ABSTRACT

CHO, EUN-AH. Bioturbation as a Novel Method to Characterize the Toxicity of Aquatic Sediment (Under the direction of Drs. W. Gregory Cope and Damian Shea.)

Bioturbation, the biological process through which many species of infaunal benthic invertebrates suspend bottom sediments into the water column through their burrowing, feeding, respiratory, and locomotor activities, may be a sub-lethal endpoint that can be exploited to assess the toxicity of aquatic sediments. Therefore, we developed a novel test method that used bioturbation (BioTurbTox test) generated by the activities of second in-star *Chironomus tentans* larvae as the toxicity endpoint (Chapter 2). To validate this method, copper (Cu) and fluoranthene were individually spiked into relatively uncontaminated aquatic sediment to assess changes in bioturbation and mobilization of the chemicals into the overlying water. Turbidity production responded to the chemicals in the sediment in a concentration-dependent manner and was an excellent indicator of sediment toxicity. Moreover, substantial concentrations of Cu were released into the overlying water from the Cu-spiked sediment, whereas little fluoranthene was mobilized into the overlying water from the fluoranthene-spiked sediment. Sediment samples were then collected from the field and used to evaluate the similarity of response of the BioTurbTox test to other more standardized toxicity tests. In the summer of 2003, sediment samples were collected at six sites in the Neuse River of North Carolina tested for toxicity, and analyzed for chemical contaminants (Chapter 3). Atrazine was the most frequently detected current-use pesticide and pyrene and fluoranthene were measured at relatively high concentrations from the Neuse River sites. Concentrations of fluoranthene were correlated with results from the *Ceriodaphnia dubia* porewater and BioTurbTox tests. We concluded that the new BioTurbTox test was useful as a rapid screening method for sediment toxicity information, but required normalization to the

clay content or to the total organic carbon content of field collected sediments. In Chapter 4, the toxicity of environmental pharmaceuticals and personal care products (PPCPs) were evaluated with the BioTurbTox and *C. dubia* reproductive tests. Fluoxetine and bisphenol A significantly affected bioturbation caused by *C. tentans*, especially at high concentrations (1-2 mg/L), and the turbidity change induced by caffeine, fluoxetine, and bisphenol A showed a concentration-response relation. Triclosan affected reproduction of *C. dubia* at relatively low concentrations (IC50: 85.4 μg/L). However, most of the tested PPCPs were not acutely toxic at environmentally relevant concentrations, but were relatively toxic at high concentrations. In Chapter 5, two sediment-spiking methods (extract mixing vs. whole sediment dilution methods) were compared with the BioTurbTox test and a gradient response was observed from both methods. Based on the similarity of the toxic response, we determined that either of the spiking methods was appropriate for estimating the toxicity of aquatic sediments in screening level assessments. The overall conclusion from this research was that the newly developed BioTurbTox test shows promise as a tool to assess the toxicity and mobilization of contaminants from aquatic sediments.

Bioturbation as a Novel Method

to Characterize the Toxicity of Aquatic Sediment

by

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BIOGRAPHY

Eun-ah Cho was born on May 17, 1973 in Pusan, Korea; the first child of Kibong Cho and Heesuk Suh. While she was in Namchun Middle School, her interest on Biology began to grow and she liked to dissect frogs, mussels, etc. and that interest was extended to environmental conservation when she was attending Dukmoon Girls' high school and she got a great influence from her high school Biology teacher, Dr. Soyoung Kim. She entered Dept. of Biology in Pusan National University in March 2, 1992 and started to work in Freshwater Ecology laboratory under the direction of Dr. Gea-jae Joo. In the lab, she collected water samples from the Nakdong River and analyzed water quality parameters, identified phytoplankton, and got an understanding on Limnology. Dr. Joo introduced her the study of Environmental Toxicology and she had a chance to visit Miami University, Ohio, in the early 1994 as a visiting student for 2 months. At the same year, she went to Monash University, Australia, as an exchange student for a semester and took Conservation Biology, getting broader understanding on the Environment Sciences and basic techniques in environmental research. She graduated Pusan National University on February 26, 1996 and continued working in the same laboratory for her Masters' degree. She married to Jonghoon Choi on June 1997. Then, she entered Dept. of Zoology, Miami Univetsity, Ohio, and started to work on the effect of environmental factors on photo-induced toxicity of fluoranthene in fathead minnow under the direction of Dr. James T. Oris from August, 1997. She got her Masters' degree on August 2000 and moved to Dept. of Environmental and Molecular Toxicology, NC State University for her Ph.D research. She started working with Dr. W. Gregory Cope and Dr. Damian Shea on the method development for sediment toxicity test using bioturbation and verified this method with contaminant spiked sediment and Neuse River, NC, sediment. The toxicity of the environmental pharmaceuticals was also tested with this novel test method.

