MICROBIAL LIFE IN VOLCANIC LAKES

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Abstract

Lakes in the craters of active volcanoes and related waters are often characterised by conditions considered extreme for life, such as high temperatures, low pH and very high concentrations of dissolved metals and minerals. Such lakes tend to be transient features whose geochemistry can change markedly over short time scales. They might also vanish completely during eruption episodes or by drainage through the crater wall or floor. These lakes and their effluent streams and springs host taxonomically and metabolically diverse microorganisms belonging in the *Archaea*, *Bacteria*, and *Eucarya*. In volcanic ecosystems the relation between geosphere and biosphere is particularly tight; microbial community diversity is shaped by the geochemical parameters of the lake, and by the activities of microbes interacting with the water and sediments. Sampling these lakes is often challenging, and few have even been sampled once, especially in a microbiological context. Developments in high-throughput cultivation procedures, single-cell selection techniques, and massive increases in DNA sequencing throughput, should encourage efforts to define which microbes inhabit these features and how they interact with each other and the volcano. The study of microbial communities in volcanic lake systems sheds light on possible origins of life on early Earth. Other potential outcomes include the development of microbial inocula to promote plant growth in altered or degraded soils, bioremediation of contaminated waste or land, and the discovery of enzymes or other proteins with industrial or medical applications.

Keywords volcanic lake, microbial communities, extremophiles, microbial diversity

1. Introduction

If asked to define a lake, most people would probably describe a body of fresh water surrounded by land. Some might attempt to distinguish a lake from a pond. Few, however, would consider the origin or nature of the lake basin, or of the water therein. In all likelihood, most people view lakes as permanent features that only affect them in terms of recreation, commerce, and biological productivity. In such terms, lakes in the craters of extinct or dormant volcanoes probably differ little from most lakes on Earth, in that their hydrological conditions may largely reflect only the surrounding air temperature, amount and nature of meteoric water, inflowing streams or rivers, and host rock chemistry (Donachie et al. 2004). In short, volcanic or geothermal forces no longer drive their circulation and chemistry, or affect their floral and faunal composition; these are the neutral dilute volcanic lakes defined by Pasternack and Varekamp (1997). Lakes in the craters of active volcanoes present very different physical and chemical characteristics, with conditions spanning broad ranges defined largely by the proximity of magma, and origin and nature of the adjacent rock and water (Pasternack and Varekamp 1997; Takano et al. 1997; Martinez et al. 2000, 2002; Jóhannesson et al. 2007). Visitors to such lakes may be surprised to find life in what are, to humans, extreme conditions, where those defined by heat and chemistry, *e.g.*, low pH and the presence of heavy metals or other toxic compounds, are especially severe. In this respect, organisms that thrive in conditions markedly different from what we might consider ‘normal’ are defined by the generic term ‘extremophile’, and their presence in such environments should be expected (MacElroy 1974). Evidence for active microbial communities in volcanic systems emerged from geochemical studies, resulting in hypotheses describing microbial roles in the oxidation/reduction of particular chemical species. Studies of elemental and isotopic profiles along with direct measurements of microbial activity

provided evidence for microbial participation in the stoichiometry of crater lake waters and sediments, and their importance in these systems (Takano et al. 1997; Wendt-Potthoff and Koschorreck 2002; Koschorreck et al. 2008; Parker et al. 2004).

Microbiologists until recently rarely visited such lakes (Takano et al. 1997; Donachie et al. 2002; Gaidos et al. 2004, 2008; Löhr et al. 2006a, b; Urbieta et al. 2012). Sampling strategies cannot always include boats, for obvious reasons, while remote sample-return vehicles have not been deployed, nor have automatic devices to collect samples at regular intervals as at hydrothermal vents (Reysenbach et al. 2000). As with other hydrothermal systems, however, the chemical composition of water in a crater lake depends on its interactions with the host rock and gas emissions. This chemistry, plus heat, generally shapes microbial community structure, so it is difficult to predict a universal microbiology for such features. One common trait of crater lakes and volcanic hot spring water is low pH, such that strong acids interact with the rock and mobilize potentially toxic metals. Here we focus on microbial diversity in volcanic acidic lakes, which represent one of the most common naturally acidic aquatic habitats on Earth (Löhr et al. 2006a). Little is known about the evolution and diversity of microbial communities in volcanic lakes, however, a fact that might be attributed to the lakes often being remote and difficult to access. To simply establish that life is present in a lake in the crater of an active volcano requires overcoming significant logistic and safety challenges (Woelfl and Whitton, 2000; Gaidos et al. 2004, 2008; Thorsteinsson et al. 2008). Hypotheses to be tested may require determination of the nature and rate of bacterial activities, or taxonomic diversity, and thus different sample collection and processing methods. However, one should bear in mind that any sample collected from such a lake may be the last before the lake vanishes during subsequent volcanic activity.

2. Methods to study environmental microbes

Defining which microorganisms are in a habitat has long challenged microbiologists. Indeed, we may yet be unable to say with certainty what is the absolute taxonomic diversity in a microbial community, regardless of the methods employed (Curtis et al. 2002). With the benefit of hindsight, supported by advances in technology, we can see how naïve we might have been when describing taxonomic diversity decades ago. For example, from the nineteenth and well into the twentieth centuries we were able only to determine which bacteria were in a sample by inoculating sub-samples to nutrient media. Tweaking the concentrations of a medium's components, the gas atmosphere, adjusting the pH, or modifying the light regime, encouraged the growth of metabolically and taxonomically different bacteria. In essence, we 'knew' which microbes were in a sample by growing them on or in such media. However, after more than a century of offering what might seem 'infinite' media and incubation conditions, it became clear to microbiologists that they could not cultivate representatives of all species from most samples. This was underscored in the 1980s by a then new technique, the analysis of nucleotide sequences of specific genes in DNA extracted from the environment and amplified in polymerase chain reactions (PCR); this approach detected bacteria (*Archaea* and *Bacteria*) for which no cultivated representatives then existed (Stahl et al. 1984; Ward et al. 1990). Extracting DNA from volcanic environments is technically challenging, and several protocols have been established (Herrera and Cockell 2007). Decades ago, advances in microscopy also showed the number of bacteria we might cultivate on growth media ranges from far less than 1% to ~50% of those counted in the same sample by microscopy, depending on the sample type (Staley and Konopka 1985; Donachie 1996; Wilson et al. 1997; Handelsman 2004). It should be noted here that these statistics refer only to the number of cells present, and not the number of 'species' or any other taxonomic unit we might define (Donachie et al. 2007). Such observations, however, encouraged use of terms

such as ‘nonculturable’ or ‘unculturable’ to describe those cells that did not grow (Xu et al. 1982; Colwell and Grimes 2000; Akob and Küsel 2011). These terms have taken on almost meme-like status. However, Amann et al. (1995) observed, perhaps wryly, “With the availability of innovative techniques, many more microorganisms will become culturable. […] After all, nature can cultivate all extant microorganisms.”

