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Earthworm bioassays and seedling emergence for monitoring toxicity, aging and bioaccumulation of anthropogenic waste indicator compounds in biosolids–amended soil

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ABSTRACT

Land application of biosolids (treated sewage sludge) can be an important route for introducing xenobiotic compounds into terrestrial environments. There is a paucity of available information on the effects of biosolids amendment on terrestrial organisms. In this study, the influence of biosolids and biosolids aging on earthworm (*Eisenia fetida*) reproduction and survival and lettuce (*Lactuca sativa*) seedling emergence was investigated. Earthworms were exposed to soils amended with varying quantities of biosolids (0, 1, 2, 3, or 4% dry mass). To investigate the influence of biosolids aging, the biosolids used in the study were aged for differing lengths of time (2 or 8 weeks) prior to exposure. All of the adult earthworms survived in the biosolids–amended soils at all concentrations that were aged for 2 weeks; however, only 20% of the adults survived in the soil amended with the highest concentration of biosolids and aged for 8 weeks. Reproduction as measured by mean number of juveniles and unhatched cocoons produced per treatment correlated inversely with biosolids concentration, although the effects were generally more pronounced in the 8-week aged biosolids–soil samples. Latent seedling emergence and reduced seedling fitness correlated inversely with biosolids concentration, but these effects were tempered in the 8-week aged *versus* the 2-week aged soil–biosolids mixtures. Anthropogenic waste indicator compounds (AWIs) were measured in the biosolids, biosolids–soil mixtures, and earthworm samples. Where possible, bioaccumulation factors (BAFs) were calculated or estimated. A wide variety of AWIs were detected in the biosolids (51 AWIs) and earthworm samples ($\leq$19 AWI). The earthworms exposed to the 8-week aged biosolids–soil mixtures tended to accumulate greater quantities of AWIs compared to the 2-week aged mixture, suggesting that the bioavailability of some AWIs was enhanced with aging. The BAFs for a given AWI varied with treatment. Notably large BAFs were determined for some AWIs. For example, the maximum BAF determined for para-cresol, methyl salicylate, bisphenol-A, and cholesterol was greater than 100 in some treatments.

1. Introduction

Increased treatment of domestic and industrial wastewaters mandated by the Clean Water Act has resulted in steadily increasing production of sewage sludge and biosolids over the last 35 plus years (USEPA, 1999, 2006; Kinney et al., 2008). For many of those years, a large percentage of the sludge produced during wastewater treatment was dumped directly into the ocean; however, the Ocean Dumping Ban Act of 1988 resulted in a shift toward land application of sewage sludge as biosolids during the 1990s that has continued through the present (NRC, 2002). Treated sewage sludge that meets regulatory standards for pathogen and metal content can be classified as biosolids. Once classified as biosolids, it may be land applied as an organic carbon- and nutrient-rich soil amendment or in land-reclamation projects (USEPA, 2012). In 2006, the United States Environmental Protection Agency (USEPA) estimated that 50% of the 7.3×10^9 kg of sludge produced in the US was land applied as biosolids (USEPA, 2012), whereas Chang et al. (2002) estimated that 37% of the 2.2×10^9 kg of biosolids produced in Europe was land applied.

Although biosolids contain nutrients and organic matter that can enhance soil properties (USEPA, 2012), they also contain varying

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concentrations of trace-elements (metals and metalloids), pathogens, and an array of organic chemicals that may pose a risk to humans and the environment (Witte, 1998; Kummerer, 2004; Kinney et al., 2006a, 2006b; Kidd et al., 2007; Markman et al., 2008; McClellan and Halden, 2010). Unfortunately, the long-term risks associated with land application of biosolids are poorly characterized and, in some cases, difficult to accurately quantify (NRC, 2002). While some research has evaluated the transfer of anthropogenic waste indicators (AWIs) commonly detected in biosolids into fish (Ramirez et al., 2007), plants (Delepee et al., 2004; Boxall et al., 2006; Herklotz et al., 2010; Wu et al., 2010; Shenker et al., 2011), and human foods (Guenther et al., 2002), relatively little is known regarding the bioavailability of AWIs to terrestrial organisms (Kinney et al., 2008).

Recently, earthworms have been found to accumulate AWIs directly from sewage effluent (Markman et al., 2007) and from biosolids applied to agricultural soils (Kinney et al., 2008). This is of concern since earthworms are at the base of the terrestrial food web, and thus biosolids-derived AWIs that accumulate in earthworms can potentially transfer to higher trophic levels within the terrestrial food chain (Harris et al., 2000; Law et al., 2006; Vermeulen et al., 2010). Markman et al. (2008) demonstrated that environmentally relevant concentrations of endocrine disrupting AWIs included in the diet of European starlings (*Sturnus vulgaris*) resulted in physiological and behavioral changes among the male starlings.

Earthworms have been used as a model organism for soil toxicity studies due to their high rates of consumption of organic matter in soil and direct contact with soil constituents, and have been useful in correlating laboratory with field study findings (Environment Canada, 2004). Because of their impact on soil aeration, moisture content, nutrient cycling, and overall soil structure, earthworms are closely linked to the physical and chemical dynamics of terrestrial environments. Field studies have provided inconsistent results with respect to the effects of manure and wastewater end-product applications on earthworm populations (Dindal et al., 1975, 1979; Cotton and Curray, 1980; Richards et al., 2000): in some cases their numbers have decreased, while in others their numbers increased or simply remained the same (Butt, 1999; Baker et al., 2002). This ambiguity likely arises from the significant variation between the applied materials with respect to both nutrient and toxicant contents and concentrations as well as differences in the pedological characteristics of the soils to which the materials were applied.

While field-based assessment of the impact of biosolids is an important tool, spatial variability and the lack of historical records or environmental control can make it difficult to accurately assess the potential impacts of biosolids application on soil-dwelling organisms. To minimize variability, laboratory assays are often employed in order to obtain a clearer picture of the potential harm that can result from exposure. One of the unaddressed issues with traditional laboratory-based earthworm bioassays, however, is the limited equilibration times typically employed. These assays are typically based on standardized procedures that were developed to test the toxicity of individual chemicals in artificial soils (ASTM, 1999) and were never adequately tested for their ability to reveal the effects of complex mixtures entrained in poorly defined matrices like sewage sludge or biosolids.

Weathering and aging have been reported to result in decreased toxicity and bioavailability of many soil-applied chemicals (White et al., 1997; Alexander and Alexander, 1999). The toxicity of some chemicals in soil has occasionally increased, likely due to biotransformations that result in the production of toxic byproducts (Vernile et al., 2007). Furthermore, aging of biosolids might be expected to result in changes in the bioavailability and therefore bioaccumulation of the AWIs when organisms are exposed to the complex mixture of organic matter that entrains the AWI burden. The purpose of this paper is to assess toxicity of biosolids using (1) earthworm survival and reproduction, and (2) a widely used plant bioassay. We also assess the bioaccumulation of AWIs in earthworms from biosolids–amended soils. We further investigate the influence of aging on these effects and processes.

