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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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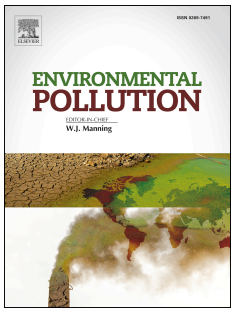

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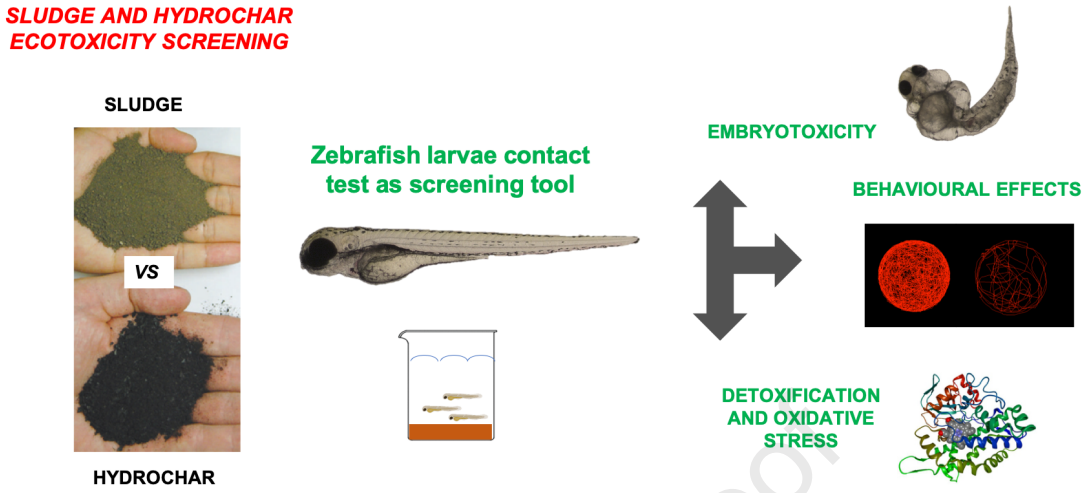

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The zebrafish (*Danio rerio*) embryo-larval contact assay combined with biochemical biomarkers and swimming performance in sewage sludge and hydrochar hazard assessment

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Abstract

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

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

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

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1. Introduction

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

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

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

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

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

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

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

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

2.3.2 Embryotoxicity test

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

The test was carried out using 20 normally developed embryos placed in beakers (50 mL), each one representing an experimental group. Control groups were exposed to 3 g of standard reference soil (composed of air-dried quartz sand, granulometry 0-2 mm). The groups exposed to SS samples (SS1 and SS2) and the respective HC (HC1 and HC2) were treated as follows: SS and HC samples were first mixed with standard reference soil, with a final ratio of 1/300 and 1/100 up to a total wet weight of 3 g and then used to expose the embryos. The two dilutions (1/300 and 1/100) were selected based on previous trials carried out in our lab showing that the 1/100 dilution was able to induce slight acute toxicity, while up to 100% lethality was observed at lower dilutions (Table S1). Reference soil and SS and HC mixture were placed in 50 mL glass beakers, then filled with 15 mL of artificial water (Instant Ocean® 0.1 g L^{-1}, $NaHCO_3$ 0.1 g L^{-1}, $CaSO_4$ 0.2 g L^{-1} and 0.1% methylene blue). Both SS and HC dilutions were prepared 24 hours before running the assay to promote the stabilization of the suspensions. Dissolved O_2 and pH of the waters in contact with soil (Ctrl, SSs and HCs mixtures) were measured using a multi-parameters probe (Aqualytic AL15), at the beginning of the exposure

(T_0) and after 96 hours (T_{96h}). Three independent exposure experiments were carried out with three replicates for each one. Every 24 hours, embryos-larvae were observed under the stereomicroscope to assess lethal and teratogenic parameters according to Schiwy et al. (2015). Dead larvae were removed to avoid fungal/bacterial infections that might originate from their degradation. At the end of the exposure period (96 h), twelve normally developed larvae from the control group, and SS and HC exposed groups from the 1/300 dilution were analyzed for behavior. The remaining larvae were collected and frozen at -80 °C for biomarkers analyses. Soil-contact waters were collected and stored at -20 °C for chemicals analysis.