That analysis of ribosomal gene sequences in environmental samples detected bacteria that had never been cultivated led many microbiologists to justify using only those techniques, now termed ‘molecular approaches’, in studies of taxonomic diversity (Schmidt et al. 1991; Barton et al. 2004; Valenzuela-Encinas et al. 2008). Such a single-method approach has been shown not to describe microbial diversity *in toto*; cultivation and molecular approaches detect very different bacteria in the same sample (Suzuki et al. 1997; Bowman et al. 1999; Kaiser et al. 2001; Donachie et al. 2002; Munson et al. 2002; Donachie et al. 2004; Shawkey et al. 2005; Donachie et al. 2007). Whatever might be the merits of one approach over another, the utility of both cultivation-based and molecular techniques in microbial diversity studies has been significantly enhanced with the development of high-throughput culturing methods and high-throughput DNA sequencing (Connon and Giovannoni 2002; Zengler et al. 2002; Donachie et al. 2007; Kalyuzhnaya et al. 2008; Kircher and Kelso 2010). The apparent ‘unculturable’ nature of some microorganisms is surely in part attributable to the medium composition or growth conditions provided not suiting the organism in question. Conditions in extreme environments such as volcanic features render it difficult to exactly reproduce the correct concentrations of nutrients and growth factors in the laboratory (Rodríguez-Valera 2002). Characterizing geochemical and physical parameters is thus essential if one is to reproduce *in situ* conditions in artificial systems, and highlights the importance of a multidisciplinary approach in microbiological studies of these

environments (Fig. 1). In addition to extrinsic factors, microbes might also require intrinsic factors such as syntrophic interactions, a mutual relationship based on nutrient exchange, or quorum-sensing, intercellular communication mediated by small molecules only when a certain population density is attained.

2.1. Cultivation approaches

Cultivating bacteria from environmental samples as a scientific objective dates back to the nineteenth century (*e.g.*, Certes 1884). Detailed reviews of the myriad requirements and often creative techniques used in the pursuit of a 'pure culture' can be found throughout the literature. Today, data from molecular methods can be of use in developing media and incubation conditions to isolate new taxa (Palleroni 1997; Giovannoni and Stengl 2007). It remains statistically prohibitive to cater to all species that may occupy a particular habitat, but given sufficient information about the habitat and with supporting molecular data we can target specific genera or metabolic groups (Huber et al. 1995; Teske et al. 1996; Tyson et al. 2005; Bomar et al. 2011; Saw 2012).

Although the exact concentration of every component in a volcanic habitat may be difficult to faithfully reproduce from scratch in the laboratory, anyone collecting samples from such sites should be able to collect enough material to provide the base of many culture media (*sensu* Rodríguez-Valera 2002). We can thus address in part the need for specific components or combinations thereof found only in the native sample.

New cell selection and cultivation strategies have attempted to overcome the limitations of diluting samples to extinction, a procedure that statistically provides a suspension of the most abundant species, while diluting out those which are less abundant but perhaps more competitive

(Fröhlich and König 2000). A drawback is that the least abundant taxa are unlikely to be present in the most dilute samples; they might also be outcompeted when inoculated to media with other species. Recent 'high-throughput' and 'gel micro-droplet' techniques operate on the same principle of dilution to provide single to a few cells that are inoculated to media in microplates. These have enabled significant expansion of the number of samples that can be processed in a given time (Connon and Giovannoni 2002; Zengler et al. 2002). However, they do not allow direct selection of any particular cell type, and given there are perhaps billion of cells in a liter of most naturally occurring waters they would still require an unfeasibly high number of microplates to sample all cells per liter. And that is before one provides different media, temperatures, or variations of any other such parameter. All is not lost, though. Cell sorting by flow cytometry targets specific cell types based upon how they scatter light, or their fluorescent signature when illuminated with particular wavelengths of light, but does not allow the direct observation and isolation of a single microbial cell. To collect single cells that one can see, and pass those cells from a sample to downstream analyses, or to media wherein they may grow free of competition from other taxa, microfluidic and laser microdissection and catapulting systems offer various capabilities (Krüger et al. 2002; Ho et al. 2005; Baret et al. 2009; Chen et al. 2009; Kang et al. 2011). Cells historically termed 'unculturable' will numerically dominate hundreds or even thousands of randomly selected cells, but today's laser systems are combined with imaging capabilities that enable us to move beyond random selection. By establishing a library of cell images we might attempt subsequently to isolate visually distinctive cells that we have previously related to specific taxa through 16S rRNA gene sequencing after whole genome amplification (*sensu* Morris et al. 2004; cf. Fig. 1); their phylogeny and physiology can thus be predicted and in subsequent selections they can be transferred to tailored growth media in

176 microplates within which they can grow free of competition from more rapidly growing or
177 antagonistic taxa (*sensu* Kovac and Voldman 2007). Laser-mediated cell selection methods have
178 already proven themselves in isolating novel microbes and single cells for transcriptome analysis
179 (Huber et al. 1995; Kang et al. 2011).