2. Materials and methods

2.1. Soil and biosolids collection and preparation

Biosolids, obtained from Cayuga Heights Sewage Plant (Ithaca, NY), were air-dried for one week at room temperature then blended in an Osterizer blender. After blending, dried biosolids were stored up to two months in a sealed, certified trace chemical free screw-capped glass jar. Field-collected Arkport soil (Ithaca, NY) from the surface layer of uncontaminated agricultural research fields at Cornell University was air-dried, pulverized by hand, sifted through a 2 mm sieve, and stored in an airtight polyethylene container at room temperature according to the same schedule as the biosolids. Characteristics of the Arkport soil have been previously reported (Kim et al., 2011) and are briefly described here. The Arkport soil has a pH of 5.63, contains 4.10% organic matter, total water holding capacity of 40% (w/w), and has an effective cation exchange capacity of 6.28 cmol kg^{-1}. Textural analysis of the soil indicated that it is 2% clay, 35.9% silt, and 62.1% sand.

Fifty-six days before commencing bioassays (T −56), "8-week" aged biosolids–amended soil samples were uniformly mixed in a Twin Shell Dry Blender (V-blender) (The Patterson-Kelley Co. Inc., East Stroudsburg, PA) as 1.5 kg batches each of 0, 1, 2, 3, and 4% biosolids per soil (w/w) on a dry mass basis, which incorporates the typical range of agronomic application rates (USEPA, 2000); hydrated to 30% moisture (w/w) with distilled/deionized (ddH_2O) water; and allowed to age for 56 days at 22 °C in sealed polyethylene containers. Fourteen days prior to conducting the assays (T −14), "2-week" aged samples were mixed with the same amounts of biosolids in an identical fashion and likewise permitted to age.

At 7 days prior to assays (T −7), a 1:1 soil to water ($ddIH_2O$) suspension was made for each batch and pH was recorded with an Orion 2-Star pH meter (Thermo Scientific, Waltham, MA) after 15 min of stirring. Where necessary, pH was adjusted to 6.6 with the addition of calcium carbonate (lime) based on calculations made using the following formula:

$$\text{Lime}_{\text{requirmement}} = \text{EA} \times 0.5 \times \frac{\text{BS}_{\text{original}}}{1-\text{BS}_{\text{original}}} \quad (1)$$

where EA is the exchangeable acidity and BS is the base saturation (Ketterings et al., 2006). EA for Arkport soil was calculated by the Cornell Nutrient Analysis Laboratory (Ithaca, NY). Dry calcium carbonate was sifted onto the soil then mixed thoroughly. Additional measurements of pH (no adjustments) were made at days T 0, T +28 and T +56 of the bioassays. Additionally, on day T 0, 10–15 g samples of all mixtures (including pure biosolids) were stored at −80 °C in trace-clean 40-mL Volatile Organic Analysis (VOA) vials (EP Scientific) for later analyses.

2.2. Earthworm preparation

Earthworm (*Eisenia fetida*) stocks were maintained with bedding and Magic Worm Food from Carolina Biological Supply (Burlington, NC). Earthworms were added to 10 L plastic containers containing moist (60% w/v) bedding that had been allowed to equilibrate for 3 days after wetting. Prior to assays, earthworms were incubated in this bedding for a minimum of 1 week at 22 °C, during which time, they were fed 15 g Magic Worm Food per week per container, with water added as needed.

2.3. Earthworm bioassays

Earthworm survival and reproduction assays followed previously published protocols (Environment Canada, 2004; ASTM, 1999). Briefly, after thoroughly mixing each batch, quadruplicate 250 g samples of prepared soil/biosolids mixture were measured and added to separate 1900 mL (2 quart) glass mason jars. Five active, mature earthworms, similar in color and size, were selected and placed in each jar. Jars were then covered with aluminum foil, containing 5–7

perforations 1 mm in diameter to allow for aeration, and secured with a rubber band. All jars were placed in a random configuration in a 22 °C dark room with controlled lighting (430–670 lx, 12 h on, 12 h off) for 56 days. These bioassays represent a closed system, which eliminates the possible runoff of toxic substances or transformation products from the soil/biosolids mixtures.

For the 14-day duration of the survival assay, moisture content for each jar was restored to 30% every 3–4 days, but earthworms were not fed. At the end of 14 days, the surviving earthworms were counted, with notes made regarding behavioral characteristics, and returned to the test jars for continuation of the reproduction assay.

For the entire 56-day reproduction assay, earthworms were fed 1 g Magic Worm Food per jar sprinkled on the surface on days T +14, T + 28 and T +42. Moisture content was restored to 30% every 3–4 days. The quantities of live earthworms (total, juveniles, and unhatched cocoons) were recorded on days T +28 and T +56. On day T +28, all adult earthworms were removed from the jars, depurated for 24 h on moist filter paper, and frozen at −80 °C for later AWI analysis. The total number of juvenile earthworms was determined on day T +56. Final soil moisture content was determined on day T +56 by drying samples for 24 h at 105 °C.

2.4. Plant bioassay-lettuce seedling emergence

Plant bioassays were adapted from a previously published protocol (Environment Canada, 2005). Emergence was tested in triplicate using the test soils specified above and lettuce seeds [*Lactuca sativa* (Buttercrunch) Agway, Ithaca, NY]. Briefly, 15 g samples of each of the prewetted and equilibrated soil preparations were added to VOA trace-clean 40-mL vials. Five lettuce seeds were placed in each vial, which was then loosely capped (to allow for aeration) and placed into a dark room with controlled lighting (430–670 lx, 12 h on, 12 h off) for 14 days at 22 °C. Every 3–4 days moisture loss was determined by mass loss due to drying and the moisture content for each vial was restored to 30%. The number of germinated seeds was recorded on days T +4, T +11 and T +14. These bioassays represent a closed system, which eliminates the possible runoff of toxic substances or transformation products from the soil/biosolids mixtures.

2.5. AWI quantification

AWI quantification for soil, biosolids, amended soils, and earthworm samples was conducted in triplicate. As has previously been reported in detail, two distinct accelerated solvent extraction (ASE; Dionex-200, Dionex Corp., Sunnyvale, CA) methods were needed to quantify the broad range of AWI compounds measured in this study (Kinney et al., 2006a, 2006b, 2008; Burkhardt et al., 2005).

