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The zebrafish (*Danio rerio*) embryo-larval contact assay combined with biochemical biomarkers and swimming performance in sewage sludge and hydrochar hazard assessment

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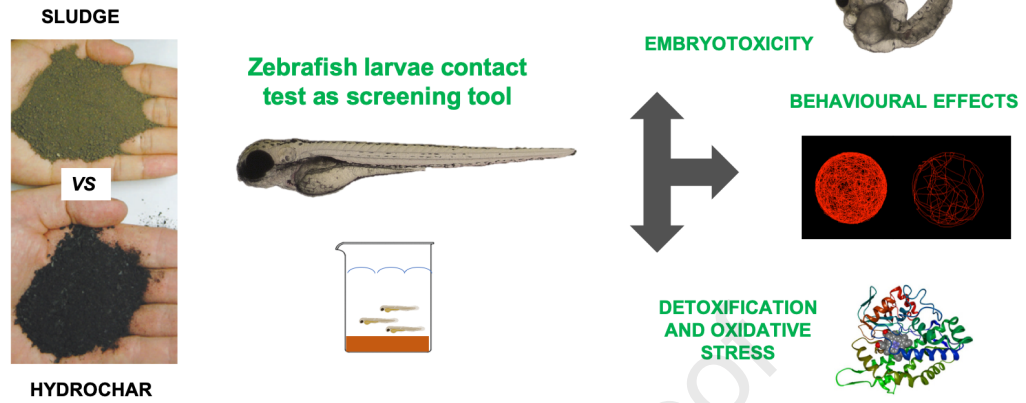
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**SLUDGE AND HYDROCHAR  
ECOTOXICITY SCREENING**



Journal Pre-proof

1 **The zebrafish (*Danio rerio*) embryo-larval contact assay combined with biochemical**  
2 **biomarkers and swimming performance in sewage sludge and hydrochar hazard assessment**

3

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21 **Abstract**

22 Hydrothermal carbonization is considered a powerful technology to convert sewage sludge (SS) into  
23 a valuable carbonaceous solid known as hydrochar (HC). Up to now criteria for landfill application  
24 of SS and HC are based only on physicochemical properties and levels of pollutant residues.  
25 Nevertheless, to ensure their safe environmental applications it is mandatory to develop biosensors  
26 which can provide relevant information on their toxic potential for natural ecosystems. Therefore,  
27 this study aimed to assess the suitability of a contact assay using zebrafish embryo/larvae combined  
28 with sub-lethal end-points to evaluate the hazard associated with SS and related HC exposure. A suite  
29 of biomarkers was also applied on larvae, related to detoxification and oxidative stress as the activity  
30 of Ethoxyresorufin-*O*-deethylase, glutathione-*S*-transferase, and catalase, the content of reactive  
31 oxygen species and the behavioral assay using the DanioVision™ chamber. Legacy priority  
32 pollutants were also measured either in SS and HC tested samples and in contact waters. The exposure  
33 to SS caused higher lethality compared to HC. No significant changes in the activity of oxidative  
34 stress markers was observed upon exposure to both matrices. The behavioral test showed a  
35 hypoactivity condition in larvae exposed to both SS and HC with the effects of SS stronger than HC.  
36 Chemical analysis revealed the presence of trace elements and halogenated compounds in either SS,  
37 HC. Heavy metals were also released in contact waters, while volatile hydrocarbons (C6-C10) and  
38 halogenated compounds resulted below LOD ( $< 0.05 \mu \text{L}^{-1}$ ).

39 Our study highlights the suitability of zebrafish embryotoxicity test, coupled with behavioral traits,  
40 as screening tool for assessing potential risks, associated with the landfill application of both SS and  
41 HC, for aquatic wildlife.

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43 **Keywords:** ecotoxicity, fish embryotoxicity assay, behavioural assay, environmental risk assessment

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## 1. Introduction

48  
49 Soil loss urgently calls for new sustainable practices aiming to cope with current perturbations which  
50 are limiting its ability to resist and adapt to future scenarios. Sewage sludge (SS) is a typical waste of  
51 the contemporary society whose recent use as a soil amendment as source of nutrients and organic  
52 matter can increase soil resilience. Recent concerns related to the presence of toxic compounds in SS,  
53 often above national and regional regulatory limits, rebound to their safe application and call for *ad*  
54 *hoc* solutions for assessing the risk for humans and the environment including reuse and disposal  
55 (Italian Legislative Decree 109/2018). Although treated and stabilized before landfill application,  
56 toxic chemicals either hydrophobic and hydrophilic, not completely degraded during the wastewater  
57 treatment processes, can be still present and pose a risk to both terrestrial and aquatic biota also being  
58 accumulated and/or transferred along food chains (Rogers, 1996; Dai et al., 2007; Sánchez-Brunete  
59 et al., 2008; Sidhu and Toze, 2009; Clarke and Smith, 2011; Venkatesan et al., 2015 Westerhoff et  
60 al., 2015). The level of risk depends on their initial concentrations in sewages, frequency of the  
61 application (cumulative effects), management practices and losses (Rorat et al., 2019).

62 To overcome such limitations and provide support for their safer agriculture applications, several  
63 recovery strategies have been developed to convert the solid organic *waste* into new resources by  
64 following the *End of Waste* principle and *Circular Economy* strategy (Tasca et al., 2019). For  
65 instance, innovative technologies such as the hydrothermal carbonization (HTC) are able to convert,  
66 at low temperature and in the aqueous phase, the organic fraction of organic wastes, including SS,  
67 into a carbonaceous solid called hydrochar (HC) (Titirici et al., 2010; Libra et al., 2011). The  
68 application of HC itself as a soil amendment has been recently proposed for its high ability to retain  
69 nutrients and then prevent their losses (*i.e.* due to run-off), thus improving soil quality and  
70 productivity (Marris, 2006; Chan et al., 2007; Maniscalco 2020; Masoumi et al., 2021). Elevated  
71 specific surface area and strong affinity for water are those properties making HC able to improve  
72 cation exchange and water retention when applied to soils (Sevilla and Fuertes, 2011). However, the  
73 balance between the effectiveness of such carbonaceous material, and environmental and human risks

74 posed by the presence of unwanted toxic chemicals coming from biomass sources (including SS)  
75 need to be adequately considered. In addition, the influence of the HTC process on chemical  
76 contaminants already present in the SS has been little explored and only addressed in few publications  
77 (Weiner et al., 2013; Tirlir et al., 2013; Brookman et al., 2018).

78 Proposed criteria such as, for instance, biochar authorization is based on physicochemical properties  
79 and levels of pollutant residues (International Biochar Initiative, European Biochar Certificate  
80 <https://www.european-biochar.org/en>). However, they are based on a chemical body burden rather  
81 than biological effects induced by their exposure, limiting our understanding of the environmental  
82 risks associated with the use of SS and HC in a predictive context, which could allow their safe  
83 application into the environment (*i.e.*, land and water).

