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Detoxification of sewage sludge by natural attenuation and implications for its use as a fertilizer on agricultural soils

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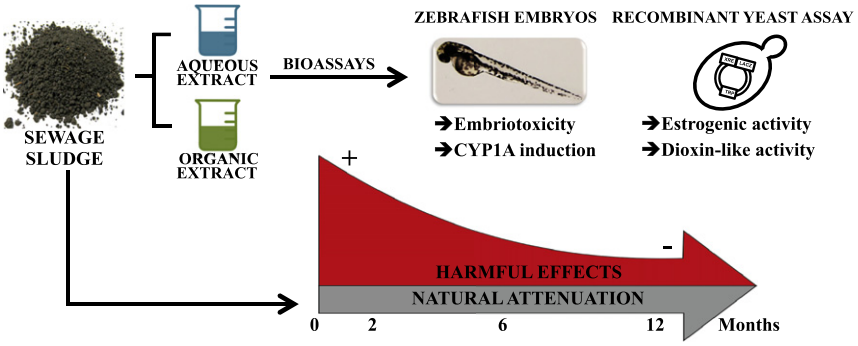
HIGHLIGHTS

- Efficiency of natural attenuation of sewage sludge performed by biological assays.
- Initial samples showed high dioxin-like activity and embryotoxicity in zebrafish.
- SS soil disposal can impact the groundwater by the presence of hydrophilic compounds.
- All the studied effects of SS significantly decreased after six months of attenuation.
- Sewage Sludge requires further decontamination before disposal into the environment.

GRAPHICAL ABSTRACT



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ABSTRACT

Sewage Sludges (SS) from wastewater treatment systems constitute a potential alternative to agricultural fertilizers. However, their use is limited by the presence of toxic substances that may represent significant hazards for the environment and for human health. To test the potential of natural processes to attenuate their putative toxic activities, actual SS samples from domestic sewage were buried in holes in a pollution-free environment for different periods of time, up to one year. Aqueous and organic extracts were obtained after each period of natural attenuation, and their respective toxicity was tested for estrogenic and dioxin-like activity by yeast-based bioassays (ER-RYA and AhR-RYA, respectively) and for general toxicity and teratogenicity in zebrafish embryos. Dioxin-like activity was also tested in zebrafish embryos by monitoring the induction of the marker gene *cyp1a*. Whereas the results showed essentially no estrogenic activity, both dioxin-like activity and embryotoxicity were observed in the initial samples, decreasing significantly after six months of attenuation. Chemical analysis of toxic SS samples showed the presence of low levels of dioxins and furans, and relatively high levels of *m*- and *p*-cresol, at concentrations that only partially justify the observed biological effects. Our data indicates the presence of largely uncharacterized hydrophilic compounds with high biological activity in SS, constituting a potential risk of groundwater pollution upon their disposal into the environment. It also shows that this potential impact may be significantly mitigated by attenuation protocols, as the one presented here.

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1. Introduction

The management of urban residues is a major environmental concern worldwide whose magnitude only tends to increase year after year (Zotos et al., 2009). Sewage sludge (SS) is one of the most worrying components of total waste, as it is generated in large quantities during wastewater treatment processes (Pathak et al., 2009). On the other hand, SS contains high concentrations of organic matter that can be used as a source of nutrients in agricultural soils, an application that has been considered one of the best alternatives for its disposal (Rathod et al., 2009; Zuloaga et al., 2013). However, the accumulation in SS of a wide variety of undesirable organic and inorganic contaminants, as well as of pathogenic microorganisms, represents a limitation for their valorization as an agricultural fertilizer (Urase and Kikuta, 2005; Gibson et al., 2005; Harrison et al., 2006).

Many countries have been encouraging the reuse of sewage sludge in agricultural lands. Nevertheless, to ensure its stability and to prevent harmful effects on the environment, limit values for concentrations of potentially toxic inorganic and organic substances (e.g. heavy metals, policyclic aromatic hydrocarbons, halogenated organic compounds, linear alkyl benzene sulphonates) as well as pathogens in sludge were established. Thus, the disposal of SS in soil must be performed in accordance with the legislation prescribed in each country, for example Directive 86/278/EEC in Europe, Regulation 40 CFR Part 503 in USA and Resolution 375 of CONAMA in Brazil.

Among environmental contaminants, emerging pollutants and endocrine disruptors are attracting much attention due to their high biological activity, usually linked to their specific interaction with vital targets in the living cells. For example, endocrine disruptors, substances able to interfere in the functioning of the endocrine system are of special concern when related to environmental contamination by SS (Giudice and Young, 2011). Among them, natural and synthetic compounds able to interact with the estrogen receptor (ER) at low concentrations may compromise fertility and reproduction of exposed animals, representing a potential risk for human populations (Fatta-Kassinos et al., 2011). Another important class of substances that can be found in SS is dioxin-like compounds, which are capable of binding to and activate the Aryl hydrocarbon Receptor (AhR) (Engwall and Hjelm, 2000). Activation of AhR constitutes the initial step of the metabolic chain leading to toxic effects of a variety of harmful pollutants, such as 2,3,7,8-tetrachlorodibenzo-(*p*)-dioxin (TCDD), co-planar PCBs and benzopyrenes. These effects include immune dysfunction, endocrine disruption, reproductive toxicity, developmental defects, and cancer in vertebrates (Nebert et al., 1993). The interaction of pollutants with both kinds of receptors can be monitored by a variety of single cell assays (Noguerol et al., 2006a, 2006b). In this work we used two yeast-based bioassays, in which yeast strains were genetically modified to reproduce the vertebrate signaling response to either ER- or AhR-ligands. These yeast-based assays, also known as RYA (Recombinant Yeast Assay), have become common tools to detect ER- and AhR-binding activities in a variety of samples and matrices (Noguerol et al., 2006a, 2006b).