Pharmaceutical compound quantification followed the method described by Cahill et al. (2004) which employed externally calibrated, high-performance liquid chromatography (HPLC) coupled with positive electrospray ionization/quadrupole mass spectrometry operated in the single ion monitoring (SIM) mode (Hewlett-Packard/Agilent model series 1100 LC/MSD, Palo Alto, CA). The broad group of nonpolar AWIs was quantified using gas chromatography/mass spectroscopy (Agilent Technologies model 5973) using electron-impact ionization (70 eV) and operated in the full-scan mode [45 to 550 mass/charge ratio (m/z)] following the method developed by Burkhardt et al. (2005). A more in-depth summary of these methods, including quality control, is available in a previous publication (Kinney et al., 2008).

3. Results and discussion

3.1. Earthworm survival and reproduction

100% of the adult earthworms in the 2-week aged biosolids–soil samples survived for all biosolids–soil concentrations (Fig. 1a), whereas only 20% of the adults in the 4% biosolids–soil survived in the 8-week aged sample (Fig. 1b). This result for the 8-week aged 4% biosolids–soil sample was statistically different from all other 8-week and 2-week aged soil–biosolids samples ($P \leq 0.006$ for ANOVA comparison to all samples). Results for all other 2-week and 8-week aged biosolids–soil samples are statistically identical ($P > 0.05$). In a related study, Artuso et al. (2011) observed mortality of adult earthworms (*E. fetida*) at the higher rates of biosolids application to soil (20 t ha^{-1} or about 1.3% biosolids) for 3 of the 5 biosolids included in their study. We also noted aestivation, a curling behavior that indicates unfavorable conditions and/or stress (Bauer and Römbke, 1997), by worms 25% of the 3% and 4% biosolids–soil samples regardless of aging.

The mean number of juveniles produced per treatment correlated inversely to biosolids concentration, although the difference was most pronounced in the 8-week aged samples. Mean number of juvenile earthworms produced per treatment (n = 4) (Fig. 2a) ranged from as high as 50 in the 2-week control to 10 in the 2-week soil with 4% biosolids, and 35 in the 8-week aged control to 3 in the 8-week aged soil with 3% biosolids (the 8-week with 4% biosolids treatment was discontinued after two weeks due low adult survival). This result is similar to previous reports that earthworm reproduction is a more sensitive measure of toxicity than adult mortality (Artuso et al., 2011). Cocoons still remaining at 56 days (Fig. 2b) were also inversely proportional to the biosolids concentration. Less than two residual unhatched cocoons were present in the 2-week control and on

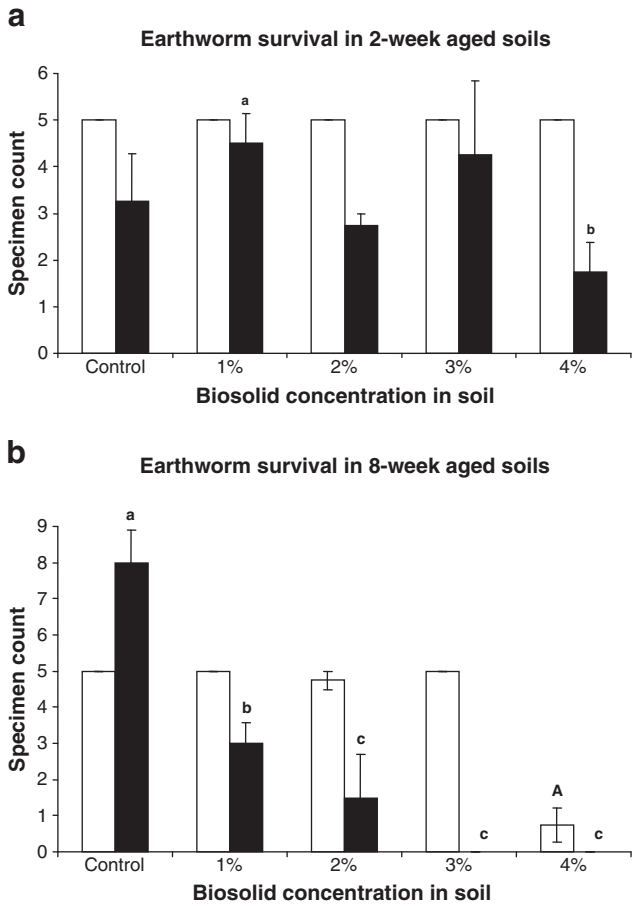

Fig. 1. (a) Adult survival (□) and cocoons (■) in 2-week aged biosolids–amended soil, reported as mean specimen counts per jar at each biosolids concentration. (b)Adult survival (□) and cocoons (■) in 8-week aged biosolidamended soil, reported as mean specimen counts per jar at each biosolids concentration. The bars represent ± one standard deviation. Statistically significant difference ($P \leq 0.05$) in the cocoon counts is indicated with different lower-case letters, and statistically significant differences ($P \leq 0.05$) in the number of adults are indicated with upper-case letters.

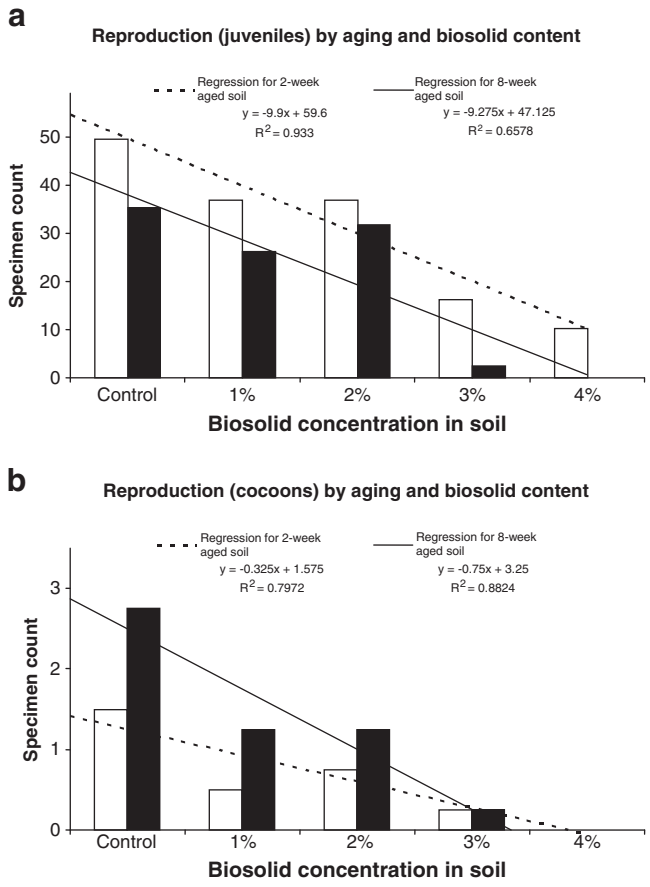

Fig. 2. (a) Mean number of juveniles produced by *E. fetida* exposed to un-amended control soils and in 2-week aged (□) and 8-week aged (■) biosolids–amended soils, reported as mean specimen counts per jar at each biosolids concentration (error bars removed for clarity). Statistical analysis (ANCOVA) indicates differing reproductive fitness relative to increased biosolids concentration and increased soil aging (more pronounced trend for 8-week aged soil–biosolids mixtures, especially above 2% biosolid content). (b) Mean number of cocoons produced by *E. fetida* exposed to un-amended control soils and in 2-week aged (□) and 8-week aged (■) biosolids–amended soils, reported as mean specimen counts per jar at each biosolids concentration (error bars removed for clarity). Statistical analysis (ANCOVA) indicates differing reproductive fitness relative to increased biosolids concentration and increased soil aging (more pronounced for 8-week).