84 Bioassays and biosensors have been largely proposed as screening tools in assessing quality of  
85 environmental complex matrices including wastewater and sludge (Barceló et al., 2020; Behnisch  
86 and Brouwer, 2020; Macova et al., 2011; Neale et al., 2020; Liberatori et al., 2022). On the other  
87 hand, most of the investigation have been carried out to assess HC ecotoxicity to soil microorganisms  
88 (Reibe et al., 2015; Madzaric et al., 2018) while aquatic systems have been overlooked, although may  
89 be still susceptible due to the release of chemicals from HC and run-off.

90 The zebrafish (*Danio rerio*) has many characteristics that make it a suitable model organism in  
91 ecotoxicity assessment, such as egg production in high manner and fast development, embryos small  
92 size and transparency, and easy handling in laboratory conditions. Moreover, the zebrafish  
93 embryotoxicity assay accomplishes the three Rs (reduction, replacement, and refinement) which  
94 characterize alternative animal testing methods. The zebrafish embryo-larvae has been successfully  
95 applied to assess both acute and chronic effects of a wide range of water pollutants, with different  
96 modes of action, such as metals (Le Fauve and Connaughton, 2017), pharmaceuticals (Leuthold et  
97 al., 2019; Parenti et al., 2019a; Pohl et al., 2019), brominated compounds (Chen et al., 2012), PAHs  
98 (Shankar et al., 2019), dioxins (Lanhan et al., 2012), nanoparticles (Canedo Pereira et al., 2019) and  
99 plastics (Cormier et al., 2019), among others. The assay is also suitable to evaluate the toxic potential

100 of complex environmental matrices, for instance wastewaters (Babic et al., 2017), sediments elutriates  
101 (Lee et al., 2020; Li et al., 2016; Babic et al., 2018), soils extracts (Legler et al., 2011; Wincent et al.,  
102 2015) and, more recently, extracts from SS (Gustavsoon et al., 2007; Cristofolletti and Mazzeo, 2016).  
103 A sediment contact assay (SCA) has also been developed (Hollert et al. 2003) and widely adopted to  
104 screen hazards associated with pollutant-bound sediment (Saiki et al., 2021 and citations therein).  
105 Indeed, in the framework of the ecotoxicological evaluation of solid matrices, an essential aspect that  
106 should be addressed concerns the effective release of pollutants in the surrounding water. Concerning  
107 aqueous-phase matrices, from one side, extracts and obtained from solid matrices using solvent-  
108 extraction might over-estimate toxic chemicals bioavailable to embryos (Bluhm et al., 2014).  
109 Conversely, the test of aqueous-phase alone, such as pore water and elutriates, might underestimate  
110 the bioavailability of some pollutants, in particular the lipophilic ones (Hollert et al., 2003). Instead,  
111 the whole-matrix assay - such as the contact test - allows taking into consideration the bioavailable  
112 fraction of chemicals bound to the sediment itself, thus providing a more realistic assessment of the  
113 sediment ecotoxicity for aquatic organisms. Even so, conflicting results are often reported, since the  
114 toxic potential of the different matrices is influenced by several factors such as organic matter content,  
115 granulometry and the different extraction procedures (Saiki et al., 2021). In this scenario, this study  
116 aimed first to evaluate the suitability of zebrafish embryo contact test for SS and HC hazard  
117 assessment for aquatic biota related to their application as soil amendments. Furthermore, from the  
118 comparison between SS vs related HC ecotoxicity, the influence of the HTC process towards toxic  
119 chemicals already present in SS and ending in HTC product (HC) is evaluated. Our hypothesis is that  
120 HC may induce a lower toxicity than SS, by virtue of the ability of this process to reduce different  
121 classes of harmful organic pollutants present in SS, although this aspect remains poorly addressed.  
122 To integrate the biological effects of chemicals mixture as the one present in both SS and HC, the  
123 SCA was coupled with the analysis of sub-lethal endpoints as activity of enzymes related to  
124 detoxification (Ethoxyresorufin-*O*-deethylase, EROD and glutathione-*S*-transferase, GST) oxidative  
125 stress (level of Reactive Oxygen Species, ROS and Catalase, CAT) and neurotoxicity

126 (Acetylcholinesterase, AChE) and behavioral alteration (swimming performances) using the  
127 DanioVision™ chamber.

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## 2. Materials and Methods

### 2.1 Sewage Sludge samples

SS samples were provided by two regional wastewater treatment plants (WWTPs) located in the Tuscany Region, named WWTP1 and WWTP2, respectively. Samples were obtained in the framework of a large project funded by the Tuscany Region (Sludge 4.0-POR FESR 2014-2020 *Circular economy for the treatment and transformation of biological sludge into biofertilizers*).

The WWTP1 layout is as briefly described as follows: pretreatments unit, denitrification tank, oxidation tank, secondary clarifier, and disinfection. Sample of SS obtained from WWTP1 and named SS1 results from the mix of the disinfection step outlet and the thickening and dewatering of the aerobic digestion of the secondary clarifier outlet. The SS2 samples obtained from WWTP2 were taken downstream of thickening and dewatering of the outlet of an anaerobic digester, which is fed by the outlet of primary clarification step prior thickening.

### 2.2 Hydrochar production

HTC trials with SS1 and SS2 samples were carried out according to previously established methodology reported in Tasca et al. (2020) in a 300 mL stainless steel reactor (AISI 316), loaded with 200 mL of sample. The reactor is equipped with a conventional electric heating system, mechanical agitation, a thermocouple for temperature control and a manometer (full scale: 1000 psi) for pressure control. Temperature inside the reactor was set by a manual controller (PARR 4842). The experimental runs were conducted at a temperature of 220 °C for 85 minutes. Samples of SS were diluted with distilled water prior loading, to reach a solid content equal to 15 wt%.

### 2.3 Zebrafish embryo-larval contact assay and sub-lethal responses

#### 2.3.1 Zebrafish husbandry and spawning

Adult zebrafish of the AB strain were bred at the Department of Biosciences, University of Milan (Italy), in flow-through conditions at water temperature of 28 °C, with a photoperiod of 14 h light and 10 h darkness. Fish were fed three times a day with small granular food (ZM Fish and Food Equipment).

156 Our facility strictly complies with the relevant European (EU Directive 2010/63/EU for animal  
157 experiments) and Italian (Legislative Decree No. 26/2014) laws, rules, and regulations, as also  
158 confirmed by the authorization issued by the municipality of Milan (PG 384983/2013). The  
159 procedures were carried out in accordance with the relevant guidelines and regulations. In compliance  
160 with National law on welfare of animals subject to scientific purposes (Legislative Decree No.  
161 26/2014), exposure experiments were carried out using embryos-larvae within 96 hours post  
162 fertilization (hpf). Embryos were collected within 3 hours post fertilization and observed under a  
163 stereomicroscope. For the exposure study poor-quality embryos were discarded. To achieve a better  
164 robustness of the exposure results, each experiment was conducted with embryos obtained from three  
165 pairs of adults.