180 **2.2. Cultivation-independent approaches**

181 Molecular approaches first used in microbial ecology almost thirty years ago were based on the
182 study of the nucleotide sequences of specific genes, those termed "molecular chronometers".
183 These are ribosomal, or rRNA genes, *e.g.*, 16S rRNA genes in *Archaea* and *Bacteria*, and 18S
184 rRNA genes in the *Eucarya*, that encode for the production of ribosomes (Kimura 1968, 1983;
185 Stackebrandt et al. 1985). The slow rate at which these genes' nucleotide sequences have
186 mutated over evolutionary time renders them valuable in classification. Such sequences also
187 form the basis of the reclassification of all life into the three currently recognized domains,
188 *Archaea*, *Bacteria* and *Eucarya* (Woese 1987). Moreover, functional genes encoding proteins
189 involved in specific metabolisms such as nitrogen fixation or sulphate reduction can be used as
190 proxies for particular bacterial groups (Moisander et al. 2006; Wagner et al. 1998). DNA-based
191 methods developed since the 1990s have provided much data on taxonomic diversity in
192 microbial communities. Such diversity in volcanic environments has to date been largely
193 investigated by denaturing gradient gel electrophoresis (DGGE) of ribosomal (rRNA) gene
194 fragments (Muyzer et al. 1993, Löhr et al. 2006, Wendt-Potthoff and Koschorreck 2002). In this
195 approach, gene fragments composed of different sequences of nucleotide in different species are
196 visualized as different 'bands' (Fig. 2). The DGGE profile, or 'community fingerprint,' of
197 samples can be statistically analysed for comparative purposes. Determining the nucleotide
198 sequence of the rRNA gene fragment in each band permits tentative assignment of the microbe

represented by that band to a taxonomic group. More recently, 'chip-based' techniques have been developed, such that specific RNA probes on a microarray can detect their complementary genes, allowing detection of potentially thousands of species in a single experiment (De Santis et al. 2003). In this respect, an acidophilic bacterial microarray was developed and tested in habitats such as Spain's Rio Tinto, where the extremely acidic conditions are similar to those in some volcanic lakes (Garrido et al. 2008). Furthermore, "-omics" approaches including metaproteomics and metatranscriptomics allow one to determine which proteins are expressed by microbial communities in the environment, and provide information on the functional diversity and/or potential of the active members of the community (Dill et al. 2010; Shi, 2011; Marchettia et al. 2012; Saw, 2012). Further advances now see these techniques being used at the single cell level, permitting evaluation of the role and spatial topology of each community member (Kang et al. 2011). Information about the presence and spatial distribution of specific populations can also be obtained through microscopic analyses that target specific phylogenetic groups. Among these, fluorescence *in situ* hybridization (FISH) has been instrumental in both significant discoveries and routine counting of specific microbial groups (Amann et al. 1990; Karner et al. 2000). Enhanced FISH-based techniques including substrate-tracking autoradiography (STAR-FISH) and catalyzed reporter deposition (CARD-FISH) have been applied to the study of microbial communities in water, *e.g.*, warm monomictic crater lake Alchichica (Mexico), and a subglacial volcanic lake beneath Iceland's Vatnajökull ice cap (Ouverney and Fuhrman 1999, 2000; Gaidos et al. 2008).

Molecular- or PCR-based approaches do have limiting factors, including the amount and quality of DNA that can be extracted from the sample, and the amount of that DNA which can be sequenced in a given time at an acceptable cost. The latter is now a minor issue given that

today's DNA sequencers can process, relatively cheaply, billions of nucleotides in a few hours. Molecular approaches today offer the possibility of quickly detecting all genes, and thus all microbes in a sample, as long as one can guarantee extracting all DNA from all cells. Just over a decade ago a multi-capillary Sanger-type sequencer was considered 'high-throughput.' Now, such a machine's performance pales into insignificance compared to that offered by a pyrosequencer, which generates hundreds of thousands of sequences in an afternoon. Such performance has revealed "unexpected" taxonomic diversity in the deep-sea (Sogin et al. 2006; Brown et al. 2009), while metagenomic analyses also showed an equally remarkable functional diversity (DeLong et al. 2006). These techniques have not yet been widely used in volcanic habitats, although Gaidos et al. (2008) showed the metagenome in anoxic water of a sub-glacial volcanic lake (Iceland) comprised an oligarchic microbial consortium suited to the lake's geochemical context. Further advances in sequencing technology, along with miniaturization and reduced cost per nucleotide sequenced, promise greater insights, with some caveats, of course (Kunin et al. 2010; Niedringhaus et al. 2011).

3. Microbial diversity in volcanic lakes

3.1 Cultivated microbes

Reports of microbes cultivated from crater lakes in active volcanoes are few and far between. Indeed, most efforts to determine which microbes are in these lakes have been based on molecular approaches. It also seems we might know more of the diversity, physiology and abundance of microbes, both cultivated and uncultivated, at submarine volcanic features than we do in their analogous terrestrial features (Karl et al. 1989; Donachie et al. 2003, 2004; Nakagawa and Takai 2008). In this respect, myriad reports of microbes in features related to volcanoes such

as geothermal springs and effluent streams appear throughout the literature (Sonne-Hansen and Ahring 1997; Hugenholtz et al. 1998; D'Imperio et al. 2008). Through just a handful of papers, however, one can appreciate what challenging environments acidic crater lakes are for microorganisms.

Takano et al. (1997) elegantly describe how sulfur-oxidizing bacteria affect the sulfate budget of the Yugama crater lake (pH 1-1.5), part of the andesitic Kusatsu-Shirane strato-volcano, Japan, noting also how pH controls distributions within the crater of two *Thiobacillus* species, *T. thiooxidans* and *T. ferrooxydans.* Due to the heat input from the bottom of the Yugama crater lake, the water temperature is generally higher than the atmospheric temperature, that can reach -15°C during winter. Members of the *Thiobacillus,* subsequently reclassified as *Acidithiobacillus*, are known to colonize extremely acidic environments characterized by high levels of reduced sulfur, which they oxidize (Kelly and Wood 2000). In the Yugama crater lake a molten sulfur pool at a then active subaqueous fumarolic vent was discharged into the water column, while sulfur particles dispersed throughout the lake as aqueous sulfur dioxide and hydrogen sulfide reacted (Takano et al. 1994b); hydrogen sulfide, polythionates, and elemental sulfur were consumed by bacteria, while reduced sulfur compounds were converted by these bacteria to sulfuric acid. An acidophilic diatom, *Pinnularia braunii* var. *amphicephala*, on the floor of a stream in the crater was identified by microscopy, showing that eukaryotes also colonize crater lake environments.