average less than one in the 2-week soil with 1, 2 and 3% biosolids, with no unhatched cocoons observed in the 2-week amended with 4% biosolids. For the 8-week aged soils, three residual unhatched cocoons were observed in the control treatment. On average less than two were observed in the soil amended to 1 and 2% biosolids, and less than one was observed in the treatment amended with 3% biosolids (Fig. 2b). Again, the 8-week aged soil with 4% biosolids treatments was discontinued after the 14-day survival period. While linear and non-linear regression analyses produce r-squared values between 0.63 and 0.93, statistical analysis reveals differing trends, and implicates 8-week aging as having a greater detrimental effect on rates of earthworm reproduction (Fig. 2a and b). In general, these results are contrary to most studies where toxicity of xenobiotic compounds is observed to decrease with increased aging or weathering (Alexander and Alexander, 1999; White et al., 1999; Sun and Li, 2005). The observed increase in toxicity to *E. fetida* with soil–biosolids mixture aging may be the result of an increase in toxicity resulting for a single or small subset of components in the biosolids. A similar observation has been reported by Vernile et al. (2007) for pentachlorophenol (PCP), where the toxicity of PCP in soil to *Eisenia andrei* increased with aging. The authors of that study hypothesized that this observation may be the result of transformation of PCP to more toxic products in the soil or *in vitro*.

3.2. Seedling emergence

Tracking lettuce seedling emergence provided a binary system to monitor general toxicological response. Germination of lettuce seed was used to monitor the toxicological trends of biosolids–amended soils. Higher biosolids concentrations resulted in obvious impairment to both latent emergence, with an obvious impairment to both emergence and seedling fitness. This influencing effect on latent emergence and seedling fitness, especially in the 1 and 2% biosolids treatment, was reduced in the longer-aged (8-week) soil (Fig. 3a and b). For the 2-week soil samples, the control had the highest percentage emergence (93%), reaching that value by day 4. Overall emergence after 14 days for the 2-week soil treatments was 73%, 40%, 60%, and 27% (biosolids concentrations of 1 to 4%, respectively). The 8-week control showed 87% emergence by day 4, but full 100% emergence by day 11. The other 8-week soil treatments exhibited 14-day emergence of 87%, 67%, 53%, and 33% (biosolids concentrations of 1 to 4%, respectively).

While the effects of higher biosolids concentrations are consistent with anticipated results, the mediated effects in the longer-aged soil indicate that aging had at least one of several possible effects; a decrease in the original levels of toxic components or a decrease in bioavailability for either the original toxic components or toxic products of transformations. In either scenario, it is clearly evident that the aging process alters the toxicity of soils amended with biosolids.

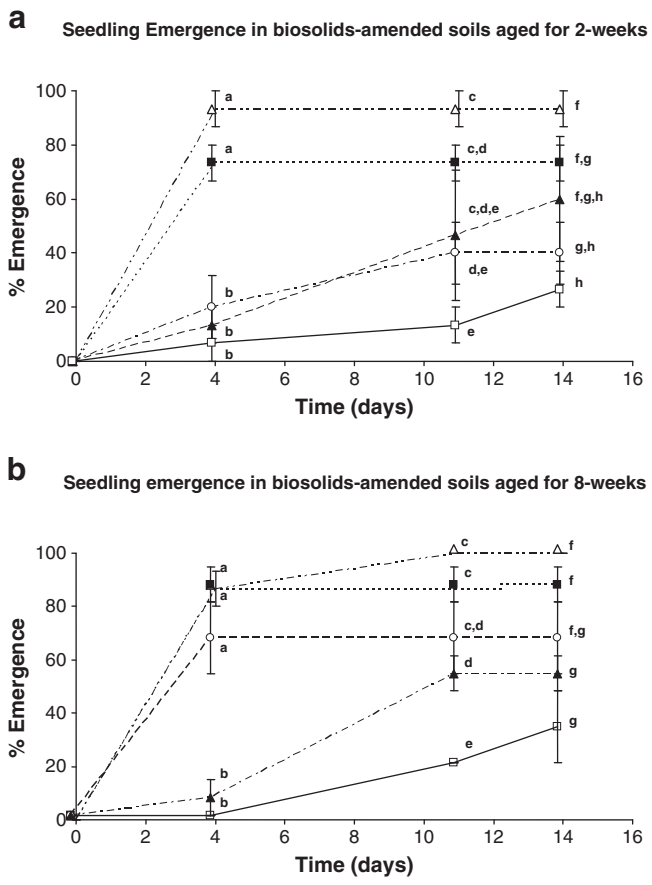

Fig. 3. (a) Percent emergence of lettuce seedling cultivated in control (Δ), 1% biosolids (■), 2% biosolids (○), 3% biosolids (▲), and 4% biosolids (□) 2-week aged biosolids–amended soils. (b) Percent emergence of lettuce seedling cultivated in control (Δ), 1% biosolids (■), 2% biosolids (○), 3% biosolids (▲), and 4% biosolids (□) 8-week aged biosolids–amended soils. Identical lower-case letters signify rates of emergence that are statistically similar ($P > 0.05$) for that particular day.

Interestingly, the germination effects associated with exposure to biosolids–soil mixtures aged longer are in contrast with the results of the earthworm survival and reproduction assays. However, the transformation and biotoxicity of certain compounds may have an organism-specific effect (see later discussion on classes of AWIs—*i.e.* sterols may have a greater effect on survival of soft-bodied invertebrates than on seedling emergence).

3.3. pH correction

Preliminary trials with biosolids–amended soils revealed a downward trend in pH during the initial aging period, likely due to microbial oxidation of reduced nitrogen and sulfur compounds in the biosolids (Qureshi et al., 2003) or insufficient calcium carbonate was added to stabilize pH. Because variations in soil pH can affect the availability of some elements (Spurgeon and Hopkin, 1996), liming was employed to minimize pH differences among the biosolids concentrations used in this study. Furthermore, pH can affect accumulation and toxicity of organic contaminants (Valenti et al., 2009; Rendal et al., 2011). This can be attributed to the fact that the neutral form of a xenobiotic organic compound is less polar and will generally cross cellular membranes more readily than the ionized form (Fent and Looser, 1995).