Besides being a direct source of soil contamination, SS disposal also contributes to the spread of pollutants in the aquatic environment, mainly by runoff or leaching, potentially affecting aquatic organisms (Eriksen et al., 2009). Zebrafish embryo assays have been widely used for the evaluation of toxicity of environmental samples related to aquatic contamination, due to the high sensitivity of these organisms to xenobiotics (Hallare et al., 2005). In addition, zebrafish embryos can be used to monitor specific toxic responses by the analysis of the induction of pollutant- or stress-related genes. A particularly useful marker is the cytochrome P450 1A gene, CYP1A, whose expression increases in zebrafish embryos (and adults) upon exposure to AhR ligands (Goksoyr and Forlin, 1992; Mcclain et al., 2003; Voelker et al., 2007; Olivares et al., 2013).

A sustainable use of SS will require further decontamination steps before its disposal into the environment (Domene et al., 2008; Ramírez et al., 2008; Rathod et al., 2009; Tas, 2010; Roig et al., 2012). Among the processes indicated for bioremediation of environmental pollutants, natural attenuation (including weathering) is considered an effective and inexpensive pre-processing practice, although its efficiency for removing some recalcitrant, highly bioactive pollutants needs to be examined (Bhupathiraju et al., 2002; Mills et al., 2003). In this context, this study aimed to evaluate the potentially toxic biological activities of SS before and after different periods of natural attenuation in order to verify the effectiveness of this process in promoting a decay of the toxic potential of SS. For this, different endpoints (embryotoxicity, estrogenicity, dioxin-like activities and activation of the CYP1A) were used.

2. Material and methods

2.1. Sludge samples

Samples of anaerobic SS dewatered by centrifugation were collected from the Jardim das Flores wastewater treatment plant (WWTP), located in Rio Claro, São Paulo, Brazil. SS samples were taken as composite samples from different depth of a sludge pile (constituted by 1–2 days production) in two independent sampling campaigns, with an interval of 15 days each.

After sampling, an amount of 8 kg of SS were placed in perforated plastic bags (holes of 0.5 mm diameter and spacing of 1 cm between them) and buried in holes prepared in an environment free of contamination, following the protocol described by Mazzeo et al. (2015). The samples remained buried for periods of 2, 6 and 12 months, in order to allow possible detoxification of the SS by natural attenuation. The periods chosen to perform the attenuation process was based on a previous pilot study, where it was observed that about 2 months of natural attenuation was the minimum period to note any alteration in the toxicity of the samples. The period of 12 months was chosen because it was considered the maximum period for the application of this process becomes feasible to be applied by the WWTP due to the high sludge produced daily amount.

The experiment was conducted in duplicate, analyzing 2 bags for each period of time.

The chemical analyses of the aqueous SS extracts were performed at the laboratory of the Global Analysis and Consultancy (São Carlos—Brazil) following the method proposed by Opeolu et al. (2010), using high performance liquid chromatography with diode array detection (HPLC-DAD). The chemical analyses of the organic SS extracts were carried out at the laboratory of the Analytical Technology Company (São Paulo—Brazil). The analyses were conducted according to the procedure described by USEPA SW-846 (1999). High concentration of *m*- and *p*-cresol and low concentrations of dioxins and furans in these extracts have been reported for these samples (Mazzeo et al. (2015); see Supplementary Table S1).

2.2. Processing and preparation of SS extracts

After each period of natural attenuation, the SS samples were freeze-dried, sieved in an 80 mm sieve and kept in glass flasks, in darkness, at −20 °C, until use.

The protocol NBR10.006 (ABNT, 2004), which is indicated to evaluate the presence of hydrosoluble substances, was used to prepare the aqueous extracts. In individual recipients, 1000 mL of ultrapure water were mixed with 125 g of each freeze-dried sample. After 7 days, the supernatant was collected and filtered in a 0.45 μm membrane.

Organic extracts from SS samples that had undergone 0, 2, 6 and 12 months of natural attenuation were obtained by the Soxhlet method, following the protocol 3540C USEPA (2008). Aliquots of 10 g of each freeze-dried sample were placed in individual cellulose thimbles

covered with glass wool. Extraction was performed using 250 mL of acetone and dichloromethane, in a proportion of 1:1 (v/v), for a period of 18 h. The extracted samples were evaporated first in a rotary evaporator (38 °C and −200/300 mmHg) (down to a volume of 2 mL) and then, in a gentle nitrogen gas flow. The solid residue was resuspended in enough volume of dimethyl sulfoxide (DMSO) to allow the solubilization of all soluble parts. Final extracts were adjusted to contain the equivalent to 0.5 g of the original SS per mL.