The first report of microbial communities in acidic waters of the White Island andesitic stratovolcano (New Zealand, 37°31'26" S; 177°11'5.6"E) applied both cultivation and molecular methods (Donachie et al. 2002). The water here is a dilute mix of sulfuric and hydrochloric acids containing dissolved andesite rock and other components. Sub-samples of water emanating from

267 a hot spring in the crater floor were inoculated to a range of media (pH 1.29 to ~8) to cater to
268 expected metabolic groups, and incubated at one of five temperatures between 30 and 60ºC for
269 up to one year. Four pure cultures affiliated with the heterotrophic and acidophilic mesophile,
270 *Acidiphilium* sp. (*Alphaproteobacteria*), which requires only low concentrations of organics and
271 cannot use sulfur or Fe^{2+} (Harrison 1981); with *Nocardia* sp., and *Nocardioides* sp.
272 (*Actinobacteria*), and *Cyanidium caldarium* (eukaryote, Rhodophyte), a photoautroph (Doemel
273 and Brock 1971). The success of the *Eucarya* in volcanic crater lakes is likely controlled by
274 temperature, even if they can tolerate the extremely low pH (Tansey and Brock 1972). For
275 example, *Cyanidium caldarium* was absent from the lake in the White Island volcano, where pH
276 was ~0 but temperature was 58ºC, yet it was isolated from slightly cooler 'soil' just meters from
277 the lake (Fig. 3) (Donachie et al. unpubl., and 2002). This photosynthetic eukaryote does not
278 grow above 56ºC (Kleinschmidt and McMahon 1970).

279 A crater lake whose microbiology was investigated in austral summer 2007 by both cultivation
280 and molecular approaches is the summit lake on Simba volcano (Demergasso et al. 2010), also
281 referred to as Lake (cf. Laguna) Aguas Calientes (Escudero et al. 2007; Cabriol et al. 2009). This
282 is an unusual lake because of its high altitude (5870 m) and concomitantly high UV radiation
283 exposure, yet rather moderate pH for a crater lake. Although water and sediment samples were
284 inoculated to enrichment media, no growth was detected by the end of the incubation period,
285 while several bacteria from just two phyla were cultivated from nearby Salar Aguas Calientes
286 and Laguna Lejía. In November of 2006, *Bacteria* were cultivated from the same lake, but they
287 belonged only to the *Gammaproteobacteria* (*Pseudomonas* spp.) and *Firmicutes* (*Staphylococcus*
288 *epidermidis*). *S. epidermidis* is unlikely to be part of the lake's autochthonous microbiota since it
289 is generally found on the human skin and membranes, and is a frequent contaminant in

290 laboratory tests (Queck and Otto 2008). Molecular methods showed a higher phylogenetic
291 diversity, revealing the presence of taxa reported from other high altitude lakes rather than acidic
292 lakes *per se*, and belonging in the *Bacteria* and *Archaea* (Demergasso et al. 2010). This work
293 demonstrates the value of coupling cultivation-dependent and molecular methods.

294 Wendt-Potthoff and Koschorrek (2001) determined the abundances of various physiological
295 groups of bacteria along the Rio Agrio on the Copahue volcano (37°51'S; 71°10.2'W; 2965 m
296 a.s.l., Argentina) an andesitic stratovolcano, and its recipient lake, Caviahue. Iron- and sulfur-
297 oxidizers, and sulphate-reducers were enumerated in enrichment media used in other acidic
298 environments. The presence of fermentative bacteria was determined by gas production at pH
299 2.0. All metabolic groups were detected, although abundance was low, and the authors posited
300 that distribution varies with rate of water flow, sediment texture, and light availability rather than
301 solely with pH. Lavalle et al. (2005) worked along the same river, but also sampled from the
302 crater lake (Laguna del Volcán) itself. In a targeted approach with specific media, acidophilic,
303 chemolithotrophic and ferrous-oxidizing bacteria were cultivated only from the river, where the
304 pH ranged from 2 to 4. These bacteria were assigned to the iron-sulphur oxidizer
305 *Acidithiobacillus ferroxidans*. A broader microbiological survey of the same system reported that
306 the lake's pH ranged from 0.2 to 1.1 (Chiacchiarini et al. 2010); these authors used diverse media
307 and incubation conditions and cultivated bacteria that use inorganic reduced sulphur compounds
308 as energy sources, and CO_2 (chemolithoautotrophs) or organic compounds
309 (chemolithoheterotrophs) as carbon sources. Among these were *Bacteria* (*Leptospirillum*
310 *ferrooxidans*, *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans*) and *Archaea*
311 (*Acidianus*, *Sulfolobus*), plus yeasts and filamentous fungi from sites that extended from a 70ºC,
312 pH ~1.0 hot spring (el Vertedero) emerging from the mountain, just below the lake, and along the

313 Upper and Lower Rio Agrio, and from Caviahue Lake. Temperature along this watercourse drops
314 to ~8ºC and pH increases to 4.2. An unidentified extremely acid tolerant (pH<2) filamentous
315 fungus was cultivated, as were over a dozen yeasts from at least three genera (*Cryptococcus*,
316 *Rhodotorula*, *Sporidiobolus*). That no microbes were cultivated from the Laguna del Volcán by
317 both Lavalle et al. (2005) and Chiacchiarini et al. (2010), although water samples were
318 inoculated to media, is consistent with observations elsewhere of there being no bacterial growth
319 in crater lakes if the pH is <1 (Satake and Saijo 1974; Takano et al. 1994a).

320 A discussion of microbes in the environment should not overlook the potential for viral
321 infections. Just as the role of viruses in marine biogeochemical cycling has been reassessed in the
322 last two decades, so too might we find that viruses are instrumental in controlling populations of
323 thermophiles and acidophiles in volcanic settings (Short and Suttle, 2002, Rice et al. 2001,
324 Snyder et al. 2003). Almost thirty years ago, Janekovic et al. (1983) described a family of viruses
325 that infect *Thermoproteus tenax*, a thermophilic *Archaea* found in hot springs. Multiple viruses
326 in *Sulfolobus*, which inhabit high temperature hydrothermal environments, are now known
327 (Zillig et al. 1994; Arnold et al. 2000). Geslin et al. (2003) reported the first virus in an
328 hyperthermophilic archaeon, *Pyrococcus abyssi* from marine hydrothermal vents. An attempt
329 about ten years ago to isolate phage from water in Ruapehu crater (pH~2, 45 - 55ºC), however,
330 found neither phage nor DNA that could be amplified (Hugh Morgan, pers. comm.).