Despite efforts to minimize pH differences, soil pH ranged from 6.1 to 7.3. During the study, the largest change in pH for any one treatment was limited to 0.8 pH units. The direct effects of these pH differences were likely minimal since prior research has shown that between 5 and 8 pH alone does not adversely affect worm survival or fitness (Spurgeon and Hopkin, 1996). With regard to differences in accumulation and potential toxic effects of organic contaminants among treatments, differences in soil mixture pH are likely to exert minimal effects and probably limited to those compounds with pK_a values similar to the pHs observed in this study. Of the AWIs for which a BAF could be determined, only miconazole (pK_a 6.65) has a pK_a in the range of pHs observed in this study. A BAF for miconazole could only be determined in the 2-week and 8-week 3% biosolids–soil samples. However, the BAF for miconazole in the 8-week 3% biosolids sample, which had a pH greater than the pK_a for miconazole, was about 2.5 times greater than that observed in the 2-week 3% biosolids sample, which had a pH below the pK_a of miconazole throughout the study.

3.4. AWI quantification and bioaccumulation

The biosolids used in these studies contained a wide variety of AWIs. Of the 59 AWIs measured in this study, 51 were detected in the biosolids (Tables 1 and 2). The Arkport soil used in this study contained trace quantities of 15 of the target AWIs above the method detection limits (MDLs). Many of these detected compounds such as indole, the sterols, and the PAHs can originate from natural sources or atmospheric deposition. As expected, the biosolids–amended soils contained a greater number of the AWIs compared to the unamended soil, but many of the compounds present in the biosolids were below detectable concentrations in these soil–biosolids mixtures, likely as a result of dilution (Tables 1 and 2). The concentration of total AWIs detected in the biosolids–amended soils does not vary greatly with aging of the soil–biosolids mixture. In fact, for a given concentration of biosolids in soil, the concentration of total AWIs was within a factor of 2 between the 2-week and 8-week aged soil–biosolids mixtures. Unexpectedly, trace quantities of some of the pharmaceuticals measured were present in the unamended soil. The source of these compounds in the control soil is unknown. Although not detected in the biosolids, fluoxetine and citalopram were detected in very small quantities ($\leq$2.2 ng/g) in a few of the biosolids–amended soil samples. Both compounds were detected in the unamended 8-week aged soil at similar concentrations.

Only five AWIs were detected above the MDLs in unexposed earthworms (zero pharmaceuticals) in measureable quantities. On a per mass basis, the sterols, beta-sitosterol and stigmastanol were the most abundant compounds measured in the unexposed earthworms. All five of the AWIs measured in the unexposed worms can originate from natural sources. Of the 25 AWIs detected in the earthworms exposed to biosolids–amended soils; acetaminophen, albuterol, and caffeine were below detection in the raw biosolids, a phenomenon previously reported, and likely the result of bioaccumulation of AWIs by the earthworms (Kinney et al., 2008). Caffeine was detected in trace quantities below its MDL in the soil used in the study. Unfortunately, since these compounds were not detected in the raw biosolids, it is impossible to make predictions for concentrations in biosolids–amended soils, thereby prohibiting any bioaccumulation factor (BAF) calculations (Table 3), which, based on the concentrations detected in exposed worms, could be significant.

The most prominent AWIs found in any sample were the sterols. As high as 812 μg/g of the sterols were detected in biosolids (85% of total AWI content), 4.3 μg/g for unamended 2-week soil and 1.9 μg/g for unamended 8-week aged soil (equates to 81 and 70% of total AWI content, respectively). The sterols were present at concentrations ranging from 9.9 to 99 μg/g of the biosolids–amended soils (72 to 93% of total AWI content), with 554–1435 μg/g being detected in worms exposed to those amended soils (96 to 99% of total AWI content). The plant sterols beta-sitosterol and stigmastanol were present in unexposed earthworms, but the fecal indicators 3-beta-coprostanol and cholesterol were below detectable concentrations in these earthworms. The ratio of 3-beta-coprostanol and cholesterol to the plant sterols beta-sitosterol and stigmastanol in the biosolids used in this study is 4.3, which is similar to the ratio of fecal to plant sterols in other biosolids previously measured (Kinney et al., 2006a, 2006b). The predominance of fecal to plant sterols is largely reflected in the biosolids–amended soils (Table 2). There was a substantial shift in the sterol composition in the earthworms from primarily the plant sterols to predominantly the fecal indicators 3-beta-coprostanol and cholesterol between the unamended and amended exposures. This shift is mainly the result of bioaccumulation of cholesterol (Tables 2 and 3). In fact, with the exception of stigmastanol in worms exposed to the 3% biosolids–soil mixtures, there is a net reduction in beta-sitosterol and stigmastanol in the worms.

The results of the earthworm survival and reproduction assays suggest a direct relationship between toxicity and biosolids exposure. Furthermore, in terms of earthworm survival, toxicity appears to increase with the aging of the soil–biosolids mixture. Likewise, the concentration of total AWIs detected in the earthworms exposed to the longer aged (8-weeks) 2% and 3% biosolids–amended soils was greater than that measured in the corresponding biosolids–soil mixtures aged for 2-weeks (Tables 1 and 2). Although the general statement cannot be made for all the compounds measured, there were notable examples of compounds measured in greater concentrations in the earthworms exposed to 3% biosolids–amended soil aged for 8-weeks compared to that aged for 2-weeks, which suggests that aging of the biosolid–soil mixtures may increase bioavailability of some compounds (Vernile et al., 2007). This includes indole, total para-nonylphenol, decafluorobiphenyl, triclosan, 3-beta-coprostanol, and cholesterol. It is unknown if the increased toxicity of biosolids to earthworms was related to the increased bioavailability of some AWI that was observed with soil–biosolids aging.