2.3. Recombinant yeast assay (RYA)

Two yeast-based bioassays, ER-RYA and AhR-RYA, were employed to analyze the biological activity of the samples. Estrogenic activity was evaluated using yeast strain BY4741, which contains the plasmids pH5HEO (hER) and pVitBX2 (ERE-LacZ). Activity due to interaction with AhR was assessed using the YCM4 yeast strain (Miller, 1997), which presents two chromosomally integrated constructs, one that express the genes AhR and ARNT under the Gal1-10 promoter, and the pDRE23-Z reporter (XRE5-CYC1-LacZ). The RYA assay was performed following the protocol proposed by Noguerol et al. (2006a, 2006b). Briefly, yeast cells were grown overnight at 30 °C in minimal medium (6.7 g/L yeast nitrogen base without amino acids, DIFCO, Basel, Switzerland, supplemented with 0.1 g/L of prototrophic markers as required). The culture was adjusted to an optical density (OD) of 0.1, and split into 100 μL aliquots in 96-well polypropylene microtiter plates (NUNC, Roskilde, Denmark; ref. 40) previously silylated by overnight exposure to an atmosphere of dimethylsilane. A serial dilution scheme was performed, by dispensing 10 μL of sample into wells of the first column (which contained 190 μL of culture) and sequentially transferring 50 μL from the previous well to the next one (1:3 dilutions). Positive (0.1 μL of 10 μM 17-β-estradiol for ER-RYA and 1 μL of 100 μM β-naphthoflavone for AhR-RYA), negative (5% methanol) and toxicity controls were also included in each plate. Samples were tested in triplicate.

Plates were incubated for 6 h at 30 °C under mild shaking. After incubation, 100 mL of YPER (PIERCE, Rockford, IL, USA) were added to each well and further incubated at 30 °C for 30 min. Then, 100 μL of MuGal solution (0.1% of 2-mercaptoethanol and 0.5% of 4-methylumbellifoerone β-D-galactopiranoside) were added in each well. After centrifugation, plates were read in a Synergy 2 spectrofluorometer (BioTek, USA) for 15 min, with an excitation filter of 355 nm and an emission filter of 460 nm. The values of β-galactosidase activity were calculated as rates of the increment in arbitrary fluorescence units with time, using standard linear regression methods.

2.3.1. Calculation of equivalent concentrations

Equivalent concentrations are defined as the reference ligand concentration (17-β-estradiol for ER or β-naphthoflavone [BNF] for AhR) required to elicit the same response as the sample in the RYA assay. Assuming that the dose-response curve follows a sigmoidal pattern, it is possible to calculate the dilution at which a certain sample elicits 50% of the maximal response of the assay (EC50 values). These values were converted to estradiol or BNF equivalents assuming a conversion value of 0.267 nM 17-β-estradiol for ER-RYA and of 47 nM BNF for AhR-RYA (Noguerol, 2006b).

2.4. Assay with D. rerio embryo

2.4.1. Maintenance of adults and embryos

D. rerio adults were maintained in tanks with reconstituted water (Instant Ocean 90 mg/L; 0.58 M $CaSO_4 \cdot 2H_2O$ in ultrapure water) at 27 °C ± 1.5 °C and a photoperiod of 12 h. One day before performing the assay, 5 females and 3 males were grouped for mating. After egg laying, which occurred in the early hours of light after grouping, viable eggs were transferred to a recipient containing reconstituted water and maintained under the same conditions as the adults.

2.4.2. Toxicity test with D. rerio embryos (FET)

In order to determine the non-toxic concentrations to be used in the gene expression analyses, an embryotoxicity test was performed with 24 hpf (hours post fertilization) embryos. Individuals were distributed in proportions of 1 embryo per well (24-well microtiter plate), containing 2 mL of the tested sample. Mortality of individuals, delay in hatching, and deformities (heart or yolk sac edemas, tail malformation, differences in pigmentation pattern and lack of movement) was scored after 48 h of exposure. Each assay was performed in duplicate, keeping a negative control line per plate (Nagel, 2002).

Organic extracts were tested in concentrations of 1:500, 1:1500 and 1:5000. Solubilized extracts were tested in concentration of 25%, 5% and 2%. Moreover, *m*-cresol and *p*-cresol, which, according to chemical analysis, were the major contaminants present in the SS, were also tested in several concentrations from 1 to 250 mg/L. All solutions were prepared with reconstituted water, in order to preserve the same concentration of salts as in the tanks. A negative control was performed with 0.2% DMSO, for the extracts obtained by Soxhlet, and with reconstituted water, for the solubilized extracts and cresols.