331 **3.2. Microbial diversity in volcanic systems by molecular methods**

332 In addition to the cultivation-based data described above for White Island, cloned 16S rRNA
333 gene fragments from the acid stream affiliated with *Pandoraea* and *Ralstonia* (Burkholderiaceae,
334 *Betaproteobacteria*), *Rhodovulum* (Rhodobacteraceae, *Alphaproteobacteria*), *Acidosphaera*

335 (Acetobacteraceae, *Alphaproteobacteria*), and the photoautotrophic *Chlorobium vibrioforme*
336 (Chlorobi) (Donachie et al. 2002). No DNA was extracted from water collected from the pH ~0,
337 58ºC lake (Donachie et al., unpubl.).

338 The largest known acidic crater lake is Kawah Ijen, Indonesia, whose pH 0.3 water is clearly
339 extreme compared to that of more typical lakes and rivers (Geller and Schultze 2009). As the
340 river Banyupahit-Banyuputih flows from Kawah Ijen, its pH increases from 0.39 to 7.62.
341 Microbial diversity in the lake and river investigated by DGGE detected no *Bacteria* or *Eucarya*
342 in the lake, although they were detected along the effluent rivers (Löhr et al. 2006a). No attempts
343 were made to cultivate microbes, nor were other sequencing strategies with larger sample sizes
344 conducted. *Bacteria* sequences detected by DGGE in the highest pH river water affiliated with
345 *Betaproteobacteria*, *Gammaproteobacteria* and *Flavobacteria*; in the most acidic sample, only a
346 sequence affiliating with the *Pseudomonadaceae* family was detected. None of the *Bacteria*
347 detected were known acidophiles. *Eucarya* were absent from low pH waters in both the Kawah
348 Ijen lake and the upper reaches of the river Banyupahit-Banyuputih. However, *Eucarya* diversity
349 was high in water at pH ≥2.66, and included both algae and diatoms (Löhr et al. 2006b). *Archaea*
350 sequences were detected, but taxonomic diversity was low; none typical of acidic environments
351 were detected, but one *Archaea* sequence from the lake and the most acidic stretch of the river
352 affiliated closely with uncultured marine non-thermophilic *Crenarchaeota*. A second *Archaea*
353 sequence retrieved from the lake was only distantly related to sequences from marine
354 hydrothermal vents. One more sequence from the most acidic stretch of the river affiliated with
355 obligate anaerobic acetoclastic methanogens in the order *Methanosarcinales* (Löhr et al. 2006a).

356 Microbial community diversity and abundance in the Copahue-Caviahue system of Argentina
357 have also been studied (Chiacchiarini et al. 2010, Urbieta et al. 2012). Here, extremely acidic

358 water (pH 0.2 - 1.1) flows from L. Copahue in the Copahue volcano into the Río Agrio. The river
359 can be divided into the Upper Río Agrio (pH 0.5 – 2.5) that flows into L. Caviahue (pH 2.1-3.7),
360 and its effluent Lower Río Agrio (pH 2.1 – 6.0). Bacterial abundance in water from the Upper
361 Río Agrio and in L. Caviahue determined by microscopy was ~2 x 10^5 cells ml^{-1}, of the order of
362 that reported in acid mine lakes, the anthropogenic homologue of volcanic lakes (Wendt-Potthoff
363 and Koschorreck 2002). Measurements of microbial activity such as oxygen consumption, and
364 iron oxidation and reduction at three points along the Upper Río Agrio, one of which was
365 immediately before the inflow to L. Caviahue, showed a gradient of microbial abundance and
366 activity. Iron- and sulphur-oxidizing bacteria and iron-reducing bacteria were present, as one
367 might expect given the abundances of iron and sulphur in this type of ecosystem (Wendt-Potthoff
368 and Koschorreck 2002, Urbieta et al. 2012). FISH detected acidophilic bacteria with a potential
369 for iron and sulphur metabolism, such as *Sulfobacillus*, *Leptospirillum*, *Acidithiobacillus* and
370 *Acidimicrobium*, in the hot spring of Copahue village (Giaveno et al. 2009). *Acidithiobacillus*
371 *ferroxidans*, *Acidithiobacillus thiooxidans* and members of the genus *Leptospirillum* and the
372 *Archaea* genus *Sulfolobus* were cultivated from different parts of the Copahue-Caviahue system
373 in a study to isolate microbes that may bioleach ores and recover precious metals (Chiacchiarini
374 et al. 2010). Bacterial diversity in four water samples from the Upper Río Agrio was recently
375 described on the basis of 16S rRNA gene clone libraries and CARD-FISH (Urbieta et al. 2012).
376 Sampling sites were characterized by increasing pH (1 - 2) and temperature (6.7 to 59°C).
377 CARD-FISH revealed *Archaea* were the most abundant members of the community, albeit not a
378 phylogenetically diverse component since 93-97% of sequences affiliated with just one species,
379 the chemolithoautotrophic, iron-oxidizing, *Ferroplasma acidiphilum* (Urbieta et al. 2012).
380 *Ferroplasma* species are key players in the sulphur cycle through their oxidation of sulphides