When possible BAFs, the ratio of AWI concentration in the earthworm to the concentration in soil–biosolids mixture on a dry mass basis, were calculated for the compounds measured in the earthworms (Table 2). A number of compounds measured in the earthworm samples were below detectable concentrations in the corresponding soil–biosolids mixtures, a phenomenon previously reported (Kinney et al., 2008). In these instances a BAF could not be directly calculated, but an estimated BAF was calculated assuming the relative mass of the AWI in the raw biosolids was conserved in the biosolids–soil mixture. The BAF or estimated BAF for para-cresol, methyl salicylate, bisphenol-A, and cholesterol was >100 in some treatments. The bioavailability of

Table 1
Concentration (ng/g) of personal care products and pharmaceuticals detected in biosolid, soil, biosolid–amended soil, and adult earthworms.[a]

	Biosolids	Unamended 2-week soil[b]	Unamended 8-week soil[b]	2-Week soil + 1% biosolid[b]	8-Week soil + 1% biosolid[b]	2-Week soil + 2% biosolid[b]	8-Week soil + 2% biosolid[b]	2-Week soil + 3% biosolid[b]	8-Week soil + 3% biosolid[b]	T0 worm[b]	Worm in 2-week soil + 1% biosolid[c]	Worm in 8-week soil + 1% biosolid[c]	Worm in 2-week soil + 2% biosolid[c]	Worm in 8-week soil + 2% biosolid[c]	Worm in 2-week soil + 3% biosolid[c]	Worm in 8-week soil + 3% biosolid[c]	Blank[d]
Personal care products																	
Galaxolide (HHCB)	7510 (34)	ND	ND	189 (37)	165 (41)	860 (57)	235 (43)	170 (91)	190 (22)	ND	1567 (38)	2310 (21)	2123 (12)	2697 (23)	6100 (19)	4500 (8)	ND
4-Tert-octylphenol	2770 (38)	ND	18[e] (73)	ND	ND	ND	ND	18[e] (73)	ND	ND	ND	ND	ND	ND	ND	ND	ND
Tonalide (AHTN)	1520 (53)	ND	ND	67 (52)	50 (29)	150 (6)	40 (23)	37 (92)	86 (11)	ND	35 (35)	47 (26)	49 (38)	27 (46)	300 (24)	350 (12)	ND
Para-nonylphenol-total	93000 (22)	ND	ND	948 (20)	627 (11)	1595 (20)	726 (16)	5900 (6)	6300 (21)	ND	1380 (23)	ND	4230 (9)	4827 (13)	23000 (40)	26000 (31)	490[e] (76)
OPEO-1	118 (18)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
OPEO-2	219 (12)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
NPEO1-total	5080 (21)	ND	ND	ND	ND	ND	ND	110[e] (73)	ND	ND	ND	ND	ND	ND	ND	ND	ND
NPEO2-total	4250 (31)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Triclosan	3240 (23)	ND	ND	ND	ND	ND	ND	ND	ND	ND	172 (14)	86 (20)	436 (22)	337 (31)	3500 (35)	4200 (75)	ND
4-Octylphenol	10159 (28)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Category subtotal (μg/g)	128	0	0	1.2	0.84	2.6	1.0	6.1	6.6	0	3.2	2.4	6.8	7.9	33	35	
Pharmaceuticals																	
Acetaminophen	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	74 (57)	96 (24)	78 (36)	100 (10)	ND	ND	ND
Albuterol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.88[e] (4)	1.9 (41)	1.2 (34)	1.5 (58)	ND	ND	ND
Cotinine	4.3 (10)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Cimetidine	0.54 (44)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Caffeine	ND	ND	1.7 (32)	0.56[e] (24)	0.53[e] (57)	0.60[e] (67)	0.59[e] (76)	ND	ND	ND	4.2 (23)	2.8 (14)	3.5 (47)	3.3 (15)	ND	ND	ND
Carbamazepine	22 (13)	ND	ND	ND	ND	ND	ND	0.60[e] (48)	0.83[e] (43)	ND	ND	ND	ND	ND	ND	ND	ND
Diphenhydramine	66 (18)	ND	ND	ND	ND	ND	ND	6.8 (37)	7.0 (30)	ND	ND	ND	ND	ND	ND	ND	ND
Diltiazem	ND	ND	ND	ND	ND	ND	ND	ND	0.02[e] (71)	ND	ND	ND	ND	ND	ND	ND	ND
Fluoxetine	ND	ND	0.17 (45)	ND	ND	ND	ND	2.2 (15)	1.9 (26)	ND	ND	ND	ND	ND	ND	ND	0.07 (46)
Miconazole	192 (7)	2.4 (14)	0.13[e] (61)	ND	ND	ND	ND	26 (27)	11 (15)	ND	ND	ND	ND	ND	23 (12)	25	0.75[e] (73)
Venlafaxine	ND	ND	ND	ND	ND	ND	ND	ND	1.6 (13)	ND	ND	ND	ND	ND	ND	ND	ND
Citalopram	ND	ND	0.66 (58)	0.20 (8)	0.30 (21)	0.40 (60)	0.60 (57)	1.7 (62)	1.9 (42)	ND	ND	ND	ND	ND	ND	ND	ND
Duloxetine	2.3 (35)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Norsertraline	93 (11)	ND	2.2 (36)	ND	ND	ND	ND	2.9 (10)	4.1 (23)	ND	ND	ND	ND	ND	ND	ND	ND
Sertraline	104 (13)	0.25 (27)	1.1 (29)	1.0 (19)	2.1 (12)	1.6 (37)	2.4 (8)	3.7 (25)	2.6 (37)	ND	ND	ND	ND	ND	8.2 (20)	5.5	0.35 (87)
Category subtotal (μg/g)	0.48	0.003	0.005	0.001	0.002	0.002	0.003	0.04	0.03	0	0.08	0.10	0.08	0.10	0.03	0.030	

AWI = anthropogenic waste indicator. ND = not detected. NA = not available.

[a] Average from replicate samples, individual compound concentrations reported as ng/g dry wt. (compound/sample) unless otherwise indicated. No data is available for earthworms from the soil + 4% biosolids because of low earthworm survival in the 8-week with 4% biosolids treatment.

[b] Value in parenthesis is the relative standard deviation.

[c] Value in parenthesis is the relative percent difference.

[d] Laboratory sand blank.

[e] Value is below the statistically calculated method detection limit based on previously published work [3, 11, 33].

Table 2
Concentration (ng/g) of sterols and other AWIs detected in biosolid, soil, biosolid–amended soil, and adult earthworms.[a]