2.3.3 Larvae swimming behavior

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

2.3.4 Biomarkers analyses

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

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

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

The following parameters were analyzed in tested SSs (SS1 and SS2) and HCs (HC1 and HC 2) and in contact-test waters: total organic carbon, major nutrients, ions, and a set of priority pollutants (heavy metals, hydrocarbons, and chlorinated organic compounds) according to several European and Italian legislations concerning fertilizing products and for soil amendments as reported in Tab. S2. Both SS and HC samples were prepared as follows: an aliquot of 50 g fresh weight of SS was placed in sterile plastic containers at -80 °C overnight and then lyophilized (Labogene, Scanvac cool safe) for 48 h until achieving a constant weight. An aliquot of 10 g of HC was placed in a glass petri dish and heated up in an oven at 105 °C for 1 h (Memmert™). All samples were then sieved (2 mm Ø) and the resulting fraction stored in plastic containers until analysis. Analytical procedures are described in detail in the supporting information section. Metals and the other elements were analyzed by Inductively Coupled Plasma Mass Spectrometry (iCAP™RQ ICP-MS, Thermo Scientific) equipped with a collision cell, according to EPA method 6020B. Analysis of polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and *dioxin-like* polychlorinated biphenyls (*dl*-PCBs) were carried out according respectively to the EPA methods 1613B and 1668C using a Trace™ 1310 Gas-chromatograph (Thermo Scientific) equipped with DFS™ Mass-spectrometer (Thermo Scientific) and a TriPlus™ RSH autosampler (Thermo Scientific). Estimated concentrations for each detected analyte of PCDD/Fs and *dl*-PCBs were expressed as of Toxic Equivalency (TEQ) and calculated according to Van den Berg et al. (2006). Analysis of PAHs was carried out according to EPA method 8270, using a 7890B Gas-chromatograph (Agilent

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

2.5 Statistical analysis

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

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3. Results and discussion

3.1 Physicochemical parameters of SS and HC samples

As shown in Table S3, pH and dissolved O_2 values of contact waters fulfilled the requirements for test validity set by OECD range of pH 6.5 - 8.5 with variations < 1.5 units during the test; O_2 ≥80% of saturation. Also, TOC contents were similar in SS1 and SS2 and in the respective HC and accomplished the limits set for SS for agricultural use, according to the European Council Directive (86/278/CEE) (≥ 20 % d.w.) (Tab. 1). Nutrients and ions showed again comparable levels of nitrates sulphates and phosphates in both SSs (Tab. 1), while differences were found in HCs. The nitrite content was below 1 mg/kg dry weight (d.w.) in all samples except SS1. As for nitrates, a significant enrichment was observed in SS1, while ten times reduced for SS2. The sulphates levels resulted about eight times higher in HC1 with respect to SS1, while were slightly increased in HC2. The phosphates content was below 50 mg/kg d.w. in all samples except SS2, in which a strong decrease of these nutrients occurred upon HTC treatment. The level of chlorides was four times higher in SS2 compared to SS1. Moreover, also HC2 showed a strong abatement of chlorides content with respect to SS2, while only minor reduction was observed in HC1. Concerning trace elements, no changes were observed for Mg content. The Ca and P were increased in both HCs with respect to SSs, while Na decreased upon HTC. The P levels accomplished the limits set for SS for agricultural use, according to the European Council Directive (86/278/CEE) (≥ 4,000 mg Kg d.w.). The content of K was also reduced in HC1 compared to SS1. The observed variation in the load of nutrients and ions might be due to the different wastewater composition and/or to the different sludge processing (described in 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
Total organic carbon (% d.w.)	34.4	38.6	40	40.9
Nitrites (mg/kg d.w.)	2.9	< 1	< 1	< 1
Nitrates (mg/kg d.w.)	30	182	26.2	2.9
Chlorides (mg/kg d.w.)	156	121	530	74
Sulphates (mg/kg d.w.)	420	3370	470	350
Phosphates (mg/kg d.w.)	<50	<50	1140	<50
Ca (mg/kg d.w.)	26,500	41,000	46,000	47,000
Mg (mg/kg d.w.)	4050	5840	3690	3510
K (mg/kg d.w.)	2110	1070	<630	<630
Na (mg/kg d.w.)	600	330	750	194
P (mg/kg d.w.)	15,100	21,800	8,700	9,900

