterium*, and *Methanoculleus*, play a controversial role in anaerobic MIC by consuming cathodically-produced hydrogen (Eq. 9.1), thereby depolarizing the cathode and potentially accelerating the overall corrosion rate.^{141,157,163,165,318} Sulfate-reducing bacteria (SRB), when present due to sulfate in feedstocks or process additives, contribute to severe localized corrosion through biogenic hydrogen sulfide production and formation of iron sulfide scales.^{256,344,345}



Fermentative and acetogenic bacteria generate organic acids (acetate, propionate, butyrate) that can locally acidify biofilm microenvironments despite bulk solution buffering, promoting aggressive dissolution of steel and corrosion products.^{346,347} Additionally, the complex organic matrix of digestate provides nutrients supporting dense biofilm development, while phosphate from biomass degradation (nucleic acids, phospholipids, ATP) and inorganic additives reaches concentrations of hundreds of mg L⁻¹, influencing corrosion product chemistry through precipitation of iron phosphate phases.

The corrosion products formed on carbon steel in anaerobic digesters differ substantially from those in sterile aqueous solutions or aerobic biological systems. While classical abiotic corrosion at neutral pH produces iron oxides and oxyhydroxides (magnetite Fe₃O₄, maghemite γ -Fe₂O₃, lepidocrocite γ -FeOOH, goethite α -FeOOH), the presence of active microbial communities and high phosphate concentrations promotes formation of mixed iron phosphate-oxide layers with complex, often poorly-crystalline structures.^{346,347} The protective character of these layers remains controversial: some studies report that biofilm-associated corrosion products provide enhanced protection compared to abiotic films,³⁴⁸ while others demonstrate that biologically-influenced layers exhibit high porosity, poor adhesion, and non-uniform coverage that sustains rather than inhibits corrosion.^{349,350} The role of biofilm extracellular polymeric substances (EPS) in templating mineral precipitation, modifying surface wettability, and creating diffusion barriers adds further complexity to understanding protection mechanisms.

Despite extensive research on MIC in various environments, fundamental questions remain regarding the specific mechanisms by which anaerobic digester microbial communities influence carbon steel corrosion and whether this influence is predominantly detrimental or can be harnessed for corrosion mitigation. Thermal treatment of digester inocula or biomass represents a potential intervention strategy with multiple practical applications: pretreatment of feedstocks to enhance biogas production, sanitation of digestate for agricultural use, and potentially modification of corrosion behavior through alteration of microbial community composition and chemical properties.^{245,285,325} Conventional thermal treatments include boiling (101 °C, atmospheric pressure) which selectively inactivates vegetative cells while preserving spores, and autoclaving (121 °C, elevated pressure) which achieves more complete sterilization. However, thermal treatment not only affects microbial viability but also fundamentally alters the chemical composition of the treated material through protein denaturation, cell membrane disruption, release of intracellular contents, thermal decomposition of organic compounds, and modification of mineral solubility and precipitation kinetics. The net effect of thermal treatment on corrosion

behavior therefore represents a complex interplay between reduced microbial metabolic activity and altered solution chemistry, with outcomes that cannot be predicted from microbial inactivation alone.

The objective of this study was to systematically investigate the influence of thermal treatment severity on microbiologically influenced corrosion of carbon steel S355 in anaerobic digester inoculum. By correlating microbial community dynamics, corrosion product characteristics, and electrochemical behavior we elucidated the mechanisms by which thermal treatment modifies corrosion processes and we identified optimal treatment strategies for corrosion mitigation in anaerobic digester applications.

9.1 Surface and morphology investigation

Optical microscopy examination of carbon steel S355 coupons after 15 days of immersion at 45 °C revealed substantial differences in corrosion morphology and product deposition among the four experimental conditions (Fig. 9.1). The biotic sample (Fig. 9.1a) exhibited heterogeneous surface coverage with green non-uniform corrosion products distributed across the entire coupon surface, accompanied by evidence of localized corrosion attack. The boiled specimen (Fig. 9.1b) displayed a similar pattern of surface degradation with a more uniform dark corrosion deposits and a slightly different texture and distribution compared to the biotic condition. The autoclaved coupon (Fig. 9.1c) showed a more uniform appearance with lighter and green colored corrosion products and reduced surface heterogeneity. The sterile control (Fig. 9.1d) presented a completely different corrosion pattern compared to the other conditions, with dark brownish corrosion products of different morphology present heterogeneously on the surface of the metal coupon.

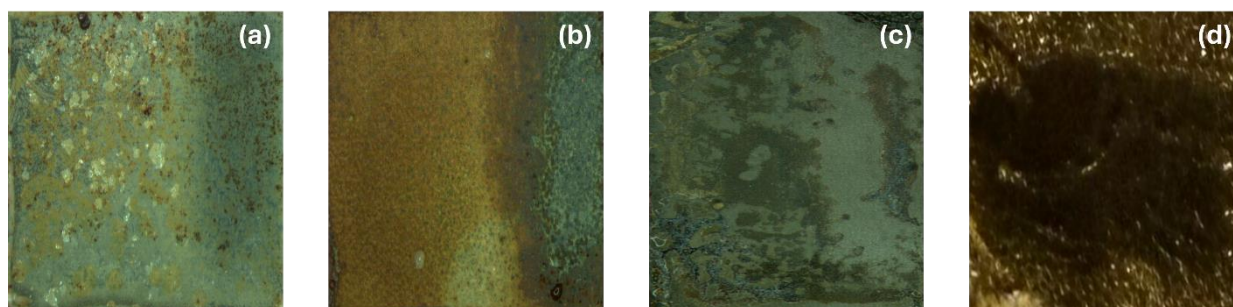


Figure 9.1. Optical microscopy images of carbon steel coupons after 15-day test in the (a) biotic, (b) boiled, (c) autoclaved and (d) sterile solutions at 45 °C.

Scanning electron microscopy provided higher magnification visualization of the surface morphology and corrosion product architecture (Fig. 9.2). The biotic sample (Fig. 9.2a) displayed

a complex, multilayered corrosion product deposit with irregular surface topography characterized by nodular formations, cracks, and porous structures. The corrosion products appeared as a continuous but non-uniform multi-layer covering the underlying steel substrate, with varying thickness across different regions of the coupon surface.

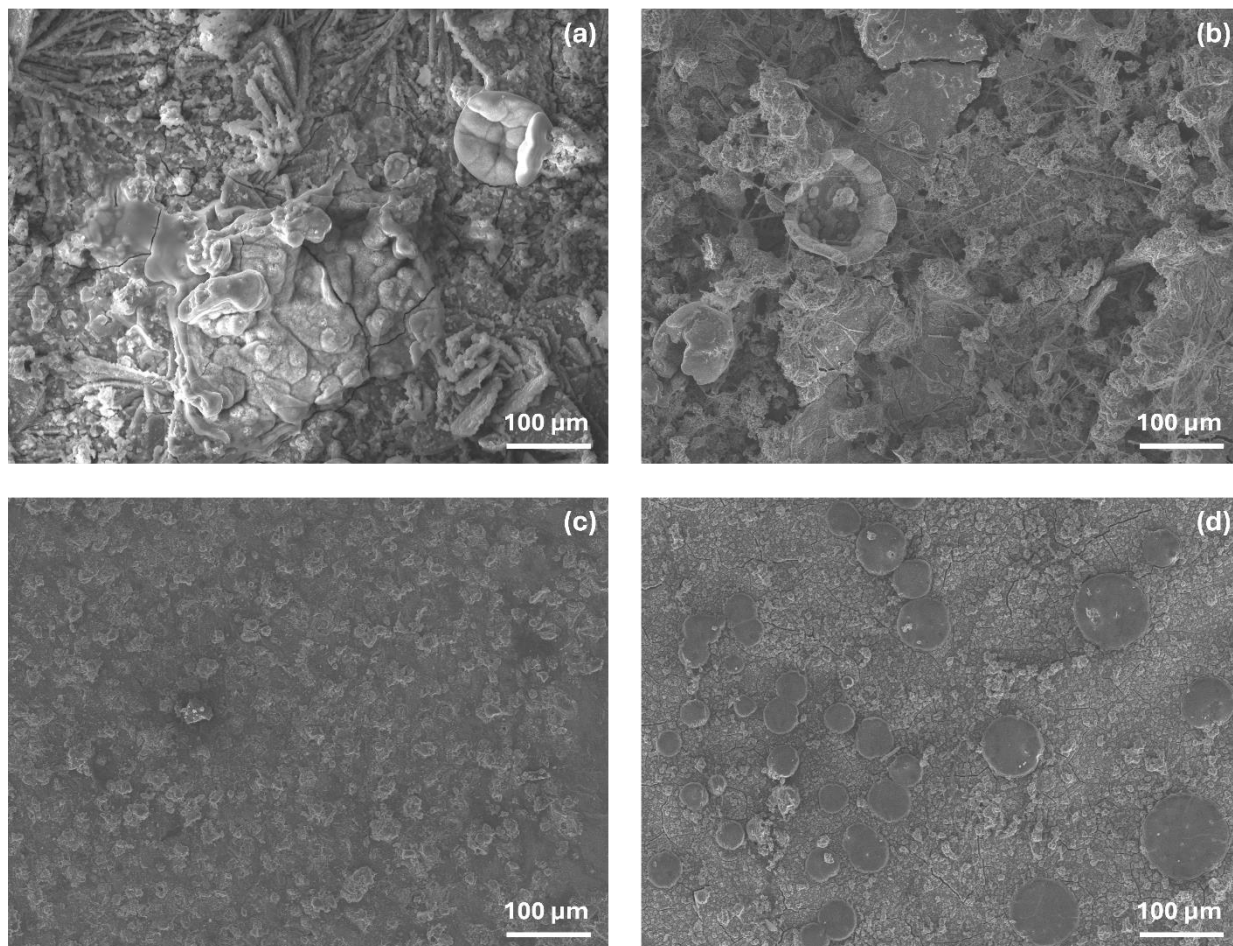


Figure 9.2. Scanning electron microscopy (SEM) images of carbon steel coupons after 15-day test in the (a) biotic, (b) boiled, (c) autoclaved and (d) sterile solutions at 45 °C.

The boiled condition (Fig. 9.2b) exhibited corrosion products with similar coverage but with distinct morphological features, including a less porous structure and more compact appearance compared to the biotic sample. Crystalline-like formations were evident in some regions, suggesting different nucleation and growth mechanisms of the corrosion products. Additionally, biofilm formation occurs under both biotic and boiled conditions on complex, multilayered corrosion products that are heterogeneously distributed. The autoclaved specimen (Fig. 9.2c) showed a markedly different surface morphology, with a more uniform and compact corrosion layer characterized by finer grain structures and reduced surface roughness. The corrosion products formed a relatively smooth, continuous film with minimal porosity and fewer surface

defects compared to both biotic and boiled conditions. The sterile control (Fig. 9.2d) presented an intermediate morphology with corrosion products forming discrete clusters and patches rather than a continuous layer, indicating different nucleation behavior in the absence of microbial activity.

Energy-dispersive X-ray spectroscopy mapping of the coupon surfaces provided detailed elemental distribution information (Figs. 9.3-9.6). In the biotic condition (Fig. 9.1), the elemental maps revealed intense and widespread phosphorus and oxygen signals across the entire analyzed surface, with phosphorus appearing as the dominant non-metallic element alongside oxygen. The iron signal was attenuated in regions of thick corrosion product coverage, consistent with the presence of a substantial overlayer. The spatial correlation between phosphorus and oxygen suggested the formation of iron phosphate-based corrosion products as the primary surface phase. Calcium signals were also detected, potentially originating from carbonate species present on the surface as protective corrosion products. The boiled sample (Fig. 9.4) exhibited a remarkably similar elemental distribution pattern, with strong phosphorus and oxygen signals distributed across the surface in a pattern comparable to the biotic condition. This similarity in surface chemistry, despite the thermal deactivation of vegetative cells, suggested that certain chemical components of the inoculum, such as phosphate species, remained active in influencing corrosion product formation even after boiling treatment. Moreover, at higher magnitude it is even more evident that both biotic and boiled samples exhibited biofilm on the surface of the metal coupon. The autoclaved sample (Fig. 9.9) displayed a distinctly different elemental composition, characterized by dominant oxygen signals with minimal phosphorus detection. The iron signal was more prominent compared to biotic and boiled conditions, indicating a thinner or less complete corrosion product coverage. The absence of significant phosphorus in the autoclaved condition suggested that the more severe thermal treatment (121 °C, elevated pressure) either decomposed or inactivated the phosphate-containing components responsible for phosphate-rich layer formation. The sterile control (Fig. 9.6) showed intermediate compositional characteristics, with oxygen as the predominant non-metallic element and scattered, localized phosphorus signals. The phosphorus distribution appeared more heterogeneous compared to biotic and boiled samples, indicating sporadic rather than uniform phosphate incorporation into the corrosion products.

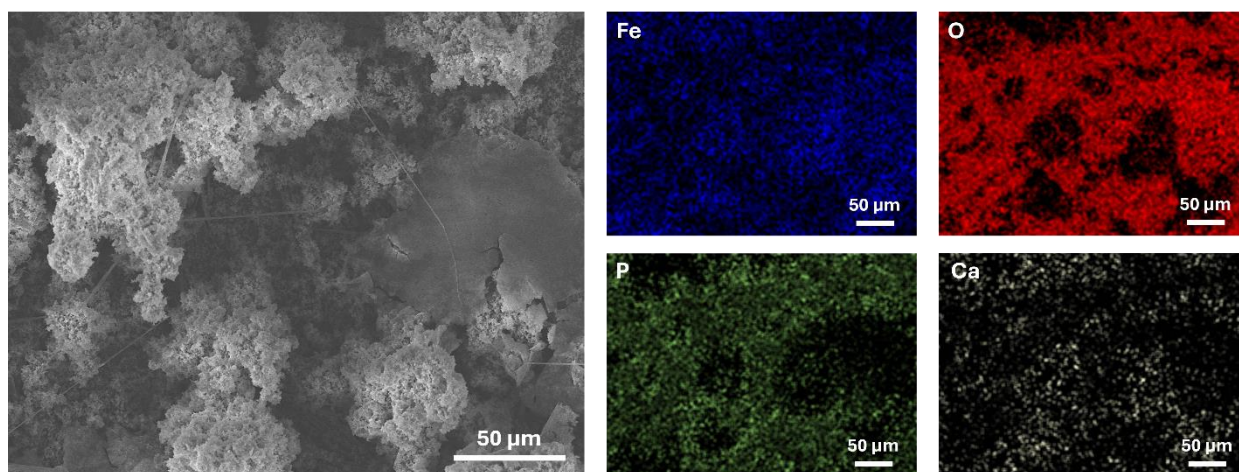


Figure 9.3. SEM-EDS images of S355 carbon steel coupon after 15-day test in the biotic solution at 45 °C.

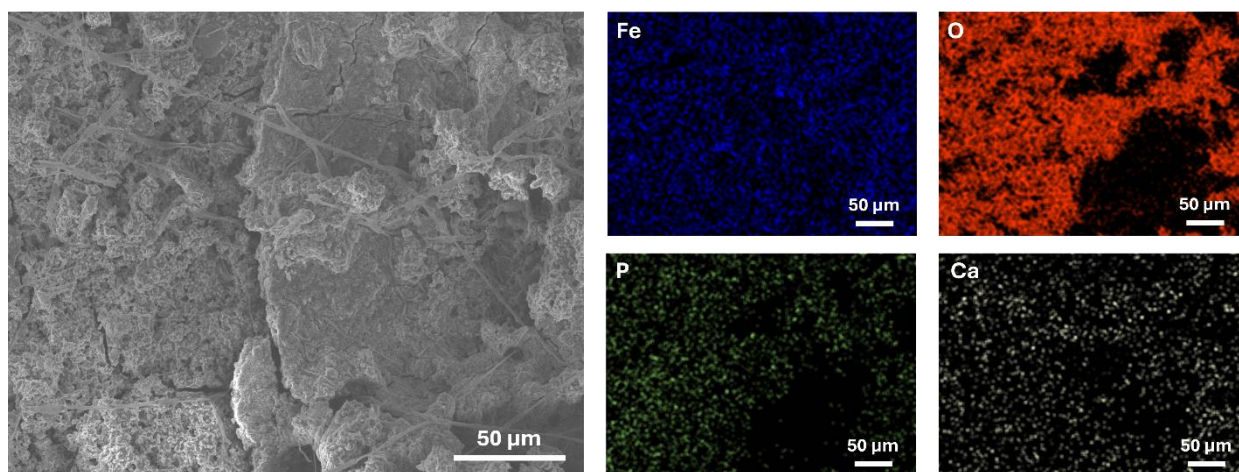


Figure 9.4. SEM-EDS images of S355 carbon steel coupon after 15-day test in the boiled solution at 45 °C.

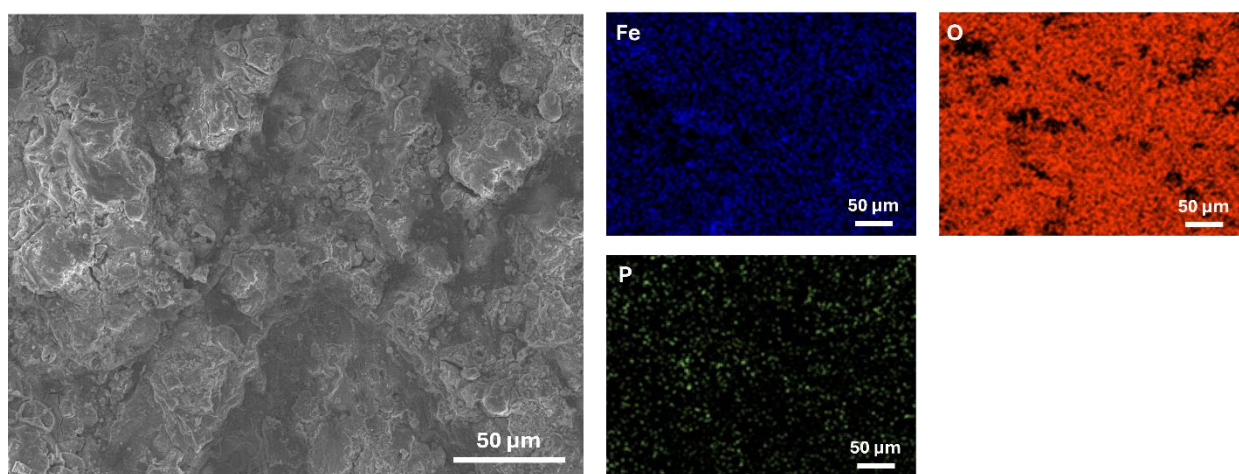


Figure 9.5. SEM-EDS images of S355 carbon steel coupon after 15-day test in the autoclaved solution at 45 °C.

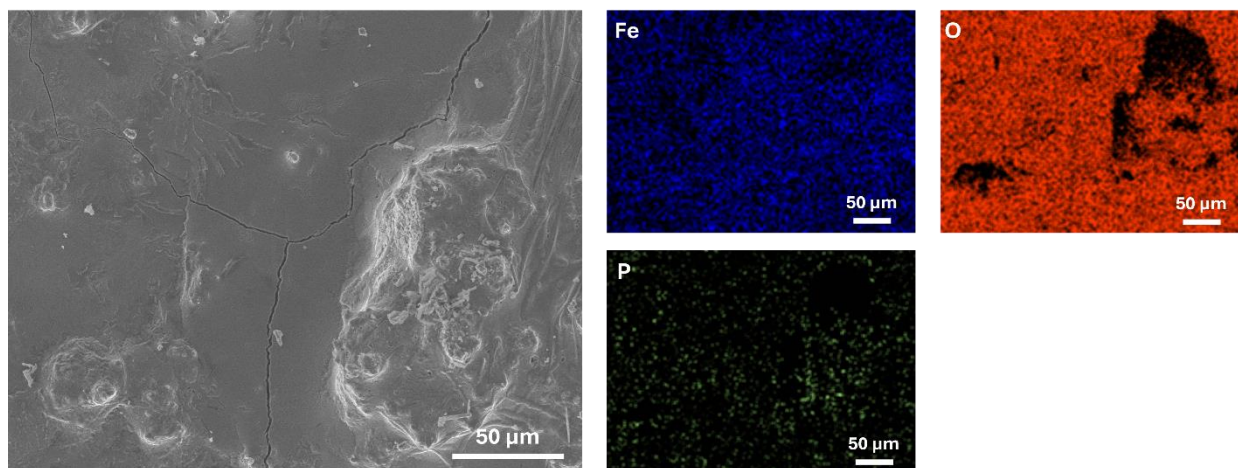


Figure 9.6. SEM-EDS images of S355 carbon steel coupon after 15-day test in the sterile solution at 45 °C.

Cross-sectional SEM-EDS analysis provided critical quantitative information regarding corrosion layer thickness, internal structure, and compositional depth profiles (Figs. 9.7-9.10). Both biotic and sterile samples (Figs. 9.7 and 9.10) developed localized pitting corrosion with maximum pit depths of approximately 10 μm, indicating significant material loss through localized attack. In contrast, the boiled and autoclaved specimens (Figs. 9.8-9.9) exhibited reduced pitting severity, with maximum pit depths limited to approximately 5 μm, suggesting that thermal treatment of the inoculum influenced the extent of localized corrosion processes.

The biotic sample cross-section (Fig. 9.7) revealed a well-developed corrosion layer with a thickness of approximately 20 μm overlying the steel substrate. The elemental maps showed that phosphorus and oxygen were distributed throughout the entire thickness of this layer, confirming that the phosphate-rich composition extended from the outermost surface down to the steel interface. The layer exhibited some internal stratification and porosity, with variations in elemental intensity suggesting compositional gradients or multiple sub-layers. The interface between the corrosion layer and the underlying steel appeared irregular, consistent with the pitting attack observed in optical microscopy. The boiled condition (Fig. 9.8) displayed a corrosion layer of comparable thickness (approximately 20 μm) with similar phosphorus and oxygen distribution throughout the layer depth. The internal structure appeared slightly more compact compared to the biotic sample, but the overall compositional profile remained remarkably similar, reinforcing the observation that boiling did not substantially alter the chemical nature of the corrosion products despite modifying their morphology. The autoclaved specimen (Fig. 9.9) exhibited a significantly thinner corrosion layer of approximately 10 μm, roughly half the thickness observed in biotic and boiled conditions. The elemental mapping revealed that this layer was composed

primarily of oxygen with iron, consistent with the formation of iron oxide/oxyhydroxide phases. Phosphorus was virtually absent from the cross-section, confirming that autoclaving prevented the formation of phosphate-containing corrosion products. The thinner layer suggested reduced overall corrosion extent under autoclaved conditions. The sterile control (Fig. 9.10) showed variable corrosion layer thickness ranging from approximately 10 to 20 μm across different analyzed locations, indicating heterogeneous corrosion product development. The elemental composition was predominantly oxygen-based, characteristic of iron oxides, with localized phosphorus presence detected in some regions. This variability in both thickness and composition suggested that in the absence of microbial activity, corrosion product formation occurred through abiotic mechanisms with less uniformity compared to the biologically influenced conditions.

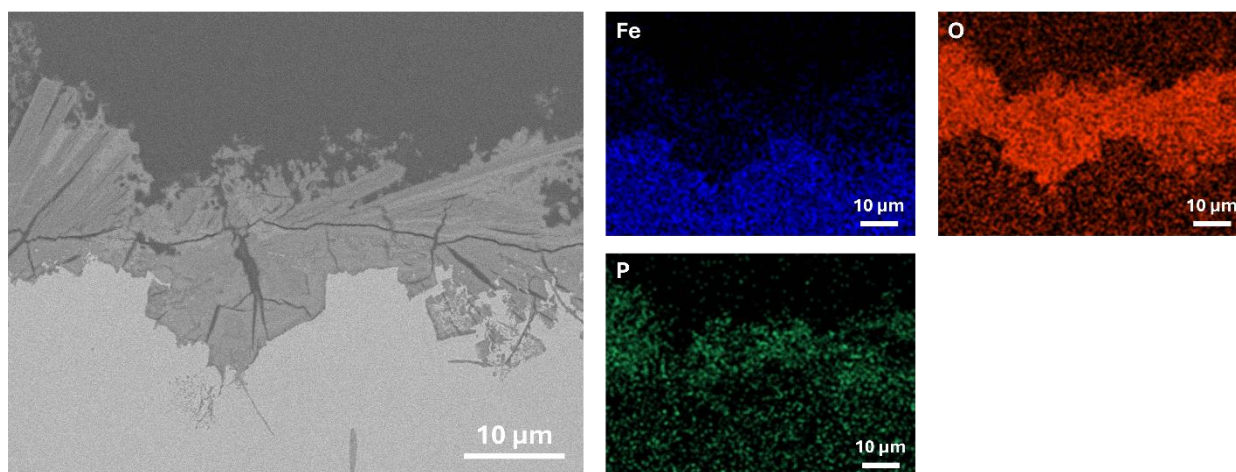


Figure 9.7. SEM-EDS images of carbon steel cross section after 15-day test in the biotic solution at 45 °C.

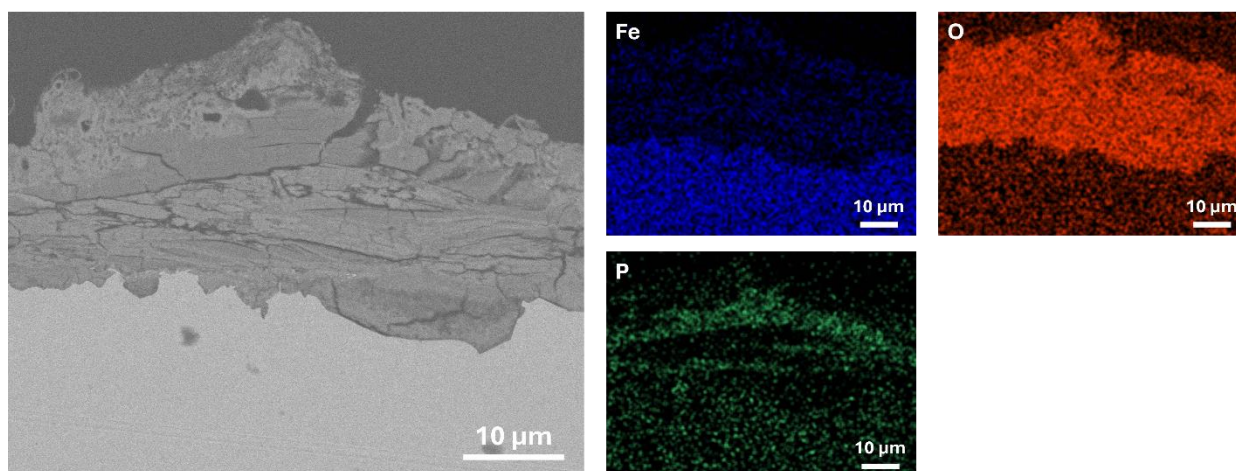


Figure 9.8. SEM-EDS images of carbon steel cross section after 15-day test in the boiled solution at 45 °C.

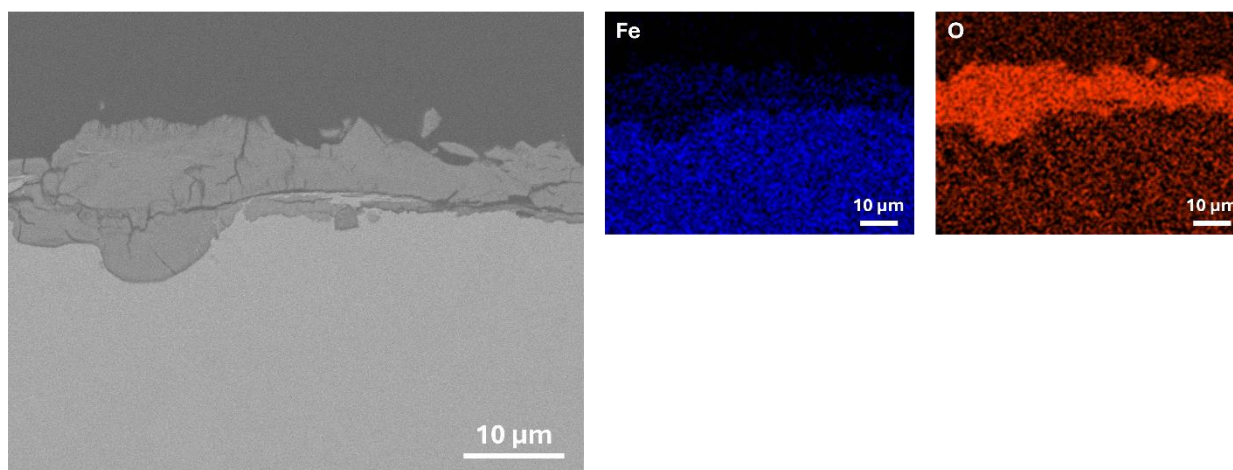


Figure 9.9. SEM-EDS images of carbon steel cross section after 15-day test in the autoclaved solution at 45 °C.

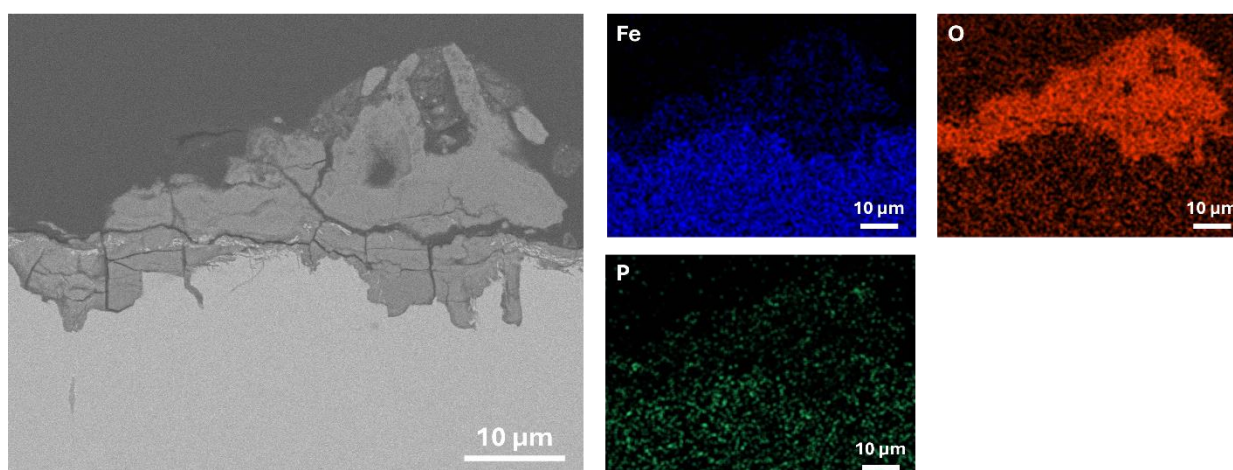


Figure 9.10. SEM-EDS images of carbon steel cross section after 15-day test in the sterile solution at 45 °C.

Raman spectroscopy analysis (Fig. 9.11) enabled crystallographic phase identification of the corrosion products, providing complementary information to the elemental composition data obtained from EDS.

The biotic sample spectrum exhibited characteristic vibrational bands at 234, 364, 515, 635, 1058, and 1288 cm^{-1} . The peaks at 234 and 364 cm^{-1} corresponded to the characteristic Raman signatures of lepidocrocite ($\gamma\text{-FeOOH}$), an iron oxyhydroxide phase commonly formed under neutral pH aqueous corrosion conditions. The bands at 515 and 635 cm^{-1} were attributed to iron phosphate compounds, consistent with the high phosphorus content detected by EDS (Fig. 9.3). The intense peaks at 1058 and 1288 cm^{-1} corresponded to P-O stretching vibrations characteristic of phosphate

groups (PO_4^{3-}), confirming the presence of substantial phosphate incorporation into the corrosion product layer.

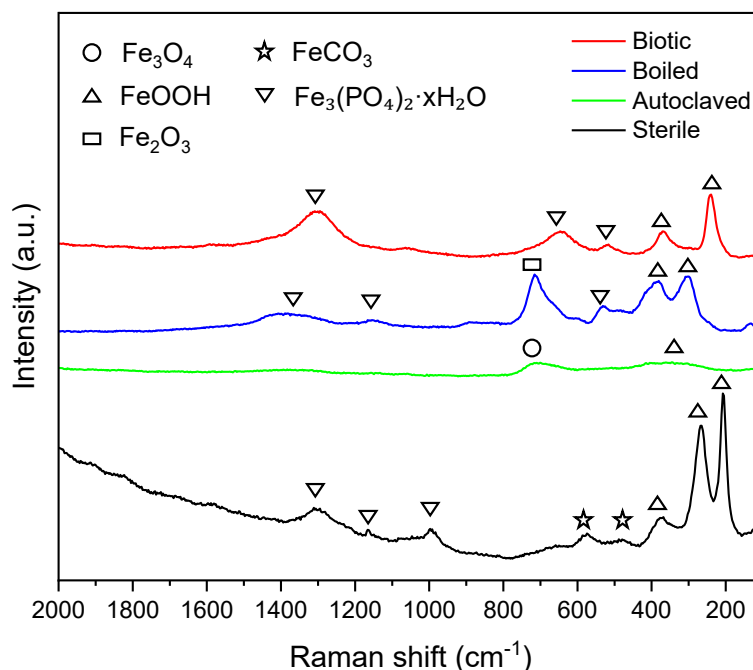


Figure 9.11. Raman spectra of carbon steel corrosion products on the surface of the coupons after 15-day test immersed in the biotic (red), boiled (blue), autoclaved (green) and sterile (gray) solutions at 45 °C.