2.4.3. CYP1A gene expression assay

Alterations in the *cyp1a* gene expression pattern were studied following the protocol of Olivares et al. (2013). Healthy 24 hpf embryos were selected and distributed in 3-cm-diameter glass Petri dishes, each dish containing 10 embryos in 2.5 mL of sample, being 5 dishes per sample. Negative controls consisted in 0.2% DMSO for organic extracts, and in reconstituted water for the aqueous extracts. Positive controls were carried out with 250 μg/L β-naphthoflavone. After 48 h, the solution was removed and the embryos were immediately frozen and kept at −80 °C until being processed.

2.4.3.1. RNA extraction. Total RNA extraction from 20 to 25 frozen embryos was performed using Trizol® (Invitrogen, Eugene), following the manufacturer's protocol. Total RNA concentration was measured by absorption spectrophotometry at 260 nm, in a NanoDrop ND-8000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA was treated with DNAse I (Ambion, Austin, Texas, USA), to remove contamination by genomic DNA. Treated RNA was reverse-transcribed to cDNA using Transcriptor First Strand cDNA Synthesis (F Hoffmann-La Roche, Basel, Switzerland) and stored at −20 °C. Specific transcripts were quantified in a LightCycler 480 Real-Time PCR System (F. Hoffmann-La Roche), using SYBR® Green Mix (F. Hoffmann-La Roche).

The primer pair of *D. rerio cyp1a* gene (GeneBank access number AB 078927) was Fw-GGTTAAAGTTCACCGGGATGC; Rv-CTGTGGTGTGACC CGAAGAAG. The *ppia2* gene (GeneBank access number BC100002) (Fw-GGGTGGTAATGGAGCTGAGA; Rv-AATGGACTTGCCACCAGTTC) was used as reference gene.

The relative abundances of mRNA were calculated from the second derivative maximum of their amplification curve (Cp, calculated in triplicate). Cp values for the target gene (*cyp1a*) were compared to the corresponding values for the reference gene (*ppia2*) to obtain ΔCp and these were used to calculate mRNA levels, expressed as the number of copies of CYP1A by 1000 copies of the reference gene ($1000 \times 2^{\Delta Cp}$).

3. Results

3.1. Recombinant yeast assay (RYA)

Yeast-based assays showed significant dioxin-like activity in essentially all aqueous and organic extracts, whereas estrogenic activities were very weak or undetectable. However, the correct detection of any putative weak estrogenic response present in organic extracts was precluded by the strong toxicity for yeast of these samples (not shown, see Table 1), which limited the sensitivity of the assay. The observed dioxin-like activity of organic extracts appeared resilient to natural attenuation, as it was only significantly reduced after 12 months

of remediation time (Table 1). In fact, values for samples taken after 2 or 6 months of attenuation showed dioxin-like activities higher than the original sample (see Table 1). The water-soluble dioxin-like activity also showed a significant increase after the 2-month attenuation period, but it decayed to essentially undetectable levels after 6 months. Although the initial weak levels make it difficult to evaluate the effects of natural attenuation on the estrogenic activity, the results suggest a decrease on water-soluble estrogenic activity during the remediation period (Table 1).

3.2. Assays with D. rerio embryos

Zebrafish toxicity assays showed a strong toxic effect of organic extracts (Fig. 1), in line with the yeast toxicity data, whereas the toxicity values of aqueous extracts were about an order of magnitude lower. Toxic effects included inability to hatch, deformities and death (Fig. 2). Toxicity of both types of extracts decreased throughout the period of SS disposal, although organic extracts from the 2-month period and aqueous extract from the 6-month still presented significant toxicity (Fig. 1).

Chemical characterization of original SS samples, and of their corresponding aqueous extracts, detected the presence of significant amounts of *m*-cresol and *p*-cresol (Supplementary Table S1). Since these compounds are recognized as toxic for zebrafish, they were tested by the zebrafish embryo toxicity test (Fig. 3). EC_{50} values for embryo toxicity were about four times lower for *p*-cresol ($EC_{50} \approx 10$ mg/L, deformities) than for *m*-cresol (Fig. 3). Since the highest concentrations actually found in SS extracts were clearly below those EC_{50} values (e.g. 4.21 mg/L in aqueous extract; see Supplementary Table S1), we consider that both compounds can only represent a minor contribution to the total toxicity of the samples, which reached 100% mortality in some cases (Fig. 1). Moreover, a typical phenotypic effect of cresol toxicity, embryo depigmentation, was not observed when the embryos were exposed to SS extracts (Fig. 2F).

All tested samples induced expression of CYP1A in fish embryos, indicating the presence of AhR ligands (aka, dioxin-like compounds) even after a long time of natural attenuation (Fig. 4). Organic extracts were more active than aqueous ones, as observed in embryo toxicity tests and RYA assays. Induction of *cyp1a* due to exposition to aqueous extracts decayed significantly after a 2-month remediation period. In contrast, the organic extract strongly induced cyp1a expression even after 12 months of natural attenuation, indicating the presence of resilient compounds with potentially harmful biological activity.