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CHAPTER I: INTRODUCTION

Review on Sediment Toxicity Tests Methods and Bioturbation

1. Sediment

Sediment is naturally occurring particulate material that has been transported and deposited in aquatic ecosystems and is normally found below the water level, including a solid and a porewater phase [ASTM, 2000; Adams et al., 2001]. It provides habitats for populations of benthic and infaunal organisms and the status of sediment reflects the quality and health of the ecosystem [Adams et al., 2001]. Sediment is a source for biologically important materials such as phosphorus, carbon, nitrogen, as well as a source of contaminants, particularly for benthic organisms [Jones and Bowser, 1978; Larsson, 1985]. When contaminants that are hydrophobic and resistant to chemical or biological degradation enter the aquatic environment, they may accumulate and concentrate in the sediment [Larsson, 1985].

Sediment environments consist of a myriad of microenvironments, redox gradients, and other interacting physicochemical and biological processes. These characteristics affect sediment toxicity, bioavailability of contaminants to sediment flora and fauna, microbial degradation, and chemical sorption [Northcott and Jones, 2000]. Infauna living in sediment, especially deposit feeders, must be able to process large amounts of sediments to obtain their nutrition as physiological adaptations to live in sediment [Lopez and Levinton, 1987]. They rapidly ventilate their burrows or obtain oxygen from the overlying waters in anoxic environment. By their burrowing, the sediment surface is extended, increasing diffusive transport of solutes between the surrounding porewater and overlying water [Timmermann et al., 2003]. Both the flux of solutes into deeper layers of the sediment and the flux from sediment porewater to overlying water are enhanced by ventilation of infaunal burrows [Timmermann, 2003]. Since this water transport enhances the flux of nutrients and oxygen [Marinelli, 1994; Kristensen and Hansen, 1999], it affects sediment redox level, enhances

organic matter degradation [Banta et al., 1999; Kristensen and Holmer, 2001], and stimulates microbial activities in the sediment [Van Duyl et al., 1992]. Insoluble forms of reduced metal complexes (e.g. FeS and FeS_2) return to oxidized zones in the sediments by bioturbation, reoxidizing them and consequently replenishing the source of oxidized metals as electron acceptors for organic matter decomposition and other oxidation reactions through metal reduction [Thamdrup et al., 1994]. Irrigation by infauna also favors the oxidation of NH_4^+ to NO_3 in the bioturbated zone and thus stimulates both nitrification and denitrification [Kristensen et al., 1991; Pelegri and Blackburn, 1995]. Both particle mixing and irrigation lead to increases in sediment porosity within the bioturbated zone [Jones and Jago, 1993] and to the oxidation of reduced sulfur compounds [Banta et al., 1999]. The potential for sediment to be a sink as well as a source of contaminants can make sediment chemistry and toxicity key components of aquatic system quality [Vigano et al., 2003].

2. *Chironomus tentans*

The family Chironomidae is a relatively and phylogenetically primitive group of flies (Diptera) in the suborder Nematocera. They are commonly called “non-biting midges” as adults and bloodworms as larvae. Chironomids are closely related to mosquitoes (Culicidae) and biting midges (Ceratopogonidae). This family is divided to 11 subfamilies and seven of them can be found in North America. Most of the Chironomidae we may come across are the members of the subfamilies Tanypodinae, Orthocladiinae and Chironominae [Epler, 2001]. The life-cycle of *C. tentans* consists of 3 stages, a larval stage composed of 4 instars (around one week in each instar), pupal stage and adult stage [ASTM, 1992].