This spectroscopic signature indicated that the corrosion products in the biotic condition consisted of a mixed phase assemblage of iron oxyhydroxides and iron phosphates. The boiled condition spectrum displayed peaks at 135, 301, 380, 521, 719, 1142, and 1382 cm^{-1} , revealing a more complex phase composition. The bands at 301 and 380 cm^{-1} were characteristic of goethite ($\alpha\text{-FeOOH}$) and lepidocrocite ($\gamma\text{-FeOOH}$), indicating the presence of multiple iron oxyhydroxide polymorphs. The peak at 719 cm^{-1} corresponded to the A_{1g} vibrational mode of maghemite ($\gamma\text{-Fe}_2\text{O}_3$), a partially oxidized iron oxide phase. The signals at 1142 and 1382 cm^{-1} confirmed phosphate presence through P-O stretching modes, similar to the biotic condition but with slightly shifted peak positions suggesting potentially different phosphate coordination environments or crystallinity. The peak at 521 cm^{-1} likely corresponded to additional iron phosphate or mixed oxide-phosphate phases. This spectroscopic profile indicated that boiling treatment promoted the formation of more oxidized iron phases (maghemite) alongside oxyhydroxides and phosphates. The autoclaved specimen presented a markedly simplified spectrum with only two prominent

peaks at 343 and 703 cm^{-1} . The band at 343 cm^{-1} was consistent with iron oxyhydroxide phases (goethite/lepidocrocite), while the peak at 703 cm^{-1} represented the characteristic A_{1g} mode of magnetite (Fe_3O_4), a mixed-valence iron oxide ($\text{Fe}^{2+}\text{Fe}_2^{3+}\text{O}_4$). The absence of phosphate-related peaks confirmed that autoclaving completely prevented phosphate incorporation into the corrosion products, resulting in a simple iron oxide/oxyhydroxide layer dominated by magnetite. The formation of magnetite, a more reduced phase compared to maghemite, suggested that autoclaved conditions promoted different redox chemistry at the steel surface. The sterile control exhibited the most complex Raman spectrum with peaks at 208, 275, 375, 479, 578, 990, 1163, and 1298 cm^{-1} . The bands at 208, 275, and 375 cm^{-1} were characteristic of iron oxyhydroxide phases, primarily lepidocrocite and goethite. The peaks at 479 and 578 cm^{-1} could be attributed to additional iron oxide phases or potentially siderite (FeCO_3), an iron carbonate that can form under certain pH and dissolved carbonate conditions. The bands at 990, 1163, and 1298 cm^{-1} confirmed phosphate presence through P-O vibrational modes, though the phosphate content appeared lower than in biotic and boiled conditions based on relative peak intensities. This complex phase assemblage suggested that abiotic corrosion in the sterile control produced a heterogeneous mixture of oxides, oxyhydroxides, and minor phosphate phases, reflecting the chemical complexity of the buffered electrolyte composition even in the absence of microbial activity.

9.2 Solution analysis

Ion chromatography analysis of the test solutions after 15 days of immersion revealed significant differences in anionic species concentrations among the experimental conditions (Table 9.1). Oxalate was the dominant organic anion detected in all conditions, with concentrations of $2213 \pm 3.9 \text{ mg L}^{-1}$ in the biotic solution, $2304 \pm 2.6 \text{ mg L}^{-1}$ in the boiled condition, and $2410 \pm 3.3 \text{ mg L}^{-1}$ in the autoclaved solution. The sterile control exhibited a substantially lower oxalate concentration of $1483 \pm 2.2 \text{ mg L}^{-1}$, representing approximately 60-65% of the values measured in conditions exposed to the inoculum (whether thermally treated or not). This significant difference indicated that components of the microbial inoculum, even after thermal treatment, contributed to elevated oxalate levels in solution. Formate was detected only in the autoclaved condition at a concentration of $43.2 \pm 1.3 \text{ mg L}^{-1}$, while remaining below the detection limit ($\text{LOD} = 0.05 \text{ mg L}^{-1}$) in both biotic and boiled conditions and was not determined in the sterile control. Acetate was detected at low concentrations in the biotic ($2.9 \pm 0.11 \text{ mg L}^{-1}$), boiled ($6.0 \pm 0.18 \text{ mg L}^{-1}$), and autoclaved ($1.4 \pm 0.08 \text{ mg L}^{-1}$) conditions, with the boiled solution showing approximately twice the concentration compared to the biotic condition. Acetate was not determined in the sterile control, confirming its origin from inoculum-related processes. The

elevated acetate in the boiled condition suggested that thermal treatment at 100°C may have released or generated acetate through partial breakdown of cellular or metabolic pathway. Propionate remained below detection limits in all analyzed conditions. Carbonate concentrations varied considerably among conditions, with $222 \pm 1.6 \text{ mg L}^{-1}$ detected in the biotic solution, $121 \pm 0.54 \text{ mg L}^{-1}$ in the boiled condition (representing approximately half the biotic concentration), and $233 \pm 0.81 \text{ mg L}^{-1}$ in the autoclaved solution. Carbonate was not determined in the sterile control. The lower carbonate concentration in the boiled condition compared to both biotic and autoclaved conditions suggested that boiling treatment affected the carbonate equilibrium in the system, potentially through CO_2 degassing during the boiling process or altered microbial metabolic activity.

Table 9.1. Concentrations of anions detected in the biotic, boiled, autoclaved and sterile inocula after 15-day test, determined by ion chromatography (IC). Results are reported in the ionic form detected by the instrument.

Analyte		Concentration ^a (mg L ⁻¹)			
		Experimental condition			
		Biotic	Boiled	Autoclaved	Sterile
Oxalate	$\text{C}_2\text{O}_4^{2-}$	2213 ± 3.9	2304 ± 2.6	2410 ± 3.3	1483 ± 2.2
Formate	HCOO^-	<LOD	<LOD	43.2 ± 1.3	n.d.
Acetate	CH_3COO^-	2.9 ± 0.11	6.0 ± 0.18	1.4 ± 0.08	n.d.
Propionate	$\text{CH}_3\text{CH}_2\text{COO}^-$	<LOD	<LOD	<LOD	n.d.
Carbonates	CO_3^{2-}	222 ± 1.6	121 ± 0.54	233 ± 0.81	n.d.

^a Values are expressed as mean $\pm$ standard deviation ($n = 2$). Concentrations below the limit of detection (LOD = 0.05 mg L⁻¹) are indicated as “< LOD”, while analytes not determined are indicated as “n.d.” in the Table.

Inductively coupled plasma optical emission spectroscopy provided quantitative data on dissolved metal and non-metal concentrations after the 15-day immersion period (Table 9.2). Iron concentrations in solution varied significantly among conditions, following the trend: sterile ($3.9 \pm 0.05 \text{ mg L}^{-1}$) > biotic ($1.9 \pm 0.10 \text{ mg L}^{-1}$) > boiled ($1.2 \pm 0.09 \text{ mg L}^{-1}$) > autoclaved ($0.7 \pm 0.04 \text{ mg L}^{-1}$). The highest dissolved iron concentration in the sterile control, which was approximately twice that of the biotic condition and more than five times that of the autoclaved condition, indicated that in the absence of microbial influence, iron dissolution occurred but the dissolved iron remained largely in solution rather than precipitating as solid corrosion products. Conversely, the progressively lower iron concentrations in biotic, boiled, and especially autoclaved conditions

suggested more extensive precipitation and incorporation of iron into surface corrosion products, consistent with the thicker corrosion layers observed in SEM cross-sections for biotic and boiled samples (Figs. 9.7-9.8). The exceptionally low iron concentration in the autoclaved solution, despite the formation of a 10 μm oxide layer (Fig. 9.9), indicated highly efficient precipitation of dissolved iron as magnetite and oxyhydroxides under these conditions. Nickel, a minor alloying element in the S355 steel (0.19 wt. %), was detected at low concentrations in biotic ($0.12 \pm 0.04 \text{ mg L}^{-1}$), boiled ($0.11 \pm 0.03 \text{ mg L}^{-1}$), and autoclaved ($0.17 \pm 0.04 \text{ mg L}^{-1}$) solutions, while remaining below detection in the sterile control. The similar nickel concentrations across the three inoculum-exposed conditions suggested preferential dissolution or selective enrichment of nickel relative to iron, which is common in steel corrosion as nickel is typically more noble than iron. Chromium, another alloying element (0.23 wt. %), was not detected in any condition, indicating either minimal dissolution or precipitation in solid phases. Copper and zinc remained at or below detection limits in all analyzed conditions.

Table 9.2. Total elemental concentration of inorganic elements detected in the biotic, boiled, autoclaved and sterile inocula after 15-day test, determined by inductively coupled plasma – atomic emission spectroscopy (ICP-AES).

Element		Concentration ^b (mg L ⁻¹)			
		Experimental condition			
		Biotic	Boiled	Autoclaved	Sterile
Chromium	Cr	n.d.	n.d.	n.d.	n.d.
Iron	Fe	1.9 ± 0.10	1.2 ± 0.09	0.7 ± 0.04	3.9 ± 0.05
Nichel	Ni	0.12 ± 0.04	0.11 ± 0.03	0.17 ± 0.04	n.d.
Copper	Cu	<LOD	<LOD	<LOD	n.d.
Zinc	Zn	n.d.	<LOD	n.d.	<LOD
Sulfur	S	4.2 ± 0.10	5.6 ± 0.11	5.5 ± 0.11	2.7 ± 0.08
Phosphorous	P	313 ± 1.1	244 ± 10.09	490 ± 1.4	55 ± 0.91

^b Values are expressed as mean $\pm$ standard deviation ($n = 2$). Concentrations below the limit of detection (LOD = 0.05 mg L^{-1}) are indicated as “< LOD”, while analytes not determined are indicated as “n.d.” in the Table.

Sulfur concentrations showed moderate variation among conditions, with values of $4.2 \pm 0.10 \text{ mg L}^{-1}$ (biotic), $5.6 \pm 0.11 \text{ mg L}^{-1}$ (boiled), $5.5 \pm 0.11 \text{ mg L}^{-1}$ (autoclaved), and $2.7 \pm 0.08 \text{ mg L}^{-1}$ (sterile). The elevated sulfur in boiled and autoclaved conditions compared to biotic suggested potential release of sulfur-containing compounds from cellular material during thermal treatment,

while the lower concentration in the sterile control reflected the absence of inoculum-derived sulfur sources. The presence of sulfur in all conditions could contribute to the formation of iron sulfide phases, though these were not definitively identified in the Raman spectra (Fig. 9.11). Phosphorus concentrations exhibited the most dramatic variation among all analyzed elements, with values of $313 \pm 1.1 \text{ mg L}^{-1}$ (biotic), $244 \pm 10.09 \text{ mg L}^{-1}$ (boiled), $490 \pm 1.4 \text{ mg L}^{-1}$ (autoclaved), and $55 \pm 0.91 \text{ mg L}^{-1}$ (sterile). The sterile control showed approximately six to nine times lower phosphorus concentration compared to the inoculum-exposed conditions, confirming that the inoculum from the anaerobic digester was the primary source of dissolved phosphate. Remarkably, the autoclaved condition exhibited the highest dissolved phosphorus concentration (490 mg L^{-1}), nearly double the biotic concentration and twice the boiled concentration. This elevated phosphorus release during autoclaving likely resulted from complete lysis of microbial cells and thermal breakdown of inorganic phosphorus compounds (nucleic acids, phospholipids, ATP) into inorganic phosphate under the severe autoclaving conditions. However, despite this high dissolved phosphate availability, the autoclaved specimens showed minimal phosphorus incorporation into corrosion products (as evidenced by EDS and Raman), indicating that the formation of iron phosphate corrosion products required not only phosphate availability but also specific chemical or biological conditions present in the biotic and boiled systems. The biotic and boiled conditions showed intermediate dissolved phosphorus levels (313 and 244 mg L^{-1} , respectively), which correlated with the extensive phosphate incorporation observed in their corrosion product layers. The lower dissolved phosphorus in these conditions compared to autoclaved suggested more efficient precipitation of iron phosphates onto the steel surface, removing phosphorus from solution and incorporating it into the $20 \text{ }\mu\text{m}$ thick phosphate-rich corrosion layers observed in cross-sections (Fig. 9.9).

9.3 Characterization of the bacterial community

High-throughput sequencing of the V3-V4 hypervariable region of the 16S rRNA gene was performed to characterize the microbial community composition in the initial inocula, in the biofilms formed on the steel coupons, and in the bulk solution after 15 days of immersion at 45°C (Fig. 9.13). Sterility was confirmed in both three cases and in particular for the biofilm (Figure S.9.1, Supplementary Information).

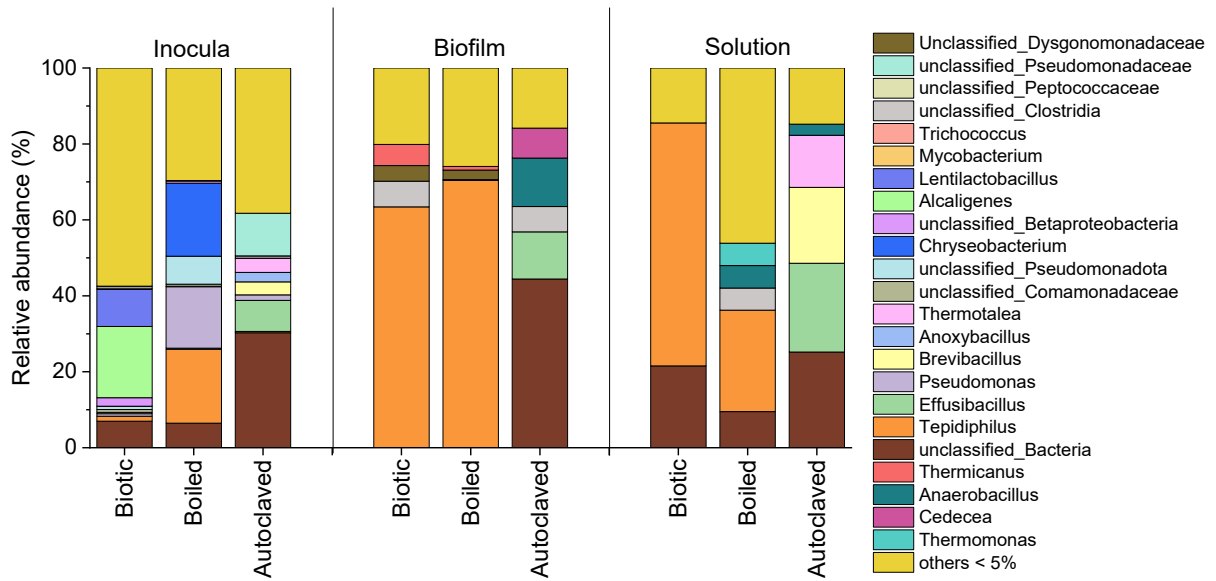


Figure 9.12. Bar plot showing the relative abundances of bacterial genera in the inocula prior the test, and in the biofilm and in solution after the test. The chart presents results for the untreated (biotic), boiled, and autoclaved conditions.

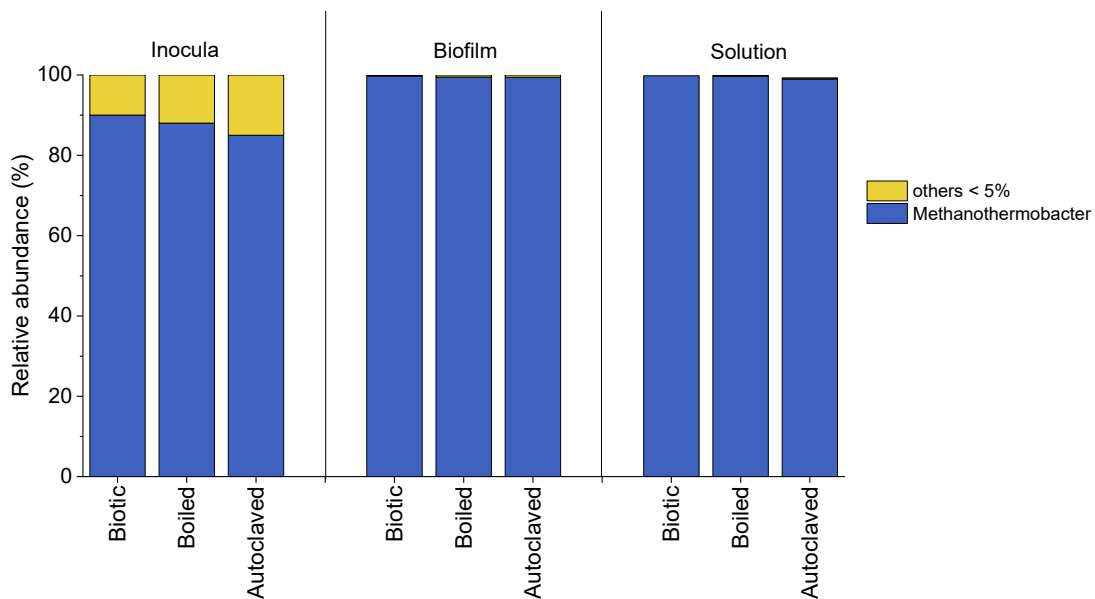


Figure 9.13. Bar plot showing the relative abundances of Archaea genera in the inocula prior the test, and in the biofilm and in solution after the test. The chart presents results for the untreated (biotic), boiled, and autoclaved conditions.

Archaeal community analysis (Fig. 9.14) revealed that *Methanothermobacter* was the predominant genus in all initial inocula, representing approximately 90% of the archaeal sequences in the biotic condition, 85% in the boiled condition, and 85% in the autoclaved

condition, with the remaining sequences attributed to low-abundance genera (collectively <15%). Remarkably, after 15 days of immersion, the archaeal communities in both biofilm and planktonic phases showed strong enrichment of *Methanothermobacter*, which constituted approximately 100% of the archaeal sequences in the biofilms formed on steel coupons under all three experimental conditions (biotic, boiled, and autoclaved). Similarly, the archaeal communities in the bulk solutions after the test were dominated by *Methanothermobacter* at approximately 100% relative abundance across all conditions. This near-complete dominance of *Methanothermobacter* in both surface-associated (biofilm) and planktonic phases indicated strong selective pressure favoring this methanogenic genus under the experimental conditions, regardless of the thermal pre-treatment applied to the inoculum.

The bacterial community composition exhibited substantially greater complexity and variability compared to the archaeal communities (Fig. 9.13). In the initial inocula, bacterial diversity varied considerably among the three conditions. The biotic inoculum showed a mixed community composition with 57.4% of sequences attributed to low-abundance genera (others <5% individually), 10% *Effusibacillus*, and 7% *Lentilactobacillus*, along with various other minor constituents. The boiled inoculum displayed a different initial composition, with 29.6% attributed to low-abundance genera, 10% *Tepidiphilus*, 10% *Pseudomonas*, and 15% *Chryseobacterium*, indicating that the boiling treatment substantially altered the bacterial community structure compared to the untreated biotic condition. The autoclaved inoculum presented 38.2% low-abundance genera, 5% *Effusibacillus*, and various unclassified genera distributed among different taxonomic groups, suggesting that the more severe autoclaving treatment further modified the bacterial community composition relative to both biotic and boiled conditions.

After 15 days of incubation, dramatic shifts in bacterial community composition were observed in the biofilms developed on the steel surfaces. The biotic biofilm became strongly dominated by *Tepidiphilus*, which represented 63% of the bacterial sequences, with the remaining 37% distributed among low-abundance genera (each <5%). The boiled biofilm showed even greater enrichment of *Tepidiphilus* at 70% relative abundance, with all other genera individually below 5%. This substantial increase in *Tepidiphilus* abundance from 10% in the boiled inoculum to 70% in the biofilm indicated strong selective enrichment of this genus during biofilm formation on the corroding steel surface following boiling treatment. In contrast, the autoclaved biofilm exhibited a markedly different composition with no clear dominance by a single genus. The autoclaved biofilm community consisted of 44% unclassified bacteria, 12% *Effusibacillus*, 13% *Anaerobacillus*, 6.7% unclassified *Clostridia*, and various other minor genera. This more diverse

and less specialized community structure in the autoclaved biofilm suggested that the severe thermal treatment (121 °C, elevated pressure) either eliminated specific bacterial groups capable of dominating under corrosive conditions or altered the physicochemical environment in ways that prevented the establishment of a dominant biofilm-forming population.

Bacterial community composition in the bulk solutions after 15 days showed distinct patterns compared to the biofilms, indicating spatial differentiation between surface-associated and planktonic bacterial populations. The biotic solution maintained high *Tepidiphilus* abundance at 63%, comparable to the biofilm, suggesting continuous exchange or similarity between biofilm and planktonic communities in the untreated condition. The boiled solution showed reduced *Tepidiphilus* representation (26%) compared to its biofilm (70%), with notable contributions from unclassified *Clostridia* (5%), *Anaerobacillus* (5%), and *Thermomonas* (5%), indicating greater community divergence between surface and bulk phases following boiling treatment. The autoclaved solution displayed a completely different bacterial profile with 23% *Effusibacillus*, 20% *Brevibacillus*, and 13.7% *Thermotalea* as the dominant genera, with no overlap with the dominant genera in the autoclaved biofilm. This pronounced spatial heterogeneity in the autoclaved condition, with distinct communities in biofilm versus solution, contrasted with the relative homogeneity observed in the biotic condition and suggested that autoclaving fundamentally altered the ecological dynamics governing microbial community assembly and distribution in the system.

Quantitative PCR (qPCR) targeting the 16S rRNA gene was performed to determine the total microbial abundance in the biofilms formed on the carbon steel coupons after 15 days of immersion under different treatment conditions (Fig. S.9.2, Supplementary Information). The qPCR results revealed dramatic differences in microbial colonization density among the experimental conditions, with microbial cell numbers spanning over four orders of magnitude depending on the thermal pre-treatment applied to the inoculum.

The biotic condition exhibited the highest microbial density, with 16S rRNA gene copy numbers reaching $(1.89 \pm 0.13) \times 10^{13}$ copies mL⁻¹ in the biofilm. This exceptionally high cell density indicated prolific microbial colonization and biofilm development on the steel surface when exposed to the untreated anaerobic digester inoculum containing viable, metabolically active microorganisms. The relatively low standard deviation (1.28×10^7 copies mL⁻¹) compared to the mean value suggested uniform biofilm coverage across replicate coupons, consistent with the extensive and homogeneous biofilm-associated corrosion products observed in SEM analysis. This high microbial load corresponded to the dominance of *Tepidiphilus* (63%) and

Methanothermobacter (~100% of Archaea) identified through 16S rRNA gene sequencing, indicating that these genera successfully established dense populations within the biofilm matrix.

The boiled condition showed substantially reduced but still considerable microbial abundance, with 16S rRNA gene copy numbers of $(3.05 \pm 0.13) \times 10^9$ copies mL⁻¹. This represented approximately 0.016% of the microbial density observed in the biotic condition. The presence of this significant microbial population despite boiling treatment at 101 °C for a defined period indicated that either: i) a fraction of the microbial community survived the thermal treatment through spore formation or thermotolerance mechanisms, ii) surviving cells underwent regrowth during the 15-day incubation period at 45 °C, or iii) thermoresistant spore-forming bacteria (such as *Bacillales*, which includes genera like *Effusibacillus* and *Anaerobacillus* detected in the community analysis) germinated and proliferated after the thermal shock. The substantial enrichment of *Tepidiphilus* to 70% in the boiled biofilm, despite the reduced total cell numbers, suggested that this genus possessed competitive advantages for colonizing the steel surface under post-boiling conditions. The relatively low standard deviation indicated reproducible biofilm formation across replicates even in the thermally stressed system.

The autoclaved condition exhibited the lowest microbial density among the inoculum-exposed conditions, with 16S rRNA gene copy numbers of $(4.60 \pm 2.99) \times 10^8$ copies mL⁻¹. The more severe thermal treatment was thus substantially more effective at reducing microbial viability compared to boiling, though complete sterilization was not achieved. The higher relative standard deviation (2.99×10^8) compared to the mean value indicated greater variability in biofilm development across replicate coupons in the autoclaved condition, possibly reflecting stochastic survival and regrowth of rare thermoresistant spore-formers or heterogeneous distribution of viable cells following the autoclaving process. The microbial community composition in the autoclaved biofilm, which showed no single dominant genus but rather a mixed assemblage of *Effusibacillus* (12%), *Anaerobacillus* (13%), unclassified bacteria (44%), and unclassified *Clostridia* (6.7%), suggested that the autoclaving process selected for a diverse consortium of spore-forming or extremely thermotolerant bacteria capable of surviving 121 °C treatment and subsequently colonizing the steel surface.

9.4 Electrochemical characterization

The temporal evolution of the open circuit potential (OCP) was monitored continuously over the 15-day immersion period to assess the thermodynamic tendency of the carbon steel surfaces under different microbial conditions (Fig. 9.15). The four experimental conditions exhibited distinctly different OCP behaviors, reflecting the influence of microbial activity and thermal treatment on the steel electrochemical state.

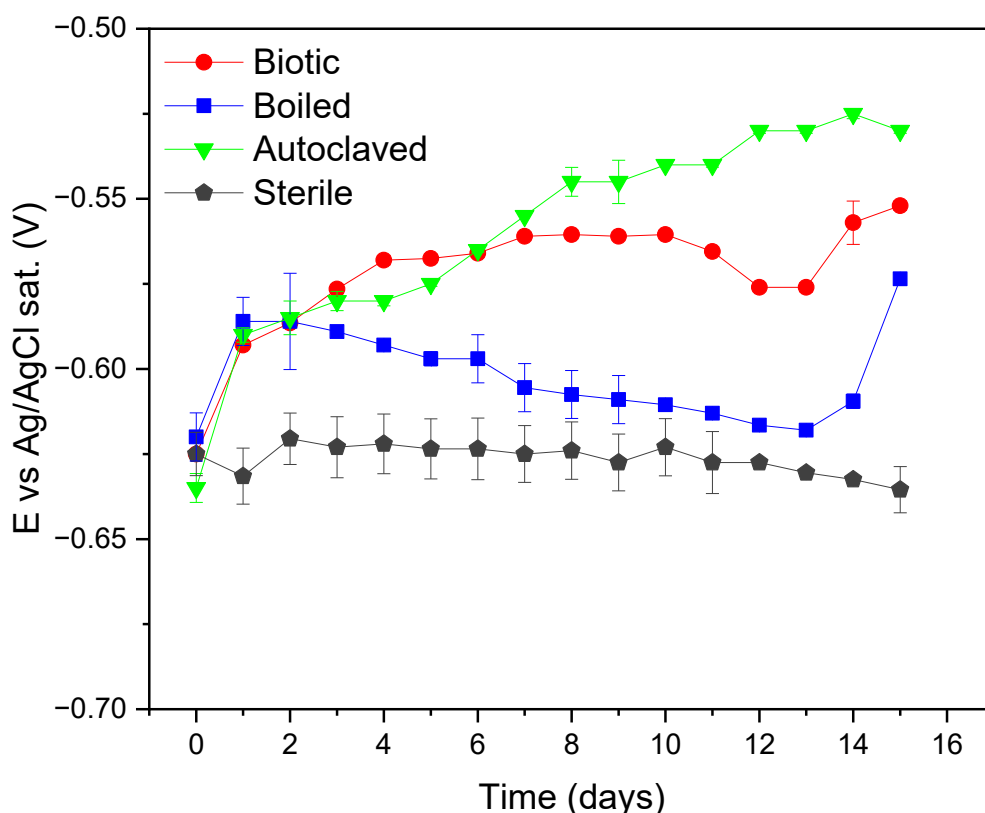


Figure 9.14. Open circuit potential (average and standard deviation, E vs Ag/AgCl sat.) of carbon steel coupons over the entire test period (15 days). Biotic (red solid line), boiled (blue solid line), autoclaved (green solid line) and sterile condition (gray solid line).

The biotic condition displayed an initial OCP of approximately -0.625 V vs Ag/AgCl at the beginning of immersion. Over the course of the experiment, the potential underwent progressive ennoblement (i.e., shift toward more positive values), reaching approximately -0.56 V by day 8 and stabilizing around -0.55 V between days 10-13. A slight potential decrease to approximately -0.55 V was observed at day 14, followed by stabilization at approximately -0.55 V at the end of the 15-day period. This ennoblement of approximately +75 mV represented a significant shift

toward more noble (i.e., less thermodynamically favorable for corrosion) potentials. The progressive nature of this ennoblement suggested time-dependent processes associated with biofilm development, microbial metabolic activity, or the formation of protective corrosion product layers. The stabilization of the potential after day 10 indicated that an electrochemical quasi-steady state was established in the biotic system, likely corresponding to mature biofilm formation and stable corrosion product layer development as observed in the morphological characterization.

The boiled condition exhibited an initial OCP of approximately -0.58 V vs Ag/AgCl, which was approximately 45 mV more noble than the biotic condition at time zero. The potential evolution in the boiled condition followed a more complex trajectory compared to the biotic system. During the first 7 days, the OCP underwent a gradual shift toward more active (i.e., more negative) values, reaching approximately -0.61 V by day 7-8. This cathodic shift of approximately -30 mV contrasted with the anodic shift observed in the biotic condition during the same period. Between days 8-13, the potential remained relatively stable in the range of -0.60 to -0.61 V, followed by a sharp ennoblement at day 14-15, with the final OCP reaching approximately -0.57 V. This oscillating behavior, characterized by initial activation followed by late-stage ennoblement, suggested dynamic changes in surface chemistry and possibly delayed formation of protective corrosion products. The final potential of -0.57 V was comparable to that of the biotic condition, despite the substantially different temporal evolution, indicating that thermal treatment at 100°C altered the kinetics but not necessarily the final electrochemical state of the corroding system.

The autoclaved condition demonstrated the most pronounced ennoblement behavior among all experimental conditions. Starting from an initial OCP of approximately -0.635 V vs Ag/AgCl (the most active initial potential among all conditions), the autoclaved system underwent continuous and substantial ennoblement throughout the entire 15-day period. The potential shifted progressively from -0.635 V to approximately -0.56 V by day 8, and continued to ennoblement to approximately -0.53 V by day 15. This total shift of approximately +105 mV was the largest potential change observed among all conditions. The continuous, monotonic nature of this ennoblement, without the plateau observed in the biotic condition or the oscillations seen in the boiled condition, suggested fundamentally different interfacial processes in the autoclaved system. The final potential of -0.53 V was the most noble value achieved in any condition, indicating that the autoclaved environment promoted the formation of surface films or oxides with greater protective character or different semiconducting properties compared to the other conditions. This ennoblement behavior was consistent with the formation of magnetite-dominated

corrosion products identified by Raman spectroscopy (Fig. 9.11) in the autoclaved condition, as magnetite is known to exhibit more noble rest potentials compared to iron oxyhydroxides and phosphate phases.

The sterile control displayed the most stable OCP evolution among all conditions. Beginning at approximately -0.625 V vs Ag/AgCl, similar to the biotic condition, the sterile system rapidly stabilized within the first 2-3 days to a potential of approximately -0.62 V. Throughout the remainder of the experiment, the OCP remained relatively constant in the range of -0.62 to -0.63 V with minimal fluctuation and only a slight drift toward more active values (approximately -0.635 V) by day 15. This stable, relatively active potential suggested that in the absence of microbial influence, the steel surface established a relatively stable electrochemical state governed primarily by abiotic corrosion processes and the formation of simple oxide/oxyhydroxide layers. The absence of significant ennoblement in the sterile condition contrasted sharply with the biotic and autoclaved systems, indicating that the presence of the inoculum (whether biotic or thermally treated) significantly influenced the electrochemical behavior of the corroding steel. The consistently more negative potentials in the sterile condition compared to all other conditions at the end of the experiment suggested that the steel surface in the sterile environment was more susceptible to anodic dissolution or that the corrosion products formed had less protective character.

Finally, the comparative analysis of OCP evolution revealed several key findings. First, all conditions exposed to the anaerobic digester inoculum (biotic, boiled, autoclaved) exhibited more noble final potentials compared to the sterile control, indicating that inoculum-derived components influenced surface electrochemistry even after severe thermal treatment. Second, the degree and kinetics of ennoblement varied substantially with treatment severity: autoclaved > biotic > boiled >> sterile. Third, the temporal patterns differed markedly: the autoclaved condition showed continuous ennoblement, the biotic condition showed rapid initial ennoblement followed by stabilization, the boiled condition exhibited complex oscillatory behavior, and the sterile condition maintained stable active potentials. These differences in OCP behavior reflected the complex interplay between microbial metabolic activity, biofilm formation, corrosion product precipitation, and surface film properties, with thermal treatment fundamentally altering but not eliminating the influence of inoculum components on steel electrochemistry.

Linear polarization resistance (LPR) measurements (Figs. 9.13 and S.9.3, Supplementary Information) were performed at day 1 and day 15 of immersion to quantify the instantaneous corrosion resistance of carbon steel S355 under the different experimental conditions (Table 9.3).

The polarization resistance (R_p) is inversely proportional to the corrosion current density and provides a direct measure of the corrosion rate at the time of measurement, with higher R_p values indicating lower corrosion rates and greater corrosion resistance.

Table 9.3. R_p values obtained from LPR measurements at the beginning and end of the test (1 and 15 days, respectively) for each experimental condition (biotic, boiled, autoclaved and sterile). Values are reported as mean $\pm$ standard deviation.