4. Discussion

SS is a highly complex residue that may contain a wide diversity of chemical compounds potentially harmful for the environment. Therefore, the suitability of a particular SS for agricultural use requires a combination of chemical analysis and bioassays (Kapanen et al., 2013). In this work we evaluated four potential biological activities, both in the original sludge and in naturally attenuated samples: embryotoxicity, estrogenicity and dioxin-like activity in yeast-based bioassays, and cyp1a induction in fish embryos. These four activities correspond to well-known endpoints for SS toxicity.

Estrogenic substances are of special concern among the contaminants present in SS, and its removal by wastewater treatment is sometimes problematic (Urase and Kikuta, 2005). The SS samples used in this work showed very low, if any, estrogenic activity, and even this weak activity became negligible after 6 or 12 months of natural attenuation. High estrogenic activity in SS samples has been linked to the presence of alkylphenols and other estrogenic substances (Murk et al., 2002; Lorenzen et al., 2004; Fernandez et al., 2009), which are likely absent or in low levels in the sludge samples studied here. In any case, and only considering the few estrogenic-positive samples, the reduction of activity shown by natural attenuation is comparable to the one obtained with active (though shorter) biological treatments in SS (e.g., Badia-Fabregat et al., 2012).

Compounds capable of binding to the AhR represent another important group of toxic pollutants, which may also be present in SS (Engwall and Hjelm, 2000). These contaminants are referred as compounds with dioxin-like activity and comprise three main groups: a) hydrophobic aromatic compounds whose planar structure and molecular size favors a perfect binding to the AhR (e.g., non-ortho polychlorinated biphenyls, polychlorinated dibenzodioxins, polychlorinated dibenzofurans); b) compounds with specific stereochemical configuration and alkylated analogs (e.g., polyhalogenated and mixed halogenated anthracenes, fluorenes, xanthones); and c) weak ligands, which do not exhibit planarity, aromaticity and hydrophobicity characteristics, but are easily degraded by detoxification enzymes (e.g., indole, heterocyclic amines, some pesticides) (Hilscherova et al., 2000). The presence of these compounds in SS has been consistently reported in several studies (Engwall et al., 1999; Sánchez-Brunete et al., 2007; Zhang et al., 2009; Clarke and Smith, 2011; Badia-Fabregat et al., 2012; Cincinelli et al., 2012; Kapanen et al., 2013). Whereas activation of ethoxyresorufin-O-deethylase (EROD) activity either in liver cells — either *in toto* or *in culture* (Engwall et al., 1999) — has been the customary procedure to analyze this activity, specific transgenic systems based either in mammalian cells, e.g., the CALUX system (Zhang et al., 2009), or in recombinant yeast strains (RYA – Badia-Fabregat et al., 2012), are the methods of choice in analyzing this kind of biological activity in SS samples.

Dioxin-like activity was present in all tested samples, detected both in the yeast AhR-RYA system and in the induction of CYP1A in fish embryos (Voelker et al., 2007). This activity showed a significant reduction after 6 months of natural attenuation, suggesting that the implicated compounds were relatively recalcitrant to the natural processes. In fact, a substantial part of the CYP1A-inducing activity (some 20%) was still present after 12 months of attenuation. In any case, this longer attenuation process was as efficient as other reported biological treatments (Badia-Fabregat et al., 2012; Kapanen et al., 2013). A consistent feature of the attenuation procedure is an apparent increase of dioxin-like activity after the first two months of treatment. The likely explanation is that many dioxin-like compounds, particularly the most hydrophobic ones, may undergo a process of bioactivation that may occur during the early stages of the treatment (Badia-Fabregat et al., 2012). However, an increase of the toxicity of SS after their composting can also be related to the formation of toxic metabolites derived from microbial degradation of organic substances present in the initial sample (Oleszczuk, 2008).

D. rerio embryos are commonly used in embryotoxicity tests, since their eggs remain transparent from the fertilization to the pharyngula phase, which facilitates the observation of the major morphological changes during early stages of development (Selderslaghs et al., 2009). Our results show consistent embryotoxicity in the initial SS extracts, particularly in the organic ones, which decrease after 6 months

Table 1
Estrogenic and dioxin-like activity of extracts from SS samples after different periods of natural attenuation.

Extract	Natural attenuation period (months)	Estrogenic activity (ER-RYA)[a]	Dioxin-like activity (AhR-RYA)[b]
Organic	0	Toxic[c]	7.8 (5.8–10.3)
	2	Toxic[c]	21.7 (18.1–26.0)
	6	Toxic[c]	16.0 (13.7–18.3)
	12	Toxic[c]	4.5 (3.4–5.9)
Aqueous	0	5.3 (3.3–8.3)	4.3 (3.3–5.4)
	2	3.15 (2.4–4.1)	17.6 (12.5–25.6)
	6	n.d.	0.95 (0.71–1.28)
	12	n.d.	n.d.

n.d.: not detected.
[a] Expressed as ng of estradiol equivalents per g of SS (95% confidence margins).
[b] Expressed as μg of BNF equivalents per g of SS (95% confidence margins).
[c] Exceeded the limit of detection (cytotoxic).