	Biosolids	Control 2-week soil[b]	Control 8-week soil[b]	2-Week soil + 1% biosolids[b]	8-Week soil + 1% biosolids[b]	2-Week soil + 2% biosolids[b]	8-Week soil + 2% biosolids[b]	2-Week soil + 3% biosolids[b]	8-Week soil + 3% biosolids[b]	T0 worm[b]	Worm in 2-week soil + 1% biosolids[c]	Worm in 8-week soil + 1% biosolids[c]	Worm in 2-week soil + 2% biosolids[c]	Worm in 8-week soil + 2% biosolids[c]	Worm in 2-week soil + 3% biosolids[c]	Worm in 8-week soil + 3% biosolids[c]	Blank[d]
Sterols																	
3-Beta-coprostanol	290000 (26)	930 (67)	ND	4590 (42)	9545 (30)	3120 (23)	4200 (16)	50000 (34)	29000 (21)	ND	7343 (14)	5767 (39)	10867 (17)	17967 (20)	14000 (18)	21000 (44)	57 (96)
Cholesterol	370000 (24)	310 (26)	360 (36)	6385 (13)	10245 (23)	4325 (31)	4190 (19)	33000 (67)	11000 (24)	ND	539667 (43)	486000 (29)	685333 (30)	910000 (18)	1000000 (8)	1300000 (13)	ND
Beta-sitosterol	109000 (20)	2600 (29)	1500 (14)	1693 (19)	3215 (25)	1935 (8)	1581 (11)	6800 (10)	6300 (16)	120000 (22)	5247 (20)	5973 (13)	8133 (18)	13133 (34)	110000 (11)	95000 (47)	ND
Stigmastanol	42900 (15)	460 (27)	200[e] (21)	645 (28)	1380 (17)	556 (24)	573 (40)	9400 (57)	1800 (17)	18000 (48)	1800 (12)	1427 (26)	2160 (27)	4080 (16)	19000 (28)	19000 (70)	ND
Category subtotal (μg/g)	812	4.3	1.9	13	24	9.9	11	99	48	138	554	499	706	945	1143	1435	
Other wastewater indicator compounds																	
Phenol	383 (45)	3.3[e] (80)	ND	ND	ND	ND	ND	15[e] (71)	ND	71 (20)	ND	ND	ND	ND	1400 (36)	1800 (25)	ND
1,4-Dichlorobenzene	24[e] (53)	ND	ND	ND	ND	ND	ND	ND	ND	14[e] (41)	ND	ND	ND	ND	18[e] (99)	65 (6)	ND
d-Limonene	249 (26)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Acetophenone	344 (47)	4.5[e] (96)	ND	ND	ND	ND	ND	16[e] (87)	ND	ND	ND	ND	ND	ND	110 (22)	ND	ND
Para-cresol	105 (37)	ND	ND	135 (46)	391 (26)	160 (62)	165 (51)	12[e] (67)	ND	ND	187 (32)	277 (14)	274 (40)	229 (46)	1100 (80)	1100 (65)	ND
Napthalene	69 (73)	ND	ND	33 (35)	31 (64)	31 (39)	30 (69)	10[e] (74)	ND	ND	ND	ND	ND	ND	150 (24)	190 (6)	20
Methyl salicylate	22 (33)	ND	ND	ND	ND	ND	ND	5.9[e] (63)	ND	ND	ND	ND	ND	ND	ND	180 (89)	ND
Indole	7130 (35)	270 (13)	230 (25)	156 (17)	175 (12)	116 (13)	121 (38)	340 (89)	330 (81)	1100 (13)	2697 (30)	3857 (44)	3973 (43)	3920 (21)	8300 (7)	11000 (43)	ND
2-Methylnapthalene	84 (41)	ND	ND	33 (23)	31 (33)	30 (46)	28 (16)	7.4[e] (47)	ND	ND	ND	29 (43)	27[e] (47)	25[e] (45)	ND	ND	ND
1-Methylnapthalene	75 (36)	ND	ND	26[e] (73)	26[e] (85)	25[e] (73)	22[e] (42)	5.9[e] (59)	ND	ND	ND	20[e] (70)	ND	22[e] (24)	11[e] (88)	64 (61)	ND
Skatol	497 (54)	3.4[e] (67)	1.5[e] (97)	64 (34)	95 (37)	37 (33)	35 (20)	ND	52 (24)	240 (11)	99 (41)	1983 (12)	1383 (21)	1460 (22)	ND	ND	ND
2,6-Dimethylnapthalene	441 (88)	ND	ND	19 (14)	19 (9)	22 (11)	17 (9)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Butylated hydroxyanisole	ND	ND	ND	ND	ND	ND	ND	140 (75)	ND	ND	ND	ND	ND	ND	ND	ND	ND
Benzophenone	1150 (20)	22[e] (69)	12[e] (73)	ND	ND	ND	ND	40 (43)	ND	ND	ND	ND	ND	ND	42 (22)	ND	30[e] (56)
4-Cumylphenol	157 (16)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Phenanthrene	1570 (19)	97 (44)	74 (28)	158 (17)	135 (46)	ND	147 (39)	66 (88)	92 (9)	ND	ND	ND	ND	ND	ND	ND	ND
Anthracene	1840 (29)	ND	19[e] (13)	66 (57)	35 (10)	ND	42 (47)	6.0[e] (73)	5.3[e] (93)	ND	ND	ND	ND	ND	ND	ND	ND
Carbazole	413 (91)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Decafluorobiphenyl	308 (37)	220 (19)	190 (34)	NA	NA	NA	NA	150 (87)	110 (97)	ND	NA	NA	NA	NA	2200 (15)	3100 (56)	ND
Pyrene	574 (27)	210 (37)	160 (19)	360 (24)	237 (20)	340 (18)	174 (46)	140 (78)	200 (13)	ND	ND	ND	ND	ND	ND	ND	ND
Fluoranthene	1910 (78)	190 (32)	130 (19)	312 (27)	209 (21)	337 (14)	159 (23)	140 (55)	210 (14)	ND	ND	ND	ND	ND	ND	ND	ND
Bisphenol A	533 (73)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1700 (26)	33 (69)
Benzo[a]pyrene	245 (27)	ND	ND	98 (54)	77 (12)	102 (40)	88 (44)	ND	ND	ND	ND	ND	ND	ND	ND	ND	24 (71)
Cumene	9[e] (65)	ND	ND	NA	NA	NA	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Isophorone	13[e] (52)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Menthol	138 (44)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dichlorvos	86 (40)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Isoquinoline	37 (58)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Tributylphosphate	888 (39)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Prometon	241 (24)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Category subtotal (μg/g)	20	0.98	0.78	1.4	1.4	1.2	1.0	1.0	0.9	1.4	3.0	6.1	5.6	5.6	13	19	

AWI = anthropogenic waste indicator. ND = not detected. NA = not available.

[a] Average from replicate samples, individual compound concentrations reported as ng/g dry wt. (compound/sample) unless otherwise indicated. No data is available for earthworms from the soil + 4% biosolids because of low earthworm survival in the 8-week with 4% biosolids treatment.

[b] Value in parenthesis is the relative standard deviation.

[c] Value in parenthesis is the relative percent difference.

[d] Laboratory sand blank.

[e] Value is below the statistically calculated method detection limit based on previously published work [3, 11, 33].

Table 3
Chemical properties and bioaccumulation factors (BAFs) [a]for AWIs detected in biosolid–amended soil earthworms.