Time days	Biotic $k\Omega \cdot cm^2$	Boiled $k\Omega \cdot cm^2$	Autoclaved $k\Omega \cdot cm^2$	Sterile $k\Omega \cdot cm^2$
1	0.31 ± 0.05	0.53 ± 0.02	0.38 ± 0.05	0.42 ± 0.03
15	0.24 ± 0.03	0.26 ± 0.04	0.66 ± 0.09	0.11 ± 0.09

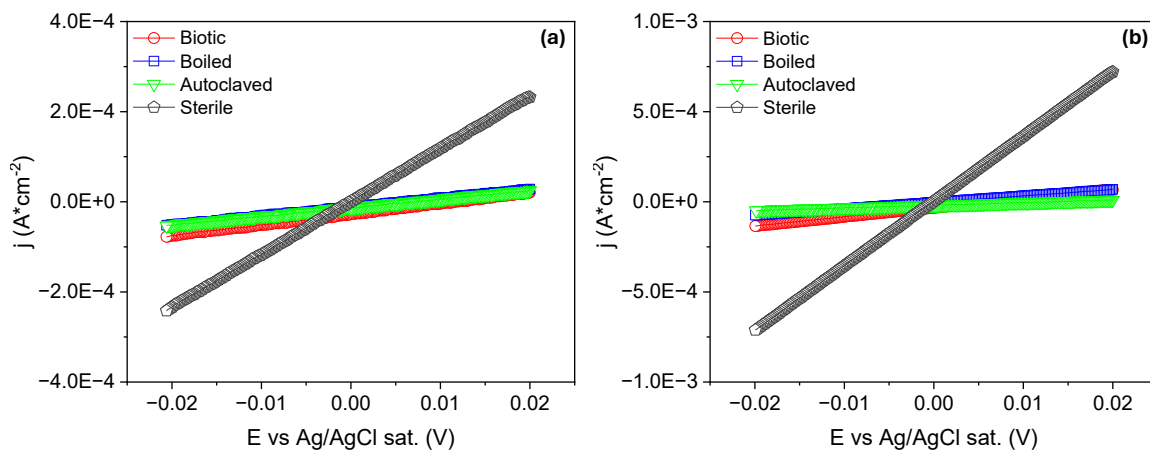


Figure 9.15. Linear polarization resistances of carbon steel coupons and replicates tested in the biotic (red), boiled (blue) and autoclaved (gray) inocula after (a) 1 day and (b) 15 days.

At day 1, the four experimental conditions exhibited relatively similar polarization resistance values within the same order of magnitude. The boiled condition showed the highest initial R_p of $0.53 \pm 0.02 k\Omega \cdot cm^2$, followed by the sterile control at $0.42 \pm 0.03 k\Omega \cdot cm^2$, the autoclaved condition at $0.38 \pm 0.05 k\Omega \cdot cm^2$, and the biotic condition at $0.31 \pm 0.05 k\Omega \cdot cm^2$. These values indicated that all systems exhibited moderate to high corrosion rates during the initial immersion period, with the boiled condition providing approximately 70% higher resistance compared to the biotic condition. The higher initial R_p in the boiled condition suggested that thermal treatment at $101^\circ C$ immediately promoted conditions more favorable for passivation or rapid formation of corrosion product layers compared to the untreated biotic inoculum. The sterile control, despite

containing no microbial components, exhibited intermediate resistance, indicating that abiotic corrosion processes in the buffered electrolyte alone resulted in corrosion rates comparable to the microbiologically influenced systems. The autoclaved condition showed R_p values similar to both biotic and sterile, suggesting that the severe thermal treatment (121°C, elevated pressure) did not confer immediate corrosion protection advantages at the onset of immersion.

By day 15, the polarization resistance values diverged dramatically among the conditions, revealing the cumulative effects of prolonged exposure to different microbiological and chemical environments. The autoclaved condition exhibited a substantial increase in R_p to $0.66 \pm 0.09 \text{ k}\Omega\cdot\text{cm}^2$, representing approximately 74% improvement from day 1 and the highest corrosion resistance among all conditions at the final time point. This progressive increase in polarization resistance indicated that the autoclaved system developed increasingly protective surface conditions over time, consistent with the formation of compact oxide layers observed in surface characterization. In contrast, the biotic condition showed a slight decrease in R_p to $0.24 \pm 0.03 \text{ k}\Omega\cdot\text{cm}^2$, representing approximately 23% reduction from the initial value. This decrease suggested that despite biofilm development and extensive corrosion product accumulation (20 μm thick phosphate-rich layer), the overall instantaneous corrosion rate remained high or even increased slightly, indicating that the phosphate-containing corrosion products formed under biotic conditions provided limited kinetic protection against ongoing corrosion. The boiled condition exhibited the most dramatic change, with R_p decreasing sharply to $0.26 \pm 0.04 \text{ k}\Omega\cdot\text{cm}^2$ at day 15, representing a 51% reduction from the initially high value at day 1. This substantial decrease in polarization resistance indicated severe degradation of the protective properties initially observed, suggesting that the boiling treatment, while conferring transient protection, ultimately resulted in conditions that promoted sustained high corrosion rates. The mechanisms underlying this deterioration may relate to the formation of defective or porous phosphate layers, incomplete biofilm development, or chemical changes in the inoculum that prevented establishment of stable protective conditions. The sterile control showed a decrease in R_p to $0.11 \pm 0.09 \text{ k}\Omega\cdot\text{cm}^2$, representing approximately 74% reduction from day 1 and yielding the lowest corrosion resistance among all conditions at day 15. This low final R_p value indicated that abiotic corrosion in the sterile buffered electrolyte resulted in sustained high corrosion rates without development of protective surface conditions, despite the formation of oxide/oxyhydroxide corrosion products. The high standard deviation ($\pm 0.09 \text{ k}\Omega\cdot\text{cm}^2$) relative to the mean value ($0.11 \text{ k}\Omega\cdot\text{cm}^2$) suggested considerable variability in corrosion behavior among replicate coupons in the

sterile condition, possibly reflecting heterogeneous oxide formation and localized corrosion attack.

The comparative analysis of polarization resistance evolution revealed important trends regarding the influence of microbial treatment on corrosion kinetics. At day 15, the R_p values followed the order: autoclaved ($0.66 \text{ k}\Omega\cdot\text{cm}^2$) $\gg$ boiled ($0.26 \text{ k}\Omega\cdot\text{cm}^2$) $\approx$ biotic ($0.24 \text{ k}\Omega\cdot\text{cm}^2$) $>$ sterile ($0.11 \text{ k}\Omega\cdot\text{cm}^2$), with the autoclaved condition exhibiting the highest resistance. This ranking indicated that severe thermal treatment (autoclaving) promoted the development of corrosion-resistant surface conditions over the 15-day period, while moderate thermal treatment (boiling) and untreated biotic exposure resulted in similarly low final resistance, and the absence of any inoculum components (sterile) yielded the poorest corrosion protection. The temporal evolution patterns were equally informative: the autoclaved condition was the only system showing positive ΔR_p (increase from day 1 to day 15), while all other conditions exhibited negative ΔR_p (decrease), indicating progressive deterioration. The magnitude of change followed the order: sterile (-74%) $>$ boiled (-51%) $>$ biotic (-23%) $>$ autoclaved (+74%), with the sterile and boiled conditions showing the most severe degradation. These data suggested that the presence of inoculum-derived components, even after thermal deactivation, influenced the long-term corrosion behavior, with autoclaving uniquely promoting conditions favorable for protective oxide development while preserving some beneficial effects of inoculum chemistry, whereas boiling created an unstable system prone to rapid deterioration.

The correlation between LPR-derived corrosion resistance and the observed corrosion morphology provided additional mechanistic insights. The low final R_p in the sterile condition ($0.11 \text{ k}\Omega\cdot\text{cm}^2$) corresponded to the possible development of localized pitting attack and heterogeneous oxide layers, indicating that high instantaneous corrosion rates in the absence of microbial influence led to non-uniform corrosion product distribution and sustained susceptibility to localized corrosion, as also evidenced by the cross-section images (Fig. 9.10). The similarly low R_p values in biotic ($0.24 \text{ k}\Omega\cdot\text{cm}^2$) and boiled ($0.26 \text{ k}\Omega\cdot\text{cm}^2$) conditions, despite the formation of thick phosphate-rich layers ($20 \text{ }\mu\text{m}$, Figs. 9.7-9.8), suggested that these layers were either porous, poorly adherent, or non-protective in nature, failing to significantly impede charge transfer and mass transport processes necessary for ongoing corrosion. In contrast, the high R_p in the autoclaved condition ($0.66 \text{ k}\Omega\cdot\text{cm}^2$) corresponded to the formation of a thinner ($10 \text{ }\mu\text{m}$, Fig. 9.9) but evidently more compact and protective magnetite-dominated oxide layer with reduced pitting severity ($5 \text{ }\mu\text{m}$ depth), indicating that the layer quality and semiconducting properties, rather than thickness alone, determined the kinetic barrier to corrosion. The reduced pitting depth

in both autoclaved and boiled conditions (5 μm) compared to biotic and sterile (10 μm) suggested some beneficial effect of thermal treatment on mitigating localized attack initiation or propagation, though the long-term protective efficacy diverged dramatically between the two thermal treatments.

Electrochemical impedance spectroscopy (EIS) measurements were performed at 1, 7, and 15 days of immersion to characterize the evolution of interfacial electrochemical properties and corrosion resistance under the different experimental conditions (Fig. 9.19 and Tables 9.4-9.7). The impedance spectra were analyzed using equivalent electrical circuits (EECs) specifically selected to represent the interfacial structure of each system based on the physical and chemical characteristics revealed by surface analysis. Three different EEC configurations were employed depending on the condition and immersion time (Fig. 9.18): Circuit I consisted of solution resistance (R_s) in series with two parallel R-CPE elements representing the corrosion product film ($R_f\text{-CPE}_f$) and the charge transfer process at the steel/electrolyte interface ($R_{ct}\text{-CPE}_{dl}$); Circuit II was a simplified version containing R_s in series with a single R-CPE element ($R_{ct}\text{-CPE}_{dl}$), representing systems where the film contribution was negligible or indistinguishable from the charge transfer process; Circuit III incorporated a Warburg impedance element (W_s) in series with R_{ct} to account for diffusion-controlled processes, in addition to R_s and CPE_{dl} . The constant phase element (CPE) was used instead of ideal capacitance to account for surface heterogeneity, roughness, and non-uniform current distribution, with the CPE impedance defined as:

$$Z_{CPE} = \frac{1}{Y_0(j\omega)^n} \quad (9.2)$$

where Y_0 is the CPE coefficient, ω is the angular frequency, and n is the CPE exponent (ranging from 0 for pure resistance to 1 for ideal capacitance). All fitting procedures yielded chi-square (χ^2) values on the order of 10^{-5} , indicating excellent agreement between experimental data and the proposed models.³⁰⁷

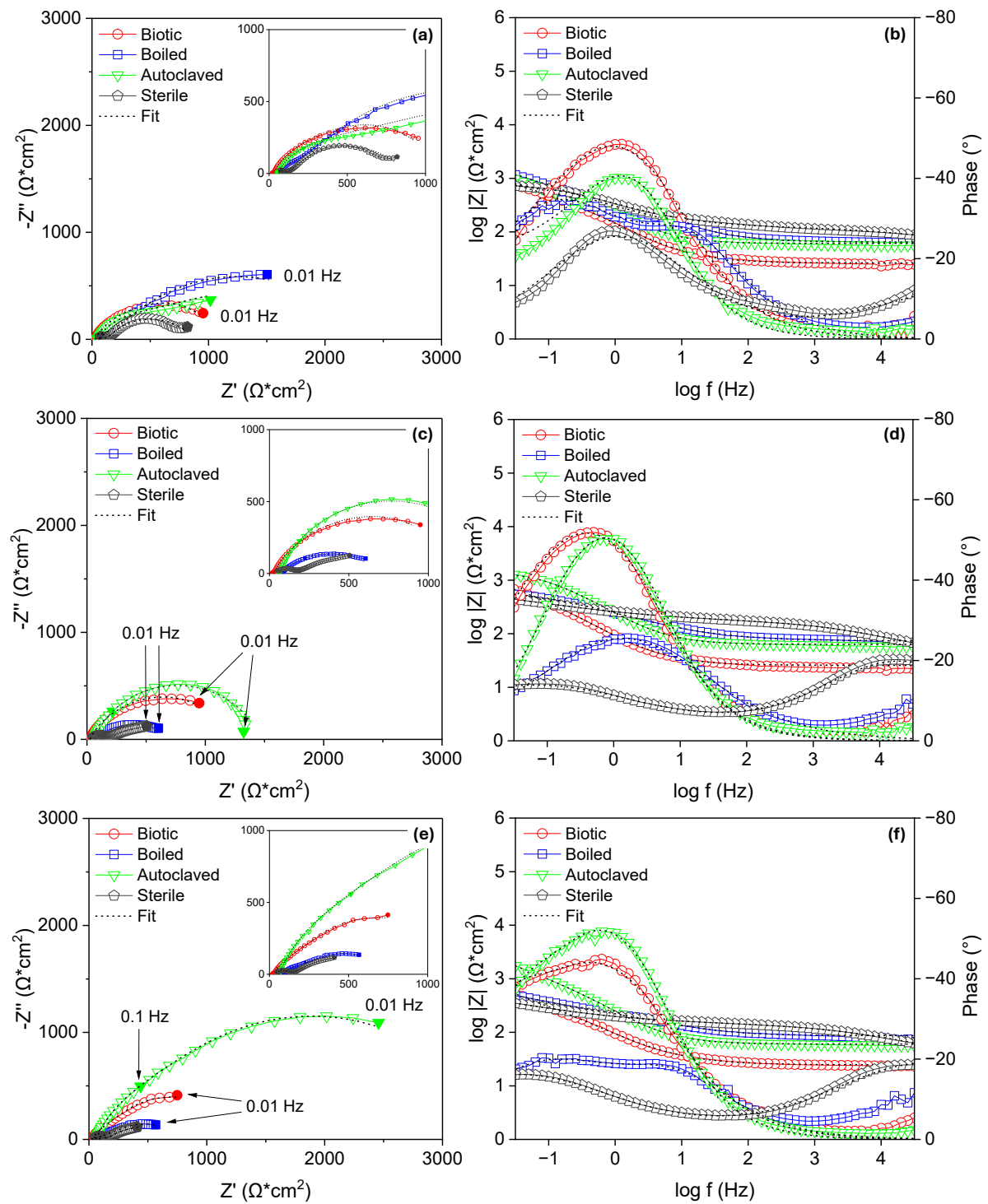


Figure 9.16. Nyquist and bode plots of carbon steel coupons tested in the biotic (red), boiled (blue), autoclaved (green) and sterile (gray) inocula after (a-b) 1 day, (c-d) 7 days and (e-f) 15 days.

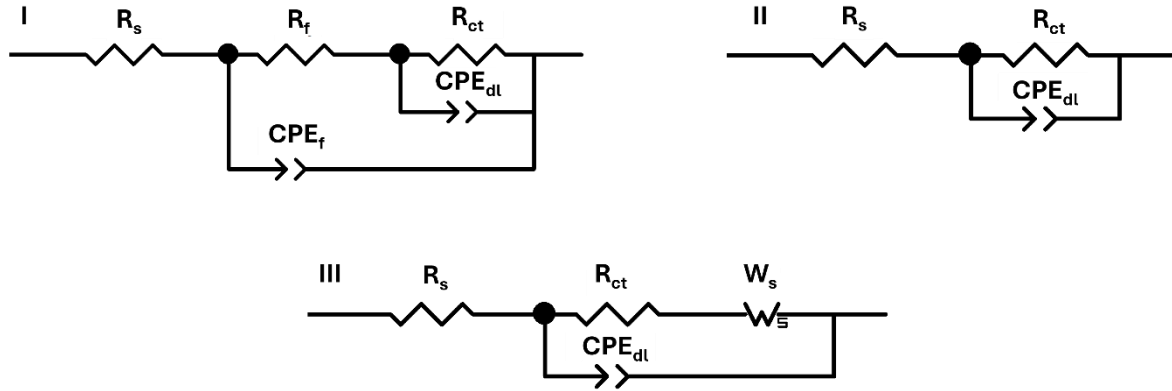


Figure 9.17. Equivalent electrical circuit (ECCs) I-III used to model impedance data.

The biotic system (Table 9.4) exhibited continuous evolution of impedance behavior throughout the 15-day immersion period, reflecting dynamic changes in biofilm development and corrosion product formation. At day 1, the impedance spectrum was best described by Circuit I, with a total polarization resistance ($R_{\text{tot}} = R_f + R_{\text{ct}}$) of $1.01 \pm 0.06 \text{ k}\Omega \cdot \text{cm}^2$. The charge transfer resistance was $0.87 \pm 0.05 \text{ k}\Omega \cdot \text{cm}^2$ and the film resistance was $0.21 \pm 0.04 \text{ k}\Omega \cdot \text{cm}^2$, indicating that in the initial stage, the charge transfer process dominated the overall impedance while a thin, relatively conductive film was already forming. The CPE_{dl} exhibited $Y_0 = 1.95 \pm 0.04 (\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$ with $n = 0.71 \pm 0.01$, while CPE_f showed $Y_0 = 12.7 \pm 4.24 (\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$ with $n = 0.98 \pm 0.06$, the latter approaching ideal capacitive behavior ($n \approx 1$). The effective capacitance was $582 \pm 1.1 \mu\text{F} \cdot \text{cm}^{-2}$, and the solution resistance was $26.1 \pm 0.15 \Omega \cdot \text{cm}^2$. By day 7, the total resistance increased to $1.20 \pm 0.09 \text{ k}\Omega \cdot \text{cm}^2$, representing an approximately 19% increase compared to day 1. The charge transfer resistance increased to $1.1 \pm 0.07 \text{ k}\Omega \cdot \text{cm}^2$, while the film resistance decreased to $0.10 \pm 0.06 \text{ k}\Omega \cdot \text{cm}^2$. The CPE_f coefficient decreased dramatically to $37.4 \pm 4.19 (\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$ with $n = 1.00 \pm 0.01$, indicating the development of a more uniform, capacitive film layer. The CPE_{dl} Y_0 increased to $3.08 \pm 0.04 (\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$ with $n = 0.74 \pm 0.01$, and the effective capacitance increased substantially to $1250 \pm 10 \mu\text{F} \cdot \text{cm}^{-2}$, suggesting increased surface area possibly due to roughening or biofilm colonization. At day 15, the total resistance reached $1.31 \pm 0.10 \text{ k}\Omega \cdot \text{cm}^2$, representing the highest value observed during the experiment and approximately 30% increase from the initial value. The charge transfer resistance decreased slightly to $0.80 \pm 0.07 \text{ k}\Omega \cdot \text{cm}^2$, while the film resistance increased substantially to $0.51 \pm 0.07 \text{ k}\Omega \cdot \text{cm}^2$, indicating that the protective character of the corrosion product layer became more significant at later immersion times. The CPE_f showed $Y_0 = 9.49 \pm 2.55 (\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$ with $n = 1.00 \pm 0.09$, maintaining ideal capacitive behavior, while CPE_{dl} exhibited $Y_0 = 3.61 \pm 0.07 (\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$ with $n = 0.66 \pm 0.01$.

The effective capacitance was $1030 \pm 9.9 \mu\text{F}\cdot\text{cm}^{-2}$, intermediate between the day 1 and day 7 values. The Nyquist plots (Figs. 9.17a, c, e) showed progressively increasing semicircle diameters from day 1 to day 15, consistent with the increasing total resistance, though the impedance magnitude remained relatively modest compared to other conditions. The Bode plots (Figs. 9.17b, d, f) revealed broad phase angle maxima at intermediate frequencies, characteristic of overlapping time constants from the film and charge transfer processes, with the phase angle reaching approximately -40° to -50° at the maximum, well below the -90° expected for ideal capacitive behavior, confirming significant surface heterogeneity.

The boiled system (Table 9.5) exhibited the most complex temporal evolution among all conditions, with substantial changes in both impedance magnitude and circuit configuration. At day 1, fitting with Circuit I yielded $R_{\text{tot}} = 2.25 \pm 0.06 \text{ k}\Omega\cdot\text{cm}^2$, more than twice the value of the biotic condition at the same time point. The charge transfer resistance was $0.23 \pm 0.02 \text{ k}\Omega\cdot\text{cm}^2$, while the film resistance was exceptionally high at $2.02 \pm 0.05 \text{ k}\Omega\cdot\text{cm}^2$, indicating that immediately after immersion, the boiled condition promoted formation of a relatively protective surface film. The CPE_f exhibited $Y_0 = 1.56 \pm 0.02 (\text{k}\Omega)^{-1}\cdot\text{s}^n\cdot\text{cm}^{-2}$ with $n = 0.63 \pm 0.01$, indicating a highly non-ideal, dispersive film, while CPE_{dl} showed $Y_0 = 0.44 \pm 0.03 (\text{k}\Omega)^{-1}\cdot\text{s}^n\cdot\text{cm}^{-2}$ with $n = 0.70 \pm 0.01$. The effective capacitance was only $89.4 \pm 6.0 \mu\text{F}\cdot\text{cm}^{-2}$, the lowest value among all conditions and time points, suggesting a compact, less porous interface. The solution resistance was notably high at $64.5 \pm 0.29 \Omega\cdot\text{cm}^2$, approximately 2.5 times higher than the biotic condition, possibly due to precipitation or aggregation of thermally denatured proteins and cellular debris from the boiling process. By day 7, the impedance behavior changed dramatically, requiring a switch to the simpler Circuit II for adequate fitting. The total resistance (equal to R_{ct} in this configuration) decreased substantially to $0.62 \pm 0.005 \text{ k}\Omega\cdot\text{cm}^2$, decreased from day 1 and indicating severe degradation of the protective properties observed initially. The CPE_{dl} showed $Y_0 = 1.59 \pm 0.01 (\text{k}\Omega)^{-1}\cdot\text{s}^n\cdot\text{cm}^{-2}$ with $n = 0.53 \pm 0.00$, the lowest n value observed in any condition, indicating highly dispersive, non-capacitive behavior possibly due to extensive surface roughening or heterogeneous corrosion attack. The effective capacitance increased to $216 \pm 1.60 \mu\text{F}\cdot\text{cm}^{-2}$, and the solution resistance increased further to $72.9 \pm 0.30 \Omega\cdot\text{cm}^2$. At day 15, the system reverted to Circuit I configuration, with $R_{\text{tot}} = 0.69 \pm 0.02 \text{ k}\Omega\cdot\text{cm}^2$, remaining substantially lower than the day 1 value. The charge transfer resistance was only $0.16 \pm 0.01 \text{ k}\Omega\cdot\text{cm}^2$, the lowest value observed in any condition at day 15, while film resistance was $0.527 \pm 0.02 \text{ k}\Omega\cdot\text{cm}^2$. The CPE_f exhibited $Y_0 = 4.75 \pm 0.15 (\text{k}\Omega)^{-1}\cdot\text{s}^n\cdot\text{cm}^{-2}$ with $n = 0.56 \pm 0.01$, indicating a non-protective, porous film, and CPE_{dl} showed $Y_0 = 0.87 \pm 0.06 (\text{k}\Omega)^{-1}\cdot\text{s}^n\cdot\text{cm}^{-2}$ with $n = 0.65 \pm 0.01$. The effective

capacitance was $177 \pm 12.3 \mu\text{F}\cdot\text{cm}^{-2}$, and the solution resistance increased to $86.6 \pm 0.49 \Omega\cdot\text{cm}^2$. The Nyquist plots (Figs. 9.17a, c, e) showed an initial relatively large semicircle at day 1 that collapsed dramatically by day 7, then partially recovered but remained depressed at day 15, reflecting the loss and incomplete recovery of corrosion resistance. The Bode plots (Figs. 9.17b, d, f) revealed that the phase angle maximum decreased from day 1 to day 7 and remained low at day 15, consistent with increasingly resistive rather than capacitive behavior.

The autoclaved system (Table 9.6) exhibited the most pronounced evolution toward high corrosion resistance, with impedance characteristics distinctly different from the other conditions. At day 1, the spectrum was best fit using Circuit III incorporating Warburg impedance, yielding $R_{\text{tot}} = 2.41 \pm 0.09 \text{ k}\Omega\cdot\text{cm}^2$, the highest initial resistance among all conditions. The charge transfer resistance was $0.17 \pm 0.02 \text{ k}\Omega\cdot\text{cm}^2$, while the Warburg coefficient was $1.25 \pm 0.09 \text{ k}\Omega\cdot\text{cm}^2$, indicating significant contribution from diffusion-controlled processes, likely related to transport through the nascent oxide film. The CPE_{dl} showed $Y_0 = 0.83 \pm 0.043 (\text{k}\Omega)^{-1}\cdot\text{s}^n\cdot\text{cm}^{-2}$ with $n = 0.84 \pm 0.02$, approaching ideal capacitive behavior more closely than the biotic or boiled conditions at day 1. The effective capacitance was $438 \pm 2.28 \mu\text{F}\cdot\text{cm}^{-2}$, and the solution resistance was $60.9 \pm 0.53 \Omega\cdot\text{cm}^2$. By day 7, the impedance behavior transitioned to Circuit II, with $R_{\text{ct}} = 1.40 \pm 0.01 \text{ k}\Omega\cdot\text{cm}^2$, representing a decrease from the total resistance at day 1 (though direct comparison is complicated by the circuit change). The CPE_{dl} exhibited $Y_0 = 1.01 \pm 0.007 (\text{k}\Omega)^{-1}\cdot\text{s}^n\cdot\text{cm}^{-2}$ with $n = 0.79 \pm 0.00$, and the effective capacitance remained stable at $487 \pm 3.59 \mu\text{F}\cdot\text{cm}^{-2}$. The solution resistance was $63.2 \pm 0.32 \Omega\cdot\text{cm}^2$. At day 15, the system returned to Circuit I configuration, demonstrating the development of a distinct film layer. The total resistance increased substantially to $3.39 \pm 0.14 \text{ k}\Omega\cdot\text{cm}^2$, approximately 2.6 times higher than the biotic condition and nearly 5 times higher than the boiled condition at the same time point, making it the most corrosion-resistant condition overall. The charge transfer resistance was $1.8 \pm 0.09 \text{ k}\Omega\cdot\text{cm}^2$, while the film resistance was $1.62 \pm 0.11 \text{ k}\Omega\cdot\text{cm}^2$, indicating that both the charge transfer kinetics and the film properties contributed significantly to the high overall resistance.

The CPE_{f} showed $Y_0 = 2.44 \pm 0.31 (\text{k}\Omega)^{-1}\cdot\text{s}^n\cdot\text{cm}^{-2}$ with $n = 0.90 \pm 0.04$, approaching ideal capacitance and indicating a relatively uniform, compact film, while CPE_{dl} exhibited $Y_0 = 1.05 \pm 0.013 ((\text{k}\Omega)^{-1}\cdot\text{s}^n\cdot\text{cm}^{-2})$ with $n = 0.78 \pm 0.00$. The effective capacitance was $474 \pm 5.69 \mu\text{F}\cdot\text{cm}^{-2}$, showing remarkable stability throughout the 15-day period. The Nyquist plots (Figs. 9.17a, c, e) revealed the largest semicircles among all conditions at day 15, with the impedance spectrum extending to approximately $2500 \Omega\cdot\text{cm}^2$ in the real axis at low frequencies. The Bode plots (Figs. 9.17b, d, f) showed well-defined phase angle maxima reaching approximately -40° to -45° , with

a clear two-time-constant structure visible at day 15, consistent with the distinct film and double-layer contributions identified in Circuit I fitting.

The sterile control (Table 9.7) exhibited intermediate impedance behavior with substantial evolution over time. At day 1, fitting with Circuit I yielded $R_{\text{tot}} = 0.73 \pm 0.006 \text{ k}\Omega \cdot \text{cm}^2$, comparable to the biotic condition but substantially lower than both boiled and autoclaved. The charge transfer resistance was $0.68 \pm 0.01 \text{ k}\Omega \cdot \text{cm}^2$, while the film resistance was minimal at only $0.05 \pm 0.002 \text{ k}\Omega \cdot \text{cm}^2$, indicating negligible film formation in the early stage under abiotic conditions. The CPE_f showed $Y_0 = 0.084 \pm 0.013 (\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$ with $n = 0.77 \pm 0.02$, while CPE_{dl} exhibited $Y_0 = 1.07 \pm 0.01 (\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$ with $n = 0.65 \pm 0.00$. The effective capacitance was $362 \pm 4.7 \mu\text{F} \cdot \text{cm}^{-2}$, and notably, the solution resistance was exceptionally high at $103 \pm 1.03 \Omega \cdot \text{cm}^2$, the highest value recorded in any condition, possibly reflecting the different ionic composition of the sterile electrolyte compared to the inoculum-containing solutions. By day 7, the system transitioned to Circuit III incorporating Warburg impedance, with $R_{\text{ct}} = 0.83 \pm 0.04 \text{ k}\Omega \cdot \text{cm}^2$, showing a modest increase from day 1. The Warburg coefficient was $0.14 \pm 0.003 \text{ k}\Omega \cdot \text{cm}^2$, much lower than in the autoclaved condition, suggesting less significant diffusion limitation. The CPE_{dl} showed $Y_0 = 4.52 \pm 0.06 (\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$ with a significantly reduced $n = 0.55 \pm 0.01$, indicating increasing surface heterogeneity. The effective capacitance decreased to $162 \pm 2.10 \mu\text{F} \cdot \text{cm}^{-2}$, and the solution resistance dropped dramatically to $32.3 \pm 1.05 \Omega \cdot \text{cm}^2$, closer to values observed in biotic conditions. At day 15, the system maintained Circuit III configuration, with $R_{\text{ct}} = 0.74 \pm 0.02 \text{ k}\Omega \cdot \text{cm}^2$, remaining essentially stable from day 7 and representing the lowest final resistance among all conditions. The Warburg coefficient decreased to $0.11 \pm 0.001 \text{ k}\Omega \cdot \text{cm}^2$, and the CPE_{dl} exhibited $Y_0 = 6.71 \pm 0.03 (\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$ with $n = 0.52 \pm 0.00$, the lowest n value at day 15 among all conditions, indicating highly non-ideal, dispersive behavior consistent with rough, heterogeneous corrosion attack. The effective capacitance increased dramatically to $607 \pm 2.40 \mu\text{F} \cdot \text{cm}^{-2}$, the highest value at day 15, suggesting extensive surface area increase through corrosion product formation or surface roughening. The solution resistance was $29.5 \pm 0.64 \Omega \cdot \text{cm}^2$. The Nyquist plots (Figs. 9.17a, c, e) showed relatively small, compressed semicircles throughout the experiment, with the most depressed appearance at day 15, and the Bode plots (Figs. 9.17b, d, f) revealed low phase angle maxima (approximately -20° to -30°), indicating predominantly resistive rather than capacitive behavior and consistent with active corrosion processes.

The comparative analysis of the EIS revealed that the total polarization resistance at day 15 followed this trend: autoclaved ($3.39 \text{ k}\Omega \cdot \text{cm}^2$) > biotic ($1.31 \text{ k}\Omega \cdot \text{cm}^2$) > sterile ($0.85 \text{ k}\Omega \cdot \text{cm}^2$, considering $R_{\text{ct}} + W_s$ contribution) > boiled ($0.69 \text{ k}\Omega \cdot \text{cm}^2$), indicating that autoclaving provided

the greatest corrosion protection despite having the lowest microbial load, while boiling paradoxically resulted in the poorest final corrosion resistance despite initially showing high resistance at day 1. The temporal evolution patterns were strikingly different: the biotic condition showed continuous, gradual improvement; the boiled condition exhibited initial protection followed by severe degradation and minimal recovery; the autoclaved condition demonstrated progressive development of high resistance; and the sterile condition showed modest, stable resistance throughout. The CPE exponents (n values) at day 15 followed the order: autoclaved ($n_{dl} = 0.78$, $n_f = 0.90$) > biotic ($n_{dl} = 0.66$, $n_f = 1.00$) > boiled ($n_{dl} = 0.65$, $n_f = 0.56$) > sterile ($n_{dl} = 0.52$), with higher n values generally correlating with more protective conditions, indicating that surface homogeneity and film quality were important factors in determining corrosion resistance. The effective capacitance values at day 15 showed the trend: sterile ($607 \mu\text{F}\cdot\text{cm}^2$) > biotic ($1030 \mu\text{F}\cdot\text{cm}^2$) > autoclaved ($474 \mu\text{F}\cdot\text{cm}^2$) > boiled ($177 \mu\text{F}\cdot\text{cm}^2$), with higher capacitance generally associated with increased surface area from corrosion product formation or roughening, though the relationship was complex due to different film dielectric properties. The solution resistance evolution was particularly informative: the initial high values in boiled ($64.5 \Omega\cdot\text{cm}^2$), autoclaved ($60.9 \Omega\cdot\text{cm}^2$), and sterile ($103 \Omega\cdot\text{cm}^2$) conditions converged toward similar final values (86.6, 60.1, and $29.5 \Omega\cdot\text{cm}^2$, respectively), while the biotic condition maintained consistently low values ($24.7\text{--}26.1 \Omega\cdot\text{cm}^2$), suggesting that thermal treatment of the inoculum initially altered solution conductivity through protein denaturation and cellular lysis, but this effect diminished over time. The requirement for different equivalent circuit configurations (Circuit I for mature biofilm/film systems, Circuit II for degraded or transition states, Circuit III for diffusion-limited systems) reflected the diverse interfacial structures developed under different conditions, with the autoclaved condition uniquely showing persistent Warburg behavior at days 1 and 7 before transitioning to a two-layer structure, consistent with the formation of a compact, diffusion-limiting magnetite layer identified by Raman spectroscopy (Fig. 9.11).