### 166 2.3.2 Embryotoxicity test

167 The contact assay has been developed according to the OECD procedures (OECD TG 236) and  
168 following the protocol developed for sediments and described by Schiwy and co-authors (2015).

169 The test was carried out using 20 normally developed embryos placed in beakers (50 mL), each one  
170 representing an experimental group. Control groups were exposed to 3 g of standard reference soil  
171 (composed of air-dried quartz sand, granulometry 0-2 mm). The groups exposed to SS samples (SS1  
172 and SS2) and the respective HC (HC1 and HC2) were treated as follows: SS and HC samples were  
173 first mixed with standard reference soil, with a final ratio of 1/300 and 1/100 up to a total wet weight  
174 of 3 g and then used to expose the embryos. The two dilutions (1/300 and 1/100) were selected based  
175 on previous trials carried out in our lab showing that the 1/100 dilution was able to induce slight acute  
176 toxicity, while up to 100% lethality was observed at lower dilutions (Table S1). Reference soil and  
177 SS and HC mixture were placed in 50 mL glass beakers, then filled with 15 mL of artificial water  
178 (Instant Ocean<sup>®</sup> 0.1 g L<sup>-1</sup>, NaHCO<sub>3</sub> 0.1 g L<sup>-1</sup>, CaSO<sub>4</sub> 0.2 g L<sup>-1</sup> and 0.1% methylene blue). Both SS  
179 and HC dilutions were prepared 24 hours before running the assay to promote the stabilization of the  
180 suspensions. Dissolved O<sub>2</sub> and pH of the waters in contact with soil (Ctrl, SSs and HCs mixtures)  
181 were measured using a multi-parameters probe (Aqualytic AL15), at the beginning of the exposure

182 (T<sub>0</sub>) and after 96 hours (T<sub>96h</sub>). Three independent exposure experiments were carried out with three  
183 replicates for each one. Every 24 hours, embryos-larvae were observed under the stereomicroscope  
184 to assess lethal and teratogenic parameters according to Schiwy et al. (2015). Dead larvae were  
185 removed to avoid fungal/bacterial infections that might originate from their degradation. At the end  
186 of the exposure period (96 h), twelve normally developed larvae from the control group, and SS and  
187 HC exposed groups from the 1/300 dilution were analyzed for behavior. The remaining larvae were  
188 collected and frozen at -80 °C for biomarkers analyses. Soil-contact waters were collected and stored  
189 at -20 °C for chemicals analysis.

### 190 *2.3.3 Larvae swimming behavior*

191 Twelve normally developed larvae at 96 hpf from Ctrl, SS and HC exposure groups (1/300 mixture)  
192 were placed in multi-well plates (24 wells) in 1 mL of artificial water and locomotor behavior was  
193 assessed using the DanioVision™ observation chamber (Noldus Inc., Wageningen, The Netherlands).  
194 The swimming performances were assessed following a light/dark transition locomotor response test  
195 developed for zebrafish larvae according to Leuthold et al. (2019) as described in details in  
196 supplementary materials.

### 197 *2.3.4 Biomarkers analyses*

198 The activities of enzymes involved in detoxification and antioxidant response were measured in  
199 pooled larvae from Ctrl, SS and HC exposed groups (1/300 mixture). Analyses were carried out on  
200 three replicates from the three independent experiments. Twenty larvae from each exposure group  
201 were homogenized in 100 mM phosphate buffer pH 7.4, KCl 100 nM, EDTA 1 nM, previously  
202 activated by the addition of 100 mM DTT and protease inhibitors (1:100 v/v), following the ratio 1  
203 embryo/10 µL. The homogenates were centrifuged for 10 min at 15,000 x g at 4 °C and the recovered  
204 supernatant used for enzyme assays. The activity of GST and CAT were measured using a 6715  
205 UV/Vis spectrophotometer (Jenway), following the protocol described by Della Torre et al. (2018).  
206 ROS levels and EROD activity were measured according to the method reported in Parenti et al.  
207 (2019a) using the Ensign™ multimode plate reader. The AChE activity was measured according to

208 Ellman et al. (1961). The reaction mixture consisted of a potassium phosphate buffer (100 mM, pH  
209 7.4) containing acetylthiocholine chloride (1 mM) and 5,5'-dithiobis-2-nitrobenzoic acid (0.5 mM).  
210 The reaction was monitored for 15 min at 412 nm using the Ensight™ multimode plate reader and  
211 AChE activity was expressed as  $\mu\text{moles of acetylcholine chloride hydrolyzed min}^{-1} \text{ mg protein}^{-1}$ . The  
212 protein content was measured according to the Bradford method (1976), calibrated on a standard BSA  
213 curve (0.1-0.5 mg mL<sup>-1</sup>).

#### 214 2.4 Chemical analysis of SS and HC samples and zebrafish contact-test waters

215 The following parameters were analyzed in tested SSs (SS1 and SS2) and HCs (HC1 and HC 2) and  
216 in contact-test waters: total organic carbon, major nutrients, ions, and a set of priority pollutants  
217 (heavy metals, hydrocarbons, and chlorinated organic compounds) according to several European and  
218 Italian legislations concerning fertilizing products and for soil amendments as reported in Tab. S2.

219 Both SS and HC samples were prepared as follows: an aliquot of 50 g fresh weight of SS was placed  
220 in sterile plastic containers at -80 °C overnight and then lyophilized (Labogene, Scanvac cool safe)  
221 for 48 h until achieving a constant weight. An aliquot of 10 g of HC was placed in a glass petri dish  
222 and heated up in an oven at 105 °C for 1 h (Memmert™). All samples were then sieved (2 mm Ø)  
223 and the resulting fraction stored in plastic containers until analysis. Analytical procedures are  
224 described in detail in the supporting information section. Metals and the other elements were analyzed  
225 by Inductively Coupled Plasma Mass Spectrometry (iCAP™RQ ICP-MS, Thermo Scientific)  
226 equipped with a collision cell, according to EPA method 6020B. Analysis of polychlorinated dibenzo-  
227 *p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and *dioxin-like* polychlorinated  
228 biphenyls (*dl*-PCBs) were carried out according respectively to the EPA methods 1613B and 1668C  
229 using a Trace™ 1310 Gas-chromatograph (Thermo Scientific) equipped with DFS™ Mass-  
230 spectrometer (Thermo Scientific) and a TriPlus™ RSH autosampler (Thermo Scientific). Estimated  
231 concentrations for each detected analyte of PCDD/Fs and *dl*-PCBs were expressed as of Toxic  
232 Equivalency (TEQ) and calculated according to Van den Berg et al. (2006). Analysis of PAHs was  
233 carried out according to EPA method 8270, using a 7890B Gas-chromatograph (Agilent