Benthic invertebrates such as chironomid larvae are important components of many freshwater systems and the dominant members of the invertebrate macrofauna in many

freshwater habitats [Deevey, 1941; Assman, 1960]. It was reported that larval populations were counted up to 100,000 per square meter in some areas. They are important fish food in lakes and rivers; they may represent up to 80 % of certain fish food during summer [Bryce and Hobart, 1972]. Thus, the stability of aquatic communities can be altered if there is anything affecting these aquatic insects [Kosalwat and Knight, 1987]. They are found in water with both poor quality and high quality [Epler, 2001].

Massive swarms of *Chironomus plumosus* (L.) and *Propsilocerus akamusi* (Tokunaga) midges have repeatedly caused nuisance for local residents in the area of Lake Suwa in central Japan [Hirabayashi, 1991a, 1991b; Hirabayashi and Okino, 1998]. Recently, substantial emergence of *Einfeldia dissidens* (Walker) has also occurred in the littoral zone during the summer and caused new problems for tourists [Nakazato et al., 1998]. It has been reported that chironomid larvae affect lake nutrient budgeting substantially [Devai, 1980]. Members of the Chironomidae, Simuliidae, Ephemeroptera, Plecoptera, Odonata, and Trichoptera are identified as significant vectors to remove contaminants from the aquatic system [Menzie, 1980]. When insect larvae emerge, bioaccumulated metals transport these elements out of the aquatic system [Lee, 1970].

Related species, *Chironomus decorus* larvae, have a thin body covering that may aid copper penetration [Kosalwat and Knight, 1987]. The penetrating copper can irritate the nerve ending in the stomach of the larvae [Venugopal and Luckey, 1978]. This irritation at the nerve endings might also cause erratic movements of the larval digestive tract and result in high incidence of defecation among the test larvae [Kosalwat and Knight, 1987]. Physiology of exposed organisms is important in their sensitivities to metals in sediment. Chironomid larvae regulate accumulation of copper, nickel, and zinc in their tissues when exposed to these metals in sediments [Krantzberg and Stokes, 1989]. Metal-binding proteins

such as metallothioneins act as sinks or sequester metals in the tissues of organisms [Petering and Fowler, 1986; Fowler, 1987; Olsson and Haux, 1986].

Lipid content of chironomid larvae in Lake Michigan ranged from 9 to 39 % [Gardner et al., 1985]. To understand the transfer and partitioning of lipid-soluble contaminants in aquatic systems, lipid levels in invertebrates needed to be known [Kenaga and Goring, 1980; Scura and Theilacker, 1977]. *C. tentans* was the more sensitive than *Hyallea azteca* and *Daphnia magna* when exposed for 10 days to fluoranthene spiked sediment (10-day LC50: 23.6 μg/L of overlying water concentration) [Suedel and Rodgers, 1996]. Harkey et al. (1994) found that the bioavailability and accumulation of selected neutral hydrophobic contaminants to *Chironomus riparius* was higher from whole sediment than from aqueous extract from the sediment. Besides chemical contamination, *C. tentans* exposed to sediment with organic matter content <0.91 % may result in increased mortality due to physical characteristics [Suedel and Rodgers, 1994]. They prefer the particle size from 1 to 25 μm (fine clay) [Rasmussen, 1984].

As chironomid larvae possess giant chromosomes, they have been extensively used in genetic research [Epler, 2001]. In addition, *C. tentans* is frequently used in sediment toxicity tests because it has a short generation time and maintains direct contact with the sediment by burrowing case building activity [Nebeker et al.,1984]. Second instar larvae are preferable for toxicity tests [Wentsel et al., 1977;Giesy et al., 1988; Nebeker et al, 1984] because tests with first instar larvae had limited success [Nebeker et al., 1984]. At second instar stage, blood gills appear simultaneously with the first moult and larvae grow very rapidly [Sadler, 1935].