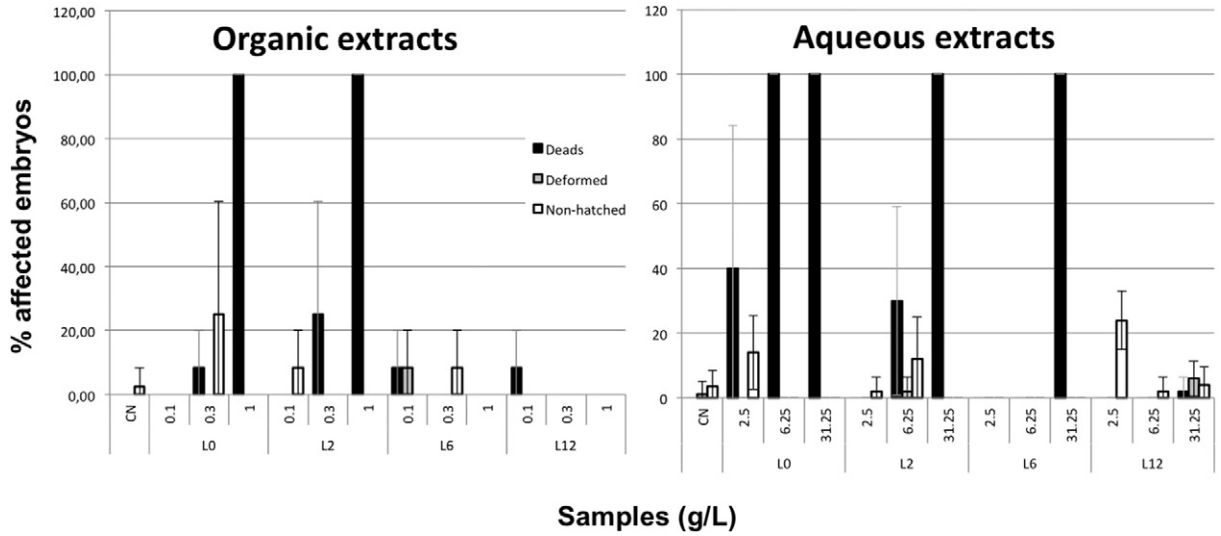

Fig. 1. Embryotoxic effect observed in the test organism *D. rerio* exposed to different amounts of sewage sludge extracts. The different doses are represented as their equivalences in g/L of the original sludge samples.

of natural attenuation. This pattern is similar to the one observed for dioxin-like activity, and suggests that the embryotoxic compounds are hydrophobic, as previously proposed (Burton, 1991; Gustavsson et al., 2007). SS is shown to be toxic for a variety of fish species, both marine and freshwater, as well as for amphibians; therefore, the release of the untreated material may represent a significant risk for the aquatic ecosystems (Costello and Gamble, 1992; Chenon et al., 2003; Gustavsson et al., 2007).

The chemical analysis revealed *m*- and *p*-cresol as major pollutants in the initial SS. Whereas both compounds are strong embryotoxins, their concentrations in the extracts seem too low to play any significant role on the overall toxicity of the samples, not to mention the absence of the decolored phenotype characteristic of their action. Neither isomer has the structural characteristics required for binding to the AhR, nor are they capable of inducing CYP1A expression in vertebrates (Letcher et al., 2005). Similarly, the levels of dioxins (0.25–1 ng/L) and furans (0.07–0.14 ng/L, Supplementary Fig. S1) present in the zebrafish toxicity assays were probably too low to account for the observed morphological and transcriptional effects. These observed concentration ranges are at least two orders of magnitude lower than the EC50 value for CYP1