Table 9.4. Fit results using the EECs from Figure 9.18 for the biotic condition.

Biotic ^c										
EEC	Time	R _s	R _{ct}	CPE _{dl} Q	CPE _{dl-n}	R _f	CPE _f Q	CPE _{f-n}	C _{eff}	R _{tot}
	days	$\Omega \cdot \text{cm}^2$	$\text{k}\Omega \cdot \text{cm}^2$	$(\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$		$\text{k}\Omega \cdot \text{cm}^2$	$(\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$		$\mu\text{F} \cdot \text{cm}^{-2}$	$\text{k}\Omega \cdot \text{cm}^2$
I	1	26.1±0.15	0.87±0.05	1.95±0.04	0.71±0.01	0.21±0.04	12.7±4.24	0.98±0.06	582±1.1	1.01±0.06
I	7	25.7±0.22	1.1±0.07	3.08±0.04	0.74±0.01	0.10±0.06	37.4±4.19	1±0.01	1250±10	1.20±0.09
I	15	24.7±0.13	0.80±0.07	3.61±0.07	0.66±0.01	0.51±0.07	9.49±2.55	1±0.09	1030±9.9	1.31±0.10

^c Values are expressed as mean ± standard deviation. Depending on the equivalent electrical circuit (EEC) used, parameters that are not detected are reported as 'n.d.', whereas parameters that are not applicable are indicated as 'n.a.'.

Table 9.5. Fit results using the EECs from Figure 9.18 for the boiled condition.

Boiled ^d										
EEC	Time	R _s	R _{ct}	CPE _{dl} Q	CPE _{dl-n}	R _f	CPE _f Q	CPE _{f-n}	C _{eff}	R _{tot}
	days	$\Omega \cdot \text{cm}^2$	$\text{k}\Omega \cdot \text{cm}^2$	$(\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$		$\text{k}\Omega \cdot \text{cm}^2$	$(\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$		$\mu\text{F} \cdot \text{cm}^{-2}$	$\text{k}\Omega \cdot \text{cm}^2$
I	1	64.5±0.29	0.23±0.02	0.44±0.03	0.70±0.01	2.02±0.05	1.56±0.02	0.63±0.01	89.4±6.0	2.25±0.06
II	7	72.9±0.30	0.62±0.005	1.59±0.01	0.53±0.00	n.a.	n.a.	n.a.	216±1.60	0.62±0.004
I	15	86.6±0.49	0.16±0.01	0.87±0.06	0.65±0.01	0.527±0.02	4.75±0.15	0.56±0.01	177±12.3	0.69±0.02

^d Values are expressed as mean ± standard deviation. Depending on the equivalent electrical circuit (EEC) used, parameters that are not detected are reported as 'n.d.', whereas parameters that are not applicable are indicated as 'n.a.'.

Table 9.6. Fit results using the EECs from Figure 9.18 for the autoclaved condition.

Autoclaved ^e											
EEC	Time	R _s	R _{ct}	CPE _{dl} Q	CPE _{dl} -n	R _f	CPE _f Q	CPE _f -n	W	C _{eff}	R _{tot}
	days	$\Omega \cdot \text{cm}^2$	$\text{k}\Omega \cdot \text{cm}^2$	$(\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$		$\text{k}\Omega \cdot \text{cm}^2$	$(\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$		$\text{k}\Omega \cdot \text{cm}^2$	$\mu\text{F} \cdot \text{cm}^{-2}$	$\text{k}\Omega \cdot \text{cm}^2$
III	1	60.9±0.53	0.17±0.02	0.83±0.043	0.84±0.02	n.a.	n.a.	n.a.	1.25±0.09	438±2.28	2.41±0.09
II	7	63.2±0.32	1.4±0.01	1.01±0.007	0.79±0.00	n.a.	n.a.	n.a.	n.a.	487±3.59	1.40±0.01
I	15	60.1±0.19	1.8±0.09	1.05±0.013	0.78±0.00	1.62±0.11	2.44±0.31	0.90±0.04	n.a.	474±5.69	3.39±0.14

^e Values are expressed as mean ± standard deviation. Depending on the equivalent electrical circuit (EEC) used, parameters that are not detected are reported as 'n.d.', whereas parameters that are not applicable are indicated as 'n.a.'.

Table 9.7. Fit results using the EECs from Figure 9.18 for the sterile condition.

Sterile ^f											
EEC	Time	R _s	R _{ct}	CPE _{dl} Q	CPE _{dl} -n	R _f	CPE _f Q	CPE _f -n	W	C _{eff}	R _{tot}
	days	$\Omega \cdot \text{cm}^2$	$\text{k}\Omega \cdot \text{cm}^2$	$(\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$		$\text{k}\Omega \cdot \text{cm}^2$	$(\text{k}\Omega)^{-1} \cdot \text{s}^n \cdot \text{cm}^{-2}$		$\text{k}\Omega \cdot \text{cm}^2$	$\mu\text{F} \cdot \text{cm}^{-2}$	$\text{k}\Omega \cdot \text{cm}^2$
I	1	103±1.03	0.68±0.01	1.07±0.01	0.65±0.00	0.05±0.00	0.084±0.01	0.77±0.02	n.a.	362±4.7	0.73±0.01
III	7	32.3±1.05	0.83±0.04	4.52±0.06	0.55±0.01	n.a.	n.a.	n.a.	0.14±0.003	162±2.10	0.97±0.04
III	15	29.5±0.64	0.74±0.02	6.71±0.03	0.52±0.00	n.a.	n.a.	n.a.	0.11±0.001	607±2.40	0.85±0.02

^f Values are expressed as mean ± standard deviation. Depending on the equivalent electrical circuit (EEC) used, parameters that are not detected are reported as 'n.d.', whereas parameters that are not applicable are indicated as 'n.a.'.

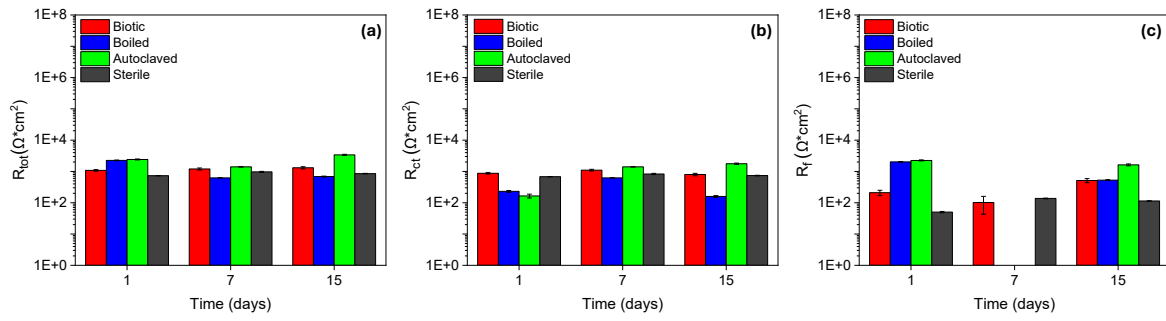


Figure 9.18. Evolution of the resistive contributions for each condition and for each time point: (a) $R_{\text{tot}} = R_{\text{ct}} + R_f$; (b) R_{ct} ; (c) R_f .

The decomposition of total polarization resistance (R_{tot}) into its constituent components (i.e., charge transfer resistance, R_{ct} and film resistance, R_f) provided detailed insights into the relative contributions of interfacial electrochemical kinetics and corrosion product layer properties to the overall corrosion resistance over the 15-day immersion period (Fig. 9.19). The temporal evolution of these parameters revealed distinctly different mechanistic pathways of corrosion protection or degradation under the four experimental conditions.

The charge transfer resistance, which reflects the kinetics of the anodic (iron oxidation) and cathodic (reduction) reactions at the steel/electrolyte interface, exhibited condition-dependent temporal patterns (Fig. 9.19b). In the biotic condition, R_{ct} increased from $872 \pm 48 \Omega \cdot \text{cm}^2$ at day 1 to $1099 \pm 70 \Omega \cdot \text{cm}^2$ at day 7, indicating progressive inhibition of charge transfer processes during the first week of biofilm development. However, by day 15, R_{ct} decreased to $797 \pm 71 \Omega \cdot \text{cm}^2$, suggesting that mature biofilm conditions paradoxically accelerated interfacial electrochemical kinetics despite extensive corrosion product accumulation. This decrease in charge transfer resistance at later stages may reflect the establishment of aggressive localized microenvironments within the biofilm, increased catalytic activity of biofilm-associated enzymes or metabolites, or development of conductive pathways through the phosphate-rich corrosion product layer. The boiled condition exhibited dramatically different behavior, with an exceptionally low initial R_{ct} of $230 \pm 16 \Omega \cdot \text{cm}^2$ at day 1, approximately 3.8 times lower than the biotic condition. This low initial charge transfer resistance indicated rapid electrochemical kinetics immediately following exposure to the boiled inoculum, possibly due to high concentrations of electroactive species released during thermal lysis of microbial cells. By day 7, R_{ct} increased substantially to $622 \pm 4 \Omega \cdot \text{cm}^2$, suggesting development of kinetic inhibition, but then collapsed to only $159 \pm 11 \Omega \cdot \text{cm}^2$ at day 15, representing the lowest final R_{ct} value among all conditions and confirming severe electrochemical activation of the steel surface. The

autoclaved condition showed a moderate initial R_{ct} of $680 \pm 6 \Omega \cdot \text{cm}^2$ at day 1, which increased progressively to $831 \pm 37 \Omega \cdot \text{cm}^2$ at day 7 and further to $1761 \pm 89 \Omega \cdot \text{cm}^2$ at day 15. This continuous increase in charge transfer resistance throughout the experiment indicated progressive development of kinetic barriers to electrochemical reactions, consistent with the formation of a compact, semiconducting magnetite layer that inhibited electron transfer across the metal/oxide/electrolyte interface. Thus, the highest R_{ct} of the autoclaved condition at day 15, represents the most effective charge transfer inhibition achieved in any condition. The sterile control exhibited an initial R_{ct} of $165 \pm 23 \Omega \cdot \text{cm}^2$, the lowest among all conditions at day 1, which increased to $830 \pm 37 \Omega \cdot \text{cm}^2$ by day 7, suggesting rapid formation of surface oxides that temporarily inhibited charge transfer. However, R_{ct} decreased to $738 \pm 16 \Omega \cdot \text{cm}^2$ at day 15, indicating that while abiotic corrosion in the sterile electrolyte eventually developed some kinetic resistance, it remained unstable and prone to deterioration.

The film resistance, representing the ionic transport resistance through the corrosion product layer, showed even more dramatic condition-dependent variations (Fig. 9.19c). In the biotic condition, R_f started at a very low value of $209 \pm 40 \Omega \cdot \text{cm}^2$ at day 1, decreased to only $101 \pm 58 \Omega \cdot \text{cm}^2$ at day 7, indicating formation of a highly porous or ionically conductive initial layer, then increased substantially to $514 \pm 75 \Omega \cdot \text{cm}^2$ at day 15. This late-stage increase in film resistance suggested that the thick phosphate-rich corrosion product layer ($20 \mu\text{m}$) observed in cross-sections (Fig. 9.7) eventually developed some ionic transport resistance, though the absolute R_f value remained relatively modest compared to the autoclaved condition. The boiled condition exhibited remarkably high initial film resistance of $2021 \pm 55 \Omega \cdot \text{cm}^2$ at day 1, higher than the biotic condition and representing the formation of an immediately protective surface layer upon exposure to the boiled inoculum. However, this film resistance could not be determined at day 7 (requiring circuit simplification to Circuit II without an R_f component), indicating complete loss of distinguishable film properties and suggesting catastrophic degradation of the initially protective layer. By day 15, R_f partially recovered to $527 \pm 20 \Omega \cdot \text{cm}^2$, comparable to the biotic final value but representing only 26% of the initially high resistance, confirming irreversible deterioration of the film's protective character. The autoclaved condition displayed exceptionally high film resistance throughout the entire experiment: $2246 \pm 87 \Omega \cdot \text{cm}^2$ at day 1 (similar to the boiled initial value), which could not be determined at day 7 (circuit transition), then increased dramatically to $1624 \pm 116 \Omega \cdot \text{cm}^2$ at day 15. Notably, while the day 15 value was lower than the day 1 value, the autoclaved condition was the only system that maintained high, stable film resistance throughout the experiment without the catastrophic collapse observed in the boiled

condition. The final R_f of $1624 \Omega \cdot \text{cm}^2$ in the autoclaved condition was the highest among the experimental conditions, indicating superior barrier properties of the magnetite-dominated oxide layer. The sterile control showed minimal film resistance throughout: $50 \pm 3 \Omega \cdot \text{cm}^2$ at day 1, increasing to $137 \pm 3 \Omega \cdot \text{cm}^2$ at day 7, then decreasing to $114 \pm 1 \Omega \cdot \text{cm}^2$ at day 15, representing consistently poor barrier properties of the abiotic oxide/oxyhydroxide layers despite their 10-20 μm thickness (Fig. 9.10).

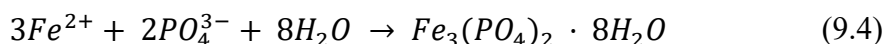
The total polarization resistance ($R_{\text{tot}} = R_{\text{ct}} + R_f$) integrated the combined effects of charge transfer kinetics and film barrier properties (Fig. 9.19a). The biotic condition showed R_{tot} increasing from $1082 \pm 63 \Omega \cdot \text{cm}^2$ at day 1 to $1312 \pm 104 \Omega \cdot \text{cm}^2$ at day 15, representing gradual, continuous improvement in corrosion resistance primarily driven by late-stage film development. The boiled condition exhibited the most complex temporal trajectory: initially high R_{tot} of $2252 \pm 58 \Omega \cdot \text{cm}^2$ at day 1, collapsing to $622 \pm 4 \Omega \cdot \text{cm}^2$ at day 7, then partially recovering to $686 \pm 22 \Omega \cdot \text{cm}^2$ at day 15. This behavior confirmed that boiling treatment conferred only transient protection that rapidly degraded under immersion conditions. The autoclaved condition demonstrated progressive resistance development: $2411 \pm 90 \Omega \cdot \text{cm}^2$ at day 1 (similar to boiled initial value), $968 \pm 37 \Omega \cdot \text{cm}^2$ at day 7, then a dramatic increase to $3385 \pm 146 \Omega \cdot \text{cm}^2$ at day 15. The final R_{tot} in the autoclaved condition was the highest value, representing the most effective overall corrosion protection. The sterile control showed R_{tot} increasing from $730 \pm 6 \Omega \cdot \text{cm}^2$ at day 1 to $968 \pm 37 \Omega \cdot \text{cm}^2$ at day 7 (33% increase), then decreasing to $852 \pm 16 \Omega \cdot \text{cm}^2$ at day 15 (lower than day 7 but higher than day 1), indicating moderate, unstable resistance development.

Global analysis of the relative contributions of charge transfer and film resistances to total resistance revealed important mechanistic insights. At day 1, the biotic and sterile conditions were dominated by charge transfer resistance ($R_{\text{ct}}/R_{\text{tot}} = 81\%$ and 23% , respectively), while boiled and autoclaved were dominated by film resistance ($R_f/R_{\text{tot}} = 90\%$ and 93% , respectively), indicating that thermal treatment immediately promoted formation of transport-limiting surface layers. By day 15, the contribution patterns evolved substantially: in the biotic condition, film resistance became more significant ($R_f/R_{\text{tot}} = 39\%$), though charge transfer still dominated; in the boiled condition, contributions became more balanced ($R_{\text{ct}}/R_{\text{tot}} = 23\%$, $R_f/R_{\text{tot}} = 77\%$), though total resistance remained low; in the autoclaved condition, both components contributed substantially ($R_{\text{ct}}/R_{\text{tot}} = 52\%$, $R_f/R_{\text{tot}} = 48\%$), with both achieving high absolute values; and in the sterile condition, charge transfer remained dominant ($R_{\text{ct}}/R_{\text{tot}} = 87\%$), with minimal film contribution. These patterns revealed that effective long-term corrosion protection (as in the autoclaved condition) required simultaneous development of both kinetic barriers (high R_{ct} through inhibition

of electron transfer) and transport barriers (high R_f through formation of compact, low-porosity films), whereas conditions exhibiting only one protective mechanism (high R_{ct} without high R_f , or vice versa) failed to achieve sustained high resistance.

9.5 Corrosion mechanisms

The comprehensive characterization of carbon steel S355 corrosion under biotic, boiled, autoclaved, and sterile conditions revealed fundamentally different mechanisms driven by the interplay between microbial activity, inoculum-derived chemistry, and electrochemical processes. Integration of morphological, microbiological, chemical, and electrochemical data enabled identification of distinct corrosion pathways for each condition. Starting from the biotic condition, it exhibited classical microbiologically influenced corrosion characterized by dense biofilm formation (1.89×10^{13} copies mL^{-1} , Fig. S.9.2), a thick phosphate-rich layer (20 μm , Fig. 9.7), moderate pitting (10 μm), and progressive OCP ennoblement (+75 mV, Fig. 9.15). The microbial community (Figs. 9.13-9.14) dominated by *Tepidiphilus* (63%) and *Methanothermobacter* (~100% of archaea) influenced corrosion through multiple mechanisms. The primary anodic reaction (Eq. 9.3) coupled with phosphate precipitation forming phases such as vivianite (Eq. 9.4) and iron phosphates (Eq. 9.5), alongside oxyhydroxide formation through Fe^{2+} oxidation producing lepidocrocite ($\gamma\text{-FeOOH}$) identified in Raman spectra.



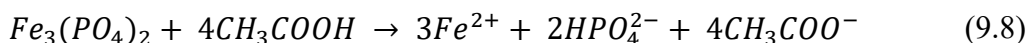
The cathodic reaction involved hydrogen evolution (Eq. 9.6) which *Methanothermobacter* consumed through methanogenesis (Eq. 9.7), depolarizing the cathode and accelerating overall corrosion.



This hydrogen consumption explains why despite substantial product accumulation, polarization resistance remained modest ($R_p = 0.24 \text{ k}\Omega \cdot \text{cm}^2$). The low film resistance ($R_f = 514 \Omega \cdot \text{cm}^2$) despite 20 μm thickness indicated a porous layer, likely due to extracellular polymeric substances creating heterogeneous structure. Pitting occurred at biofilm heterogeneities where organic acids (2.9 mg

L⁻¹ acetate, Table 9.1) created locally aggressive conditions. The moderate dissolved iron (1.9 mg L⁻¹, Table 9.2) reflected dynamic equilibrium between dissolution and phosphate precipitation.

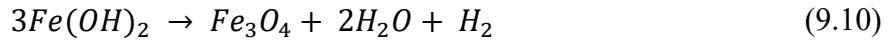
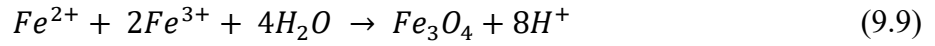
The boiled condition exhibited paradoxical behavior: initially high resistance ($R_{\text{tot}} = 2.25 \text{ k}\Omega \cdot \text{cm}^2$ at day 1, $R_f = 2.02 \text{ k}\Omega \cdot \text{cm}^2$) followed by catastrophic degradation ($R_{\text{tot}} = 0.62 \text{ k}\Omega \cdot \text{cm}^2$ at day 7) and failure to recover ($R_p = 0.26 \text{ k}\Omega \cdot \text{cm}^2$ at day 15, lowest final value). Despite poor electrochemical performance, a 20 μm phosphate-rich layer (Fig. 9.8) formed containing lepidocrocite, goethite, maghemite, and phosphates, with reduced pitting (5 μm). Boiling selectively inactivated vegetative cells while spores survived, yielding significant colonization (3.05×10^9 copies mL⁻¹, Fig. S.9.2) dominated by *Tepidiphilus* (70%). Critically, boiling lysed cells, releasing intracellular contents including elevated acetate (6.0 mg L⁻¹, Table 9.1) and phosphate. Initial high resistance resulted from rapid precipitation of compact phosphate-organic complexes and maghemite ($\gamma\text{-Fe}_2\text{O}_3$, 719 cm⁻¹, Fig. 9.11), a more oxidized phase than in biotic conditions. However, this layer proved unstable, failing through mechanisms including delamination due to poor adhesion, phase transformation, mechanical breakdown from volume changes, and metabolic acidification after spore germination. Elevated acetate supported acid attack on phosphates (Eq. 9.8).



After failure, extremely low charge transfer resistance ($R_{\text{ct}} = 159 \Omega \cdot \text{cm}^2$ at day 15) indicated highly active surfaces with rapid electrochemical kinetics. Partial recovery at day 15 ($R_f = 527 \Omega \cdot \text{cm}^2$) showed new product formation, but sustained low resistance confirmed non-protective character. Lower dissolved iron (1.2 mg L⁻¹, Table 9.2) despite higher corrosion rates suggested efficient but non-protective precipitation in the lysate-rich environment. Reduced pitting depth despite poor resistance indicated more uniform general corrosion rather than localized attack.

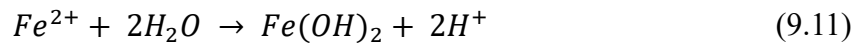
On the contrary, the autoclaved condition achieved optimal protection: highest resistances ($R_p = 0.66 \text{ k}\Omega \cdot \text{cm}^2$; $R_{\text{tot}} = 3.39 \text{ k}\Omega \cdot \text{cm}^2$), greatest ennoblement (+105 mV to -0.53 V, Fig. 9.15), and reduced pitting (5 μm , Fig. 9.9). The thinner layer (10 μm) was magnetite-dominated (Fe_3O_4 , 703 cm⁻¹, Fig. 9.11) with minimal phosphate despite highest availability (490 mg L⁻¹ dissolved P, Table 9.2). Autoclaving at 121°C achieved most complete inactivation (4.60×10^8 copies mL⁻¹, Fig. S.9.2), with diverse surviving thermoresistant community rather than single-species dominance. Severe treatment caused complete lysis and thermal decomposition, evidenced by unique formate appearance (43.2 mg L⁻¹, Table 9.1) and exceptional phosphate release (Table 9.2). The absence

of phosphate incorporation despite high availability revealed that phosphate precipitation requires organic templating agents (proteins, polysaccharides) present in biotic/boiled but destroyed by autoclaving. Without templating, thermodynamically stable magnetite formed through Eqs. 9.9-9.10.



Magnetite formation rather than oxidized phases indicated reducing conditions from low dissolved oxygen post-autoclaving, H_2 production maintaining reducing atmosphere, and limited microbial hydrogen consumption. Magnetite provided protection through its compact inverse spinel structure (high $R_f = 1624 \Omega \cdot cm^2$) and semiconducting properties creating Schottky barriers inhibiting electron transfer (high $R_{ct} = 1761 \Omega \cdot cm^2$). The noble OCP directly reflected magnetite's rest potential from Fe^{3+}/Fe^{2+} equilibrium. Continuous monotonic ennoblement indicated progressive layer densification without degradation. Warburg impedance at early times indicated diffusion-limited growth, transitioning to distinct film/charge-transfer structure by day 15. Lowest dissolved iron (0.7 mg L^{-1} , Table 9.2) confirmed efficient stable incorporation into adherent magnetite.

The most severe corrosion behavior is observed for the sterile condition. Indeed, it exhibited poorest resistance ($R_p = 0.11 \text{ k}\Omega \cdot cm^2$; $R_{tot} = 0.85 \text{ k}\Omega \cdot cm^2$), stable active OCP ($\sim -0.62 \text{ V}$, Fig. 9.15), severe pitting ($10 \mu m$), and heterogeneous layers ($10\text{-}20 \mu m$ variable thickness, Fig. 9.10). Despite substantial thickness, minimal film resistance ($R_f = 114 \Omega \cdot cm^2$) indicated highly porous products. Raman (Fig. 9.11) identified mixed oxyhydroxides (lepidocrocite, goethite) with possible siderite ($FeCO_3$) and minor phosphates from background buffer ($55 \text{ mg L}^{-1} \text{ P}$, Fig. 9.2). Classical abiotic corrosion proceeded through Equation 9.3 followed by hydrolysis (Eq. 9.11) and oxidation to oxyhydroxides (Eqs. 9.12-9.13) or carbonate precipitation (Eq. 9.13).



Highest dissolved iron (3.9 mg L^{-1} , Table 9.2) indicated inefficient precipitation forming poorly adherent products that spalled rather than building protective layers. Without organic nucleation sites, homogeneous solution precipitation competed with surface precipitation. Variable thickness and heterogeneous EDS distribution (Figs. 9.6 and 9.10) reflected steel microstructure dominating local behavior through grain boundaries and inclusion-induced galvanic cells. Severe pitting propagated through autocatalytic acidification (Eq. 9.15) with anion migration maintaining pit stability.



Stable active OCP (Fig. 9.15) without ennoblement confirmed absence of passivation, with potential remaining near active dissolution region.

Systematic comparison revealed that inoculum presence and thermal state fundamentally determined corrosion mechanisms. Phosphate incorporation required biological or biologically-derived organic components: biotic and boiled formed $20 \text{ }\mu\text{m}$ phosphate layers (Figs. 9.7-9.8) consuming dissolved phosphate ($313, 244 \text{ mg L}^{-1}$ remaining), while autoclaved (490 mg L^{-1}) and sterile (55 mg L^{-1}) showed minimal incorporation (Table 9.2). This demonstrated that phosphate precipitation required organic templating molecules (proteins, polysaccharides, EPS) present in viable biofilms, partially preserved after boiling, but completely decomposed by autoclaving. Thermal treatment severity determined stability: boiling provided transient protection through rapid metastable precipitation prone to failure, while autoclaving enabled slow thermodynamic equilibration toward stable magnetite. High microbial density (biotic: $10^{13} \text{ copies mL}^{-1}$, Fig. S.9.2) promoted product formation but sustained corrosion through H_2 depolarization and acidification, while low density (autoclaved: $10^8 \text{ copies mL}^{-1}$) eliminated detrimental metabolic effects while preserving beneficial chemical influences. Ennoblement mechanisms differed: biotic involved complex biofilm-mediated pH/redox changes and mixed products, while autoclaved reflected intrinsic magnetite potential representing fundamental thermodynamic ennoblement. Layer thickness did not correlate with protection: sterile $10\text{-}20 \text{ }\mu\text{m}$ ($R_f = 114 \text{ }\Omega\cdot\text{cm}^2$) and biotic/boiled $20 \text{ }\mu\text{m}$ ($R_f = 514, 527 \text{ }\Omega\cdot\text{cm}^2$) provided poor protection versus autoclaved $10 \text{ }\mu\text{m}$ magnetite ($R_f = 1624 \text{ }\Omega\cdot\text{cm}^2$). Phase composition and crystallinity dominated: magnetite's compact spinel structure with low defects provided superior ion-blocking versus layered oxyhydroxides or amorphous phosphates. Solution iron inversely correlated with resistance (sterile: 3.9 mg L^{-1} , $R_p = 0.11 \text{ k}\Omega\cdot\text{cm}^2$; autoclaved: 0.7 mg L^{-1} , $R_p = 0.66 \text{ k}\Omega\cdot\text{cm}^2$), confirming that efficient stable precipitation into adherent films was critical.

9.6 Concluding remarks

This study revealed that thermal treatment severity of anaerobic digester inocula critically determines corrosion protection mechanisms and long-term performance on carbon steel. The thermal regimes investigated produced fundamentally different outcomes through their differential effects on microbial viability and organic matter chemistry. Autoclaving emerged as the optimal treatment, achieving superior corrosion resistance through complete thermal decomposition of organic templating molecules, which eliminated biologically-mediated phosphate precipitation and enabled formation of compact, protective magnetite layers. The severe treatment reduced microbial colonization while promoting continuous OCP ennoblement and minimal localized attack. Conversely, boiling represented an ineffective compromise that provided only transient protection: despite achieving initially high resistance, partial organic denaturation produced metastable phosphate-organic precipitates that catastrophically failed by day 7, resulting in the poorest final resistance. The untreated biotic condition yielded intermediate performance, where dense biofilm formation templated extensive phosphate precipitation but simultaneously sustained corrosion through metabolic hydrogen depolarization and acidification, achieving only moderate protection. The key mechanistic insight is that effective thermal treatment must completely destroy organic macromolecules to prevent formation of porous, biologically-templated corrosion products and instead promote thermodynamically stable inorganic oxides. These findings establish autoclaving as the preferred thermal pretreatment for anaerobic digester environments where steel corrosion is problematic, demonstrating that incomplete sterilization through boiling is not merely less effective but actively detrimental compared to no treatment, while complete thermal decomposition through autoclaving fundamentally redirects corrosion chemistry and activate spore thermotolerant species toward protective passivation.

Chapter 10

10 Bioelectrochemical CO₂RR

The rapid increase in atmospheric carbon dioxide (CO₂) concentrations remains one of the principal drivers of global climate change, emphasizing the urgent need for effective and sustainable mitigation strategies. Carbon Capture and Sequestration (CCS) technologies have emerged as critical approaches to reduce CO₂ emissions,^{351,352} with several field-scale projects deployed worldwide. However, high costs, technical limitations, and the large storage capacity requirements have constrained their widespread development, shifting increasing interest toward Carbon Capture and Utilization (CCU) strategies that convert CO₂ into value-added products.³⁵³ Within the CCU framework, bioelectrochemical systems (BES) represent a promising biological route for CO₂ conversion into valuable chemicals and fuels. Bioelectrochemical CO₂ reduction (CO₂RR) can occur through direct electron uptake from a cathode^{354,355} or indirectly via electrochemically generated intermediates such as hydrogen,^{114,356} with evidence suggesting that both electron transfer mechanisms may operate simultaneously. These systems have demonstrated capability for producing organic chemicals including acetate³⁵⁷ and gaseous fuels such as methane,³⁵⁸ offering potential pathways for renewable energy storage and chemical synthesis.

Despite technological progress, capturing CO₂ from industrial point sources remains limited by high energy demand, complex gas separation processes, and infrastructure constraints, particularly for low-concentration or dispersed emissions.³⁵⁹ These challenges have stimulated interest in alternative CO₂ sources that are naturally concentrated and continuously emitted, such as geogenic CO₂ released from geothermal and volcanic systems. In geothermal areas, CO₂ is continuously emitted through soil degassing, fumaroles, and diffuse vents,^{173,360} representing a largely underexplored opportunity for localized CO₂ capture and utilization without the energy-intensive separation processes required for dilute industrial streams. Geothermal environments are characterized not only by high CO₂ availability but also by unique microbial communities adapted to extreme conditions including elevated CO₂ concentrations, low pH, high sulfate levels, and variable redox gradients.³⁶¹ Laboratory incubation experiments indicate that CO₂-rich mofette soils supplied with H₂ gas exhibit markedly higher CO₂ fixation potential compared to control soils, suggesting that these environments harbor specialized microbial populations capable of autotrophic CO₂ reduction.³⁶¹ However, the functional capacity of these communities to support bioelectrochemical CO₂ conversion, and the geochemical constraints governing their activity in electrochemical systems, remain poorly characterized.

Italy hosts several geothermal areas with significant CO₂ emissions, including the Monte Amiata volcanic complex in southern Tuscany, where diffuse soil CO₂ fluxes create steep geochemical gradients over spatial scales of tens of meters.³⁶² These gradients generate microenvironments with contrasting sulfate concentrations, nutrient availability, and microbial community compositions, offering opportunities to investigate how soil geochemistry influences the establishment and performance of electromethanogenic consortia. Despite this potential, geothermal soils remain an unexplored inoculum source for bioelectrochemical systems, and systematic evaluation of the geochemical and operational parameters governing their application is lacking.

The successful implementation of bioelectrochemical CO₂ conversion using geothermal inocula requires addressing fundamental questions regarding: i) the effect of competing electron-accepting processes (sulfate reduction, denitrification) on methanogenesis establishment, ii) the role of reactor configuration (membrane presence, electrode materials) in supporting productive CO₂-reducing communities, iii) the temporal dynamics of community assembly and electrochemical interface development under sustained cathodic polarization, and iv) the metabolic pathways and microbial populations responsible for observed product profiles.

This study investigates the feasibility of using geothermal CO₂ emissions and associated soil microbial communities for bioelectrochemical methane production. Specifically, we: i) characterize soil samples collected along a natural CO₂ emission gradient at the Monte Amiata geothermal area to assess geochemical constraints on microbial inoculum suitability; ii) evaluate the influence of inoculum sulfate content, reactor configuration (membrane-equipped vs. membrane-free), and metabolic inhibition strategies on electromethanogenesis performance over a 90-day preliminary screening; iii) validate and characterize the enriched methanogenic consortium through replicated 31-day experiments with detailed electrochemical, chemical, and microbiological analysis; and iv) elucidate the mechanisms governing microbial community assembly, interfacial development, and metabolic pathway competition in soil-based bioelectrochemical systems. The results provide mechanistic insights into the factors controlling electromethanogenesis from geothermal CO₂ and establish a foundation for developing integrated carbon capture and utilization strategies at geothermal sites.