3. Sediment toxicity tests

The goal of sediment toxicity tests is to prioritize sediments and sites in terms of most concern with the greatest toxicity, or in monitoring recovery-related, e.g., remedial activities. Thus, the inability to quantify toxicity would be problematic [DeFoe and Ankley, 2003]. Because of a wide range of sensitivity of test endpoints to aqueous and sediment-bound contaminants [Ingersoll and Nelson, 1990; Winner 1988], selection of suitable test endpoints is essential for accurate assessment of sediment toxicity [Suedel et al., 1996].

There have been many standardized sediment toxicity tests, which use endpoints as survival/death, growth (dry weight and length), behavior or reproduction of benthic organisms such as *Chironomus* spp., *Hexagenia, Tubifex, Lumbriculus* spp, mussels, *etc*. [USEPA 2000]. Among those, 10-day survival of *Chironomus* spp. or *Hyalella azteca*. is the most commonly reported endpoint [USEPA 2000]. However, there is a need for standard laboratory tests that can estimate the potential chronic effect of contaminated sediment, since population or community-level impacts by sediment-associated contaminants often reflect chronic and sublethal effects rather than acute toxicity [Benoit et al., 1997]. Kemble et al (1994) proved that sublethal endpoints of sediment toxicity tests were better estimates of responses to contaminants in the field by benthic communities because the survival on short-term exposures may not be able to identify moderately contaminated sediments [Sibley et al. 1997, Benoit et al 1997, Ingersoll et al. 1998].

There are some other ways to test whole sediment toxicity using commercialized toxicity testing instruments such as BioTox™ flash assay or Microtox™ solid-phase test, which use bioluminescent microorganisms, *Vibrio fisheri*, and measure the inhibition of the bioluminescence by *V. fisheri* in the presence of contaminants [Lappalainen et al., 1999].

BioTox ™ flash assay has an advantage in that it contacts directly to the sediment and the results from the test can represent true bioavailability [Kwan and Dutka, 1995].

Another way to assess the sediment quality is porewater tests, because porewater represents a major route of contaminant exposure to sediment-dwelling organisms [Whiteman et al., 1996; Carr et al., 1989] and substantially influences the bioavailability of contaminants in sediment [Ankley et al., 1994; Di Toro et al., 1991]. With the extracted porewater, acute or chronic tests such as water flea (*Ceriodaphnia dubia*) survival/reproduction, sea urchin (*Arbacia punctulata*) embryological development, polychaete (*Dinophilus gyrociliatus*) reproduction, *etc* [Carr et al., 1996] can be conducted.

4. Bioturbation

Because many benthic invertebrates cause bioturbation through the resuspension of sediment into the water column by their burrowing, feeding, locomotive, respiratory, and excremental activities, bioturbation can play an important role in mediating both physical and chemical processes near the sediment-water interface [McCall and Fisher 1980, Matisoff et al, 1985, Krantzberg and Stokes 1985]. The role of bioturbation on the sediment-to-water flux of chemicals has been recognized by a number of authors [Petr, 1977; McCall and Tevesz, 1982; Krantzberg and Strokes, 1985] and bioturbation increases sediment-to-water flux of organic (and inorganic) compounds in aquatic ecosystems.

Oligochaetes, as conveyor belt species, forage below the surface egestion and excrete particles onto the surface of the sediment so that the worms secure that their food supply is replenished continually and not diluted by recently digested materials [Alsterberg, 1925; Brinkhurst et al., 1972]. The pollutant flux into the water caused by bioturbation, Fp,, can be described as follows:

$$Fp = Fs \cdot S^{f} \cdot R,$$

where Fs = the flux of oligochaetes fecal pellets to the surface;

S^{f} = the sorbed pollutant concentration on the transported sediment; and

R = the pollutant re-lease from the fecal pellet during its residence time at the surface [Karickhoff and Morris, 1985].

However, Karickhoff and Morris (1985) did not see that the water-to-sediment flux was enhanced by bioturbation, since bioturbation resulted in a net upward transport of sediment organic chemicals. The presence of oligochaetes considerably expanded the depth of sediment that interacted with the water over a short time. Their burrowing activity increased porosity and enhanced porewater movement. Thus, diffusion rate may be affected by bioturbation [Karickhoff and Morris, 1985].