and regeneration of Fe^{3+}, the primary oxidant of pyrite at low pH (Urbieta et al. 2012). In contrast to what one might expect in an 'ecological dogma' context, that more extreme conditions exert a stronger selective pressure and thus a less complex community, a *Bacteria* 16S rRNA gene clone library from the sample with the lowest pH (1) and highest temperature (59°C) contained the most phylogenetically diverse community. Moreover, a 'less extreme' habitat, characterized by pH 2 and 15.9°C, showed the lowest diversity indices. *Bacteria* clones in the Upper Río Agrio water samples so analysed affiliated with *Alpha-, Beta-* and *Gammaproteobacteria*, and the phyla *Actinobacteria*, *Firmicutes* and *Nitrospirae*, the latter represented only by the genus *Leptospirillum*, typically found in acid environments with high concentrations of reduced iron and sulphur. The distribution of the known iron-sulphur oxidizing bacterium *Acidithiobacillus ferrooxidans* along the Upper Río Agrio correlated with the water's iron content and pH value, and confirmed previous cultivation-dependent experiments of the site (Chiacchiarini et al. 2010). Clone libraries also contained an *Acidiphilium* sp., an important observation given they may scavenge organic molecules that would otherwise be toxic to chemolithoautotrophs such as *Acidithiobacillus ferrooxidans* (Johnson, 1995; Urbieta et al. 2012). Sulphur-oxidizing species affiliating with *Acidithiobacillus thiooxidans* and *Acidithiobacillus albertensis* were also detected in the URA; these species, along with *Sulfobacillus* spp., are typically found in natural or man-made acidic habitats, *e.g.*, the Rio Tinto in the Iberian Pyritic Belt, or in acid mine drainage.

As with studies of the Upper Río Agrio, a DGGE survey of the El Chichón crater lake (17°21'36''N; 93°13'40''W¸ 1100 m a.s.l., Mexico) revealed the presence of chemolithoautotrophic bacteria able to synthesize organic carbon from carbon dioxide (Fig. 2). *Alpha-, Beta-* and *Gammaproteobacteria* along with *Aquificae*, *Firmicutes* and *Acidobacteria*

were detected in acidic, sulphate-rich waters of the lake (Peiffer et al. 2011; Mapelli, pers. comm.). The presence of *Sulfurihydrogenibium*, *Acidithiobacillus*, *Thiomonas* and *Hydrogenophilus,* members of which are involved in sulphur and iron cycling, and hydrogen oxidation, reflects the chemical composition of El Chichón's lake and thermal springs.

Volcanic lakes present unique challenges for both microbes and microbiologists. After a few decades of cultivation- and microscopy-based studies, plus a decade or so of molecular methods, it seems we might conclude that microbial communities in the few lakes studied comprise a handful of microbial eukaryotes, and several *Archaea* and *Bacteria* (Schleper et al. 1995; Donachie et al. 2002). On the other hand, these lakes have been so rarely investigated in microbiological terms we should rather reserve judgment until more extensive data are available.

4. Potential applications of microbiological studies of volcanic lakes

Interest in microbial diversity in crater lakes is both scientific and economic in nature. For example, knowledge of ecological processes in natural and artificial acidic systems provides leads for established and novel biotechnological applications (Antranikian et al. 2005; Liu and Zhang 2008). Such studies may allow improvement of or development of new applications, such as in biomining, and the development of strategies to restore waters and ecosystems affected by acidification. Applications based on biological resources from volcanic systems include reclaiming or scavenging metals from contaminated substrates or toxic mixtures. Chiacchiarini et al. (2010) showed a putative *Acidothiobacillus ferrooxidans* from the Upper Rio Agrio could reduce chromium and zinc concentrations in municipal sludge to below permitted levels; consortia of bacteria from Copahue–Caviahue could also extract more gold from a sulphide concentrate than could pure cultures of *Acidothiobacillus* ferrooxidans DSM 11477 *and*

426 *Leptospirillum* ferrooxidans ATCC 29047^{T} (Giaveno et al. 2009b). These strains and related
427 species have long been the focus of bioleaching studies (Porro et al. 1989; Donati et al. 1996).

428 Molecules derived from microorganisms originating in extreme volcanic habitats have
429 significant potential through unique applications in biotechnology. For example, the activities of
430 'extremozymes' (enzymes synthesized by extremophiles) can be enhanced by extreme
431 temperature, pH and heavy metal content, and are stable under several environmental extremes
432 (reviewed in Morozkina et al. 2010). Such enzymes may enable industrial processes in
433 conditions under which conventional proteins are denatured or inefficient.

434 An innovative application of microbiological research in volcanic systems is that of Plant
435 Growth Promoting (PGP) bacteria, those that help sustain plant growth in extreme geochemical
436 settings. Only certain pioneer plants, mainly those in the *Poaceae* family (Baldantoni et al.
437 2009), can tolerate the severe environmental conditions that typify soils affected by volcanic
438 activity, *e.g.*, high temperature, anomalous gas fluxes through soils and sediments, low pH, and
439 presence of potentially toxic elements in aerosols and water. A PGP bacterial activity that may be
440 widespread and important to plants colonizing volcanic systems is the detoxification of
441 phytotoxic compounds, such as heavy metals. PGP bacteria isolated from pioneer plants in
442 volcanic areas also have the potential to be incorporated into 'biofertilizers' to sustain plant
443 growth in altered and degraded soils during phytoremediation and land restoration practices, *e.g.*,
444 in acid mine waste remediation. Bacteria associated with the roots of a pioneer plant growing in
445 a hot, acidic stream emanating from the crater lake of El Chichón volcano (Mexico) have been
446 isolated and characterized *in vitro* for known PGP activities, resistance to abiotic stressors, and
447 root colonisation (Fig. 4). To develop an effective 'biofertilizer' it is essential to determine if the
448 bacteria are rhizocompetent, *i.e.*, able to colonize the plant root. Rhizocompetence can be

449 evaluated by engineering cells to express ‘green fluorescent protein’ (GFP), and by observing
450 them in root sections by epifluorescence microscopy (Fig. 4) (Marasco et al. 2012). The utility of
451 GFP-tagged rhizobacteria does depend on the transformation efficiency and use of strain-specific
452 genetic systems. However, microbial inocula with such detoxification or growth stimulation
453 activities can enhance stress tolerance in plants in polluted ecosystems, and promote
454 rhizoremediation, phytoremediation and phytostabilization techniques (Regvar et al. 2006; Ma et
455 al. 2011). The cultivation of microbes from volcanic systems, or the application of genetic
456 information from these and uncultivated taxa, may yet contribute to socially acceptable,
457 environmentally friendly practices in land restoration.