10.1 Chemical characterization

10.1.1 Soil inocula (HCS and LCS) characterization

Physicochemical characterization of the two soil inocula (HCS and LCS) collected along the CO₂ emission gradient revealed marked compositional differences that reflect the distinct geochemical environments at the sampling site (Table 10.1). Both soils exhibited strongly acidic pH values (4.2-4.3), characteristics of geothermal CO₂-enriched systems where continuous degassing generates carbonic acid and drives soil acidification. Despite this pH similarity, the soils differed substantially in nutrient content and sulfur speciation.

Table 10.1. Chemical characterization of soil inocula collected along the CO₂ emission gradient at the Acquapassante 1 ex SMI site.

Inoculum ^a	Soil type	pH	Sulfates (mg kg ⁻¹)	C tot (mg kg ⁻¹)	N tot (mg kg ⁻¹)	P tot (mg kg ⁻¹)	P ass. ^b (mg kg ⁻¹)
HCS	High CO ₂ /SO ₄ ²⁻	4.2	6650	54500	3470	0.80	9.06
LCS	Low CO ₂ /SO ₄ ²⁻	4.3	110.0	91500	6050	1.2	21.5

^a See Sections 3.3.1 and 3.3.4

^b P ass. = assimilable phosphorus (Olsen method, Section 3.3.4).

The high-CO₂ proximal soil (HCS), collected immediately adjacent to the emission vent, contained significantly elevated sulfate concentrations (6650 mg kg⁻¹) compared to the low-CO₂ distal sample (LCS, 110 mg kg⁻¹). This sulfate enrichment likely reflects oxidation of reduced sulfur species (H₂S, elemental sulfur) present in the geothermal gas emissions, combined with limited microbial sulfate reduction under the extreme CO₂-saturated, low-pH conditions prevailing near the vent. The high sulfate content presents a potential challenge for electromethanogenesis, as sulfate-reducing bacteria (SRB) exhibit higher hydrogen affinity than methanogens (Michaelis constants^{345,363} K_m for H₂: 1-6 μM for SRB vs. 5-50 μM for methanogens) and thus outcompete methanogens for cathodically supplied electrons.

In addition to sulfate enrichment, the high-CO₂ soil (HCS) displayed substantially reduced organic matter and nutrient content relative to the distal sample. Total carbon was 40% lower (54500 mg kg⁻¹ vs. 91500 mg kg⁻¹), total nitrogen was 43% lower (3470 mg kg⁻¹ vs. 6050 mg kg⁻¹), and total phosphorus was 33% lower (0.8 mg kg⁻¹ vs. 1.2 mg kg⁻¹). Assimilable phosphorus, the plant-available fraction critical for supporting microbial growth, was similarly depleted (9.06 mg kg⁻¹ vs. 21.5 mg kg⁻¹), representing a 58% reduction. These compositional differences indicate that the

extreme geothermal conditions near the CO₂ vent impose nutrient-limited conditions that constrain microbial biomass accumulation and metabolic activity.

The low-CO₂ distal soil (LCS), by contrast, exhibited higher organic matter content, greater nutrient availability, and substantially lower sulfate concentrations. These characteristics suggest a more favorable nutritional environment for supporting diverse microbial metabolisms, including methanogenesis. The elevated bioavailable phosphorus in LCS inoculum is particularly significant, as phosphorus limitation can constrain methanogen growth and activity in anaerobic systems. Based on these contrasting geochemical profiles, we hypothesized that the low-CO₂ soil would serve as a superior inoculum source for establishing productive electromethanogenic biofilms, while the high-CO₂ soil would present challenges due to sulfate competition and nutrient scarcity.

These initial soil characterization results provide critical context for interpreting the subsequent reactor performance data and highlight the importance of inoculum selection in bioelectrochemical systems targeting CO₂ conversion. The striking geochemical gradients observed over a spatial scale of only 30 meters underscore the heterogeneity of geothermal environments and the need for systematic site characterization when developing microbial inocula for biotechnological applications.

10.1.2 Preliminary screening experiment (90 days): catholyte and anolyte chemistry

Chemical analysis of catholytes and anolytes at the conclusion of the 90-day screening experiment revealed substantial reactor-specific differences in product accumulation, electron acceptor utilization, and inorganic ion distribution (Table 10.2). These profiles provide insight into the competitive metabolic pathways active under different operational configurations and highlight the factors governing carbon and electron flow in soil-based bioelectrochemical systems. Acetate emerged as the dominant reduced carbon product across most reactors, with concentrations ranging from 18 to 10969 mg L⁻¹ (0.3 to 182 mM). The highest acetate accumulation was observed in reactor R6 (10969 mg L⁻¹), which operated with LCS inoculum, membrane separation, and molybdate-mediated sulfate reduction inhibition (Section 3.3.2). This exceptionally high concentration indicates that suppression of sulfate-reducing bacteria (SRB) through molybdate addition successfully redirected electron flow toward acetogenic CO₂ reduction, consistent with the intended metabolic selection.

Table 10.2. Chemical characterization (IC and ICP-AES) of catholytes (R1, R2, R3, R4, R5 and R6) and anolytes (R2 and R5) at the end of the experiment (90 days).

		Concentration (mg L ⁻¹) ^c							
Compounds		R1	R2	R3	R4 ^d	R5	R6	R2	R5
		catholyte						anolyte	
Formates	$HCOO^-$	< LOD	n.d.	< LOD	n.d.	n.d.	n.d.	n.d.	n.d.
Acetates	CH_3COO^-	209	18	161	1033	1598	10969	n.d.	1025
Propionates	$CH_2CH_3COO^-$	n.d.	n.d.	n.d.	375	n.d.	n.d.	n.d.	n.d.
Carbonates	CO_3^{2-}	1700	1293	9077	300.0	1019	2901	1104	160
Oxalates	$C_2O_4^{2-}$	222.0	187.3	40.2	65.50	112.3	26.3	186.5	133.8
Sulfates	SO_4^{2-}	7520	4610	132	11.00	13.0	6.00	2716	11.00
Nitrates	NO_3^-	63.00	8.00	2271	31000	3370	2448	9.00	2290
Fluorides	F^-	14.00	2.00	10.00	n.d.	14	342	1.00	11.00
Chlorides	Cl^-	34.00	2734	4.00	24.00	152	5.00	1595	161.0
Sulfides	S^{2-}	n.d.	n.d.	33.1	13.10	55.9	0.700	666.8	42.10
Iron	Fe	0.750	2.00	1.50	11.50	30	3.50	4.250	20.50
Manganese	Mn	n.d.	4.25	n.d.	n.d.	n.d.	4.50	3.25	n.d.
Copper	Cu	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total Sulfur	S	2490	1481	77.15	16.75	60.25	2.75	1574	45.75

^c Concentrations below the limit of detection (LOD = 0.05 mg L⁻¹) are indicated as “< LOD”, while analytes not determined are indicated as “n.d.” in the Table.

^d Used as inoculum for reactors R7, R8, R9, R_{control}.

Reactor R5, also inoculated with low-sulfate soil (LCS inoculum) and equipped with a membrane but without SRB inhibition, similarly accumulated substantial acetate (1598 mg L⁻¹), though at

significantly lower levels than R6. In contrast, reactor R4, which ultimately produced the highest methane yield, exhibited moderate acetate concentrations (1033 mg L⁻¹ in catholyte), suggesting active consumption of acetate via acetoclastic methanogenesis or syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis. The presence of propionate exclusively in R4 (375 mg L⁻¹) indicates secondary fermentation activity, potentially arising from the degradation of organic matter introduced with the soil inoculum or from syntrophic metabolism within the established microbial consortium.

Formate was detected only at trace levels in R1 and R3, indicating that this two-carbon intermediate either was not produced in significant quantities or was rapidly consumed by downstream metabolic processes. The absence of detectable butyrate, valerate, or other higher-order VFAs suggests that fermentative pathways producing these compounds were not active or that their products were efficiently converted to acetate and H₂ through syntrophic oxidation.

Carbonate concentrations varied widely (160-9077 mg L⁻¹), with the highest levels observed in R3 (9077 mg L⁻¹). Elevated carbonate reflects cathodic alkalization resulting from CO₂ reduction processes, particularly acetogenesis and methanogenesis, which consume protons and generate hydroxide equivalents according to the stoichiometry of reductive carboxylation reactions. The correlation between high carbonate and substantial acetate production in R6 supports this interpretation. Conversely, reactors with lower carbonate accumulation (e.g., R4: 300 mg L⁻¹) may reflect either lower CO₂ reduction activity or effective pH buffering that prevented alkalization.

Oxalate, a two-carbon dicarboxylic acid, was detected across all reactors at concentrations ranging from 26 to 222 mg L⁻¹. Oxalate formation likely arises from incomplete CO₂ reduction or as a byproduct of oxidative stress response in biofilm microorganisms. Its relatively low and uniform concentrations suggest that oxalate is a minor metabolic endpoint in these systems.

Sulfate concentrations exhibited pronounced reactor-dependent variability, ranging from 6 to 7520 mg L⁻¹. Reactors inoculated with high-sulfate soil (R1, R2) retained elevated sulfate throughout the experiment (4610-7520 mg L⁻¹), indicating limited sulfate reduction despite continuous cathodic polarization. In contrast, reactors inoculated with low-sulfate soil displayed substantially lower final sulfate concentrations, particularly R4, R5, and R6 (6-13 mg L⁻¹), suggesting near-complete sulfate depletion through dissimilatory sulfate reduction or initial scarcity in the inoculum.

The effectiveness of molybdate inhibition is clearly demonstrated by comparing R3 (with inhibitor) and R1 (without inhibitor), both inoculated with high-sulfate soil and equipped with membranes (Section 3.3.2). Reactor R3 exhibited dramatically reduced sulfate levels (132 mg L⁻¹ vs. 7520 mg L⁻¹ in R1) and substantial sulfide accumulation (33.1 mg L⁻¹ as sulfide-S), confirming that molybdate did not completely abolish sulfate reduction but significantly attenuated it. This partial inhibition likely relieved competitive pressure on H₂ and electrons, enabling alternative CO₂-reducing pathways (acetogenesis) to dominate, as evidenced by the moderate acetate accumulation in R3 (161 mg L⁻¹).

In low-sulfate reactors, sulfide production varied: R4 accumulated 13.1 mg L⁻¹ sulfide-S despite nearly complete sulfate depletion, whereas R5 showed higher sulfide levels (55.9 mg L⁻¹) alongside residual sulfate, indicating active but incomplete sulfate reduction. Reactor R6, with molybdate addition, displayed minimal sulfide (0.7 mg L⁻¹), confirming effective SRB suppression and supporting the hypothesis that molybdate-mediated inhibition facilitated the exceptional acetate accumulation observed in this reactor.

Total sulfur measurements (ranging from 2.75 to 2490 mg L⁻¹) corroborate these trends, with high-sulfate reactors retaining the majority of sulfur in oxidized forms, while low-sulfate systems exhibited predominantly reduced sulfur species.

Nitrate concentrations displayed extreme reactor-specific variability, ranging from 2 to 31000 mg L⁻¹. Reactor R4 (i.e., the best methane producer) exhibited exceptionally high nitrate levels (31000 mg L⁻¹ in catholyte), substantially exceeding initial concentrations expected from the soil inoculum. This anomalous accumulation may reflect: concentration effects due to water loss via evaporation during the 90-day experiment, nitrification activity in the anode chamber (in membrane-equipped reactors, nitrate can diffuse from anode to cathode), or incomplete nitrate reduction coupled with oxidative nitrogen cycling within the biofilm.

In contrast, reactor R3 showed elevated but intermediate nitrate levels (2271 mg L⁻¹), while R1, R2, R5, and R6 displayed substantially lower concentrations (8–2448 mg L⁻¹). The presence of significant nitrate in several reactors indicates that denitrification, a thermodynamically favorable electron-accepting process, competed with CO₂-reducing pathways for cathodically supplied electrons. Denitrifying bacteria, possessing higher H₂ affinity than methanogens^{345,363} (K_m for H₂: ~1–10 μM for denitrifiers vs. ~5–50 μM for methanogens), likely outcompeted methanogenic archaea for available electron donors in nitrate-rich environments.

Nitrate reduction to N₂ via denitrification does not directly contribute to reduced carbon product formation, representing an electron sink that diminishes coulombic efficiency for target products. The highest methane production in R4 occurred despite (not because of) high residual nitrate, suggesting that the methanogenic consortium established in this reactor was sufficiently robust to coexist with denitrifiers, possibly through spatial segregation within the biofilm or temporal niche differentiation.

Chloride concentrations varied from 4 to 2734 mg L⁻¹, with pronounced enrichment in R2 and the membrane-free anolyte of R2 (2734 and 1595 mg L⁻¹, respectively). Elevated chloride likely reflects concentration of soil-derived salts, evaporative water loss, or release from soil minerals during the extended operational period. Chloride itself is electrochemically inert under cathodic conditions and primarily affects ionic strength and osmotic potential, which can influence microbial activity at extreme concentrations.

Fluoride was detected at low levels in most reactors (0-14 mg L⁻¹), with the notable exception of R6 (342 mg L⁻¹). This enrichment may arise from fluoride release from soil minerals (e.g., fluorapatite, biotite) under the altered pH conditions generated by cathodic CO₂ reduction and alkalization. Fluoride at elevated concentrations can be inhibitory to certain microbial metabolisms,^{364,365} though the concentrations observed here are generally within tolerable ranges for most environmental microorganisms.

Trace metal concentrations (Fe, Mn, Cu) remained relatively low across all reactors. Iron ranged from 0.75 to 30 mg L⁻¹, with moderate elevation in R5 (30 mg L⁻¹) and the membrane-free anolyte R5 (20.5 mg L⁻¹). Manganese was detected sporadically (0-4.5 mg L⁻¹), while copper remained below detection limits in all samples. These low trace metal concentrations indicate that the soil inocula did not introduce substantial dissolved metal burdens and that electrode corrosion (e.g., titanium anode dissolution) was negligible.

Comparison of catholyte and anolyte compositions in membrane-free reactors (R2 and R5) reveals distinct chemical compartmentalization despite the absence of a physical barrier. In reactor R2, anolyte contained no detectable acetate, while catholyte showed modest accumulation (18 mg L⁻¹), indicating limited cross-compartment diffusion of reduced carbon products. Similarly, anolyte in R5 accumulated substantial acetate (1025 mg L⁻¹) despite higher concentrations in the corresponding catholyte (1598 mg L⁻¹), suggesting that the soil matrix itself provided some degree of physical separation and diffusion resistance.

Sulfate concentrations in anolytes (2716 mg L⁻¹ in R2; 11 mg L⁻¹ in R5) paralleled their respective catholytes, supporting the hypothesis that in membrane-free systems, the soil inoculum functions as a porous separator that permits ionic conduction while restricting bulk electrolyte mixing. However, the elevated chloride in both anolyte and catholyte of R2 suggests some degree of salt redistribution driven by electrophoretic migration or osmotic gradients.

10.1.3 Validation experiment (31 days): enriched methanogenic consortium

Based on the preliminary screening results, reactor R4 (LCS inoculum, membrane-equipped, no inhibitor) was identified as the optimal configuration and its catholyte was used as enriched inoculum for a 31-day validation experiment comprising three replicate polarized reactors (R7, R8, R9) and one unpolarized control (R_{control}).

Chemical analysis at day 31 revealed substantial acetate accumulation in the polarized reactors, with concentrations of 420, 7250, and 6103 mg L⁻¹ in R7, R8, and R9, respectively (Table 10.3). These values represent 96-59% reductions relative to the inoculum R4 (10333 mg L⁻¹), indicating active acetate consumption during the validation period. Reactor R7 exhibited the greatest acetate depletion, while R8 and R9 retained higher residual concentrations.

The reduction in acetate levels correlates inversely with methane production rates: R9, which produced the highest methane yield (12% headspace composition, 4.8 L m⁻² d⁻¹), retained 6103 mg L⁻¹ acetate, suggesting incomplete acetate conversion. In contrast, R7, with the lowest residual acetate (420 mg L⁻¹), produced less methane (6.5% headspace, 2.6 L m⁻² d⁻¹), possibly indicating that acetate was diverted toward biomass synthesis, alternative oxidation pathways, or that acetoclastic methanogenesis was kinetically slower in this reactor.

The unpolarized control (R_{control}) exhibited minimal acetate accumulation (53 mg L⁻¹), representing a 99.5% reduction from inoculum levels. This near-complete acetate depletion without electrochemical driving force suggests that in the absence of continuous electron supply, acetate was oxidized via respiratory metabolism (using residual oxygen, nitrate, or other electron acceptors introduced during reactor handling) or consumed by non-electrotrophic fermentative microorganisms.

Propionate, present in the inoculum at 375 mg L⁻¹, was completely absent in all reactors at day 31, indicating efficient propionate oxidation. Propionate degradation in anaerobic systems

typically occurs via syntrophic oxidation to acetate and H₂, requiring low hydrogen partial pressure to remain thermodynamically favorable.

Table 10.3. Catholyte chemical characterization (IC and ICP-AES analysis of the three reactors and control at the end of the experiment (31 days).

Compounds		Concentration (mg L ⁻¹) ^e				
		Inoculum ^f	R7	R8	R9	R _{control}
Formates	$HCOO^-$	n.d.	n.d.	n.d.	n.d.	n.d.
Acetates	CH_3COO^-	10333	420.0	7250	6103	53.00
Propionates	$CH_2CH_3COO^-$	375.0	n.d.	n.d.	n.d.	n.d.
Carbonates	CO_3^{2-}	300.0	524.0	1973	2199	241.0
Oxalates	$C_2O_4^{2-}$	65.50	70.80	154.2	133.0	n.d.
Sulfates	SO_4^{2-}	11.00	13.00	11.00	8.00	n.d.
Nitrates	NO_3^-	31000	13899	11930	2433	n.d.
Fluorides	F^-	n.d.	176.0	212.0	228.0	n.d.
Chlorides	Cl^-	24.00	10.00	13.00	13.00	12.00
Sulfides	S^{2-}	13.10	3.16	2.82	4.58	52.75
Iron	Fe	11.50	13.5	3.25	3.00	1.750
Manganese	Mn	n.d.	n.d.	n.d.	n.d.	n.d.
Copper	Cu	n.d.	n.d.	n.d.	n.d.	n.d.
Total Sulfur	S	16.75	7.50	6.50	7.25	52.75

^e Concentrations not determined are indicated as “n.d.” in the Table.

^f R4 catholyte, preliminary (90-day) experiment (Table 10.2)

The consumption of propionate across all reactors, including the control, suggests that H₂-scavenging processes (methanogenesis in polarized reactors, alternative electron-accepting processes in the control) maintained sufficiently low H₂ levels to enable propionate oxidation.

Carbonate concentrations increased markedly in all polarized reactors (524-2199 mg L⁻¹) compared to the inoculum (300 mg L⁻¹), reflecting sustained cathodic CO₂ reduction activity. Reactor R9 exhibited the highest carbonate accumulation (2199 mg L⁻¹), consistent with its superior methane production and indicating extensive proton-consuming CO₂ reduction reactions. The strong correlation between carbonate buildup and methane production supports the interpretation that alkalinization is directly coupled to reductive methanogenesis.

In contrast, the control reactor (R_{control}) showed minimal carbonate accumulation (241 mg L⁻¹), confirming that electrochemical polarization is the primary driver of CO₂ reduction and associated alkalinization. The modest carbonate increase in R_{control} relative to the inoculum may reflect residual CO₂ dissolution from atmospheric exposure during sampling or minor fermentative CO₂ production.

Oxalate concentrations increased moderately in polarized reactors (70.8-154.3 mg L⁻¹ vs. 65.5 mg L⁻¹ in inoculum), with R8 showing the highest accumulation. Oxalate formation may arise from incomplete two-electron reduction of CO₂ or as a metabolic byproduct of stress-responsive pathways. Its relatively low and stable concentrations indicate that oxalate remains a minor carbon endpoint in these enriched systems.

Sulfate concentrations remained uniformly low across all polarized reactors (8-13 mg L⁻¹), comparable to inoculum levels (11 mg L⁻¹), indicating minimal sulfate availability and consequently limited potential for sulfate-reducing competition. The control (R_{control}) exhibited undetectable sulfate, likely due to complete reduction during the 31-day period in the absence of electrochemical CO₂ reduction that would otherwise outcompete SRB for electrons.

Sulfide levels were correspondingly low in polarized reactors (2.82-4.58 mg L⁻¹ as sulfide-S), representing partial reduction of residual sulfate. Notably, total sulfur in polarized reactors (6.5-7.5 mg L⁻¹) closely matched sulfate plus sulfide, indicating minimal organic sulfur accumulation. In stark contrast, the control exhibited exceptionally high total sulfur and sulfide (52.75 mg L⁻¹ for both), suggesting either accumulation of sulfide through abiotic processes (e.g., metal sulfide precipitation followed by acid-extractable release during analysis), sulfur disproportionation reactions, or concentration effects combined with heterogeneous sulfur distribution in the non-polarized system.

Nitrate concentrations declined substantially in all polarized reactors (2433-13899 mg L⁻¹) relative to the inoculum (31000 mg L⁻¹), with R9 exhibiting the greatest depletion (2433 mg L⁻¹). This 92% nitrate reduction in R9 indicates extensive denitrification activity, which likely competed with methanogenesis for cathodically supplied electrons, particularly during the initial phase of the experiment when nitrate concentrations were high. The progressive depletion of nitrate over the 31-day period likely contributed to the observed increase in methane production rates over time, as competitive electron-accepting processes diminished and methanogenic pathways became increasingly dominant.

The control (R_{control}) showed complete nitrate depletion, consistent with the absence of sustained electron input that would support continuous nitrate reduction. This suggests that residual nitrate was consumed early in the experiment through respiratory denitrification or assimilatory reduction.

Fluoride concentrations increased markedly in all polarized reactors (176-228 mg L⁻¹) compared to the inoculum (n.d.), indicating ongoing release from soil minerals under the alkaline conditions generated by cathodic CO₂ reduction. The uniformity of fluoride enrichment across replicates suggests a common geochemical process driven by elevated pH and extended contact time with the soil inoculum.

Chloride concentrations remained stable and low (10-13 mg L⁻¹ in polarized reactors; 12 mg L⁻¹ in control), indicating minimal salt accumulation or concentration effects during the shorter 31-day validation period.

Iron concentrations varied from 3.0 to 13.5 mg L⁻¹ in polarized reactors, with R7 exhibiting the highest level (13.5 mg L⁻¹). These modest concentrations likely reflect dissolution of iron oxyhydroxides under reducing conditions established at the biocathode. Iron can serve as an electron shuttle in bioelectrochemical systems, potentially enhancing electron transfer between the electrode and microorganisms.³⁶⁶⁻³⁶⁸ The control displayed lower iron (1.75 mg L⁻¹), consistent with less strongly reducing conditions.

Manganese and copper remained below detection limits in all reactors, confirming negligible trace metal mobilization or contamination during the validation experiment.

10.2 Prokaryotic community structure and dynamics

10.2.1 Community composition in preliminary screening experiment (90 days)

High-throughput 16S rRNA gene sequencing revealed distinct microbial community structures that correlated strongly with inoculum sulfate content and reactor configuration (Fig. 10.1). The taxonomic profiles demonstrate that electrochemical selection pressure, combined with geochemical constraints imposed by the soil inoculum, fundamentally shapes community assembly and functional potential in bioelectrochemical systems.

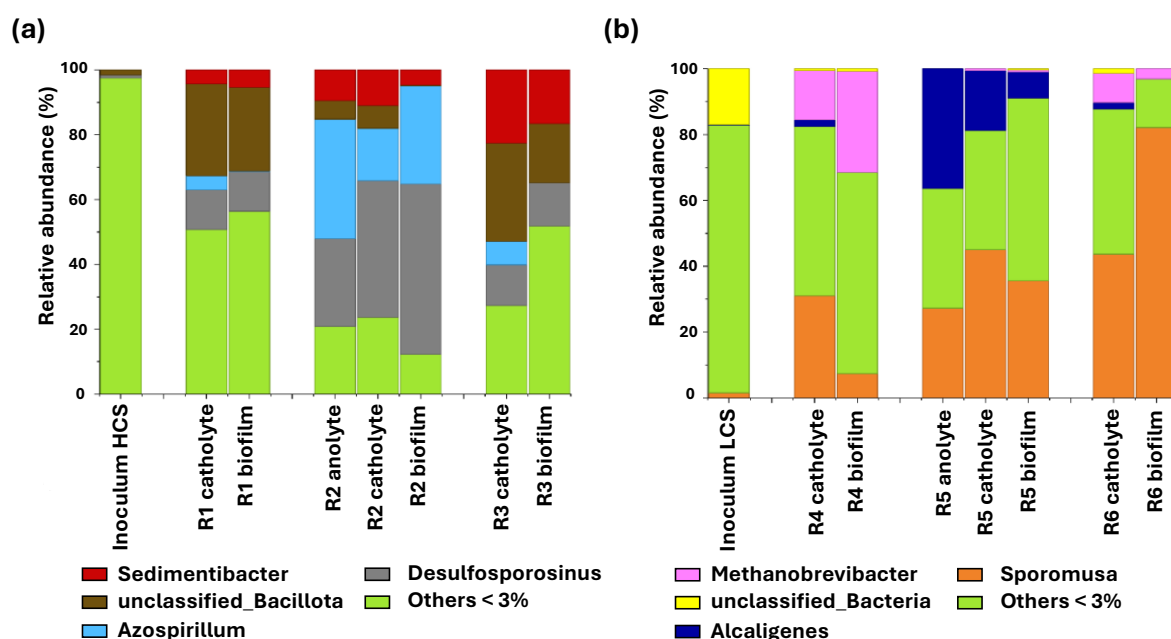


Figure 10.1. Bar plots showing the relative abundances of the five most abundant prokaryotic genera of 16S rRNA gene sequencing from (a) R1-R3 (HCS, high-sulfate soil) and (b) R4-R6 (LCS, low-sulfate soil), including catholytes, anolytes and cathodic biofilm, together with the corresponding inocula after 90 days of operation.

Reactors (R1, R2 and R3) inoculated with high-sulfate soil (HCS, Fig. 10.1a) developed communities dominated by sulfate-reducing and fermentative taxa, with minimal representation of acetogenic or methanogenic populations. The initial soil inoculum (HCS) exhibited high taxonomic diversity with no single genus exceeding 3% relative abundance, reflecting the complex microbial assemblages characteristic of geothermal soils subjected to extreme environmental stresses.

Following 90 days of cathodic polarization, reactor R2 (membrane-free configuration) displayed the most pronounced taxonomic selection, with *Desulfosporosinus* dominating both catholyte (approximately 40% relative abundance) and cathode biofilm (approximately 55%). *Desulfosporosinus* species are obligate anaerobic sulfate-reducing bacteria capable of coupling H₂ oxidation to dissimilatory sulfate reduction, making them highly competitive for cathodically generated hydrogen. The enrichment of *Desulfosporosinus* in R2 is consistent with the chemical data showing substantial sulfate depletion and sulfide accumulation (Table 10.2), confirming active sulfate respiration as the dominant terminal electron-accepting process. *Azospirillum*, a facultatively anaerobic diazotroph capable of nitrogen fixation and organic acid metabolism, constituted a secondary population in R2 (approximately 15% in catholyte), potentially benefiting from syntrophic interactions with sulfate reducers.

Reactor R1 (membrane-equipped, high-sulfate) maintained greater taxonomic diversity compared to R2, with notable representation of *Bacillota* (formerly *Firmicutes*) members including *Clostridium* and *Desulfosporosinus* at more balanced proportions (each 15-25%). This suggests that the physical separation imposed by the proton exchange membrane may have created distinct microenvironments within the catholyte and biofilm, supporting coexistence of multiple functional guilds rather than complete dominance by sulfate reducers.

Reactor R3 (membrane-equipped, high-sulfate, with molybdate inhibitor) showed elevated proportions of *Sedimentibacter* (approximately 30% in catholyte), an obligately anaerobic, fermentative bacterium that degrades amino acids and carbohydrates to produce acetate, butyrate, and H₂. The enrichment of *Sedimentibacter* in R3, despite the presence of sulfate, suggests that molybdate-mediated suppression of sulfate reduction relieved competitive pressure, allowing fermentative populations to proliferate. However, the absence of substantial methanogenic populations indicates that molybdate inhibition alone was insufficient to enable methanogen establishment in high-sulfate systems, likely due to persistent low-level sulfate reduction and the inherently slower growth kinetics of methanogens compared to fermenters.

Critically, *Methanobrevibacter*, the hydrogenotrophic methanogen ultimately responsible for methane production, represented less than 1% of the microbial community in all high-sulfate reactors, consistent with the negligible methane yields observed in these systems (Table 10.2). This near-absence of methanogens confirms that elevated sulfate concentrations create insurmountable competitive pressure that prevents methanogenic colonization, even under continuous cathodic H₂ supply.

In stark contrast, reactors inoculated with low-sulfate soil (LCS, Fig. 10.1b) developed communities dominated by acetogenic and methanogenic taxa, consistent with the successful electromethanogenesis observed in these systems. The low-sulfate inoculum similarly exhibited high initial diversity with no dominant taxa, but enrichment under cathodic polarization selected for CO₂-reducing populations.

Sporomusa, an acetogenic bacterium capable of autotrophic growth via the Wood-Ljungdahl pathway using H₂ and CO₂ as sole substrates to produce acetate,^{369–371} emerged as the dominant taxon across all low-sulfate reactors. In reactor R6 (membrane-equipped, with molybdate), *Sporomusa* constituted approximately 80% of the cathode biofilm and 60% of the catholyte, representing one of the highest reported enrichments of acetogens in bioelectrochemical systems. This exceptional dominance correlates with the extraordinarily high acetate accumulation observed in R6 (10969 mg L⁻¹, Table 10.3), confirming that *Sporomusa* was the primary driver of electrosynthetic acetate production.

Methanobrevibacter, a strictly anaerobic hydrogenotrophic methanogen that reduces CO₂ to CH₄ using H₂ as electron donor,³⁷² exhibited variable but substantial abundances across low-sulfate reactors. In reactor R4, which produced the highest methane yields, *Methanobrevibacter* constituted 20% of the catholyte and 30% of the cathode biofilm, demonstrating successful establishment of a methanogenic population at the electrode-biofilm interface. The higher relative abundance in the biofilm compared to the bulk catholyte suggests that *Methanobrevibacter* preferentially colonizes the cathode surface, where H₂ concentrations are highest due to direct electrochemical generation.

Reactor R6, despite extremely high *Sporomusa* abundance, maintained *Methanobrevibacter* at 10% in catholyte and only 3% in biofilm. This taxonomic profile explains the chemical observations: R6 produced copious acetate but minimal methane, indicating that acetogenesis outcompeted methanogenesis for available H₂ and electrons. The lower methanogen abundance in R6 may reflect either kinetic limitations (acetogens establishing dominance before methanogens could colonize) or active exclusion through competition for H₂, which *Sporomusa* can scavenge at lower threshold concentrations than *Methanobrevibacter* (K_m for H₂: 1–5 μM for acetogens vs. 5–50 μM for methanogens).

Alcaligenes, a versatile facultative anaerobe capable of denitrification, organic compound degradation, and nitrogen cycling, represented a minor but consistent component (5–15%) across low-sulfate reactors. Its presence may reflect residual denitrifying activity utilizing the elevated

nitrate concentrations observed in catholytes (Table 10.2), representing an alternative electron sink that competed with acetogenesis and methanogenesis.

The contrasting community structures between high- and low-sulfate systems (HCS and LCS) demonstrate that sulfate availability is the primary determinant of community assembly in these soil-based bioelectrochemical systems. Even under identical electrochemical conditions, high sulfate drives exclusive selection for sulfate reducers, whereas low sulfate permits establishment of acetogenic-methanogenic consortia capable of productive CO₂ conversion.

10.2.2 Community stability and enhancement in validation experiment (31 days)

The validation experiment, conducted with enriched inoculum derived from the best-performing reactor R4, confirmed the stability and further enhancement of the acetogenic-methanogenic consortium under sustained cathodic polarization (Fig. 10.2). The catholyte inoculum was pre-enriched in *Sporomusa* (approximately 50%) and *Methanobrevibacter* (approximately 25%), providing a favorable starting community composition.

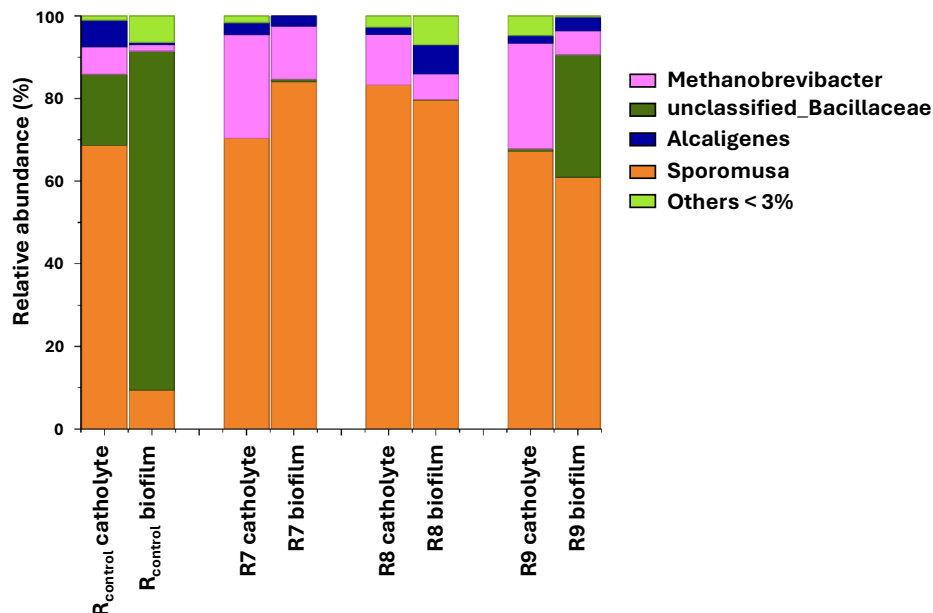


Figure 10.2. Bar plots showing the relative abundances of the five most abundant prokaryotic genera of 16S rRNA gene sequencing from reactors R7-R9 and from the control (R_{control}) after 31 days of operation.