Bioturbation did not increase sediment accumulation of 2,2',4,4'-tetrachlorobiphenyl (TCB) by oligochaetes [Ewald et al. 1997]. Instead, the sediment accumulation could be explained by retarded diffusion, a combined effect of the processes of adsorption and diffusion. No effect of clay content on the accumulation was found [Ewald et al. 1997]. Diffusion of lipophilic substances is highly retarded by sorption [Wang et al. 1991]. In other research with dissolved Cd (water source), bioturbation by lugworms transferred dissolved Cd to sediment [Rasmussen et al. 1998] because of the increase in suspended particles which have increased availability of binding sites such as metal oxides, mineral, and organic matter surfaces by bioturbation [Banta and Andersen 2003].

Studies have indicated that bioturbation occurs at the interface of sediment and overlying water. Bioturbation can release many chemicals bound to the sediment into the overlying water. For example, bioturbation by estuarine amphipods (*Corophium volutator*) significantly increased total suspended solids and particulate organic carbon/particulate

organic matter concentrations in overlying water and it increased uptake of sediment-bound fluoranthene to by filter-feeding mussels (*Mytilus edulis*) [Ciarelli, 1999]. Likewise, bioturbation by *Chironomus riparius* resulted in a remobilization of particle-associated lindane to the interstitial and overlying water, implying an increase in the bioavailability of the lindane [Goedkoop and Peterson, 2003]. Similarily, it was found that sediment-associated Cd was mobilized into the overlying water by nymphs of a mayfly through burrowing and respiratory activities [Bartsch et al. 1999]. In the presence of *C. riparius*, the pH of the sediment substantially rose [Edwards, 1958]. These results were attributed to the removal of acid metabolites such as free amino acid, purine, and urea and uric acid by flushing action of tube irrigation may explain this [Johannes and Webb 1970]. Ganapati (1949) found that *C. riparius* larvae elevated the release of free ammonia from the sediment and Edwards (1958) similarly showed increased ammonia concentrations and decreased nitrate-N concentrations in overlying waters of colonized sludge. The burrowing infauna promoted a return of mineralized nutrients (e.g. NH_3^+ and NH_4) to the overlying seawater at a greater rate than molecular diffusion alone [Rhoads et al., 1977]. A major route for the release of nitrate from the sediment is thought to be the deposition and subsequent decomposition of faecal pellets. By burrowing activities, rates of sediment-water exchange of ammonium-N are increased [Chatarpaul et al., 1980]. According to Neame (1977), dissolved phosphorus was released across the sediment-water interface largely by the stirring activities of the macrofauna. Excretion from *Chironomus* spp. was confirmed for most of the phosphorus release from aerobic sediment [Gardner et al., 1981].

Banta and Andersen (2003) summarized the positive and negative effects of infauna and their bioturbation on the fate of the particle-reactive pollutants in their review paper. One of the positive effects is that the conditions for microbial degradation of persistent organic

pollutants is stimulated by bioturbation due to the increased availability of O_2, NO_3^-, and metal oxides [Rockne and Strand 1998]. Alternatively, the infauna themselves may metabolize pollutants leading to remediation of sediment contamination. On the other hand, one of the negative effects is increased mobility of the contaminants in sediment by bioturbation [Banta and Andersen 2003]. Banta and Andersen (2003) concluded that the results of the opposing effects of bioturbation on the fate of contaminants depend on the physicochemical characteristics, concentration, distribution, and source of the pollutants, kinds of infauna and their mode of bioturbation, and physiological processes by infauna.

From many studies on the effect of bioturbation on the polycyclic aromatic hydrocarbons (PAHs) fate, Banta and Andersen (2003) concluded that, depending on where the contaminants are found, bioturbation by the lugworm *Arenicola marina* could lead to different effects on the fate of PAHs in the sediment. When PAHs are found at or near the sediment water interface or in the water, the effect is enhanced burial and incorporation of PAHs within the sediment, and a decrease in the rate of release and mineralization of PAHs. On the contrary, if PAHs are already distributed throughout the sediment, then lugworm bioturbation increases the removal of PAHs from the sediment, both as untransformed compounds to the overlying water and as metabolic products [Banta and Andersen 2003].