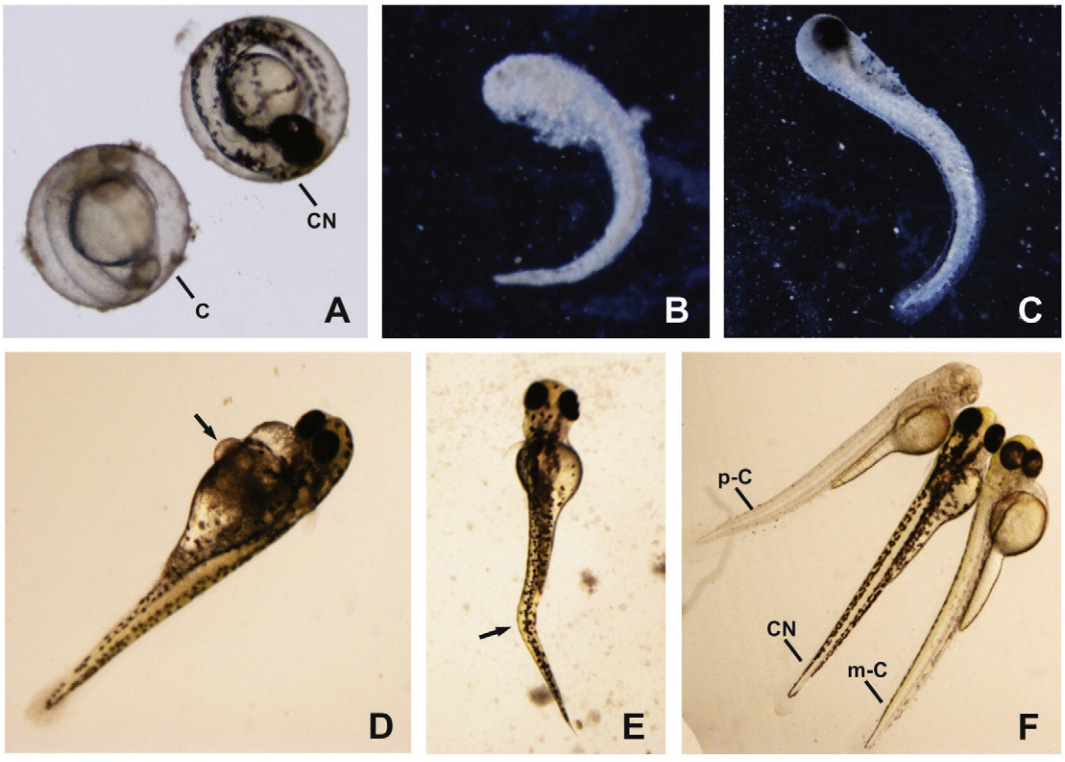

Fig. 2. Alterations observed in *D. rerio* embryo-larval stages after exposure to SS aqueous extracts, *m*-cresol and *p*-cresol. **A.** 48 h embryos with difference in pigmentation pattern induced by exposure to cresol (depigmented embryo); **B.** and **C.** Dead individuals as a result of exposure to SS and cresol; **D.** Eleutheroembryo presenting yolk sac edema induced by the action of SS and cresol (arrow); **E.** Eleuthero embryo with tail malformation induced by the action of SS and cresol (arrow); **F.** Eleuthero embryos of the same age with different patterns of pigmentation. NC: Negative control - Normal pigmentation pattern; C: cresol - change in pigmentation pattern (depigmentation); m-C: depigmentation induced by *m*-cresol; p-C: depigmentation induced by *p*-cresol.

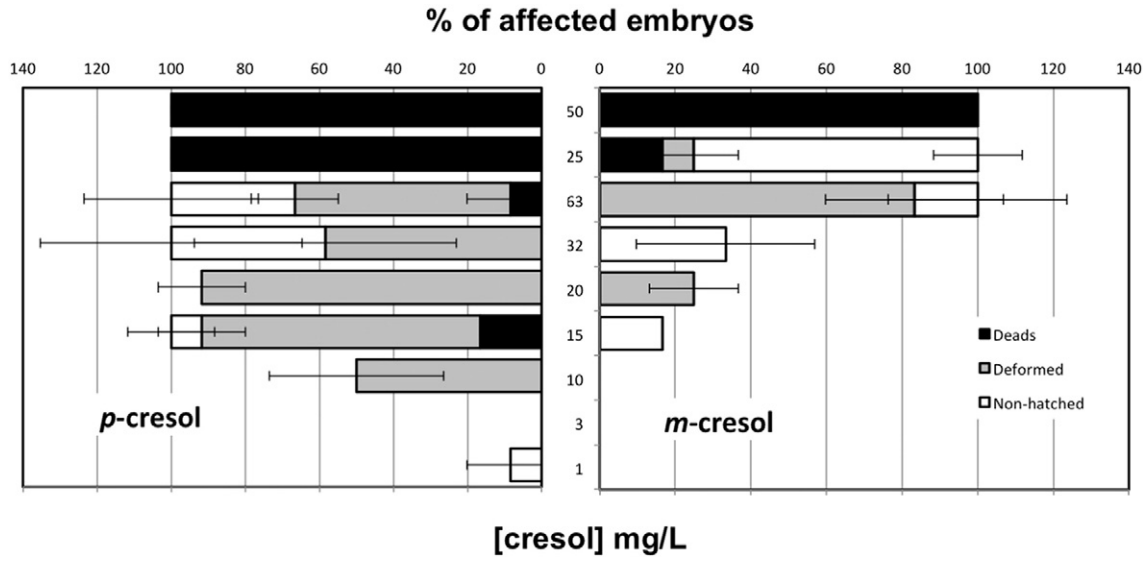

Fig. 3. Embryotoxic effect observed for the test organism *D. rerio*, exposed to different concentrations of *m*- and *p*-cresol.

activation (64 ng/L, Seok et al., 2008) reported for the most powerful dioxin congener (2,3,7,8-TCDD), whereas the corresponding LC50 value for embryo toxicity is some 25 times even higher (17 μg/L, Seok et al., 2008). In addition, our data indicate an increase, rather than a decrease, in the amount of dioxins and furans present in the samples, even though the biological effects were significantly reduced after the longer periods of natural attenuation of SS (Supplementary Fig. S1). This is consistent with the already reported presence of these substances in domestic and industrial effluents, even after standard sewage treatment (Horstmann and Mclachlan, 1995). Taken together, our results suggest the presence of other unknown compounds that contributed significantly to the observed biological effects. Additionally, since SS is a complex matrix, different effects (antagonistic, additive or synergistic) may be occurring due to chemical interactions between different substances present in it (Oleszczuk, 2008).