After 31 days of operation, all three polarized reactors (R7, R8, R9) maintained or increased the relative abundances of both *Sporomusa* and *Methanobrevibacter*, demonstrating reproducible community enrichment across biological replicates. *Sporomusa* abundance remained consistently high (60-75%) in catholytes and biofilms of polarized reactors, confirming sustained acetogenic activity. The slightly lower *Sporomusa* proportions compared to R6 in the preliminary experiment (which reached 80%) likely reflect the presence of a more balanced methanogenic population that consumed acetate, preventing the extreme acetate accumulation that characterized R6.

Methanobrevibacter was detected in all polarized reactors at substantial abundances, with a consistent pattern of higher representation in catholytes (15-25%) than in biofilms (10-20%). This distribution differs from the preliminary experiment (where biofilm enrichment was observed in R4), possibly reflecting temporal dynamics: early colonization may favor biofilm attachment, while mature systems develop planktonic methanogen populations that utilize H₂ diffusing from the cathode into the bulk liquid.

Bacillaceae (family-level classification, primarily *Bacillus* and related genera) showed variable representation, with notable enrichment in reactor R9 biofilm (28%). *Bacillaceae* are metabolically versatile aerobes and facultative anaerobes capable of fermentation, suggesting they may occupy microniches where residual oxygen or alternative electron acceptors permit their persistence^{302,328}. Their presence does not appear detrimental to system performance, as R9 produced the highest methane yield despite elevated *Bacillaceae* abundance.

The unpolarized control (R_{control}) exhibited a fundamentally different community structure, particularly in the cathode-associated biofilm. Sequencing revealed that *Bacillaceae* dominated the control biofilm (approximately 70%), while *Sporomusa* represented only 7% and *Methanobrevibacter* was barely detectable (< 3%). This stark compositional shift demonstrates that sustained electrochemical polarization is essential not only for driving CO₂-reducing metabolism but also for maintaining selective pressure that favors electrotrophic populations over heterotrophic competitors. In the absence of continuous electron input via cathodic polarization, the control system reverted to fermentation-based metabolism supported by organic carbon introduced with the soil inoculum or derived from microbial turnover. *Bacillaceae*, with their rapid growth rates and metabolic versatility, outcompeted the slower-growing acetogens and methanogens that require continuous H₂ or direct electron supply. The control catholyte maintained somewhat higher *Sporomusa* abundance (approximately 30%) compared to the biofilm, suggesting that planktonic populations may have persisted temporarily by fermenting

residual acetate or other organics, whereas the biofilm community collapsed completely without electrochemical driving force.

These results conclusively demonstrate that applied cathodic potential is the primary selective force structuring microbial communities in bioelectrochemical CO₂ conversion systems. Without sustained polarization, even pre-enriched consortia rapidly deteriorate as heterotrophic competitors displace electrotrophic specialists.

10.2.3 Quantitative microbial abundance

Quantitative PCR targeting the bacterial 16S rRNA gene and the methanogen-specific *mcrA* gene provided absolute quantification of total bacteria and methanogens in catholytes and cathode biofilms (Fig. 10.3a-b). These data complement the relative abundance information from 16S sequencing by revealing actual population densities and confirming the functional capacity for methanogenesis.

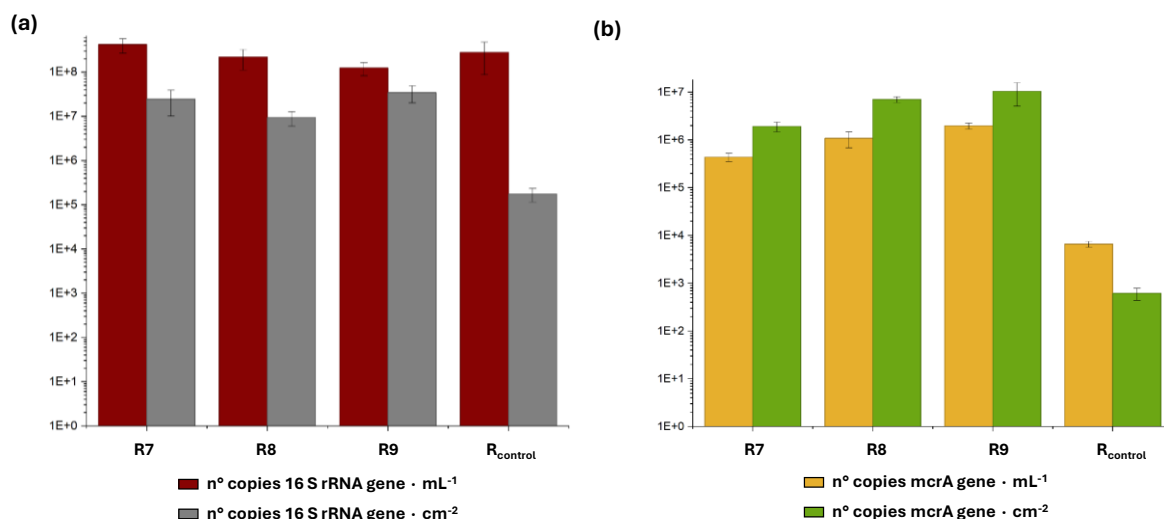


Figure 10.3. (a) 16S rRNA gene copy numbers normalized to sample volume (red) and electrode surface area (grey). (b) *mcrA* gene copy numbers normalized to sample volume (yellow) and electrode surface area (green). Error bars represent standard deviation ($n = 3$).

In the 90-day preliminary experiment, *mcrA* gene copy numbers remained below the detection limit ($\sim 10^3$ copies per reaction) in all reactors except R4, confirming that only the low-sulfate, membrane-equipped reactor successfully established a quantifiable methanogenic population. This selective methanogen enrichment in R4 validates the reactor selection strategy for subsequent experiments.

The enriched inoculum (R4 catholyte) contained approximately 1.2×10^7 copies mL⁻¹ of 16S rRNA gene and 8×10^5 copies mL⁻¹ of mcrA gene, representing a methanogen-to-bacteria ratio of approximately 6.7%. On the electrode biofilm, the inoculum exhibited 5×10^7 copies cm⁻² (16S rRNA) and 6×10^6 copies cm⁻² (mcrA), indicating preferential methanogen colonization of the cathode surface (methanogen proportion: 12% of total bacteria).

After 31 days of operation, total bacterial abundances in catholytes remained consistently high across all polarized reactors ($1\text{--}5 \times 10^8$ copies mL⁻¹). This substantial bacterial population expansion, compared to the inoculum, indicates active microbial growth supported by continuous electron and carbon supply from cathodic processes. Reactor R9 exhibited the highest bacterial abundance (4.8×10^8 copies mL⁻¹).

Methanogen abundances in catholytes ranged from 3×10^5 to 1.2×10^6 copies mL⁻¹ across polarized reactors, with reactor R9 again showing the highest values (1.2×10^6 copies mL⁻¹). While methanogen absolute numbers increased modestly compared to the dramatic bacterial expansion, the methanogen-to-bacteria ratio remained relatively stable (0.3-2.5%), indicating that methanogens maintained their niche within the growing community without being displaced by faster-growing heterotrophs.

Cathode biofilms exhibited substantially higher cell densities than catholytes, as expected for surface-attached communities. Total bacterial abundances on polarized electrodes ranged from 1×10^7 to 8×10^7 copies cm⁻². Reactor R9 displayed the highest biofilm density (7.5×10^7 copies cm⁻²), consistent with its superior electrochemical performance and methane production.

mcrA gene abundances in biofilms ranged from 8×10^6 to 2×10^7 copies cm⁻² across polarized reactors, with reactor R9 exhibiting the highest methanogen colonization (1.8×10^7 copies cm⁻²). These values represent a great increase (1.3-3%) from inoculum levels, confirming continued methanogen enrichment on cathode surfaces. The methanogen-to-bacteria ratio in biofilms (8-24%) was consistently higher than in catholytes (0.3-2.5%), corroborating the sequencing data and supporting the hypothesis that methanogens preferentially colonize the electrode-biofilm interface where H₂ availability is maximal.

The unpolarized control exhibited drastically reduced methanogen abundances: catholyte mcrA levels declined to 1×10^4 copies mL⁻¹ (98.8% reduction from inoculum), and biofilm mcrA dropped to 5×10^5 copies cm⁻² (91.7% reduction). These represent near-complete methanogen population collapse, consistent with the sequencing data showing <3% *Methanobrevibacter* abundance in the control. In contrast, total bacterial counts in the control remained substantial (2

$\times 10^8$ copies mL⁻¹ in catholyte; 3×10^7 copies cm⁻² in biofilm), indicating that heterotrophic bacteria persisted in the absence of polarization while methanogens disappeared.

The differential survival of bacteria versus methanogens in the control underscores that methanogenesis is strictly dependent on continuous H₂ supply or direct electron uptake from the cathode, neither of which occurs without applied potential. Heterotrophic bacteria, by contrast, can persist on organic substrates derived from the soil inoculum or microbial turnover, explaining their retention in the unpolarized system.

10.3 BES performances

After 90 days of operation, only R4, with the membrane and inoculated with low sulphate concentration soil (LCS) exhibited substantial methane production (approximately 10%). In contrast, none of the reactors (R1, R2 and R3) inoculated with high sulphate concentration soil (HCS) produce detectable methane, including the one (R3) supplemented with 2 mM sodium molybdate. The absence of detectable methanogenesis can be explained by the predominance of competing metabolisms which exhibit higher affinity for H₂ and thus outcompete methanogens for the available electron donor under the tested conditions. R5 and R6 produced only minor amounts of methane (around 1% and 4%, respectively) by the end of the experiment. Overall, R4 yielded the highest methane production, indicating that the most favorable configuration in this preliminary study was the combination of low-sulfate soil and a proton exchange membrane separating the anode and cathode chambers. Based on this outcome, the catholyte of R4 was selected as inoculum for three replicates (R7, R8, R9), and a control (R_{control}) which were operated for 31 days. Across the three reactors, the mean methane daily production was approximately 213 mmol CH₄ m⁻² day⁻¹ at 25 °C and at atmospheric pressure (Table 10.4). This value assumes a 24-hour accumulation period and complete CH₄ retention in the headspace. Among the tested reactors, reactor R9 exhibited the highest methane production, reaching 30% of the gas headspace on day 31. This corresponds to a methane production of approximately 294 mmol m⁻² day⁻¹. This value significantly exceeds the average production calculated across all three reactors, highlighting reactor R9's superior performance under the same operational conditions.

Table 10.4. Reactors R7, R8 and R9 electrochemical performance and corresponding CH₄ and acetate production at the end of the test (31 days).

Reactor	I (mA)	CH ₄ productivity ^g (mmol m ⁻² day ⁻¹)	CE CH ₄ (%)	Acetate ^g (mmol L ⁻¹)	CE Acetate (%)	Total CE (CH ₄ + acetate) (%)
R7	22.4 ± 1.56	151	15.5	7.12	1.54	23.9
R8	20.2 ± 1.93	193	20.1	102	21.9	42.0
R9	22.0 ± 1.79	294	30.0	123	26.6	56.6

^g Values are means of triplicate analyses (RSD < 1%).

10.4 Electrochemical characterization

Preliminary tests provided useful information regarding cathode potential settings. Indeed, a cathode potential of -1.0 V vs. Ag/AgCl sat. KCl yielded negligible current generation, indicating insufficient driving force for measurable electrochemical activity under these mild polarization conditions. Consequently, all experiments conducted in this study were performed at an elevated cell voltage of -6.6 ± 0.05 V (anode-to-cathode), which consistently generated stable and quantifiable current signals. Figure 10.4 presents the temporal evolution of current density for the three polarized reactors (R7, R8, R9) over the 31-day operational period.

All reactors exhibited reproducible current profiles characterized by initial instability followed by progressive stabilization. During the first 5–7 days, current density remained relatively low (0.75–0.85 mA cm⁻²) and displayed pronounced fluctuations, reflecting the initial formation and equilibration of the electrode-electrolyte interface. After 18 days, all reactors transitioned toward a more stable operational regime, with current density fluctuating within a narrower range (0.80–0.95 mA cm⁻²), consistent with the possible development of an electrochemically active biofilm. The three biological replicates maintained comparable current envelopes throughout the experiment, demonstrating reproducible electrochemical response to the applied polarization. Although transient current drops were occasionally observed (e.g., around day 15), these events were followed by rapid recovery to baseline levels, suggesting resilient charge-transfer processes at the cathode-biofilm interface. Notably, the unpolarized control (R_{control}, open-circuit conditions) exhibited no measurable current generation, confirming that the observed currents in polarized reactors arose directly from the imposed electrochemical gradient rather than spontaneous biochemical processes or instrumental drift.

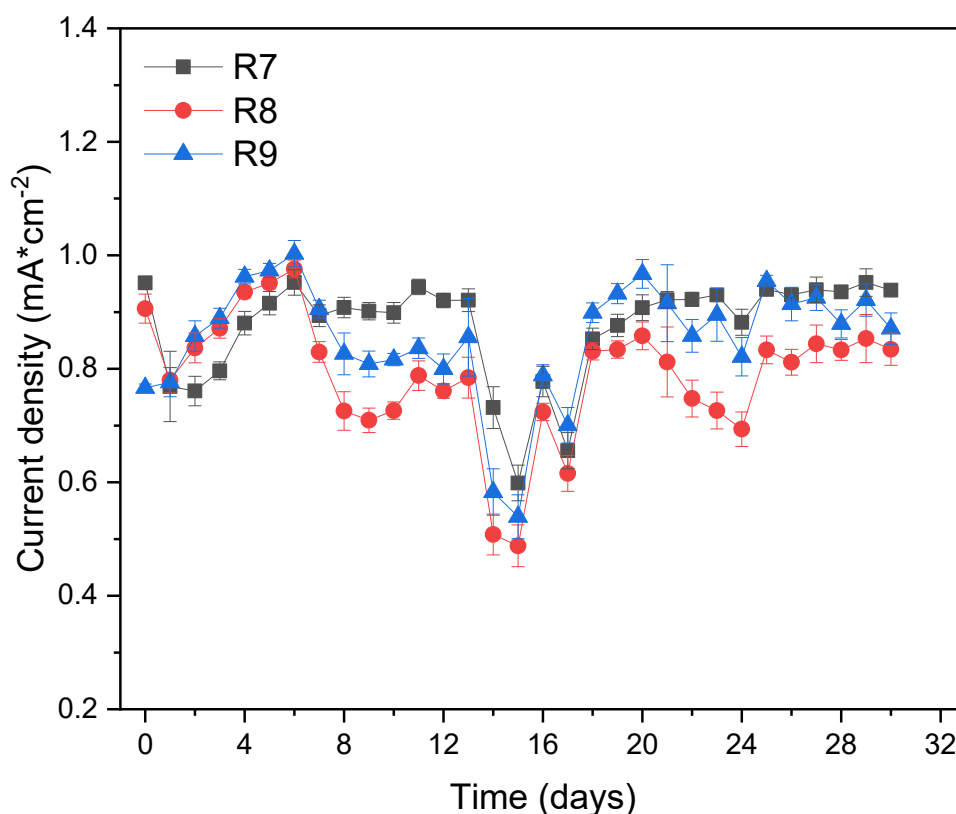


Figure 10.4. temporal evolution of the average current density of reactors R7, R8 and R9 recorded continuously over 31 days.

The temporal evolution of cyclic voltammograms (Fig. 10.5) reveals distinct and reproducible electrochemical signatures that differentiate the polarized reactors (a) R7, (b) R8, and (c) R9 from the (d) unpolarized control (R_{control}). All voltammograms were recorded relative to each reactor's open circuit potential (E_{OC}), which exhibited temporal variation reflecting the evolving redox state of the cathode-biofilm interface.

In the three polarized systems, the CVs underwent a progressive transformation from relatively narrow, featureless profiles at day 17 to substantially broader, quasi-rectangular envelopes by day 31. This temporal progression indicates marked expansion of the electrochemically active surface area, consistent with biofilm maturation and increasing electrode colonization. The enlargement of the current response, coupled with increasingly symmetric anodic and cathodic branches, is characteristic of systems developing enhanced capacitive behavior dominated by electrical double-layer charging processes. The shift from narrow early-stage CVs to later-stage voltammograms exhibiting wider hysteresis and larger enclosed areas reflects sustained increases

in charge-storage capacity. Notably, the progressive lateral displacement of CV profiles toward more negative potentials (when referenced against the Ag/AgCl sat. KCl electrode) reflects the temporal evolution of E_{OC} toward increasingly reducing conditions. This shift is consistent with biofilm establishment, accumulation of reduced metabolic intermediates (e.g., H_2 , reduced cofactors), and the development of a strictly anoxic microenvironment at the cathode surface. The absence of sharp, well-defined Faradaic peaks in the polarized reactors suggests that electron transfer occurs through distributed redox processes, likely mediated by endogenous electron shuttles (e.g., cytochromes, flavins, quinones) embedded within the biofilm matrix, rather than through direct electron transfer to discrete electroactive species.

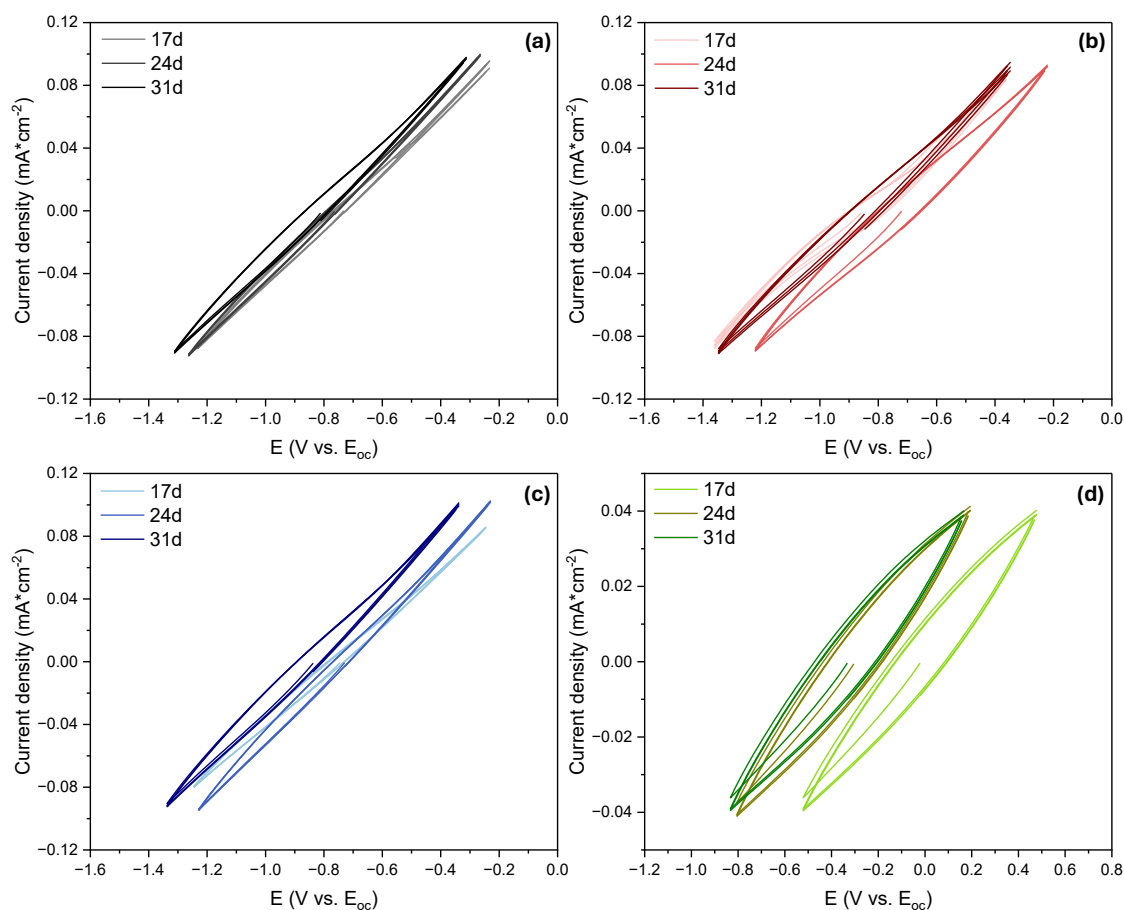


Figure 10.5. Cyclic voltammetry of (a) R7, (b) R8, (c) R9 and (d) $R_{control}$ after 17, 24 and 31 days.

Despite operating as independent biological replicates, reactors R7, R8, and R9 exhibited remarkably similar developmental trajectories. By day 24, each reactor demonstrated discernible enhancement in capacitive contribution across the full potential window, accompanied by increasingly pronounced quasi-rectangular distortion, a signature of double-layer-dominated

current response. By day 31, the voltammograms converged toward a reproducible shape characterized by symmetric anodic and cathodic limbs and elevated current density across the entire scan range. This inter-replicate reproducibility underscores the robustness of the electrochemical response under sustained polarization and demonstrates that interfacial activation proceeds in a highly consistent manner when driven by comparable bioelectrochemical conditions. In marked contrast, the CVs of the unpolarized control (Fig. 10.5d) exhibited fundamentally different electrochemical behavior. Throughout the 31-day period, the control voltammograms retained narrow current envelopes with substantially reduced capacitive character. Additionally, the entire CV profile underwent a systematic shift toward more positive potentials over time when referenced to E_{OC} . This positive potential shift, distinct from the negative shift observed in polarized reactors, reflects progressive oxidation of the electrode-electrolyte interface in the absence of continuous cathodic polarization. The shift likely results from accumulation of oxidized surface species, electrode passivation, or changes in the resting potential of the non-polarized system as it equilibrates with ambient conditions rather than being maintained under reducing bias. The absence of systematic temporal evolution in the control, evidenced by minimal changes in voltammogram shape, current magnitude, or capacitive character from day 17 to day 31, confirms that the transformations observed in polarized reactors arise directly from the imposed electrochemical gradient rather than from spontaneous electrode aging, passive biofilm accumulation, or electrolyte compositional drift. This divergence underscores that active polarization is the principal driver of interfacial development, promoting both structural reorganization of the biofilm and functional enhancement of electrocatalytic activity.

Overall, the evolution of the cyclic voltammograms reflects the emergence of an increasingly active and structurally complex electrode, biofilm interface under sustained cathodic polarization. The progressive broadening of capacitive envelopes, the enhancement of current responses over time, and the convergence toward stable, reproducible voltammetric shapes collectively indicate that continuous polarization drives the development of high-surface-area, electrochemically responsive biofilms capable of mediating efficient charge transfer. The stark electrochemical contrast between polarized reactors and the unpolarized control, manifested in both the magnitude of capacitive response and the nature of observable redox processes, highlights the central role of applied potential in establishing and maintaining long-term bioelectrocatalytic functionality.

Electrochemical surface area (ECSA) was quantified through double-layer capacitance (C_{dl}) measurements derived from cyclic voltammetry at multiple scan rates (10-50 mV s⁻¹) in non-

Faradaic potential windows (Table 10.5). C_{dl} values were obtained from the linear regression of capacitive current (Δi) against scan rate (v), with coefficients of determination (R^2) ranging from 0.856 to 0.960, indicating generally reliable capacitance determination. ECSA was subsequently calculated assuming a specific capacitance (C_s) of $20 \mu\text{F cm}^{-2}$ for carbon electrodes in aqueous electrolytes, consistent with literature values for unmodified carbon materials in bioelectrochemical systems.³⁷³ The roughness factor (R_f) was defined as the ratio of ECSA to the geometric electrode area (25 cm^2).

The three polarized reactors (R7, R8, R9) exhibited substantial but non-uniform temporal evolution of interfacial capacitance over the 31-day operational period. On day 17, C_{dl} values ranged from 1.70 to 2.95 mF, corresponding to ECSA values of 85.2-147.5 cm^2 and roughness factors of 3.41-5.90. These initial values, substantially elevated above unity, confirm that the carbon cloth electrodes exhibited intrinsic three-dimensional surface accessibility beyond their nominal geometric area, consistent with the fibrous, porous microstructure of the electrode material. By day 24, all three reactors displayed increased capacitance, with C_{dl} values reaching 2.77-3.75 mF (ECSA: 138.6-187.4 cm^2 , R_f : 5.54-7.50). Reactor R7 exhibited the most pronounced enhancement, achieving a capacitance of 3.75 mF and a roughness factor of 7.50. However, between day 24 and day 31, temporal trends diverged substantially among replicates. Reactor R9 maintained progressive growth throughout the entire period (C_{dl} increasing from 2.95 to 3.44 to 3.60 mF; ECSA from 147.5 to 172.0 to 180.2 cm^2), achieving a final roughness factor of 7.21. In contrast, reactors R7 and R8 exhibited maximum capacitance at day 24, followed by declines by day 31 (R7 from 187.4 to 147.2 cm^2 ; R8 from 138.6 to 110.0 cm^2).

This divergent behavior among biological replicates reflects the inherently dynamic nature of bioelectrochemical interfaces. The non-monotonic profiles observed in R7 and R8 suggest that interfacial development involves not only progressive biofilm accumulation but also counteracting processes such as: partial biofilm detachment or sloughing events in response to hydrodynamic stress or nutrient limitation; localized electrode fouling or pore blockage by metabolic byproducts (e.g., extracellular polymeric substances, mineral precipitates); temporal fluctuations in microbial community composition affecting overall electrochemical activity; or differential rates of biofilm maturation among replicates due to stochastic colonization dynamics. Despite these fluctuations, all polarized reactors maintained roughness factors substantially elevated above unity throughout the experiment ($R_f = 4.40$ -7.21 at day 31), demonstrating sustained three-dimensional electrochemical activation relative to pristine electrodes.

Table 10.5. Electrochemical surface area parameters calculated from double-layer capacitance measurements.

Reactor	Day	C_{dl} (mF) ^h	ECSA ⁱ (cm ²)	Roughness Factor ^j	R^2 fit ^k
R7	17	1.70	85.2	3.41	0.956
	24	3.75	187.4	7.50	0.960
	31	2.94	147.2	5.89	0.856
R8	17	2.38	118.8	4.75	0.873
	24	2.77	138.6	5.54	0.874
	31	2.20	110.0	4.40	0.919
R9	17	2.95	147.5	5.90	0.936
	24	3.44	172.0	6.88	0.923
	31	3.60	180.2	7.21	0.940
R _{control}	17	1.48	73.9	2.96	0.920
	24	0.27	13.5	0.54 ^l	0.954
	31	0.21	10.6	0.42 ^l	0.929

^h C_{dl} is the double-layer capacitance.ⁱ ECSA is the electrochemical surface area calculated assuming $C_s = 20 \mu\text{F cm}^{-2}$ for carbon electrodes in aqueous electrolytes;^j Roughness Factor is calculated considering the geometric area equal to 25 cm².^k R^2 = coefficient of determination for linear regression of capacitive current vs. scan rate.^l The anomalously low roughness factors observed in the unpolarized control at days 24 and 31 ($R_f < 1$) indicate severe electrode passivation, restricting electrochemical accessibility to a small fraction of the geometric surface area (see main text for detailed discussion).

The observed ECSA enhancements are consistent with progressive wetting and electrical integration of the internal fibrous structure of carbon cloth. Biofilm colonization, accompanied by extracellular polymer deposition and potential surface roughening through microbial metabolic activity, collectively contributes to expansion of the electrochemically addressable interface. The magnitude of roughness factors observed here (4-7) aligns with values reported for carbon cloth and carbon felt electrodes in microbial fuel cells and bioelectrochemical systems, though remains lower than highly porous activated carbon materials where R_f values exceeding 10 are common.^{374,375} This suggests that substantial portions of the internal carbon cloth structure remain electrochemically inaccessible, potentially due to incomplete electrolyte penetration, limited biofilm colonization of interior fibers, or mass transport constraints within the porous matrix.

On the contrary, the unpolarized control (R_{control}) exhibited markedly different temporal evolution, characterized by substantial decline in double-layer capacitance. On day 17, the control displayed $C_{\text{dl}} = 1.48 \text{ mF}$ ($\text{ECSA} = 73.9 \text{ cm}^2$, $R_f = 2.96$), comparable to values observed in polarized reactors at early time points. However, by day 24, C_{dl} had decreased dramatically to 0.27 mF ($\text{ECSA} = 13.5 \text{ cm}^2$, $R_f = 0.54$), representing an 82% reduction in capacitance and an 82% loss of electrochemically accessible surface area. This decline persisted through day 31 ($C_{\text{dl}} = 0.21 \text{ mF}$; $\text{ECSA} = 10.6 \text{ cm}^2$; $R_f = 0.42$), indicating progressive and severe interfacial degradation. The calculated roughness factors for the control at days 24 and 31 (0.54 and 0.42, respectively) are substantially below unity, indicating that the electrochemically active area has become smaller than the geometric electrode surface. While physically counterintuitive, this phenomenon can occur when severe passivation restricts electrochemical accessibility to only the outermost tips of protruding fibers, effectively isolating the majority of the carbon cloth structure from electrochemical processes.³⁷⁵ This behavior represents a departure from the typical assumption that all geometric surface area is electrochemically accessible, revealing that the control electrode has undergone profound interfacial transformation. The dramatic capacitance loss in the control suggests severe interfacial degradation through one or more mechanisms: i) formation of insulating deposits (e.g., biominerals, recalcitrant organic precipitates, or metal oxide/hydroxide layers) that electrically passivate the electrode surface; ii) non-electroactive biofilm accumulation that physically blocks ion access to the carbon surface without contributing to charge storage; iii) electrolyte contamination or pH drift in the absence of electrochemical buffering provided by continuous polarization, leading to surface chemistry changes that reduce capacitance; or iv) collapse or compaction of the carbon fiber network, though this is unlikely given the mechanical stability of carbon cloth.

The progressive passivation of the control electrode contrasts sharply with the sustained or increasing capacitance observed in polarized reactors, even accounting for temporal fluctuations. This divergence suggests that applied polarization may actively prevent fouling through electrochemical mechanisms such as: localized pH modulation that inhibits mineral precipitation, continuous reduction of surface oxides that maintains carbon surface activity, electrochemical promotion of biofilm architectures that enhance rather than inhibit ion transport, or periodic electrochemical "cleaning" effects that prevent accumulation of passivating layers. Alternatively, the absence of an electrochemical driving force may have permitted accumulation of dead biomass, metabolic waste products, or abiotic precipitates that progressively insulated the electrode from the electrolyte.

10.5 Surface and morphology investigations

Scanning electron microscopy revealed distinct biofilm development patterns that correlated with reactor performance and microbial community composition (Fig. 10.6).

The cathode electrodes from all three polarized reactors (Fig. 10a-c) exhibited extensive biofilm coverage, with carbon fibers almost completely obscured by dense microbial mats. The biofilms displayed characteristic multi-layered architecture with cells embedded in abundant extracellular polymeric substance (EPS) matrix. Cell morphologies were predominantly rod-shaped bacteria ranging from 1-3 μm in length and 0.5-0.8 μm in diameter, often arranged in chains or small clusters. This morphology is consistent with *Sporomusa* species,³⁷⁶ which are typically elongated rods that form chains during active growth (Fig. 10.2). Intercalated among the dominant rod-shaped cells were smaller coccoid to short-rod forms (0.5-1 μm), likely representing *Methanobrevibacter*, which typically exhibits coccobacillary morphology.³⁷² However, morphological identification from SEM images alone is not taxonomically definitive and should be interpreted in conjunction with the molecular data present in Section 10.2. The presence of two distinct morphotypes in proportions roughly consistent with sequencing results (dominant rods ~70%, minor cocci ~20%) supports the interpretation that the visible biofilm structure reflects the acetogen-methanogen consortium characterized by 16S sequencing (Section 10.2).

The EPS matrix appeared as fibrous networks and amorphous material surrounding and connecting cells, indicative of active biofilm secretion. EPS production in bioelectrochemical systems serves multiple functions: promoting cell adhesion to the electrode surface, facilitating electron transfer through conductive pili or redox-active compounds embedded in the matrix, protecting cells from environmental stresses, and maintaining optimal microenvironmental conditions (pH, redox potential, H₂ concentration) within the biofilm microarchitecture.