458 Volcanic activity has always been a part of Earth’s history, so investigating microbial life in
459 volcanic crater lakes and their effluent rivers may provide clues to the origin of Life on Earth,
460 and how it has adapted over time to extreme conditions. In this respect, the multidisciplinary
461 field of astrobiology focuses on the origin, evolution and pattern of Life on Earth and
462 extraterrestrial bodies. The only life we know of in the universe is that on Earth. We thus define
463 conditions required for life’s development and persistence according to observations of life here.
464 Clearly, the extremes of pH, temperature and chemistry we see in terrestrial volcanic systems
465 that harbor life provide clues about the conditions in which life could occur in extraterrestrial
466 habitats.

467 Geochemical features in volcanic crater lakes such as pH, and sulphur and iron species, appear to
468 be the principal forces shaping the composition of autochthonous microbial communities. Iron in
469 particular plays a fundamental role in the biogeochemistry of many volcanic ecosystems, and
470 was probably also important on the early Earth (Huber et al. 1997; Cody et al. 2000; Weber et al.
471 2006). Signatures of acidic aqueous systems with iron- and sulfur-based redox cycles, *e.g.*, the

iron oxide mineral jarosite, were detected on the Martian surface by the rover 'Opportunity' (Bibring et al. 2007); jarosite's presence on Mars only implies that aqueous processes were once at work there (Klingelhofer et al. 2004), while that found in leaching waters of the Kawah Ijen volcano on Earth may be biogenic, such as that generated from pyrite by *Acidithiobacillus ferrivorans* cultivated from iron bioweathered soil, and El Chichón crater lake (Borin et al. 2009; van Hinsberg et al. 2010; Mapelli, pers. obs.). One can see why *Acidithiobacillus* spp. and other extremophiles have been proposed as biomarkers for life detection strategies on planetary bodies (Gómez and Parro 2012). Extreme crater lakes such as those in the Chilean Altiplano are eminently suited to investigations of microbial physiology and community dynamics in extreme and rapidly changing environments (Demergasso et al. 2010). Considerable technical challenges await those who choose to work on the microbiology of volcanic crater lakes, but the field will surely yield both exciting methodological developments and scientific discoveries.

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820 **Figure legends**
821

822 **Fig. 1.** Schematic representation of approaches used by microbiologists to investigate diversity,
823 function and potential applications of microbes in environmental samples.

824 **Fig. 2.** DGGE gel of amplified 16S rRNA gene fragments from El Chichón crater lake and
825 associated thermal springs. Bands in each lane represent a different bacterial species. Symbols
826 show which band corresponds to a particular *Bacteria* genus or family.

827 **Fig. 3.** Sampling at White Island, New Zealand. Acidic gases require use of respirators. The
828 water's green coloration is due to elemental sulfur. (Photo, SPD)

829 **Fig. 4.** Extremophilic bacteria can be isolated from pioneer plants, studied under laboratory and
830 *in vivo* conditions, and may be used in environmental biotechnology, *e.g.*, to clean and restore
831 land.

832

Fig. 1. Mapelli *et al*.