234 Technologies) equipped with a 7000B triple-quadrupole mass-detector (Agilent Technologies).  
235 Analysis of anions was carried out according to EPA method 9056A, using a Dionex™ ICS-1100  
236 Ionic Chromatograph (Thermo Scientific), working with a conductivity detector Dionex™ DS6  
237 (Thermo Scientific). Analysis of the volatile fraction of the hydrocarbons on the zebrafish contact-  
238 test waters was carried out according to EPA method 8015, using a 7890A Gas-chromatograph  
239 (Agilent Technologies) equipped with an FID detector. For the insert of the sample in the gas-  
240 chromatograph a Purge & Trap unit AtomX (Teledyne Tekmar) was used, according to EPA method  
241 5030. The determination of total organic carbon (TOC) on the dried SS and HC samples was carried  
242 out according to UNI EN 13137 method, using a CHS 580 elemental analyzer (ELTRA), working at  
243 a combustion temperature of the furnace between 900 and 1400°C. The determination of total  
244 phosphorus was carried out using a double-ray Cary 100 Scan (Varian) spectrophotometer, and a cell  
245 with 1 cm optical-length.

#### 246 *2.5 Statistical analysis*

247 Results from toxicity, behavioural assay and biomarkers were statistically analysed. Analyses were  
248 based on three data points originated from the three independent experiments. The analyses were  
249 carried out using STATISTICA 7.0 software package. One-way analysis of variance (ANOVA) was  
250 applied to verify the presence of statistically significant differences between the different treatments.  
251 The ANOVA assumptions were checked using the Kolmogorov-Smirnov test for data normality and  
252 Levene's test for homogeneity of variances. Significant differences were identified by the Fisher LSD  
253 post-hoc test, considering  $p \leq 0.05$  as significant cut-off.

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### 255 3. Results and discussion

#### 256 3.1 Physicochemical parameters of SS and HC samples

257 As shown in Table S3, pH and dissolved O<sub>2</sub> values of contact waters fulfilled the requirements for  
258 test validity set by OECD range of pH 6.5 - 8.5 with variations < 1.5 units during the test; O<sub>2</sub> ≥80%  
259 of saturation. Also, TOC contents were similar in SS1 and SS2 and in the respective HC and  
260 accomplished the limits set for SS for agricultural use, according to the European Council Directive  
261 (86/278/CEE) (≥ 20 % d.w.) (Tab. 1). Nutrients and ions showed again comparable levels of nitrates  
262 sulphates and phosphates in both SSs (Tab. 1), while differences were found in HCs. The nitrite  
263 content was below 1 mg/kg dry weight (d.w.) in all samples except SS1. As for nitrates, a significant  
264 enrichment was observed in SS1, while ten times reduced for SS2. The sulphates levels resulted about  
265 eight times higher in HC1 with respect to SS1, while were slightly increased in HC2. The phosphates  
266 content was below 50 mg/kg d.w. in all samples except SS2, in which a strong decrease of these  
267 nutrients occurred upon HTC treatment. The level of chlorides was four times higher in SS2 compared  
268 to SS1. Moreover, also HC2 showed a strong abatement of chlorides content with respect to SS2,  
269 while only minor reduction was observed in HC1. Concerning trace elements, no changes were  
270 observed for Mg content. The Ca and P were increased in both HCs with respect to SSs, while Na  
271 decreased upon HTC. The P levels accomplished the limits set for SS for agricultural use, according  
272 to the European Council Directive (86/278/CEE) (≥ 4,000 mg Kg d.w.). The content of K was also  
273 reduced in HC1 compared to SS1. The observed variation in the load of nutrients and ions might be  
274 due to the different wastewater composition and/or to the different sludge processing (described in  
275 paragraph 2.1).

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**Table 1** Levels of organic carbon, nutrients and ions measured in SS and HC from the plant 1 and 2.

	SS1	HC1	SS2	HC2
<b>Total organic carbon (% d.w.)</b>	34.4	38.6	40	40.9
<b>Nitrites (mg/kg d.w.)</b>	2.9	< 1	< 1	< 1
<b>Nitrates (mg/kg d.w.)</b>	30	182	26.2	2.9
<b>Chlorides (mg/kg d.w.)</b>	156	121	530	74
<b>Sulphates (mg/kg d.w.)</b>	420	3370	470	350
<b>Phosphates (mg/kg d.w.)</b>	<50	<50	1140	<50
<b>Ca (mg/kg d.w.)</b>	26,500	41,000	46,000	47,000
<b>Mg (mg/kg d.w.)</b>	4050	5840	3690	3510
<b>K (mg/kg d.w.)</b>	2110	1070	<630	<630
<b>Na (mg/kg d.w.)</b>	600	330	750	194
<b>P (mg/kg d.w.)</b>	15,100	21,800	8,700	9,900

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### 285 3.2 Toxic chemicals in SS and HC samples

286 Chemical characterization was carried out on solid matrices and waters collected at the end of the  
287 contact assay.

288 Concerning heavy metals in solid samples, the two SS showed similar levels (Tab. 2) while an  
289 enrichment is observed for the HCs. All samples exceeded the limits set for Cu, Hg, Zn according to  
290 Reg (UE) 2019/1009. The levels of Mn and V were higher than the limits set by the DM 22/2013 in  
291 all samples. The Cd levels exceeded the limits set by Dlgs. 75/2010 and 2019/1009 for growing  
292 medium, but only in HC1. The levels of Ni were higher than the limit set by DM 22/2013 in all  
293 samples except SS1. All samples exceeded the limits set by DM 22/2013 for Sb. Both HCs exceeded  
294 the limit set by Reg (UE) 2019/1009 for Pb. On the contrary, the levels of all heavy metals were  
295 below the limits set by the European (86/278/CEE) and Italian Law (Dlgs 130/2018) for the reuse of

296 SS in the agricultural soils. Levels of PCDD/Fs including *dl*-PCBs resulted below the limit of 25  
 297 ng/kg TEQ<sub>WHO</sub> d.w. set by Dlgs 130/2018 for both SSs. Nevertheless, if the same legislation were  
 298 applied to the HC samples, the HC1 would show a value very close to the threshold level. Although  
 299 not included in Regulatory Limits, the DDx levels were strongly reduced in both HCs with respect to  
 300 SSs. Total PCBs were below the National Regulatory Limits set by the Dlgs 130/2018 ( $\leq 0.8$  mg/kg  
 301 d.w), resulting 100 times lower, both in the SSs and HC1, while they were slightly higher only for  
 302 HC2 .