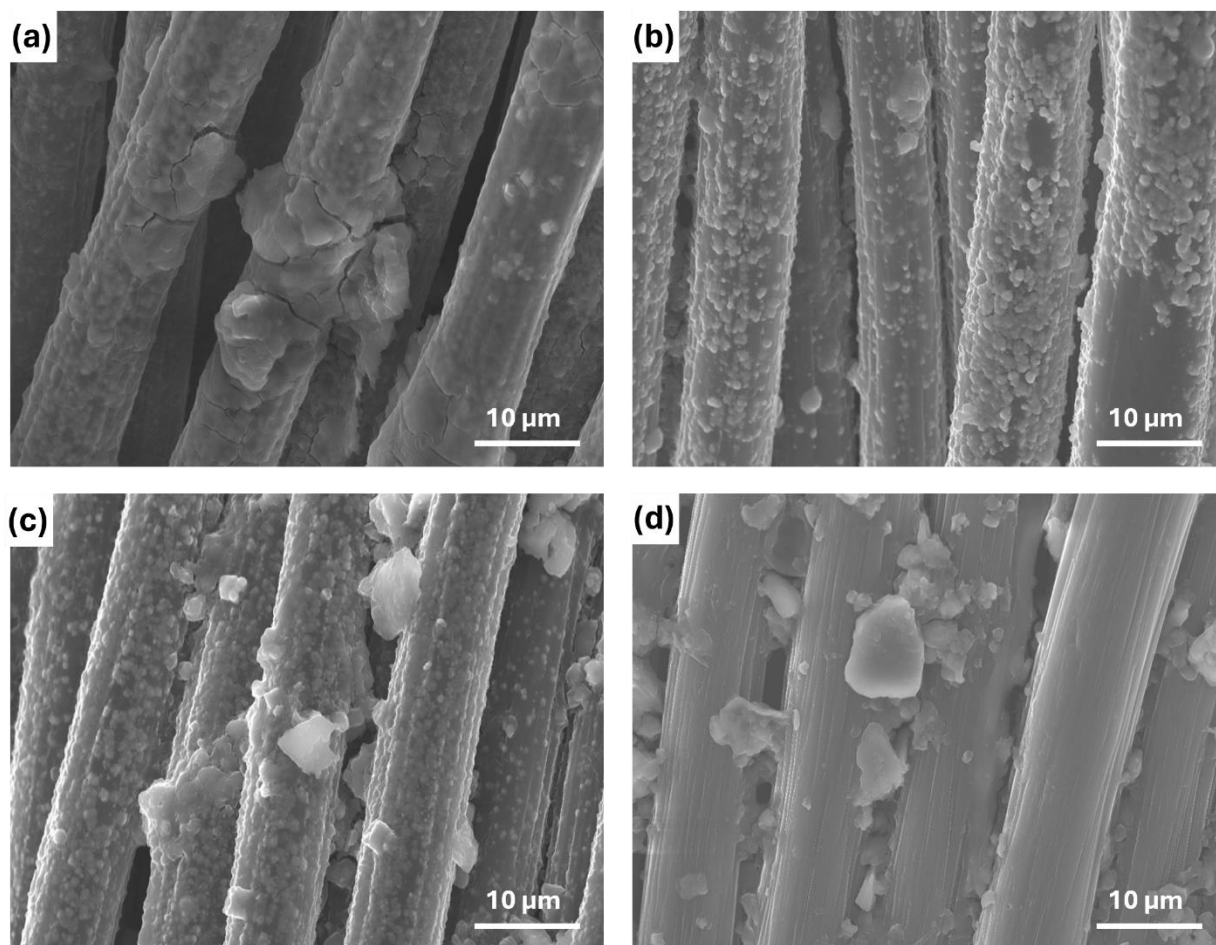


Figure 10.6. SEM images of biofilm development on reactors (a) R7, (b) R8, (c) R9 and (d) Rcontrol.

Notably, no evidence of biofilm disruption or cell lysis was observed despite the high applied voltage (-6.6 V cell potential) and vigorous H₂ evolution at the cathode. This demonstrates that the established biofilm architecture is mechanically robust and that H₂ bubble formation does not prevent stable colonization. The dense, continuous biofilm coverage observed in polarized reactors correlates with the high bacterial and methanogen abundances quantified by qPCR (10^7 - 10^8 copies cm⁻², Table 10.3) and the substantial electrochemical surface area enhancements (roughness factors 4-7) derived from capacitance measurements (Table 10.5).

In stark contrast, the control electrode (Fig. 10.6d) displayed minimal biofilm development, with most carbon fibers remaining exposed and only sporadic cell attachment visible. Where cells were present, they appeared as isolated individuals or small microcolonies rather than continuous biofilm layers. The sparse colonization pattern is consistent with the low *mcrA* abundances measured by qPCR (5×10^5 copies cm⁻², representing <10% of polarized electrode levels) and the

dramatically reduced electrochemical surface area ($R_f = 0.42$, Table 10.5) indicative of electrode passivation.

The control biofilm morphology differed qualitatively from polarized reactors: cells appeared as shorter rods and cocci in more uniform size distribution, consistent with *Bacillaceae* dominance (70% relative abundance) revealed by sequencing (Section 10.2). The absence of extensive EPS matrix and the failure to form multi-layered structures suggest that without continuous electron input, the biofilm development pathway observed in polarized systems does not proceed. Instead, the control electrode experiences only transient, heterogeneous colonization by opportunistic heterotrophs that cannot establish stable, functional biofilms.

The close physical association between cells and carbon fiber surfaces observed in polarized reactors, combined with the abundant EPS matrix, is consistent with multiple potential electron transfer mechanisms operating simultaneously: direct electron uptake via outer membrane cytochromes or conductive pili in direct contact with the electrode, H₂-mediated indirect transfer where electrochemically generated H₂ diffuses short distances through the biofilm to hydrogenotrophic populations, and electron shuttling via redox-active compounds (flavins, quinones, humic substances) embedded in or secreted into the EPS matrix.

The layered biofilm architecture observed in SEM images suggests spatial organization that could support syntrophic metabolic coupling: acetogens (*Sporomusa*) located in the inner layers closest to the electrode surface where H₂ concentrations are highest could produce acetate, which diffuses outward to methanogen populations (*Methanobrevibacter*) in outer layers or planktonic phase that convert acetate to CH₄. This spatial segregation would be consistent with the higher methanogen proportions observed in catholytes versus biofilms in some reactors.

10.6 Discussion

The temporal evolution of electrochemical surface area reveals complex, non-linear dynamics driven by competing processes of biofilm accumulation and reorganization. Polarized reactors achieved roughness factors of 4-7 (Table 10.5), indicating an enhancement of electrochemically accessible area relative to geometric projection. However, complete utilization of the carbon cloth's porous structure (theoretical $R_f > 10-20$, Table 10.5) was not achieved, likely due to incomplete electrolyte penetration, mass transport limitations, or restricted biofilm colonization in electrode interiors. The divergent temporal trajectories among replicates, with R9 showing continuous ECSA growth (Table 10.5) while R7 and R8 peaked at day 24 before declining, reflect the stochastic nature of biofilm establishment and local microenvironmental variations. Cyclic

voltammetry (Fig. 10.5) confirmed progressive interfacial activation, with transformation from narrow early-stage profiles to broad quasi-rectangular envelopes indicating enhanced capacitive character. The shift toward more negative potentials (vs. Ag/AgCl) tracks evolution of open circuit potential toward reducing conditions (Fig. 10.4), consistent with biofilm maturation and development of an anoxic microenvironment. In stark contrast, the unpolarized control exhibited severe passivation (82% capacitance loss; $R_f < 1$), indicating that sustained polarization serves dual functions: driving productive biofilm establishment while preventing electrode fouling. The control's minimal temporal CV evolution and narrow current envelopes confirm that without electrochemical driving force, electrodes undergo progressive functional deterioration rather than beneficial development.

Chemical characterization (Table 10.2) reveals that electron flow partitioning is governed by kinetic competition rather than strict thermodynamic hierarchy. Despite methane formation being thermodynamically favorable ($E^0 \approx -0.24$ V vs. -0.29 V for acetogenesis), sulfate-reducing bacteria (K_m for H_2 : 1-6 μ M) consistently outcompeted methanogens (K_m : 5-50 μ M) in high-sulfate reactors, preventing methanogenesis despite 90 days of polarization. The 60-fold difference in inoculum sulfate content (6650 vs. 110 mg kg⁻¹) determined whether productive methanogenesis could establish, demonstrating that inoculum geochemistry sets boundary conditions for electrochemical selection.

In low-sulfate reactor R4, progressive depletion of competing electron acceptors (e.g., sulfate 11 mg L⁻¹ residual and nitrate from 31000 to 2433 mg L⁻¹) relieved competitive pressure, enabling *Methanobrevibacter* colonization and achieving 12% headspace methane. The acetate decline from 10333 mg L⁻¹ (inoculum) to 420-7250 mg L⁻¹ in validation reactors, coupled with methane production, indicates sequential metabolic coupling where acetogenic CO₂ reduction feeds downstream methanogenesis. Coulombic efficiencies (24-57% total for CH₄ + acetate) reveal substantial electron diversion toward unmeasured sinks including biomass synthesis, hydrogen evolution, and unquantified products. While molybdate inhibition successfully redirected electrons toward acetogenesis (R6: 10969 mg L⁻¹ acetate, 80% *Sporomusa*), uninhibited R4 achieved superior methanogenesis, suggesting complete community establishment outperforms targeted metabolic blockade for stable CH₄ production.

16S rRNA sequencing demonstrates that sustained cathodic polarization imposes powerful selective pressure favoring electrotrophic specialists. In high-sulfate reactors, *Desulfosporosinus* dominated (40-55%), monopolizing electrons for sulfate reduction and preventing *Methanobrevibacter* establishment (<1%). Conversely, low-sulfate reactors developed

Sporomusa-dominated consortia (60-80% in biofilms) with substantial *Methanobrevibacter* populations (10-30%), achieving one of the highest reported acetogen enrichments in bioelectrochemical systems. The validation experiment confirmed consortium stability: across three replicates, *Sporomusa* maintained 60-75% while *Methanobrevibacter* stabilized at 15-25% in catholytes. The higher methanogen proportions in biofilms versus catholytes (8-24% vs. 0.3-2.5%) indicates preferential electrode colonization where H₂ concentrations are maximal. The unpolarized control exhibited community collapse: *Bacillaceae* dominated (70%), *Sporomusa* declined to 7%, and *Methanobrevibacter* became undetectable (<3%), demonstrating that continuous polarization is essential for maintaining electrotrophic populations against fast-growing heterotrophs.

qPCR (Fig. 10.3) revealed methanogen population collapse in the control (98.8% reduction in catholyte; 91.7% in biofilm) versus modest total bacterial decline (~50%), confirming that methanogens, as obligate H₂-utilizing autotrophs, cannot persist without continuous electron supply. SEM (Fig. 10.6) confirmed these patterns: polarized reactors displayed dense biofilms with rod-shaped cells (consistent with *Sporomusa*) embedded in EPS matrix, while the control showed minimal colonization and no structured biofilm development.

The achieved methane production rates (mean 213 mmol CH₄ m⁻² day⁻¹; maximum 294 mmol CH₄ m⁻² day⁻¹ in R9) demonstrate proof-of-concept for electromethanogenesis using geothermal CO₂ and soil-derived microbial inocula, but remain substantially below rates reported for optimized systems using defined cultures or pre-enriched consortia (20-100 L m⁻² day⁻¹ in recent literature).³⁷⁷ This performance gap reflects several addressable limitations in the current experimental design.

First, the relatively low coulombic efficiencies (13-46% total for CH₄ + acetate) indicate substantial electron loss to competing pathways. Strategies to improve electron recovery include a more complete depletion of competing electron acceptors (nitrate, residual sulfate) through extended pre-enrichment or chemical removal, the optimization of applied potential to minimize hydrogen evolution losses while maintaining sufficient driving force for CO₂ reduction, an enhanced mass transfer through improved reactor mixing or electrode configuration to minimize diffusion limitations, and lastly, operational parameter optimization (temperature, pH, CO₂ partial pressure) to favor methanogenic over competing metabolisms.

Second, the incomplete utilization of available electrode surface area (roughness factors 4-7 vs. theoretical maximum >10-20) suggests that substantial capacity for current density enhancement

remains unexploited. Approaches to maximize electrochemically active area can be associated with electrode pretreatment to enhance hydrophilicity and promote complete wetting of internal pore networks, selection of alternative carbon materials with more open pore structures (reticulated vitreous carbon, carbon felts with optimized pore size distributions), surface modification with conductive polymers or metal nanoparticles to enhance electron transfer kinetics, or three-dimensional electrode architectures that maximize surface area per unit reactor volume.

Third, the extended time required for community establishment (90 days preliminary screening + 31 days validation) limits practical deployment timelines. Indeed, this study highlights the importance of using pre-enriched inocula from established reactors (demonstrated successful in validation phase) to maximize the efficiency of the overall process.

10.7 Concluding remarks

This study provides mechanistic insight into the factors governing microbial community assembly, interfacial development, and metabolic pathway competition in soil-based bioelectrochemical systems for CO₂ conversion. Sustained cathodic polarization drives progressive electrochemical surface area enhancement and selective enrichment of electrotrophic acetogenic-methanogenic consortia, while inoculum geochemistry, particularly sulfate and nitrate content, establishes boundary conditions determining whether productive methanogenesis can establish dominance over competing electron-accepting processes.

The successful conversion of geothermal CO₂ to methane using soil-derived inocula demonstrates proof-of-concept for integrating bioelectrochemical technologies with existing geothermal infrastructure for carbon capture and utilization. However, substantial performance improvements, particularly in coulombic efficiency, methane production rate, and electrode surface area utilization, will be required before this approach achieves economic competitiveness with conventional natural gas or alternative CO₂ conversion technologies.

FINAL REMARKS AND FUTURE PERSPECTIVES

The transition toward a decarbonized energy economy requires technologies capable of coupling renewable electricity with sustainable carbon conversion. This doctoral research addressed key challenges within the Power-to-X framework by combining electrocatalyst design for water electrolysis, the study of microbiologically influenced corrosion in bioelectrochemical systems, and the bioelectrochemical conversion of geothermal CO₂ to methane. Together, these investigations highlight both the opportunities and the constraints associated with integrating electrochemical and biological processes for energy storage and carbon utilization.

A central and unexpected outcome of this work is the finding that microbiologically influenced corrosion in bioelectrochemical systems differs fundamentally from classical industrial corrosion. Contrary to conventional mitigation strategies based on sterilization, experimental results across multiple cathode materials demonstrated that complete microbial removal consistently exacerbates corrosion, while selective thermal enrichment of thermoresistant microorganisms promotes surface passivation. This behavior arises from microbially mediated control of solution chemistry, particularly carbonate and phosphate speciation, which governs the formation of protective inorganic oxides and carbonates. As a result, corrosion resistance was found to depend more strongly on bulk solution chemistry than on microbial metabolic activity itself, reframing microbial communities as potential allies rather than liabilities in reactor operation.

The feasibility of bioelectrochemical CO₂ conversion was demonstrated using geothermal emissions from the Monte Amiata volcanic complex, establishing proof-of-concept for integrating natural CO₂ sources with renewable electricity. However, reactor performance was shown to be critically constrained by geochemical factors, especially sulfate availability, which strongly suppresses methanogenesis through competitive sulfate reduction. Only low-sulfate inocula enabled stable methane production, underscoring the need for careful site selection and geochemical characterization prior to deployment. Furthermore, the positive role of the *Bacillaceae* family was validated, both in terms of corrosion mitigation through inhibitory effects and in enhancing methane production during bioelectrochemical tests.

Integrating these findings with advances in low-iridium oxygen evolution catalysts highlights the importance of holistic reactor design, in which anodic efficiency, cathodic stability, microbial

ecology, and solution chemistry are jointly optimized. Overall, this thesis demonstrates that solution chemistry is a unifying control parameter linking corrosion behavior, microbial community assembly, and bioelectrochemical performance. By shifting the design paradigm from sterilization and component-level optimization toward chemically and biologically informed system integration, this work provides a foundation for developing scalable Power-to-X technologies that couple renewable electricity with biological CO₂ conversion in geothermal environments.

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Appendix

Supplementary Information

CHAPTER 4

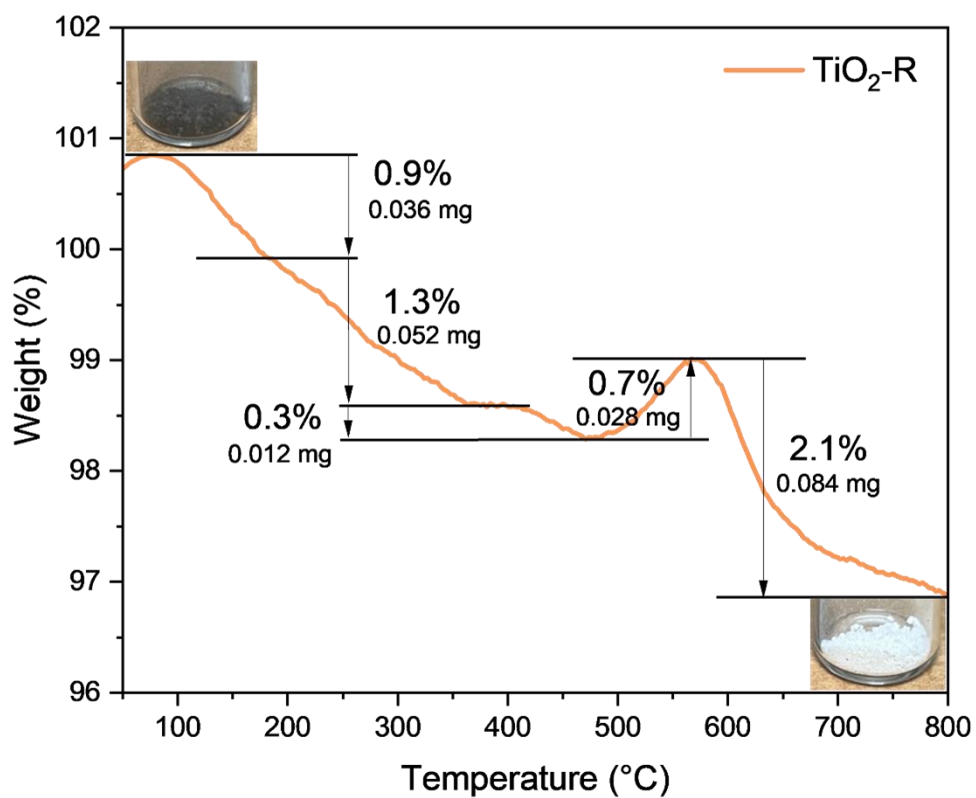


Figure S.4.1. Thermogravimetric analysis (TGA) of TiO₂-R in synthetic air over the temperature range of 50 - 800°C with a heating rate of 3 °C min⁻¹.

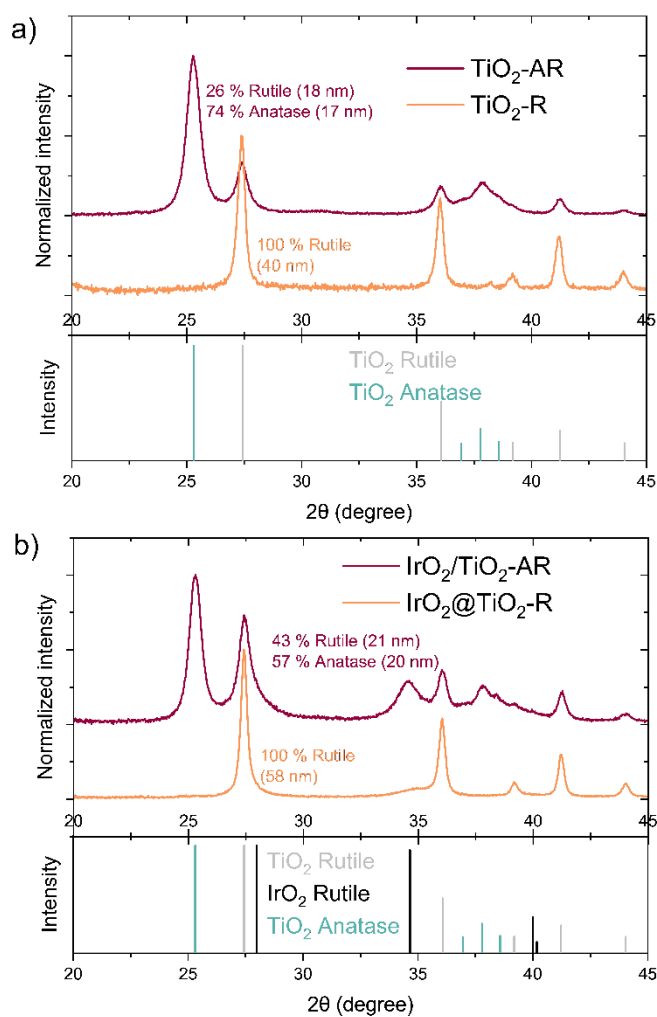


Figure S.4.2. XRD patterns of (a) the bare titania support materials and (b) the final electrocatalysts. Catalysts with titania support in the rutile phase (designated as R) and in both anatase and rutile phase (designated as AR) are shown in orange and red respectively.

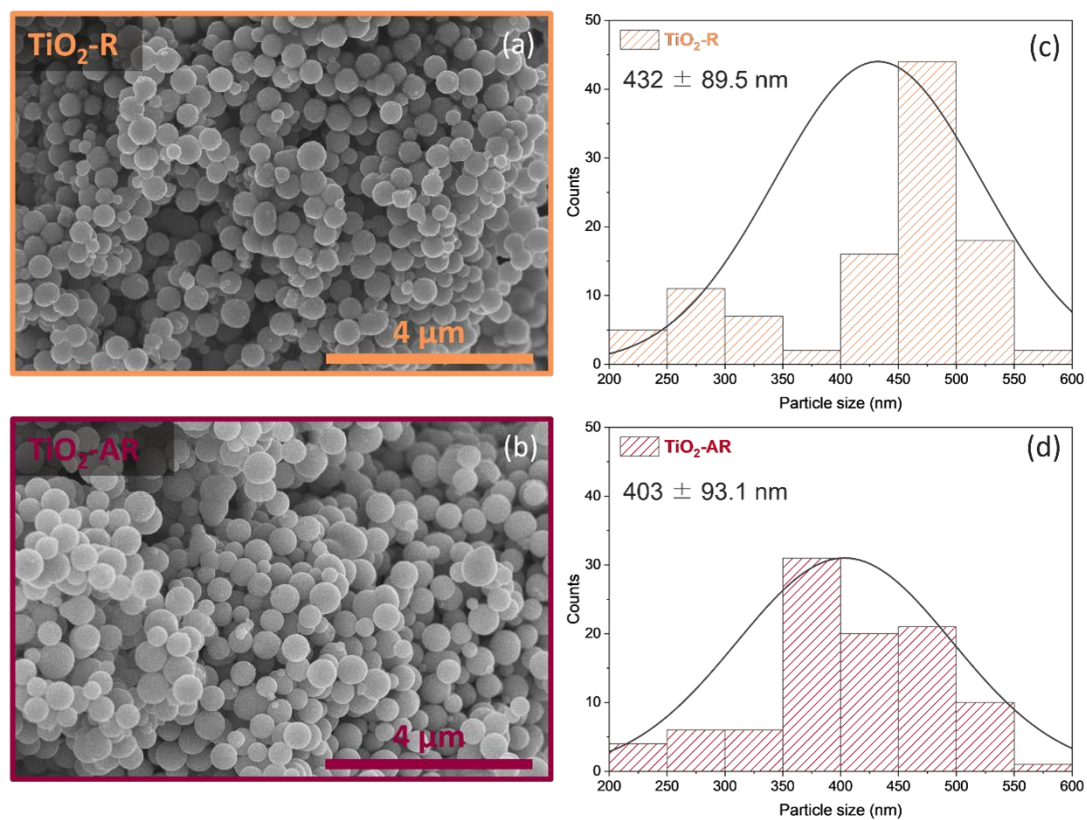


Figure S.4.3. (a-b) SEM images of $\text{TiO}_2\text{-R}$ and $\text{TiO}_2\text{-AR}$. (c-d) Nanoparticles size distribution in evaluated through SEM images. At least 100 particles are measured for each support using ImageJ.

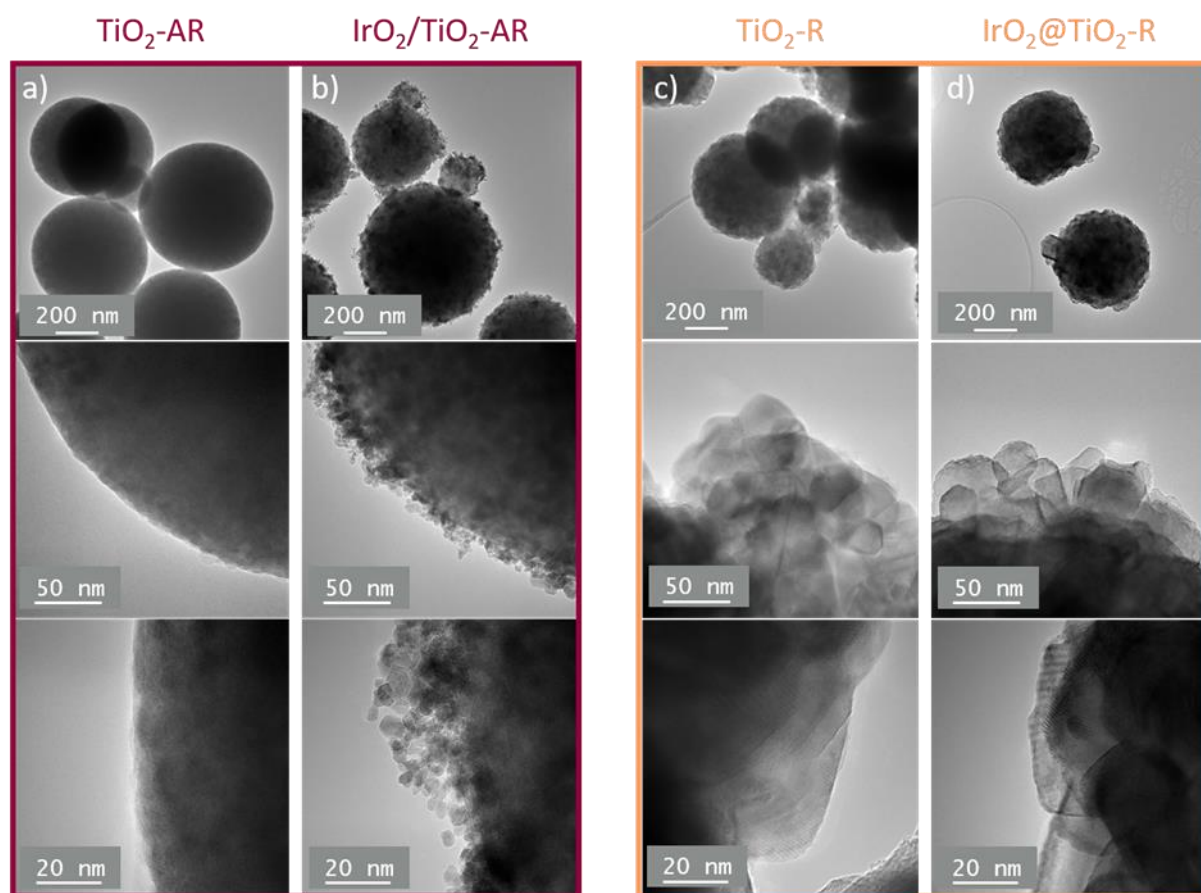


Figure S.4.4 TEM images of a) $\text{TiO}_2\text{-AR}$, b) $\text{IrO}_2/\text{TiO}_2\text{-AR}$, c) $\text{TiO}_2\text{-R}$, and d) $\text{IrO}_2@\text{TiO}_2\text{-R}$.

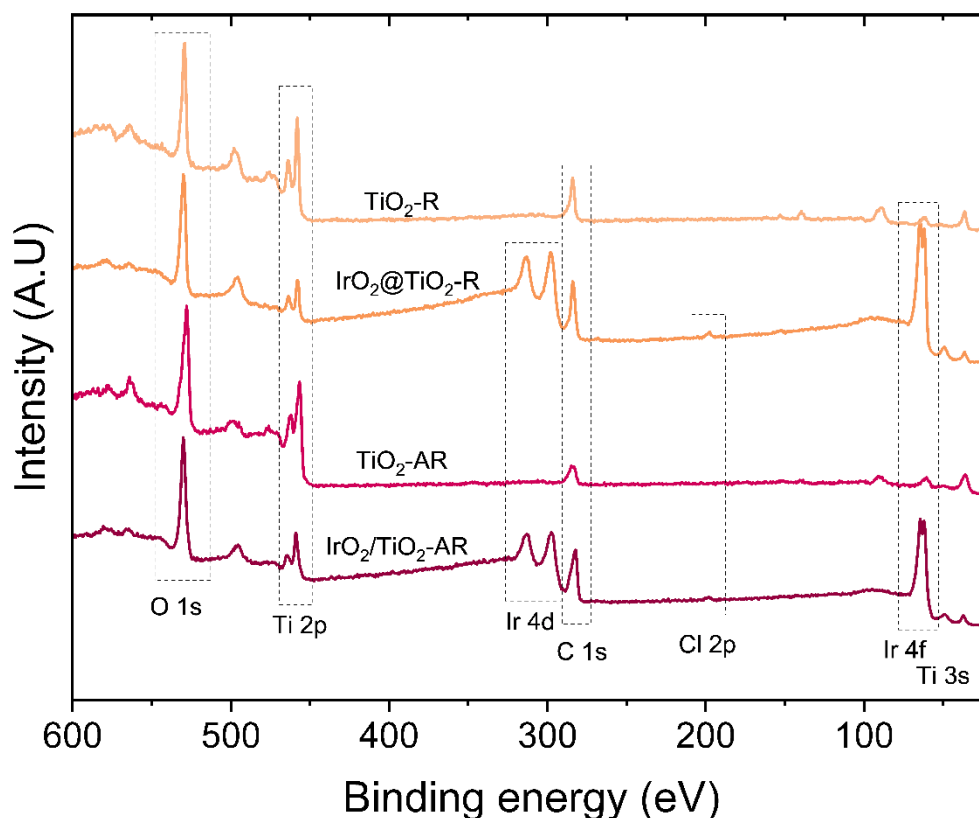


Figure S.4.5. Survey XPS spectra for the bare supports and the IrO₂/TiO₂ materials.

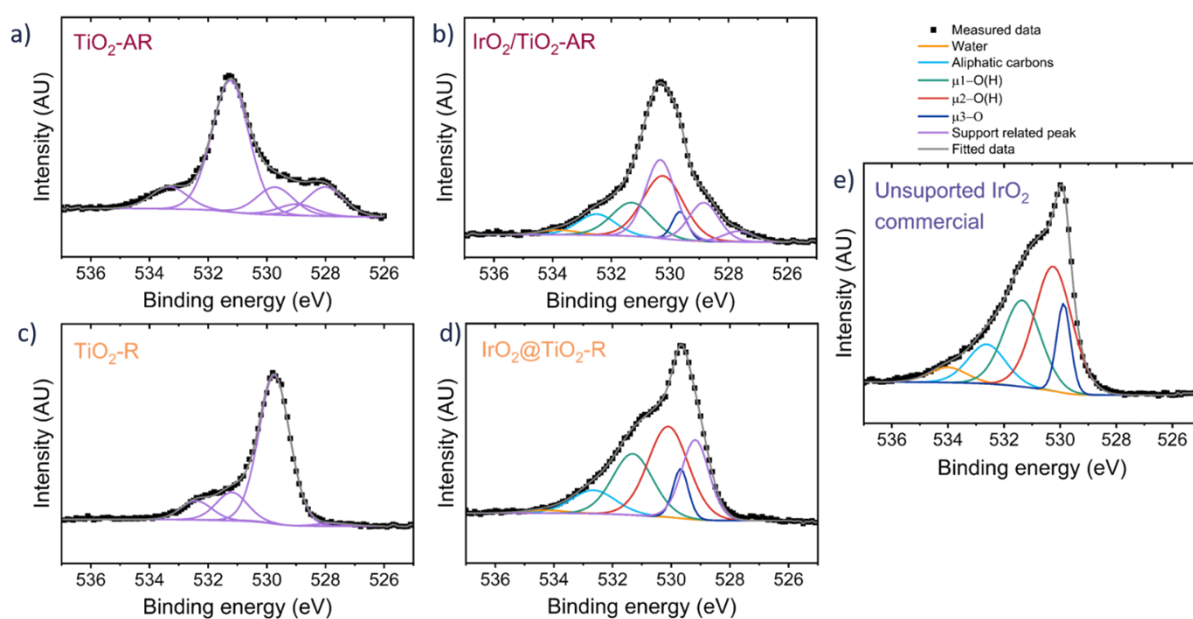


Figure S.4.6. O1s spectra and corresponding fine analysis of (a) TiO₂-AR, (b) IrO₂/TiO₂-AR, (c) TiO₂-R, (d) IrO₂@TiO₂-R, and (e) IrO₂ unsupported rutile TKK.

Non-linear model for incorporation of an additional resistance

The electrochemical analysis is based on three key equations. First, the ohmic resistance was fully compensated, with 85% corrected dynamically and the remaining 15% adjusted post-measurement. The current between 1.35 and 1.45 V was averaged and subtracted to obtain capacitance-free data. This current was then normalized by the iridium oxide mass loading. The Tafel region was identified by determining the linear potential range, and a linear fit of the current within this region was performed. From this fit, the mass-normalized exchange current density and the charge transfer coefficient (assuming a two-electron rate-limiting step) were extracted (Eq. S.4.1). These parameters were subsequently incorporated into a Butler-Volmer model across the full potential range (Eq. S.4.2), revealing deviations from the ideal Tafel behavior at higher overpotentials. To account for this deviation, an additional resistive term was introduced, inducing an extra overpotential. The modified Butler-Volmer equation (Eq. S.4.3) was fitted non-linearly, allowing extraction of the effective resistance, reported in $\Omega \cdot \text{mg}$.