In general, both aqueous and organic extracts showed similar responses to the different evaluated parameters, i.e., elevated values for early disposal of the SS and subsequent reduction along the increase in the period of natural attenuation. In the aqueous extracts, the presence of compounds capable of binding to AhR or provoking toxic effects indicates that the disposal of SS in soil may be responsible for the dispersion of these pollutants into groundwater, since water soluble compounds can percolate into the deeper layers by rainwater entrainment. According to Tewari et al. (2005), urban SS leachates constitute heterogeneous mixtures that may contain metals as well as a wide variety of organic substances, which, even in very small concentrations, may result, by a joint action, in a harmful effect to exposed organisms. Thus, the decay in the SS toxicity could be at least partially due to the leaching of substances during the deposition period in an open environment. However, the decrease of potentially active substances in the organic extract over time suggests a possible degradation of hydrophobic contaminants in raw SS samples, as these compounds are less likely to leach than hydrophilic ones.

Our data stresses the need for identification of emerging compounds in SS before its disposal in the environment, as toxic effects were observed even in the absence of recognized active pollutants, like polycyclic aromatic hydrocarbons, organochlorine pesticides, chlorinated benzenes, phthalate esters, and others, as determined by a thorough chemical analysis. Thus, since SS may contain several different compounds and the chemical identification of all these compounds is not

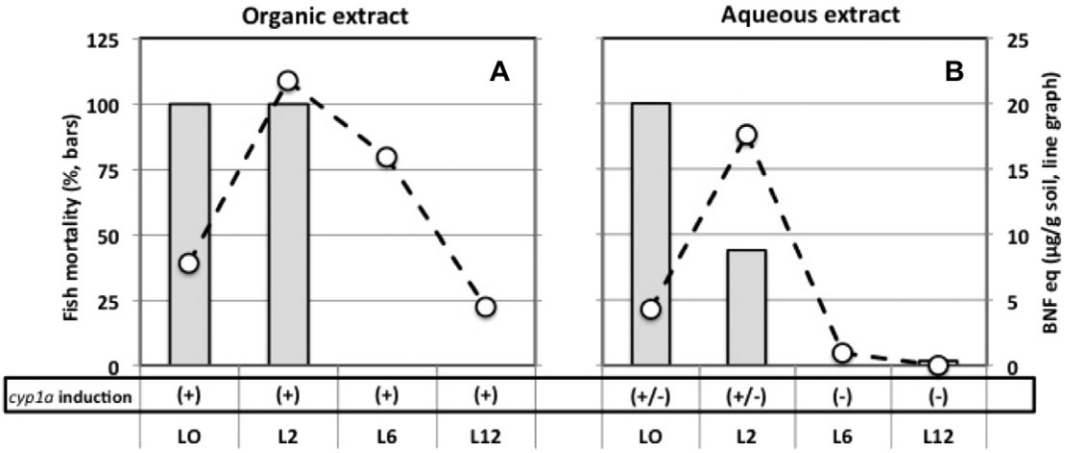

Fig. 4. Mortality and *cyp1a* induction in zebrafish. **A.** Effects of organic extract on mortality (grey bars) of zebrafish embryos, corresponding to 1 g/L of sewage sludge. **B.** Effects of aqueous extract on mortality (grey bars) of zebrafish embryos, corresponding to 6 g/L of sewage sludge. L0 to L12 indicate samples after different times of natural attenuation (L0, initial sample; L2, 2 months; L6, 6 months; L12, 12 months). Animals from the non-toxic exposures were tested for *cyp1a* mRNA induction; the results are shown as symbols on the box below the graphs (+, positive induction; ±, weak induction; −, no observed increase on mRNA levels). For comparison, AhR ligand activity levels on the same extracts are represented as BNF eq (empty circles, data from Table 1).

feasible (Chenon et al., 2003), the use of biological assays for the detection of biologically active substances will help to better define the potential human and environmental risks associated to SS.

5. Conclusion

Due to the different responses of SS observed in the several performed bioassays, it is possible to conclude that the studied SS presents a diverse and complex composition, which can induce harmful effects to exposed organisms. The disposal of SS without treatment to remove its toxic contaminants can directly contribute to soil contamination, as well as to the dispersion of pollutants to surface and groundwater, since the aqueous extract obtained from SS samples showed the presence of substances capable of inducing both embryotoxicity and, at lower doses, ectopic activation of the AhR. Even more pronounced were the responses observed for the organic extract, and the environmental fate of the corresponding active pollutants is presently unclear. The estrogenic activity observed in some aqueous extracts seems to be too weak to constitute a matter of concern, at least for the samples analyzed in this study.

The natural decay method used here for SS detoxification appeared effective in reducing the presence of contaminants with biotoxic effects, although some significant dioxin-like activity remained even after 12 months of treatment. Our data indicates that six months may be a convenient minimum attenuation time, and that this burying protocol constitutes an efficient, low-cost tool for the detoxification process of SS. They also demonstrate that a battery of bioassays for the detection of the toxic potential of SS provides a valuable complement of the data from chemical analysis to evaluate the risks associated to SS.

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The following are the supplementary data related to this article.

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