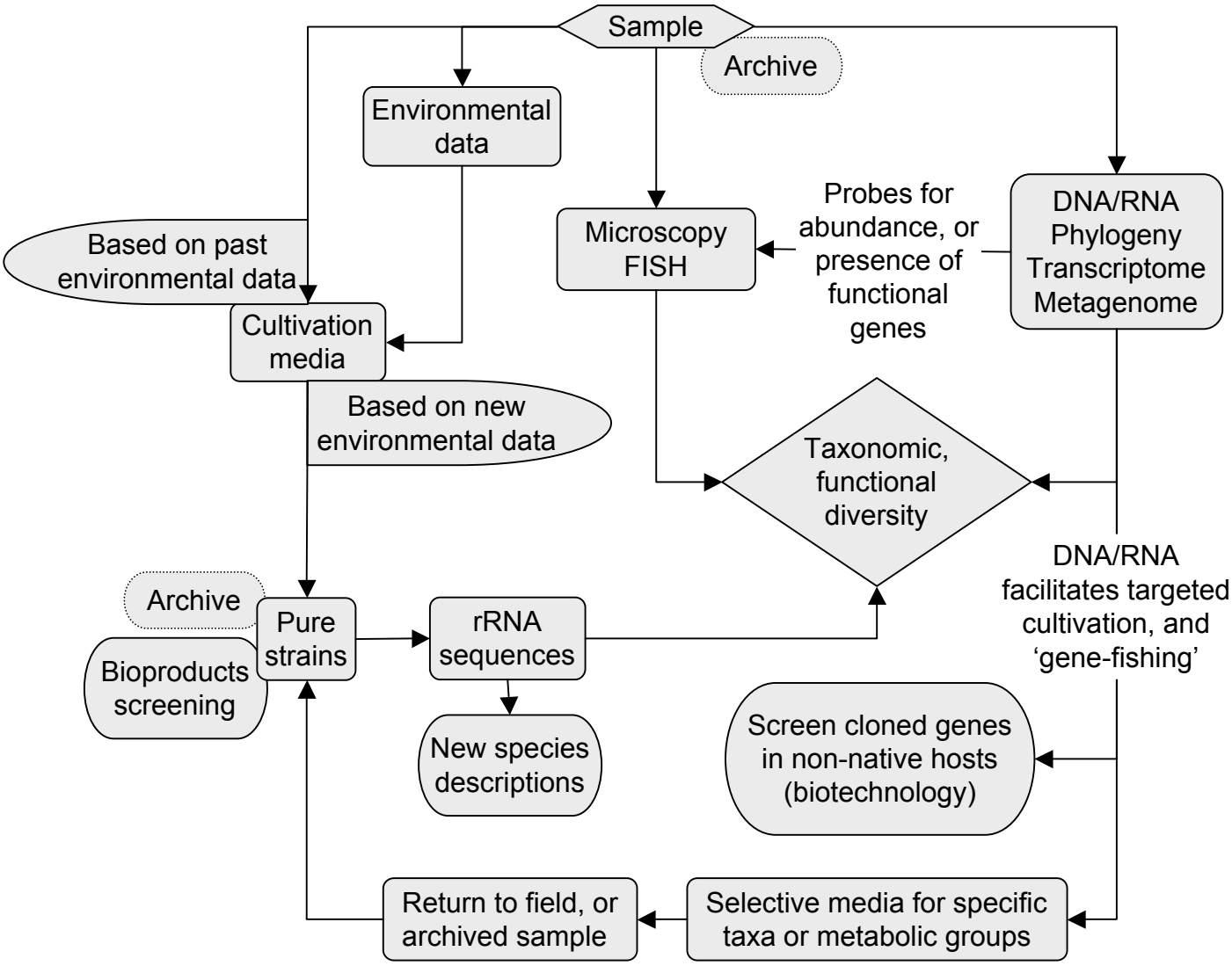


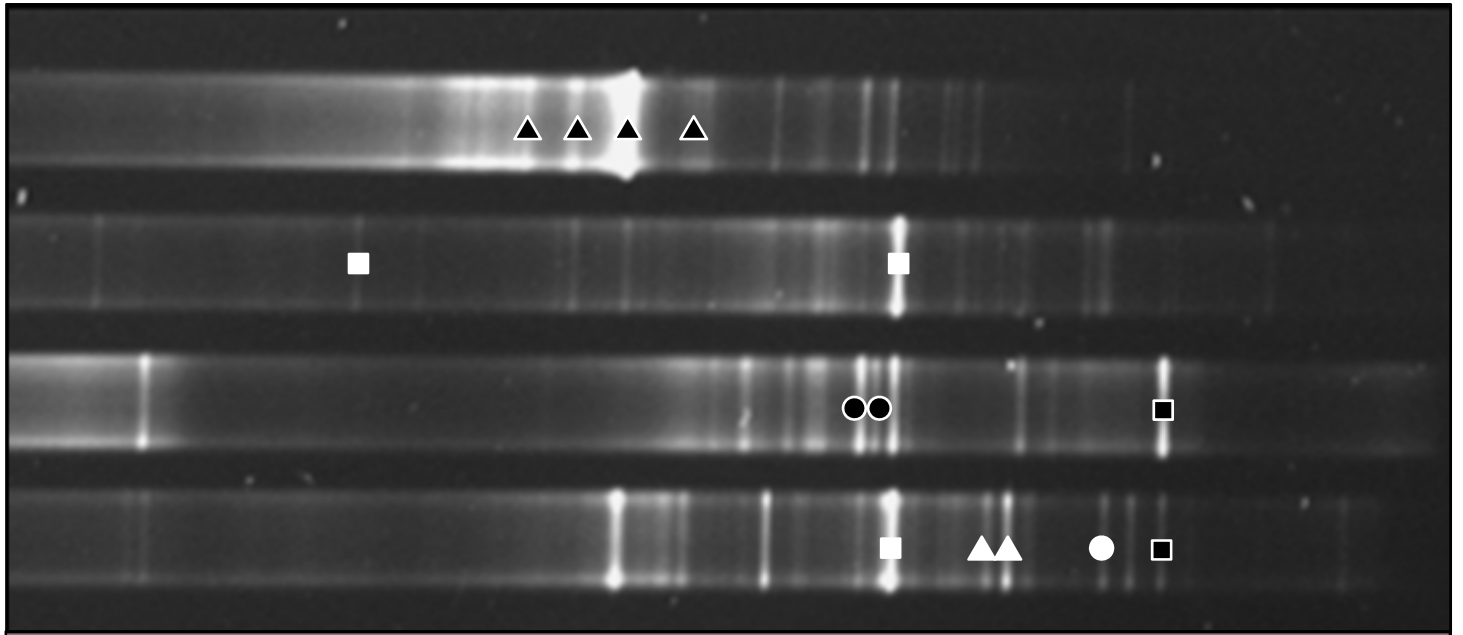
Fig. 2. Mapelli *et al*.

Steam heated pools
(pH 4.1, SO_4-type , T 42°C)

Crater lake
(pH 2.3, SO_4-Cl type)

Saline thermal springs
(pH 2.8, Cl-SO_4 type, T 55°C)

Saline thermal springs
(pH 2.8, Cl-SO_4 type, cold)

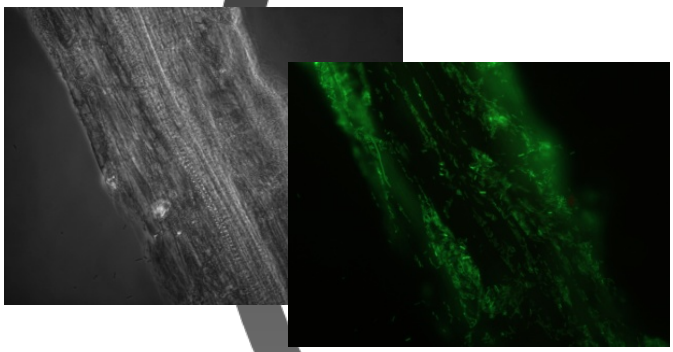


Pioneer plant

Isolation of potential PGP bacteria

Screening for PGP activity *in vitro*

Plant recolonization *in vivo*



USE FOR LAND RESTORATION

Table 1. Volcanic lakes and streams investigated by microbiological approaches.

Volcanic lake system	Country	Methods	Reference
Yugama (L)	Japan	Activity measurement; cultivation; microscopy-based techniques	Takano et al. 1997
White Island (L, S)	New Zealand	Cultivation; clone libraries; microscopy-based techniques	Donachie et al. 2002
Simba summit lake (L) Salar de Aguas Calientes (L) Laguna Lejía (L)	Chile	Cultivation; DGGE; clone libraries	Demergasso et al. 2010
Laguna del Vólcan (L) Upper Rio Agrio (S) Caviahue Lake (L) Lower Rio Agrio (S)	Copahue-Caviahue system, Argentina	Cultivation Activity measurement; cultivation; microscopy-based techniques Cultivation; clone libraries Clone libraries; microscopy-based techniques	Lavalle et al. 2005 Wendt-Potthoff et al. 2002 Chiacchiarini et al. 2010 Urbieta et al. 2012
Kawah Ijen (L) Banyupahit-Banyuputih (S)	Indonesia	DGGE	Löhr et al. 2006a Löhr et al. 2006b

L: Volcanic lake; S: Stream