Tafel equation on the anodic branch (OER)

$$\eta = \frac{R \cdot T}{\alpha \cdot n \cdot F} 2.303 \log \left(\frac{j}{j_0} \right) \quad (\text{Eq. S.4.1})$$

- j : IrO₂ mass normalized current density (A/mg)
- j_0 : IrO₂ mass normalized exchange current density (A/mg)
- η : overpotential (V), defined as $E - 1.23$ V
- α : charge transfer coefficient (dimensionless)
- n : number of electrons transferred (2 electrons)
- F : Faraday constant (96485 C/mol)
- R : gas constant (8.314 J/mol·K)",
- T : temperature in Kelvin (298 K)

Linear Butler-vomer equation

$$j = j_0 \cdot \left[\exp \frac{\alpha \cdot n \cdot F \cdot \eta}{R \cdot T} - \exp \frac{-(1-\alpha) \cdot n \cdot F \cdot \eta}{R \cdot T} \right] \quad (\text{Eq. S.4.2})$$

- j : IrO₂ mass normalized current density (A/mg)
- j_0 : IrO₂ mass normalized exchange current density (A/mg)
- η : overpotential (V), defined as $E - 1.23$ V
- α : charge transfer coefficient (dimensionless)
- n : number of electrons transferred (2 electrons)

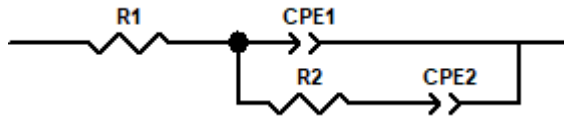
- F: Faraday constant (96485 C/mol)
- R: gas constant (8.314 J/mol·K)",
- T: temperature in Kelvin (298 K)

Non-linear Butler-vomer equation

$$j = j_0 \cdot \left[\exp^{\frac{\alpha \cdot n \cdot F \cdot (\eta - j \cdot R_{fit})}{R \cdot T}} - \exp^{\frac{-(1-\alpha) \cdot n \cdot F \cdot (\eta - j \cdot R_{fit})}{R \cdot T}} \right] \quad (\text{Eq. S.4.3})$$

- j: IrO₂ mass normalized current density (A/mg)
- j₀: IrO₂ mass normalized exchange current density (A/mg)
- η: overpotential (V), defined as E – 1.23 V
- α: charge transfer coefficient (dimensionless)
- R_{fit}: Fitted additional resistance (Ω.mg)
- n: number of electrons transferred (2 electrons)
- F: Faraday constant (96485 C/mol)
- R: gas constant (8.314 J/mol·K)",
- T: temperature in Kelvin (298 K)

The potentiostatic electronic impedance spectroscopy at 1.0 V is used to obtain the characteristic frequency of the capacitive behavior of the catalysts. The following model is used with R1 the ionic resistance, CPE1/R2 the charge transfer component and CPE2 the capacitive behavior. The characteristic frequency is calculated using the CPE2 parameters.



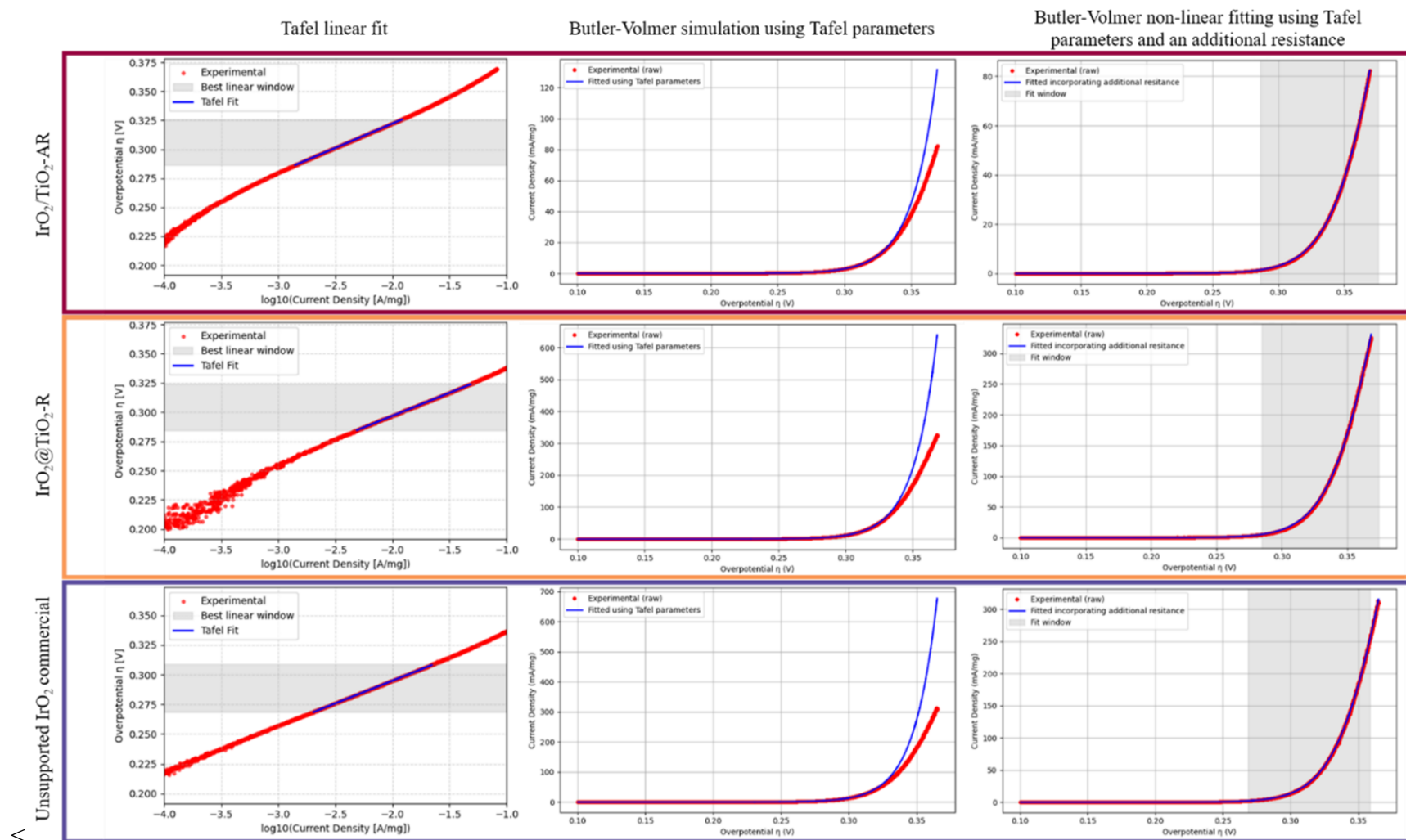


Figure S.4.7. Fitted data using the methodology described above on representative electrodes of each catalyst.

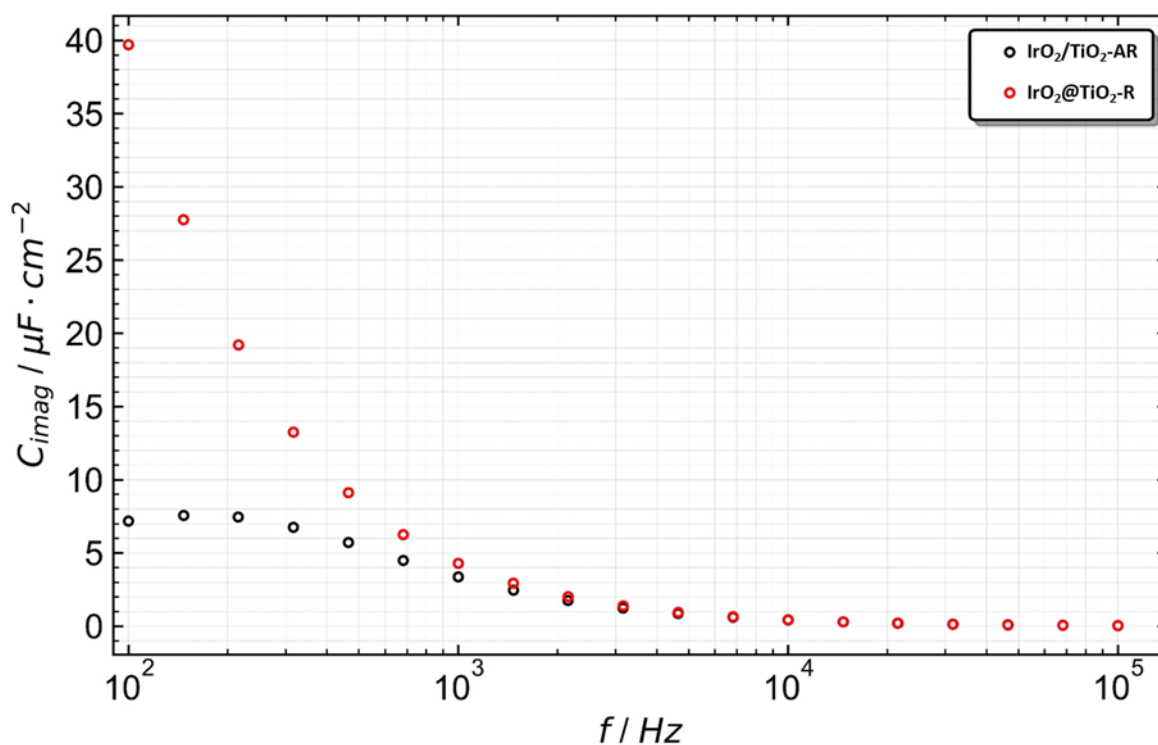


Figure S.4.8. Bode-phase plot of the electronic impedance spectroscopy for the two supported materials. The characteristic frequency could be read as the x-position of the C_{imag} maxima. In the case of the core-shell material it is higher than the scanning range.

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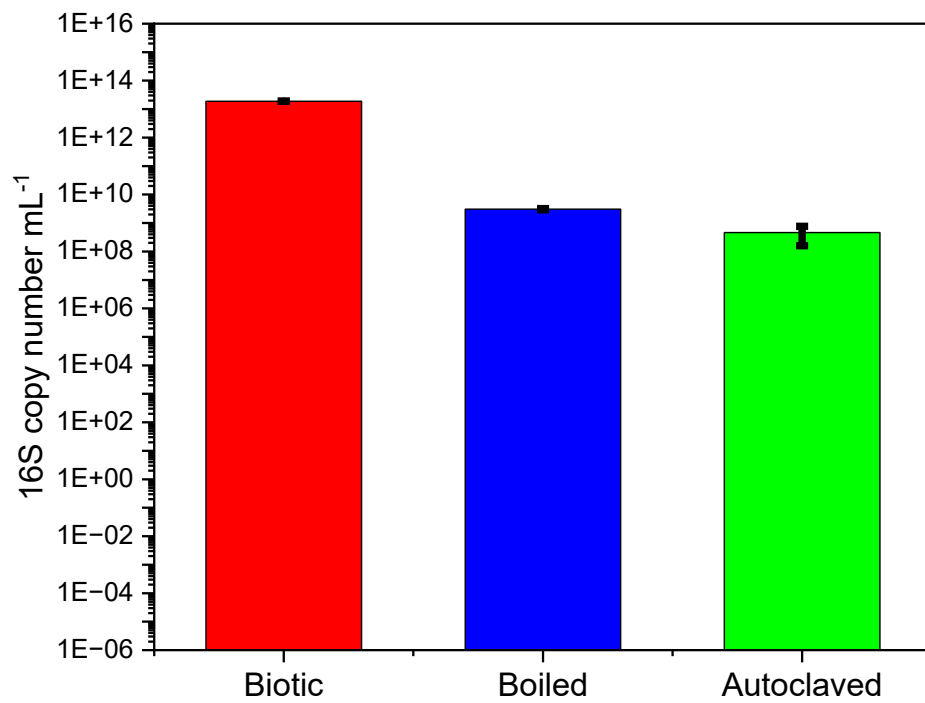


Figure S.5.1. qPCR analysis of the biofilm after 15 days of testing with biotic (red), boiled (blue) and autoclaved (gray) inocula.

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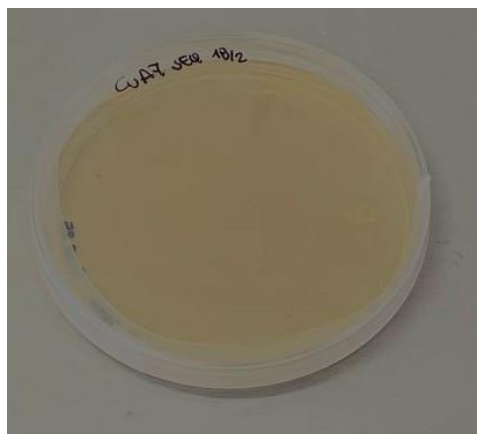


Figure S.6.1. Demonstration of sterility, shown by the absence of microbial growth on LD agar under sterile conditions.

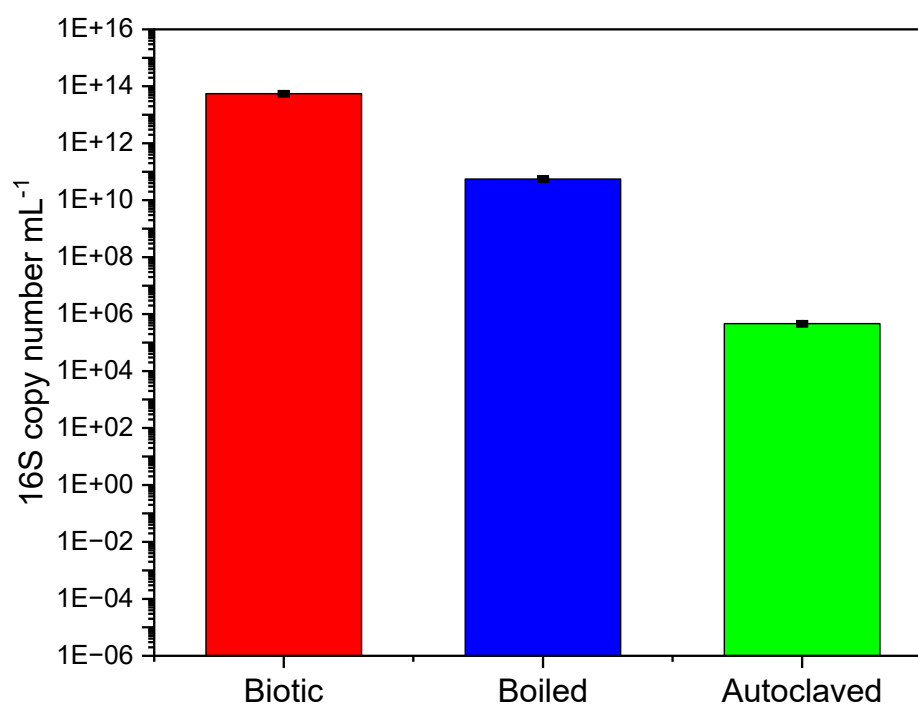


Figure S.6.2. qPCR analysis of the biofilm after 15 days of testing with biotic (red), boiled (blue) and autoclaved (gray) inocula.

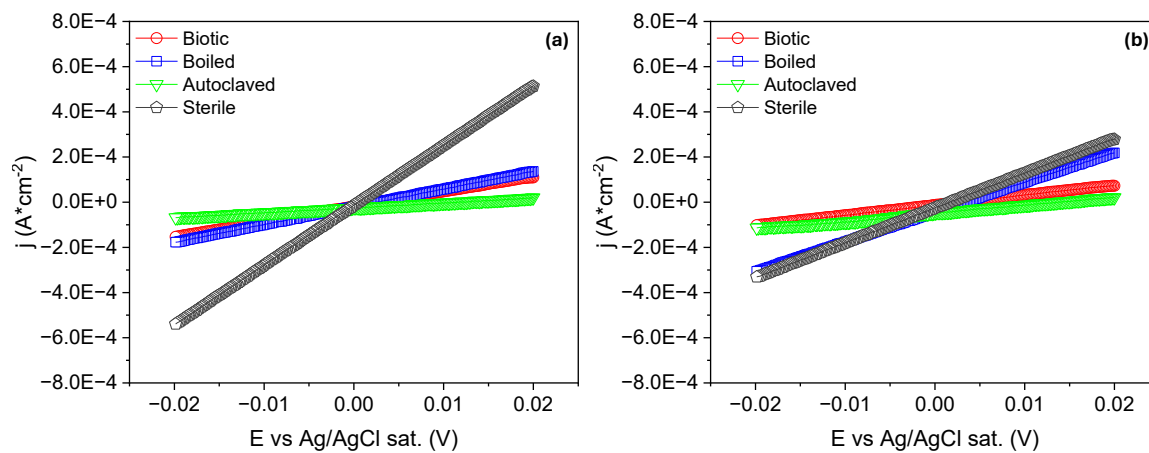


Figure S.6.3. Linear Polarization Resistance (LPR) of Cu replicas tested in biotic (red), boiled (blue), autoclaved (green), and sterile (gray) inocula after (a) 1 day and (b) 15 days.

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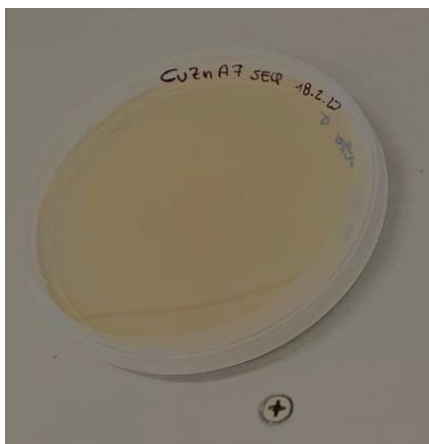


Figure S.7.1. Demonstration of sterility, shown by the absence of microbial growth on LD agar under sterile conditions.

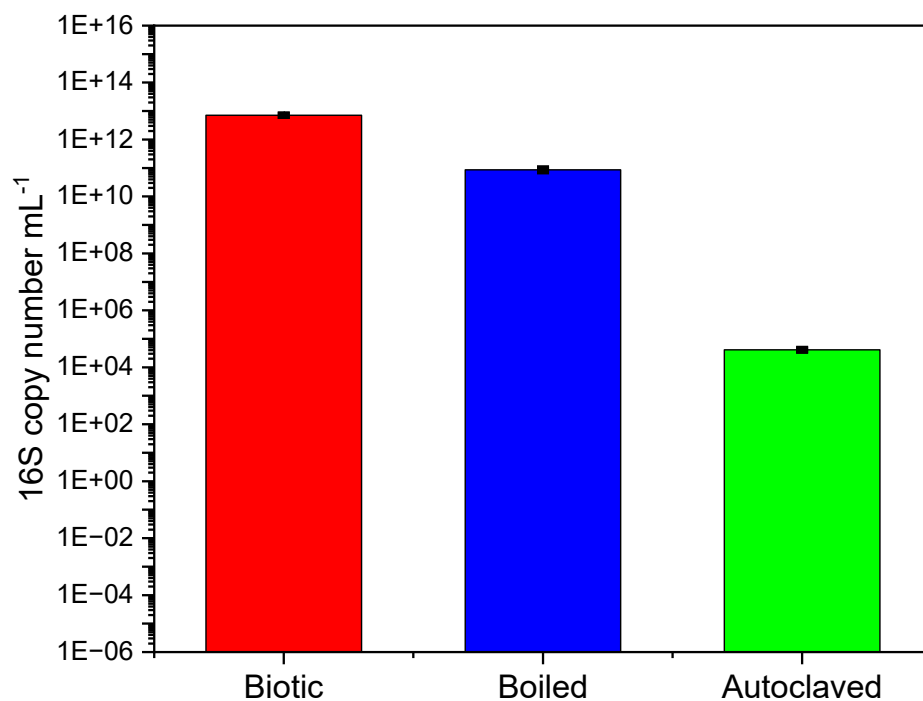


Figure S.7.2. qPCR analysis of the biofilm after 15 days of testing with biotic (red), boiled (blue) and autoclaved (gray) inocula.

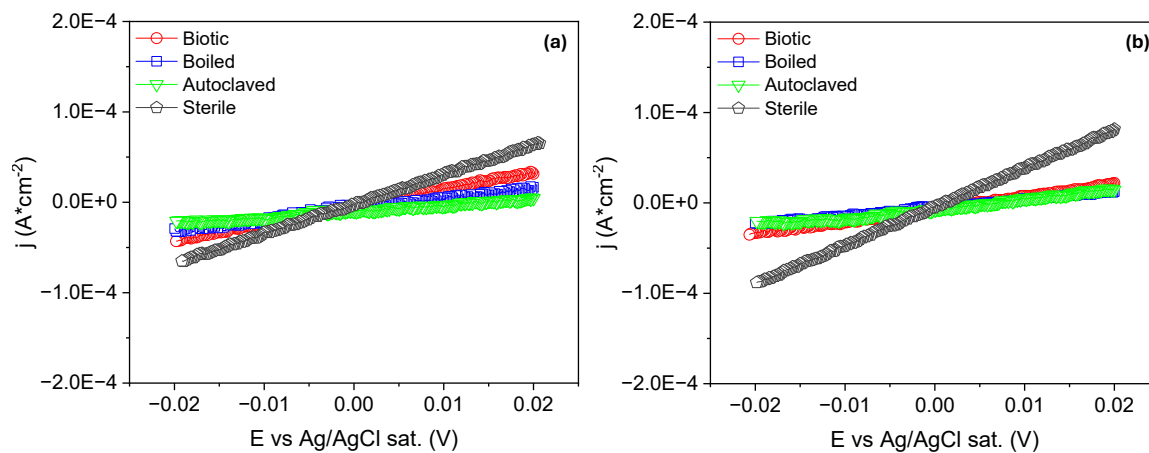


Figure S.7.3. Linear Polarization Resistance (LPR) of Cu-Zn alloys replica tested in biotic (red), boiled (blue), autoclaved (green), and sterile (gray) inocula after (a) 1 day and (b) 15 days.

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Figure S.8.1. Demonstration of sterility, shown by the absence of microbial growth on LD agar under sterile conditions.

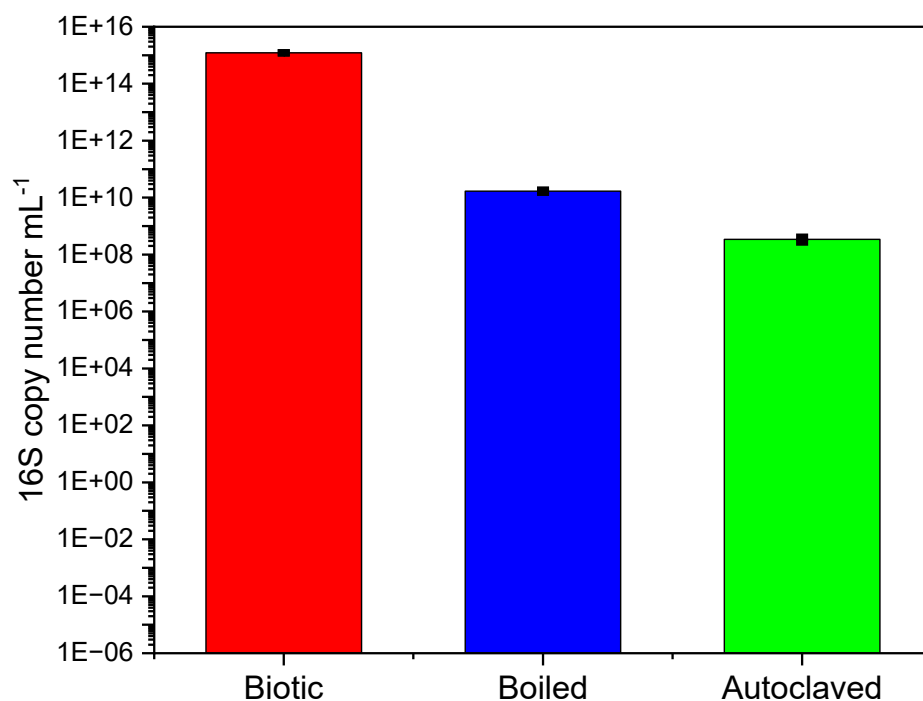


Figure S.8.2. qPCR analysis of the biofilm after 15 days of testing with biotic (red), boiled (blue) and autoclaved (gray) inocula.

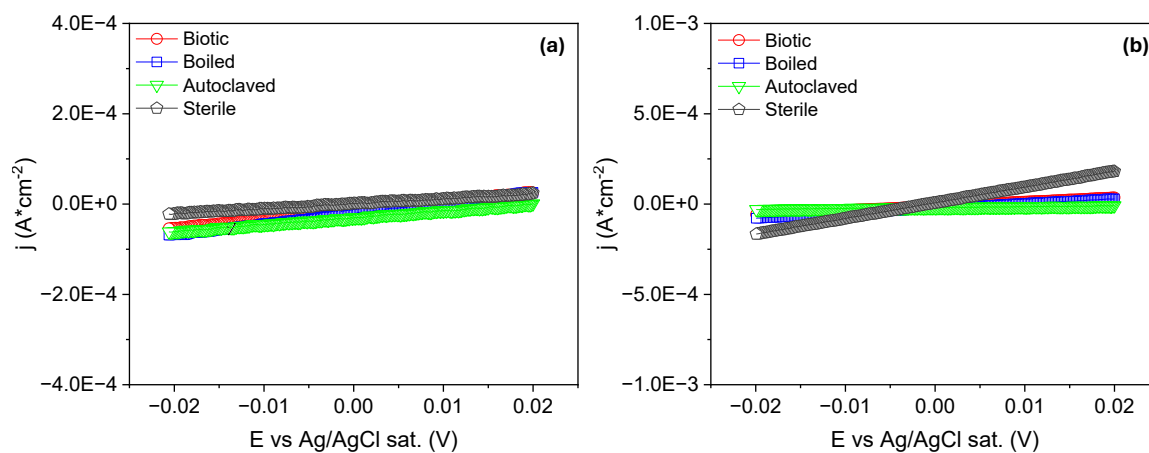


Figure S.8.3. Linear Polarization Resistance (LPR) of ARMCO iron replicas tested in biotic (red), boiled (blue), autoclaved (green), and sterile (gray) inocula after (a) 1 day and (b) 15 days.

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Figure S.9.1. Demonstration of sterility, shown by the absence of microbial growth on LD agar under sterile conditions.

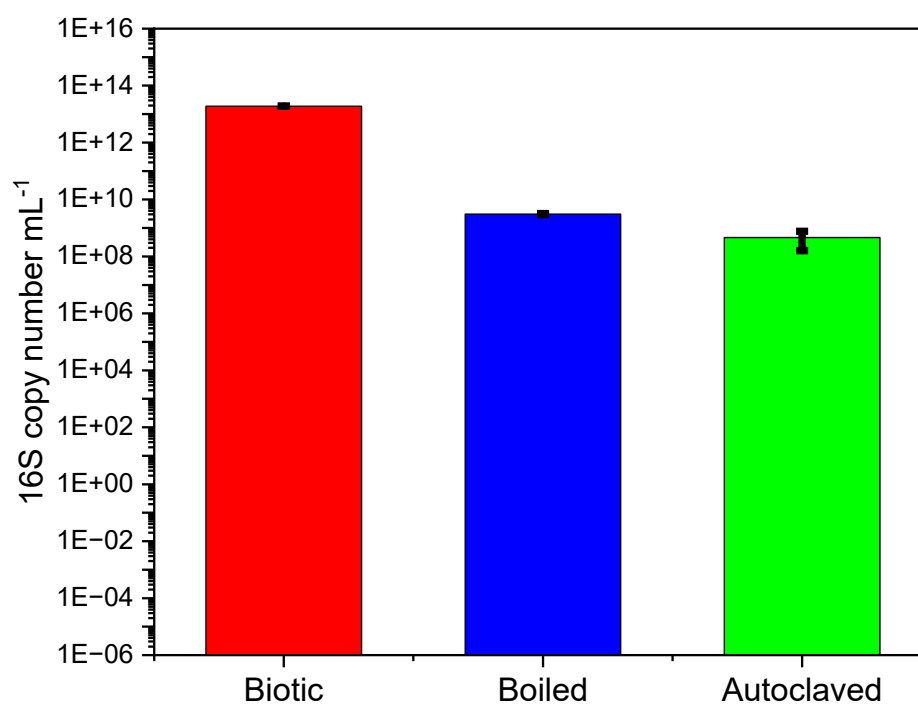


Figure S.9.2. qPCR analysis of the biofilm after 15 days of testing with biotic (red), boiled (blue) and autoclaved (gray) inocula.

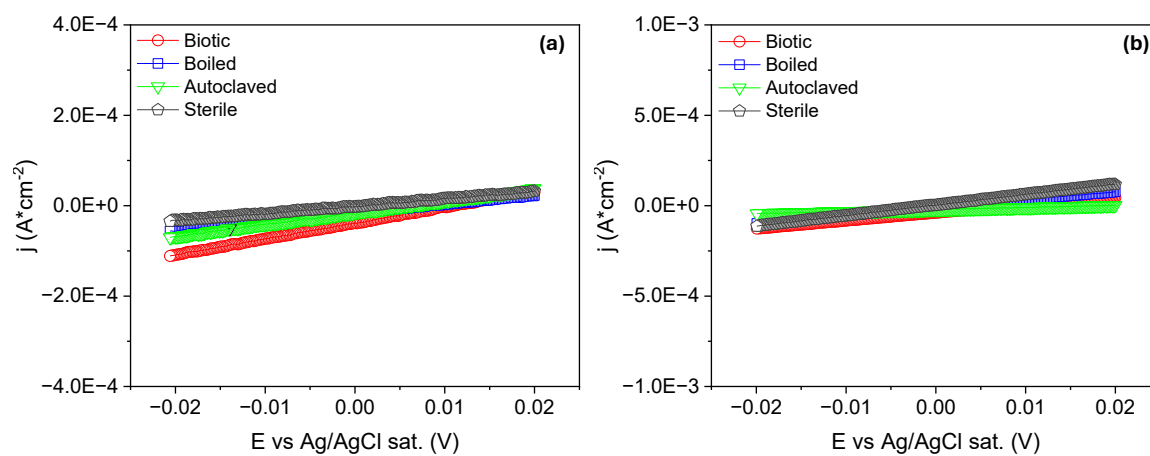


Figure S.9.3. Linear Polarization Resistance (LPR) of carbon steel replicas tested in biotic (red), boiled (blue), autoclaved (green), and sterile (gray) inocula after (a) 1 day and (b) 15 days.

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E. Cazzulani, G. Ghiara, G. Caucia, A. Franzetti, M. Balordi, P. Fedeli, F. Pini, P. Cristiani, G. L. Chiarello, Thermal Selection of Microbial Consortia Modulates Copper Corrosion toward Mineral Stabilization, *J. Clean. Prod.* **2026** (*Under review*).

Attended congresses, schools, workshops and research activities

BessyII@HZB – An In Situ NEXAFS Investigation of Copper-Cobalt Oxides for the Oxygen Evolution Reaction in Acidic Media, accepted synchrotron beamtime proposal (Proposal No. 251-13173-ST), Helmholtz Zentrum Berlin (24th – 30th March **2025** – Berlin, Germany).

E. Cazzulani, P. Atanassov, C. Roiron, P. Cristiani, G. L. Chiarello. Oxygen Evolution Catalysts with Iridium Oxide Supported on Doped Titanium Oxide: Role of Dopants on Catalysts Reactivity and Stability. ECS Conference PRiMe 2024 (6th – 11th October **2024** – Honolulu, Hawaii - USA) – Oral communication.

E. Cazzulani, P. Cristiani, A. Franzetti, G. L. Chiarello. Corrosion Enhanced by Hydrogenotrophic Methanogens Differently Enriched on Copper Alloys. ECS Conference PRiMe 2024 (6th – 11th October **2024** – Honolulu, Hawaii - USA) – Poster contribution.

E. Cazzulani, G. Ghiara, S. Massardo, F. D’Acapito, G. L. Chiarello, P. Cristiani. X-Ray Absorption Spectroscopy Study on Methanogens Influenced Corrosion of Metallic Components from the Natural Gas Network. Eurocorr 2024 (1st – 4th September **2024** – Paris, France) – Oral communication.

LISA@ESRF – Methanogens-Influenced Corrosion Mechanisms of Metallic Components in the Natural Gas Network, accepted synchrotron beamtime proposal (Proposal No. 20232102), European Synchrotron Radiation Facility (26th – 29th October **2023** – Grenoble, France).

E. Cazzulani, G. Ghiara, A. Aiello, A. Franzetti, G. L. Chiarello, P. Cristiani. Corrosion Enhanced by Bacteria and Archaea Differently Enriched on Copper and Copper Alloys. GEI 2023 (17th – 21st September **2023** – Cefalù, Italia) – Poster contribution.

E. Cazzulani, G. Ghiara, G. L. Chiarello, P. Cristiani. Corrosion Enhanced by Hydrogenotrophic Methanogens Differently Enriched on Steel Alloys. Eurocorr 2023 (27th August – 31st August **2023** – Bruxelles, Belgium) – Oral communication.

MIC Industry Workshop & Training Course EUROMIC Cost Action 20130 (29th May – 2nd June **2023** – Duzce, Turkey).

“Cecilia Monticelli” School AIM - Electrochemical techniques for corrosion studies (7th -9th February **2023** – Ferrara, Italy).

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