3.2 Toxic chemicals in SS and HC samples

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

Concerning heavy metals in solid samples, the two SS showed similar levels (Tab. 2) while an enrichment is observed for the HCs. All samples exceeded the limits set for Cu, Hg, Zn according to Reg (UE) 2019/1009. The levels of Mn and V were higher than the limits set by the DM 22/2013 in all samples. The Cd levels exceeded the limits set by Dlgs. 75/2010 and 2019/1009 for growing medium, but only in HC1. The levels of Ni were higher than the limit set by DM 22/2013 in all samples except SS1. All samples exceeded the limits set by DM 22/2013 for Sb. Both HCs exceeded the limit set by Reg (UE) 2019/1009 for Pb. On the contrary, the levels of all heavy metals were 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_{WHO} 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 (≤ 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_6-C_{10}) and PAHs
304 resulted below LOD (respectively < 0.05 µg L^{-1} for volatile hydrocarbons (C6-C10) and < 0.1 µgL^{-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
Metals (mg/kg d.w.)				
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	**1.86**^{B,E}	1.06	1.45
Co	3.6	4.8	4.3	4.3
Cr_{TOT}	34.3	51	50	69
Cu	**326**^{B,D,E}	**603**^{B,D,E}	**347**^{B,D,E}	**446**^{B,D,E}

Fe	7200	9800	10000	10500
Hg	**2.1**[B,C,D,E]	**3**[B,C,D,E]	**2.17**[B,C,D,E]	**3**[B,C,D,E]
Mn	**820**[A]	**1370**[A]	**356**[A]	**392**[A]
Mo	8.6	15.1	7	28.8
Ni	27.5	**67**[A]	**39.4**[A]	**150**[A]
Pb	74	**124**[C,D,E]	111	**143**[C,D,E]
Sb	1.42	0.82	2.26	2.15
Se	3.9	5.1	2.18	3
Sn	**23.9**[F,G]	**47**[F,G]	**24.7**[F,G]	**35**[F,G]
Sr	177	294	167	181
Tl	<0.63	<0.63	<0.63	<0.63
Te	<0.3	<0.3	<0.3	<0.3
V	**11.1**[A]	**17.2**[A]	**12.3**[A]	**14.4**[A]
Zn	**970**[B,D,E]	**1840**[B,D,E]	**990**[B,D,E]	**1340**[B,D,E]
Hydrocarbon & Σ PAHs (mg/kg d.w.)				
C>12	2,460	13,200	7,800	12,900
Σ PAHs	0.2238	0.2028	1.6723	0.3279
Chlorinated organic compounds				
ΣPCDD, PCDF (ng WHO-TEQ/kg d.w.)	2.9	13.1	3.6	9.1
Σ *dl*-PCB DL (ng WHO-TEQ/kg d.w.)	4.8	11.7	2.2	4
ΣPCDD, PCDF, *dl*-PCB (ng WHO-TEQ/kg d.w.)	7.7	24.8	5.8	13.1
ΣPCB (mg/kg d.w.)	0.00997	0.0185	0.00657	0.00907
ΣDDx (mg/kg d.w.)	0.146	0.0045	0.151	0.0036