303 Concerning the contaminant levels in contact waters, volatile hydrocarbons (C<sub>6</sub>-C<sub>10</sub>) and PAHs  
 304 resulted below LOD (respectively  $< 0.05 \mu\text{g L}^{-1}$  for volatile hydrocarbons (C<sub>6</sub>-C<sub>10</sub>) and  $< 0.1 \mu\text{g L}^{-1}$   
 305 for PAHs) either SSs and HCs. conversely, heavy metals were present in waters from SSs and HCs.  
 306 An enrichment of Cr was observed in both HCs compared to SSs, while higher levels of Co, B, Mn,  
 307 Ni, Cu, Se, V and Zn were observed only in waters from HC2 with respect to SS2. Al and As levels  
 308 resulted lower in exposure water comparing both SS samples to HCs (Tab. S4).

309 **Table 2** Levels of pollutants measured in solid matrix samples of SSs and HCs (a) and in contact  
 310 waters collected after exposure (b). Values exceeding regulatory limits for sludge and other solid  
 311 substrates for landfill applications are in bold (details in Table S2) (A) DM 22/2013, (B) Dlgs  
 312 75/2010, (C) Reg (UE) 2019/1009 for fertilizers, (D) Reg (UE) 2019/1009 for soil conditioners, (E)  
 313 Reg (UE) 2019/1009 for growing medium, (F) Council Directive 86/278/CEE, G Dlgs 130/2018.  
 314  
 315

(a)

	SS1	HC1	SS2	HC2
<i>Metals (mg/kg d.w.)</i>				
Al	3,700	6,600	4,500	6,400
As	3.9	4.2	4.3	4.5
Ba	164	287	171	209
Be	<0.63	<0.63	<0.63	<0.63
B	32.3	21.9	19.3	8.5
Cd	0.99	<b>1.86<sup>B,E</sup></b>	1.06	1.45
Co	3.6	4.8	4.3	4.3
Cr <sub>TOT</sub>	34.3	51	50	69
Cu	<b>326<sup>B,D,E</sup></b>	<b>603<sup>B,D,E</sup></b>	<b>347<sup>B,D,E</sup></b>	<b>446<sup>B,D,E</sup></b>





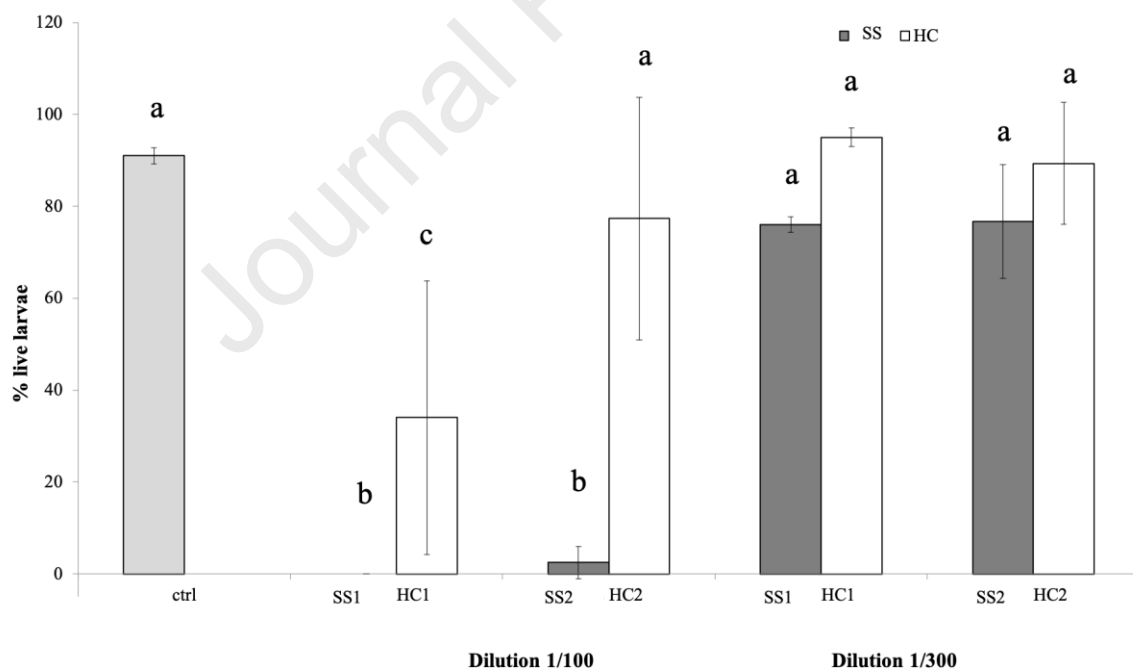
Co (mg/L)	< 0.0005	0.00117	0.00064	0.00112	0.00125	0.00083	< 0.0005	0.00208	0.00099
Cr (mg/L)	< 0.0005	< 0.0005	0.00058	0.00124	0.00066	< 0.0005	< 0.0005	0.00083	0.00057
Cu (mg/L)	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	0.122	< 0.05	< 0.05	< 0.05
Fe (mg/L)	< 0.000125	< 0.000125	< 0.000125	< 0.000125	< 0.000125	< 0.000125	< 0.000125	< 0.000125	< 0.000125
Hg (mg/L)	0.0107	0.0184	0.0056	0.0106	0.00206	0.00344	0.00095	0.053	0.0084
Mn (mg/L)	0.00174	0.0109	0.0075	0.006	0.00359	0.0104	0.0059	0.0078	0.0051
Mo (mg/L)	0.00083	0.0042	0.00341	0.0068	0.00301	0.0066	0.00247	0.0092	0.0047
Ni (mg/L)	< 0.0005	< 0.0005	< 0.0005	< 0.0005	0.00055	< 0.0005	< 0.0005	< 0.0005	< 0.0005
Pb (mg/L)	< 0.0005	0.00072	0.00052	0.00067	< 0.0005	< 0.0005	0.00054	0.00099	0.00053
Sb (mg/L)	0.00071	0.00097	0.00091	0.00124	0.00081	< 0.0005	< 0.0005	0.00073	0.00058
Se (mg/L)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Sn (mg/L)	0.344	0.65	0.49	0.54	0.4	0.51	0.52	0.55	0.49
Sr (mg/L)	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005
Tl (mg/L)	< 0.0025	< 0.0025	< 0.0025	< 0.0025	< 0.0025	< 0.0025	< 0.0025	< 0.0025	< 0.0025
Te (mg/L)	0.0049	0.0163	0.013	0.0085	0.0058	0.00253	0.0053	0.0048	0.0062
V (mg/L)	< 0.005	0.0096	0.0077	0.009	0.0222	0.0083	< 0.005	0.0231	0.0074
Zn (mg/L)	0.0138	0.0251	0.0193	0.0243	0.0138	0.0249	0.0204	0.0133	0.0115
Ca (mg/l)	60	109	83	78	68	108	81	83	74
Mg (mg/l)	16.6	25.7	25.6	26	19.8	20.8	20.7	19.1	19.4
K (mg/l)	190	84	78	26.3	15.1	13.5	14.7	13.8	15.6
Na (mg/l)	164	194	204	215	174	164	189	162	172
C>12 (µg/L)	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Σ PAHs (µg/L)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
ΣPCDD/PCDF (µg/L)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
ΣPCB (µg/L)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
ΣPCB (µg/L)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1