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CHAPTER II: Performance of BioTurbTox: A New Bioturbation-Based Toxicity Test with *Chironomus tentans* Larvae for Assessing the Toxicity of Aquatic Sediments

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ABSTRACT

Bioturbation is caused by the burrowing, feeding, respiratory, and locomotor activities of many species of infaunal benthic invertebrates. A reduction in bioturbation (turbidity generation) may be indicative of contaminant exposure and subsequent toxic effects on normal physiological function in these organisms. Therefore, we developed a novel test method that used bioturbation generated by the activities of second in-star *Chironomus tentans* larvae as the toxicity endpoint. Because bioturbation is also an important biological activity that influences physical and chemical processes at the sediment-water interface, including contaminant transport and availability, we assessed the mobilization of two model contaminants (copper and fluoranthene) into the water column during these experiments. Initial tests were conducted to evaluate the influence of sediment texture (particle size distribution) and the number of midge larvae in a given experimental unit on the bioturbation response. We found that a clay-dominated sediment with five *C. tentans* larvae per experimental unit provided the optimum conditions for future testing. To further validate the performance of the test, copper (Cu) and fluoranthene were individually spiked into a relatively uncontaminated aquatic sediment to assess changes in bioturbation and the mobilization of the chemicals into the overlying water. Turbidity of overlying water decreased with increasing Cu concentrations (0, 10, 20, 30, 40 μg/g) and fluoranthene concentrations (0, 2.5, 5.0, 7.5, 10, 12.5 μg/g) in the sediment compared to treatments without larvae. Substantial concentrations of Cu were released into overlying water from the Cu-spiked sediment, whereas little fluoranthene was mobilized into the overlying water from the fluoranthene-spiked sediment. Based on our results, the bioturbation test shows promise as a tool to assess toxicity and mobilization of contaminants from aquatic sediments.

INTRODUCTION

Recent studies have shown that sub-lethal endpoints such as growth, reproduction, and behavior provide accurate and ecologically meaningful estimates of the toxic responses by benthic organisms exposed to contaminated aquatic sediments both in the laboratory [Sibley et al. 1997] and in the field [Kemble et al. 1994]. Bioturbation, the biological process through which many species of infaunal benthic invertebrates suspend bottom sediments into the water column through their burrowing, feeding, respiratory, and locomotor activities, may be one such sub-lethal endpoint that can be exploited to assess the toxicity of aquatic sediments. For example, a reduction in bioturbation (as measured as turbidity generation) may be indicative of contaminant exposure and subsequent toxic effects on normal physiological function in these organisms.

To our knowledge, the concept of using bioturbation as an indicator of sediment toxicity has been tested only twice; once in a study with a freshwater burrowing mayfly [Bartsch et al. 1999] and once in a study with a marine amphipod [Briggs et al. 2003]. In the study by Bartsch et al. (1999), nymphs of the burrowing mayfly *Hexagenia bilineata* were exposed to cadmium-spiked sediment for 21 d. Their test was conducted in 4-L beakers and required large volumes of sediment (750 g wet weight), overlying water (3 L), and repeated removal-based sampling of the overlying water for the analysis of turbidity. Moreover, the extensive experimental design of their relatively long (21 d) study required sizable laboratory space and relied upon field-collected organisms to conduct the test. They found that turbidity progressively decreased as cadmium concentration in the sediment increased up to 7µg/g, but that turbidity in their greatest exposure concentration (15µg/g) was not significantly different from the control. They determined that size variation in nymphs may have impaired detection of significant differences and concluded that bioturbation may not be

a suitable endpoint of toxicity with this test organism and experimental design.