316

317 **(b)**

	CTL	SS1 1/100	SS1 1/300	HC1 1/100	HC1 1/300	SS2 1/100	SS2 1/300	HC2 1/100	HC2 1/300
Al (mg/L)	0.0138	0.0251	0.0193	0.0243	0.0138	0.0249	0.0204	0.0133	0.0115
As (mg/L)	0.0093	0.079	0.052	0.0203	0.0122	0.0179	0.0119	0.007	0.0087
Ba (mg/L)	0.0178	0.0055	0.0045	0.004	0.0026	0.007	0.0041	0.0093	0.0048
Be (mg/L)	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005
B (mg/L)	1.05	0.42	0.43	0.53	0.49	0.52	0.414	0.94	1.73
Cd (mg/L)	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005	< 0.0005

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

3.3 Larval Toxicity of SC and HC samples

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

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

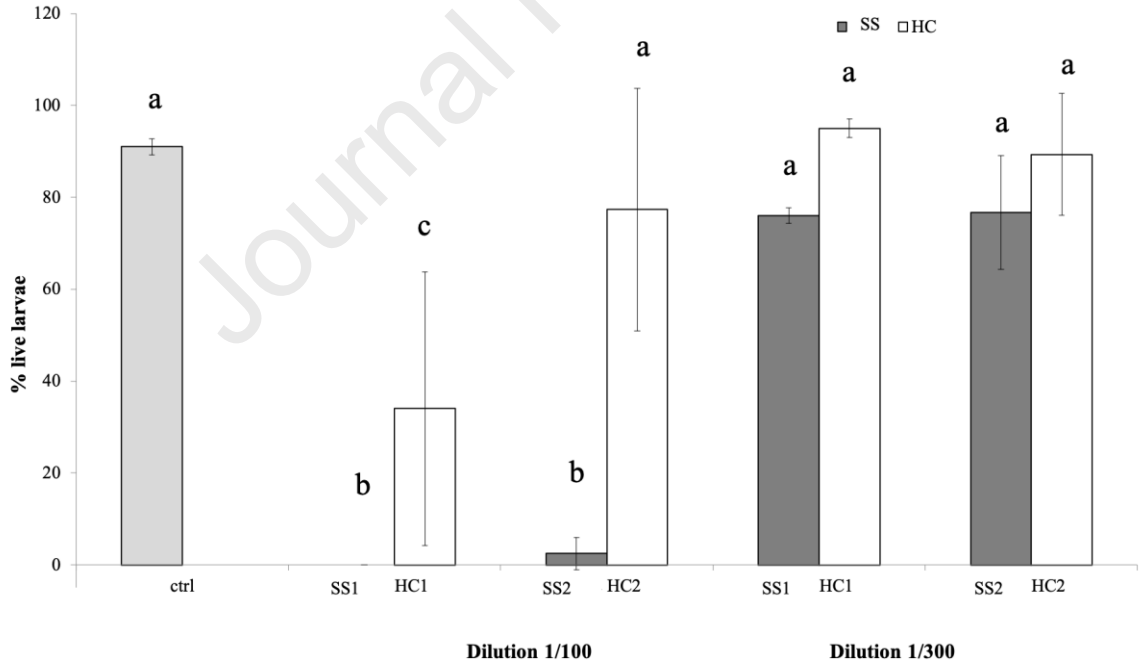

Fig. 1 Mortality rate (shown as % of live larvae) of zebrafish larvae exposed to SSs and HCs at (1/100 and 1/300) for 96 h. Results are presented as means ± standard dev of three independent experiments. Different letters mean statistically significant differences among exposure groups (one-way ANOVA,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

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).