318

319 *3.3 Larval Toxicity of SC and HC samples*

320 Larvae exposed to SS and HC samples showed several lethal effects such as: coagulation, whole  
321 deformations and not detachment of the tail from the yolk sac (Fig. S1). These effects occurred within  
322 the first 24 h of exposure. Both SS and HC samples, at the highest dilution tested (1/300), did not  
323 cause any acute toxicity with the treated larvae displaying a Ctrl phenotype. However, at the lower  
324 dilution (1/100), a high percentage of mortality > 90% was observed for both SS samples (Fig.1),  
325 while lower mortality was observed for HCs, 34 % for HC1 ( $p = 0.0002$ ) and 77% for HC2 ( $p =$   
326  $0.0001$ ), respectively. Larvae exposed to HC2 showed the same viability as Ctrl ( $p = 0.27$ ). The

327 observed toxic effects can be attributable to acute toxicity since no sub-lethal morphological  
 328 anomalies were detected in larvae exposed either to SS and HC samples at both dilutions (1/100 and  
 329 1/300). The observed toxicity upon exposure to SS and HC could be due to heavy metals exposure in  
 330 agreement with their presence in SS and HC and in contact waters and considering the known acute  
 331 effects reported in fish models, including zebrafish (Alsop and Wood, 2011 and citation therein;  
 332 Wang et al., 2013). The presence of anions and nutrients could also contribute to the observed acute  
 333 toxicity, although acute toxicity it is generally observed at higher exposure concentrations (Camargo  
 334 et al., 2005; Luo et al., 2016; Axton et al., 2019). Notwithstanding, as larvae were exposed to a  
 335 complex mixtures of toxic chemicals that might act synergistically, the adverse effects might occur  
 336 at much lower levels.  
 337

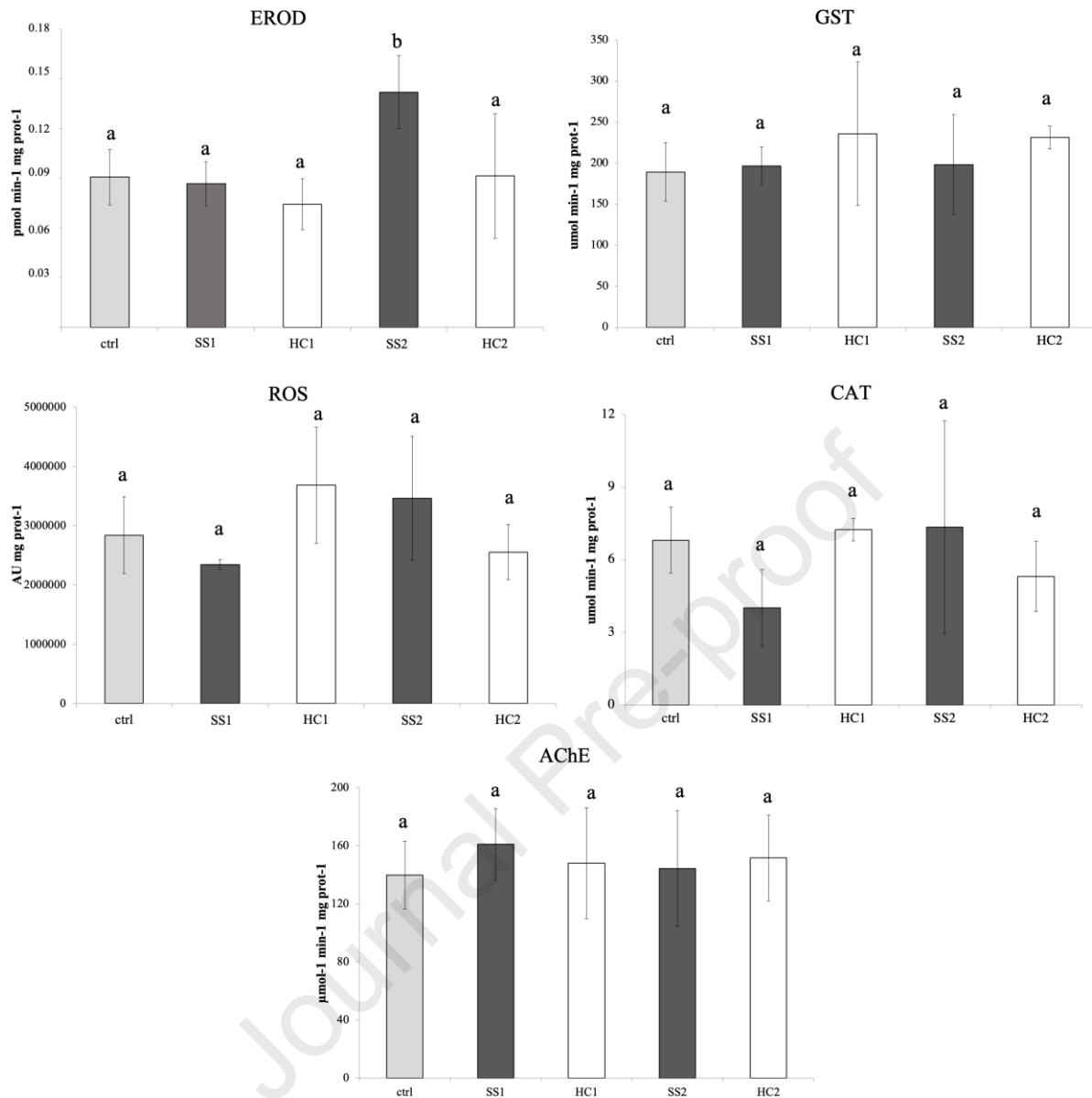


338

339 Fig. 1 Mortality rate (shown as % of live larvae) of zebrafish larvae exposed to SSs and HCs at (1/100  
 340 and 1/300) for 96 h. Results are presented as means  $\pm$  standard dev of three independent experiments.  
 341 Different letters mean statistically significant differences among exposure groups (one-way ANOVA,  
 342 LST post-hoc test  $p < 0.05$ ).  
 343

344 *3.4 Sub-lethal effects: detoxification, oxidative stress, and behavioral effects*