	Log K_{ow}[b]	Water solubility (mg/L)[b]	Vapor pressure (mm Hg)[b]	BAF Worm in 2-week Soil + 1% biosolids	BAF Worm in 8-week Soil + 1% biosolids	BAF Worm in 2-week Soil + 2% biosolids	BAF Worm in 8-week Soil + 2% biosolids	BAF Worm in 2-week Soil + 3% biosolids	BAF Worm in 8-week Soil + 3% biosolids
Wastewater compounds									
Phenol	1.46	8.28×10^{4}	0.35	–	–	–	–	16[c]	21[c]
1,4-Dichlorobenzene	3.44	81.3	1.74	–	–	–	–	–	NC[d]
Acetophenone	1.58	6.13×10^{3}	0.397	–	–	–	–	6.9[c]	–
Para-cresol	1.94	2.15×10^{4}	0.11	1.4	0.71	1.7	1.4	522[c]	348[c]
Napthalene	3.3	31	0.085	NC	NC	NC	NC	94[c]	82[c]
Methyl salicylate	2.55	700	0.0343	–	–	–	–	–	279[c]
Indole	2.14	3.56×10^{3}	0.0122	1.3	2.2	2.4	2.3	5.0	6.9
2-Methylnapthalene	3.86	24.6	0.055	NC	0.93	NC	NC	–	–
1-Methylnapthalene	3.87	25.8	0.067	–	–	–	–	–	28[c]
Skatol	2.6	498	5.55×10^{-3}	(−0.46)[e]	5.2	4.1	4.2	NC	NC
2,6-Dimethylnapthalene	4.31	2	4.3×10^{-3}	NC	NC	NC	NC	–	–
Butylated hydroxyanisole	3.5	213	2.48×10^{-3}	–	–	–	–	NC	–
Benzophenone	3.18	137	1.93×10^{-3}	–	–	–	–	1.1	–
Para-nonylphenol-total	5.92	5×10^{3}	9.42×10^{-5}	1.5	NC	2.7	6.6	3.9	4.1
Phenanthrene	4.46	1.15	1.21×10^{-4}	NC	NC	NC	–	NC	NC
Anthracene	4.45	0.0434	6.53×10^{-6}	NC	NC	–	NC	–	–
Galaxolide (HHCB)	5.9	1.75	5.45×10^{-4}	8.3	14	2.5	11	36	24
Tonalide (AHTN)	5.7	1.25	5.12×10^{-4}	0.53	0.93	0.33	0.68	8.1	4.1
Decafluorobiphenyl	5.76	0.176	0.0979	NA	NA	NA	NA	15	28
Pyrene	4.88	0.135	4.5×10^{-6}	NC	NC	NC	NC	NC	NC
Triclosan	4.76	10	6.45×10^{-7}	5.3[c]	2.7[c]	6.7[c]	5.2[c]	36[c]	43[c]
Fluoranthene	5.16	0.26	9.22×10^{-6}	NC	NC	NC	NC	NC	NC
Bisphenol A	3.32	120	3.91×10^{-7}	–	–	–	–	–	104[c]
Benzo[a]pyrene	6.13	1.62×10^{-3}	5.49×10^{-9}	NC	NC	NC	NC	–	–
Sterols									
3-Beta-coprostanol	8.82	2.03×10^{-4}	5.47×10^{-10}	1.6	0.60	3.5	4.3	0.28	0.72
Cholesterol	8.74	0.095	7.79×10^{-10}	85	47	158	217	30	118
Beta-sitosterol	9.65	1.3×10^{-5}	3.77×10^{-10}	(−0.94)[e]	(−0.93)[e]	(−0.92)[e]	(−0.88)[e]	(−0.79)[e]	(−0.20)[e]
Stigmastanol	9.73	9.78×10^{-6}	5.33×10^{-10}	(−0.87)[e]	(−0.86)[e]	(−0.85)[e]	(−0.85)[e]	0.04	0.05
Pharmaceuticals									
Acetaminophen	0.46	1.4×10^{4}	7×10^{-6}	NC[d]	NC[d]	NC[d]	NC[d]	–	–
Albuterol	0.64	1.41×10^{4}	8.89×10^{-9}	–	NC[d]	NC[d]	NC[d]	–	–
Caffeine	−0.07	2.16×10^{4}	7.33×10^{-9}	NC[d]	NC[d]	NC[d]	NC[d]	–	–
Diphenhydramine	3.27	3.06×10^{3}	3.7×10^{-5}	–	–	–	–	NC	NC
Fluoxetine	4.05	60.3	2.52×10^{-5}	–	–	–	–	NC	NC
Miconazole	6.25	0.0111	1.77×10^{-10}	–	–	–	–	0.90	2.3
Venlafaxine	3.28	267	2.46×10^{-7}	–	–	–	–	–	NC
Citalopram	3.74	31.1	1.13×10^{-7}	NC	NC	NC	NC	NC	NC
Norsertraline	2.18	93.8	1.11×10^{-11}	–	–	–	–	NC	NC
Sertraline	5.29	3.52	1.17×10^{-6}	NC	NC	NC	NC	2.2	2.1

NA = not available, data for this analyte were not available for the soil or earthworm.
[a] NC = not calculated, analyte was detected in soil; it was below detection in earthworm tissue. – = not present in soil or earthworm tissue.
[b] Physicochemical properties are from the following online database: http://www.syrres.com/esc/physdemo.htm (last viewed February 2009).
[c] Analyte was detected in earthworm tissue; it was below detection in soil; however, it was detected in raw biosolids so the concentration in soil could be mathematically estimated.
[d] Analyte was detected in earthworm tissue; it was below detection in soil and below detection in raw biosolids so estimation was not possible.
[e] BAF calculates as a negative number; this is because analyte was detected at higher levels in T0 earthworms than in worms exposed to biosolids–amended soils.

some AWIs, as measured by BAF, increased with increasing biosolids content in the biosolids–soil mixture, such as for para-cresol, triclosan, galaxolide, and tonalide. Despite the relatively high content of sterols in the samples tested, only cholesterol showed significant bioaccumulation (3-beta-coporstanol gave confounding results). Although a consistent trend for all AWIs does not exist, the BAFs for some AWIs in the treatments aged for 8 weeks do exceed those for the treatments aged for 2-weeks, which again might suggest that greater bioavailability and bioaccumulation of some AWIs was occurring for the earthworms exposed to the 8-week aged soils.

4. Conclusions

It appears that when biosolids were applied to soils at levels above 3%, the toxic components were lethal. Furthermore, AWI content in the exposed earthworms along with BAF values suggests that aging may alter the bioavailability and bioaccumulation of many AWIs as well as the toxicity of biosolids. It therefore seems reasonable to conclude that bioassays with seedling emergence and *E. fetida* are useful laboratory tools with respect to assess the toxicity of biosolids , and the associated potential for bioaccumulation of AWIs. Specifically, the accumulation of AWIs below detectable concentrations in soil matrices demonstrates the usefulness of *E. fetida* as a test organism for terrestrial monitoring of the effects of land applied biosolids and as a sentinel for accumulation of AWIs in the terrestrial food web. Although further work is needed, the insights from increased toxicity of aged biosolids-soil mixtures may provide additional utility, permitting further characterization of the transformations following land application of biosolids, as well as assessment of the risks to other terrestrial organisms that may be susceptible to AWIs.

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