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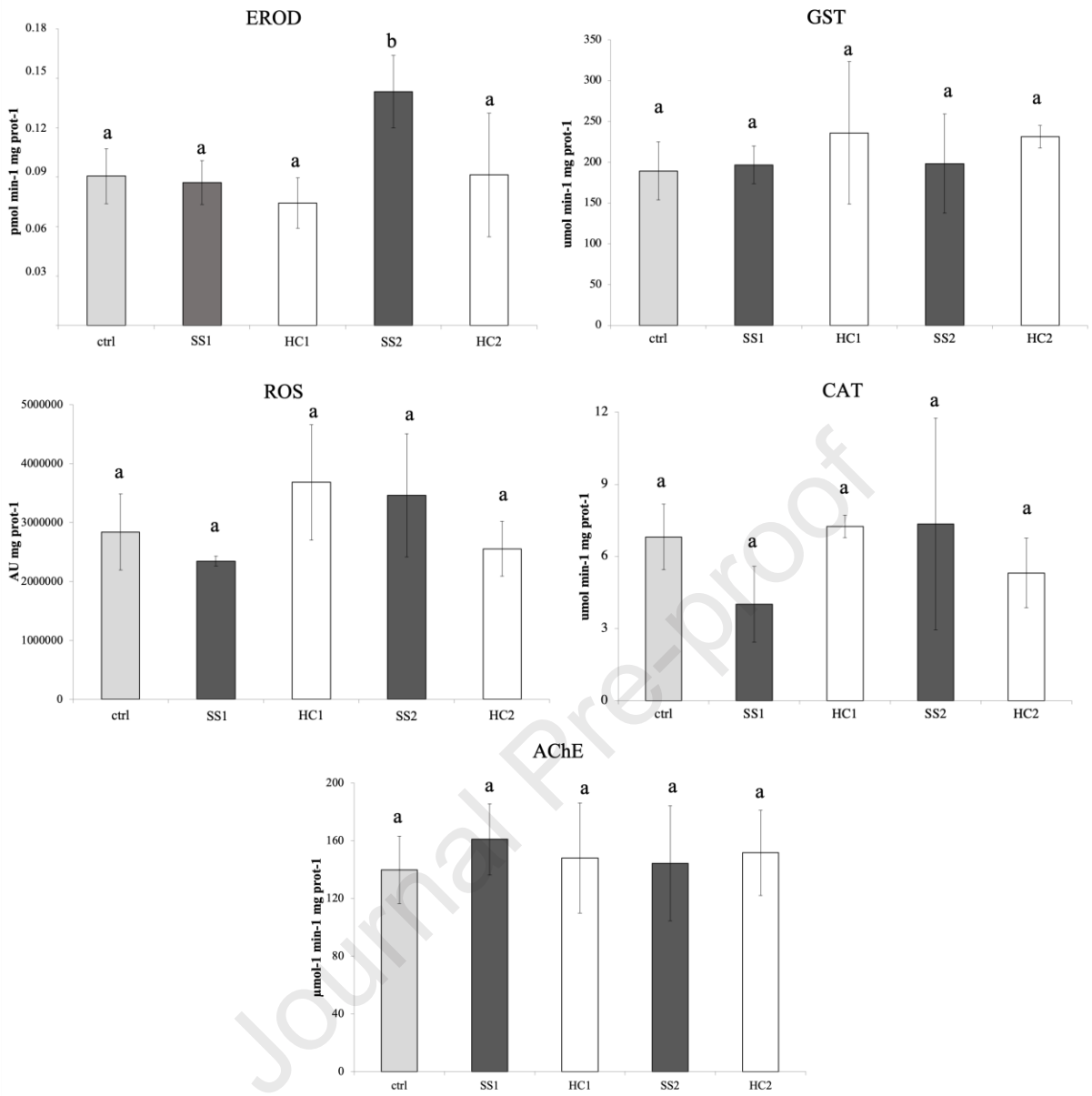

Figure 2 Biomarkers measured in zebrafish larvae exposed to SSs and HCs at 1/300 for 96 h. Results are presented as means ± standard dev of three independent experiments. Different letters mean statistically significant differences among exposure groups (one-way ANOVA, LST post-hoc test $p < 0.05$).