345 The exposure to SS and HC samples did not affect larvae EROD activity except for those exposed to  
346 SS2, in which a significant increase vs Ctrl was observed ( $p = 0.02$ ) (Fig. 2). While such findings are  
347 in agreement with the general levels <LOD of *AhR* agonists - as PCDD/Fs and dl-PCBs- in SS and  
348 contact waters, in SS2 other compounds rather than organohalogen might have caused the observed  
349 increase. As a further confirmation, the phase II enzyme GST was not modulated in SSs and HCs  
350 exposed larvae as well as oxidative stress response, with similar levels of ROS content and CAT  
351 activity in larvae exposed to either SS or HC. (Fig. 2). Levels measured in exposure water from both  
352 SS and HC highlighted a release from the solid matrices of Al, Co, Mn, Cr, Pb, for which it was  
353 reported the ability to trigger oxidative stress conditions in zebrafish (Cai et al., 2012; Jin et al., 2015;  
354 Gao et al., 2019; Marins et al., 2019; Shaw et al., 2020; Capriello et al., 2021). Nevertheless, results  
355 of oxidative stress parameters suggest that metals released from SS and HC within 96 h of  
356 exposure, are not enough to generate a significant oxidative stress response in larvae.  
357 The larval AChE activity was not affected upon all exposure conditions (Fig. 2).



358

359 Figure 2 Biomarkers measured in zebrafish larvae exposed to SSs and HCs at 1/300 for 96 h. Results  
 360 are presented as means  $\pm$  standard dev of three independent experiments. Different letters mean  
 361 statistically significant differences among exposure groups (one-way ANOVA, LST post-hoc test  $p$   
 362  $< 0.05$ ).

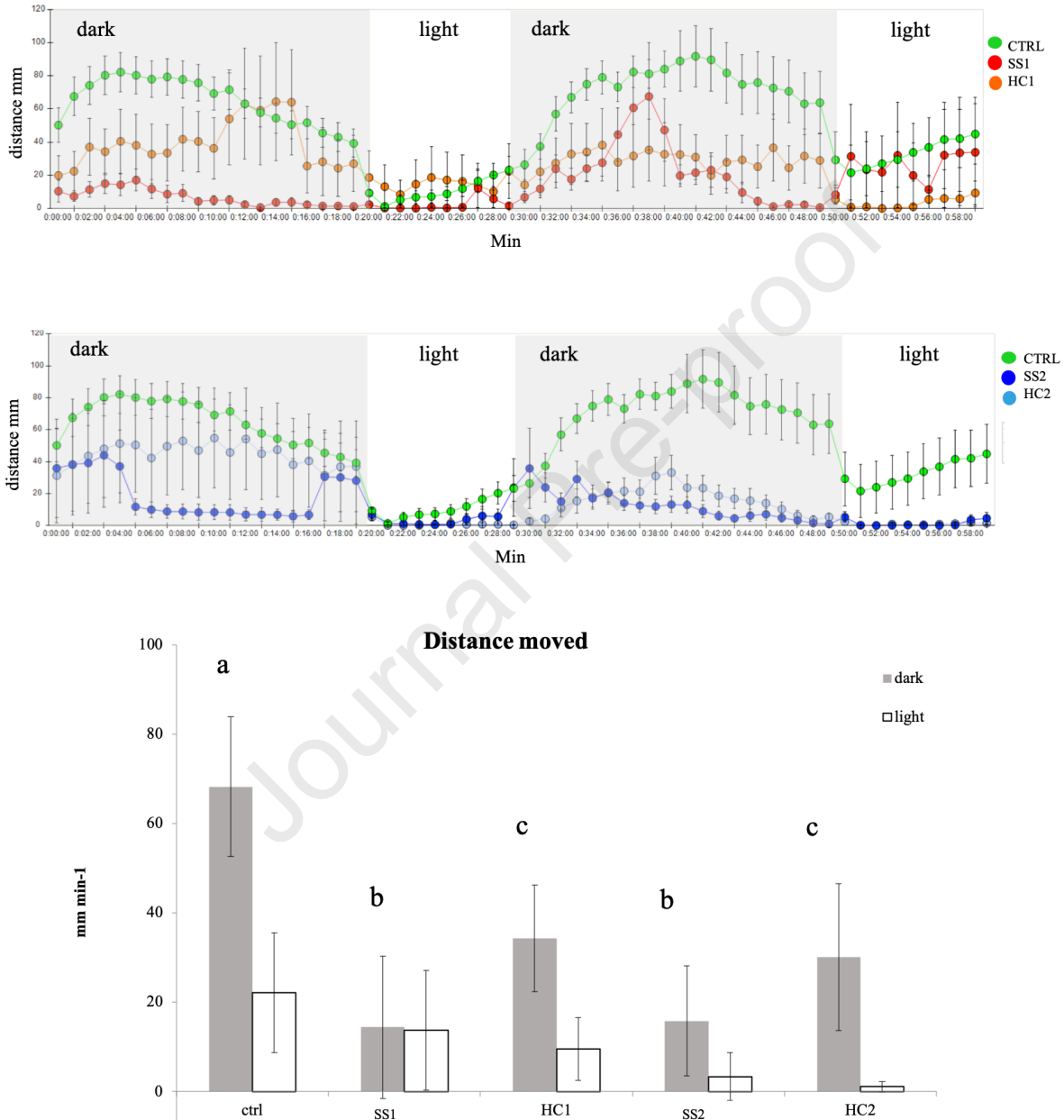
363

364

365

366 The exposure to both SS and HC samples significantly affected zebrafish larvae swimming  
 367 performance by inducing a clear hypoactivity effect, with lower distance moved by exposed larvae  
 368 compared to Ctrl (Fig. S2). Moreover, larvae exposed to SS and HC did not show any change in  
 369 activity profile by switching from light to dark conditions, while a clear shift from basal to increasing  
 370 locomotor activity was observed in Ctrl (Fig. 3). Indeed, the distance moved during the dark phase

371 resulted significantly reduced in larvae exposed to SS samples compared to Ctrl ( $p < 0.0001$ ). Such  
 372 effect was partially recovered in larvae exposed to HCs, even if still significantly lower distance  
 373 travelled was measured in these groups with respect to Ctrl ( $p < 0.0001$ ) (Fig. 3).



374

375 Figure 3. Behavioural effects in zebrafish larvae exposed to SSs and HC at dilution 1/300 for 96 h,  
 376 measured as distance moved per minute during the dark/light transition test. Results are presented as  
 377 means  $\pm$  standard dev of three independent experiments. Different letters mean statistically significant  
 378 differences among exposure groups (one-way ANOVA, LST post-hoc test  $p < 0.05$ ).

379

380 These results provide evidence of exposure of zebrafish larvae to chemicals able to affect behavioral  
381 traits thus supporting their hypothesis of their release from SS and HC in exposure water. With the  
382 aim to understand the potential mechanism underlying the reduced swimming activity we assessed  
383 AChE activity. Cholinesterase activities are biomarkers for neurochemical alterations induced by  
384 different pollutants in fish (van der Oost et al., 2003). In zebrafish, the AChE plays a key role in the  
385 development of the neuromuscular apparatus at early life stages (Behra et al., 2002). Therefore, this  
386 endpoint could be considered a potential bridge to link the sub-cellular effects with the impairment  
387 of locomotor activity (Beauvais et al., 2001). The absence of AChE modulation observed in larvae  
388 exposed to both SS and HC suggests that the alteration of embryo swimming performance does not  
389 involve the inhibition of this enzyme activity, but is rather due to the presence of chemicals with  
390 different toxic mechanisms in both matrices. Among pollutants detected in SS and HC, heavy metals  
391 have known neurotoxic potential. For instance, Le Fauve and Connaughton (2017), showed that Cd  
392 and Ni significantly impacted on visually guided behavior of zebrafish embryos, decreasing the larval  
393 opto-motor responses and a lowering the sensitivity of the larvae to the day/night variations. Also,  
394 the reduction of locomotor activity has been reported in zebrafish embryos exposed to Cd and Al  
395 (Capriello et al., 2019) and CrVI (Jin et al., 2015) at  $\mu\text{M}$  range. A similar result was also observed in  
396 zebrafish larvae exposed to Mn and Pb, which showed reduced swimming distance and velocity  
397 combined with several developmental defects (Tu et al., 2017). In addition, organohalogen  
398 compounds including hydrocarbons, are known to generate behavioral disturbances in fish models  
399 (Legradi et al., 2018). For instance, neurotoxic effects such as hypoactivity in fish exposed to single  
400 and PAH mixtures have been previously reported (Goncalves et al., 2008; Oliveira et al., 2012; Vignet  
401 et al., 2014a;b). A recent study by Johann and co-authors (2020), showed that zebrafish embryos  
402 exposed to both native and chemically dispersed oils had a low baseline swimming activity and were  
403 less sensitive to the light/dark transitions.

404 The alteration of swimming performance could also be due to the presence of neuroactive drugs such  
405 as pharmaceuticals (Carlsson et al., 2009; David and Pancharatna, 2009; Xia et al., 2017; Nogueira

406 et al., 2019; Oliveira de Faria et al., 2019; Zindler et al., 2020), known to be present in urban sludges  
407 and in SS, although quite often below detection limits.

408 The lower behavioral effect observed in larvae exposed to the HC suggests that the HTC could reduce  
409 the load and/or bioavailability of some neuroactive molecules able to impact on swimming  
410 performances. Indeed, some studies showed that HTC reduces a relevant percentage of  
411 pharmaceuticals such as antibiotics, carbamazepine, and diclofenac, from raw sludges (vom Eyser et  
412 al., 2015;), therefore the toxic potential of this carbonaceous matrix should be significantly reduced  
413 (Tasca et al., 2016).

414

#### 415 *3.4 Environmental implications*

416 According to the European Law 86/278/CEE and Italian Legislative Decree 99/1992, the amount of  
417 SS that is allowed for agricultural soil application should not exceed 15 t/ha of dry matter in a three-  
418 year period. Therefore, with the aim to assess if SS and HC samples tested in the present study will  
419 satisfy such requirements an attempt was made to calculate the maximum threshold concentration of  
420 contaminants that could occur, considering as legal limits those imposed by Italian Legislative Decree  
421 109/2018. Based on the levels of heavy metals, the highest were observed for Zn and the lowest for  
422 Cd. An almost double enrichment was observed from SS to HC in WWTP1 (14.55-27.60 kg/ha d.w.  
423 for Zn and 14.85-27.9 g/ha d.w. for Cd, respectively) and a slightly lower in WWTP2 (14.85-20.1  
424 kg/ha d.w. for Zn and 15.9-21.7 g/ha d.w. for Cd, respectively). Same trend is observed for chlorinated  
425 organic compounds, with the sum of PCDD/Fs and *dl*-PCBs more than 3-times higher in HC  
426 compared to SS in WWTP1 (0.115-0.372 mg/ha d.w.) and more than 2-times higher in WWTP2  
427 (0.087-1.95 mg/ha d.w.). A relevant enrichment was also observed for total PCBs, where the highest  
428 values were again found in WWTP1 with respect to WWTP2. In detail, WWTP1 showed an almost  
429 twice enrichment in PCBs between SS and HC (0.15-0.27 g/ha d.w.) while in WWTP2, the  
430 enrichment resulted less than 1.5 (0.01-0.13 g/ha d.w.). Based on such calculation, current regulatory  
431 limits are not exceeded by both SS and HC samples. On the other hand, in case of a massive supply



432 over time to agricultural soil, HC could still affect the quality of the crops yields, the food, and the  
433 environmental safety due to an enrichment of toxic chemicals (Protano et al., 2019). This concern is  
434 also supported by the results achieved through the zebrafish bioassays and reported in this manuscript,  
435 which highlight the toxic potential of both SS and HC samples to aquatic organisms. Anyway, a  
436 reduction of adverse effects was observed in larvae exposed to HC with respect to SS, likely because  
437 the HTC process could have a "cage-like effect" on several chemicals, changing their release from  
438 the solid matrix to the water column, making their toxic action on larvae less effective.

#### 439 **4. Conclusions**

440 Our study contributed to fill the current analytical and cognitive gap, on the assessment of the health  
441 risk associated to the environmental application of solid residues such as SS and HC, using a  
442 biological model that is well representative of the aquatic system such as zebrafish. The zebrafish  
443 embryo-larval contact assay emerged as a suitable and sensitive screening tool for assessing  
444 ecotoxicity for natural ecosystems of SS and HC. In future applications, this assay could be combined  
445 with an effect-directed analysis that fractionates the aqueous samples and analyses the received  
446 fractions for chemical constituents and observed biological effects (Hecker and Hollert, 2009). This  
447 approach can be helpful to finally figure out which constituents lead to the observed lethal and  
448 behavioral effects. Comparing results of sub-lethal parameters, the locomotor activity resulted in the  
449 most sensitive endpoint. As the swimming behavior is a key factor in prey/predator relationship, as  
450 well as to get food, the swimming impairment could have implications at the population level,  
451 underlining the importance of including behavioral endpoints within the framework of protocols for  
452 environmental risk assessment of complex matrices.

453

454

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460

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Bioassays are needed to ensure the safe environmental application of hydrochar

Contact test with zebrafish as valid screening tool for sludge and hydrochar

Sludges and hydrochars induced acute toxicity and hypoactivity on zebrafish larvae

Journal Pre-proof



## **Author contributions**

Camilla Della Torre: Conceptualization, Data Curation, Methodology, Investigation, Supervision, Writing-original draft

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Anna Ghilardi: Resources, Formal analysis

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**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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