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

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

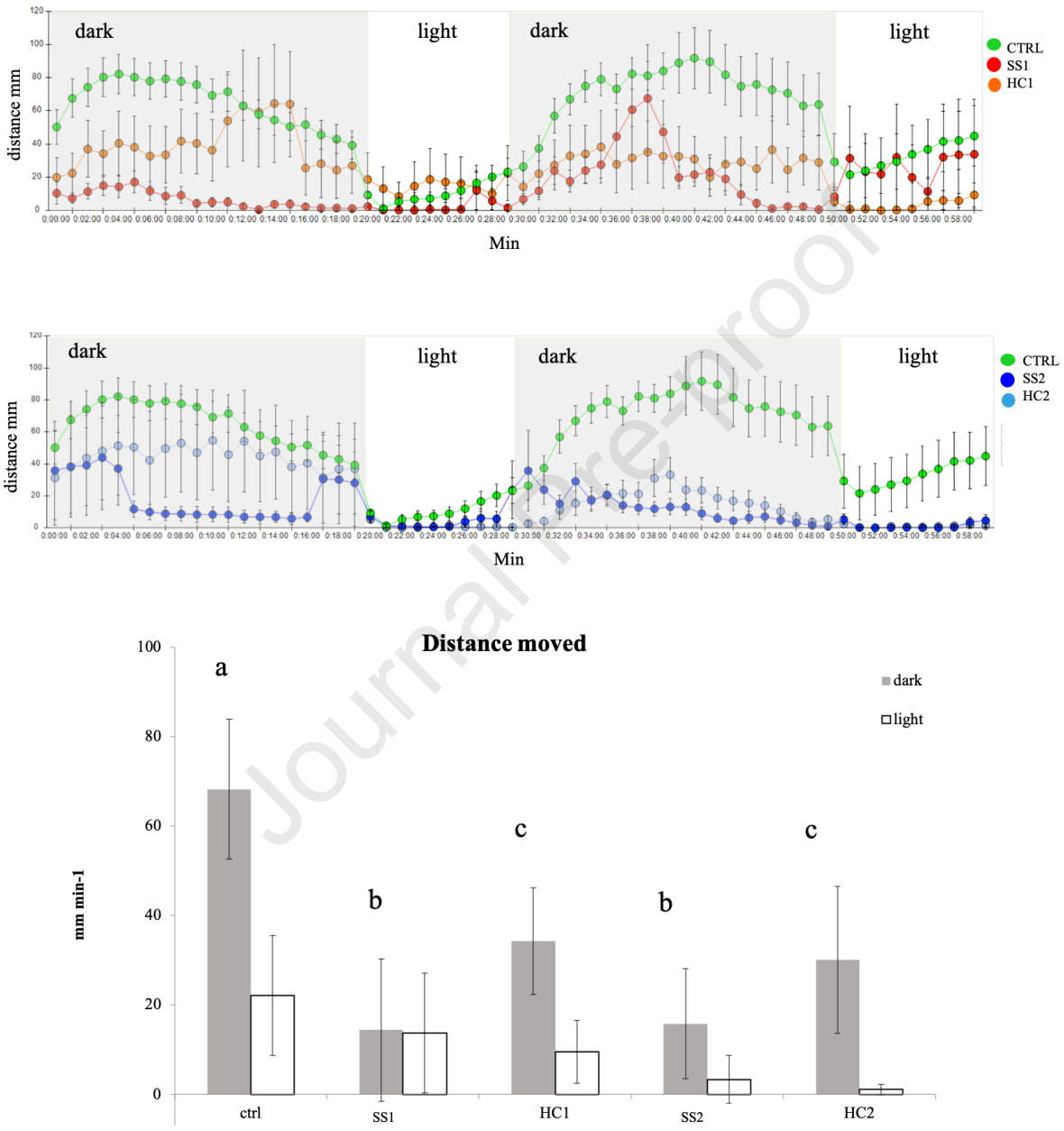

Figure 3. Behavioural effects in zebrafish larvae exposed to SSs and HC at dilution 1/300 for 96 h, measured as distance moved per minute during the dark/light transition test. Results are presented as means ± standard dev of three independent experiments. Different letters mean statistically significant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

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

The alteration of swimming performance could also be due to the presence of neuroactive drugs such 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

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

4. Conclusions

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

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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

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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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