In contrast, the study by Briggs et al. (2003) evaluated whether bioturbation caused by the marine amphipod *Corophium volutator* was indicative of exposure to copper or hydrocarbon spiked sediments in 10 d tests. Their experimental units were smaller than those of Bartsch et al. (1999), but still required 1-L beakers, 150 mL of sediment, 650 mL of overlying seawater, sizable laboratory space, and collection of organisms from the field to conduct the test. However, a major advance of this study was that repeated, removal-based sampling of the overlying water for the analysis of turbidity was not required. Their turbidity measurements were performed by placing the beaker directly in a photovoltaic cell connected to a multimeter, similar to a standard turbidity analysis with a commercial turbidimeter. They found that turbidity increased as contaminant concentrations in the sediment increased, before decreasing due to the effects of mortality, and that turbidity measured at 24 h was correlated with mortality at 10 d. They concluded that bioturbation-caused turbidity measurements have potential for estimating the toxicity of sediments, but that the effects of variables such as sediment particle size require further testing for this to be a suitable and reproducible endpoint of sediment toxicity.

Incorporating the positive attributes and results of these two studies, we developed and tested a small-scale, rapid, high throughput type screening test that utilizes easily cultured or commercially available standard test organisms with commercially available turbidimeter equipment. In this paper, we report the optimization and performance of this novel test method that uses bioturbation generated by the activities of second in-star *Chironomus tentans* larvae as the toxicity endpoint. Because bioturbation is also an important biological activity that can influence physical and chemical processes at the sediment-water interface, including contaminant transport and availability, we also quantify and report the

flux of sediment-associated contaminants caused by chironomid bioturbation during tests with two model compounds (copper and fluoranthene). Therefore, the specific objectives of this study were to develop and refine a novel toxicity test method for freshwater sediment that uses bioturbation generated by second in-star *C. tentans* larvae as a sub-lethal endpoint and to evaluate the relation between bioturbation generated by *C. tentans* and contaminants mobilized from the sediment into the overlying water through bioturbation.

MATERIALS AND METHODS

Development and Optimization of BioTurbTox Test

We chose to develop this test with *C. tentans* because it and other species in the genus Chironomus have been shown to cause bioturbation through their direct contact with sediment in their burrowing and case building activities [Matisoff et al. 1985; Goedkoop and Peterson 2003]. Moreover, this species is commonly used in standard sediment toxicity tests [ASTM 1992], it is easily cultured in the laboratory or is commercially available and has a short life span. It is also an ecologically relevant choice because larval chironomids are often dominant members of the invertebrate macrofauna in many freshwater habitats and are important in the diet of many species of fish [Deevy 1941; Assman 1960]. Originally, chironomids of the species *C. tentans* were purchased from Aquatic Bio Systems Inc. (Fort Collins, Colorado) and cultures were maintained in dechlorinated tap water at around 21 °C, with aeration and a 16:8 h light:dark photoperiod and fed with ground Tetramin fish food everyday. Surficial sediment was collected from Apex Community Park Lake in Apex, NC, placed into acid and solvent cleaned buckets, transported to the laboratory in coolers with ice, and refrigerated at 4°C. As soon as the sediment was brought to the laboratory, it was homogenized with a stainless steel spoon, and passed through a stainless steel sieve with 2

mm openings (without the use of additional water) to remove large particulate matter and indigenous organisms and stored refrigerated at 4 °C until the tests began. To optimize the number of larvae tested, ~15 g wet weight of sediment (2.6 cm depth in the testing cells) and ~ 20 mL dechlorinated tap water were very carefully filled into HACH sample cells for ratio turbidimeter using Pasteur pipette to reduce the turbidity caused by handling and allowed to settle for 1 day before addition of larvae to the cells. Second in-star (around 14-day old) larval *C. tentans* were used in the test and 0, 1, 2, 4, 8, 16, and 32 larvae were placed into the sediment (n=1-2) per treatment. Turbidity (NTU) was measured after 0, 6, 20, 25, and 48 hours using 2100 AN turbidimeter (HACH company, Loveland, CO). Larvae were not fed during the exposure, no aeration was supplied to reduce the turbidity in the vials, and 16:8 h light:dark photoperiod were provided during exposure time. To assess the effect of sediment type, further tests with sandy type sediment (sand 89 %, silt 7.6 %, clay 3.3 %) and clay type sediment (sand 45.5 %, silt 35.1 %, clay 19.3 %) were conducted. Both types of the sediment were also collected from Apex Community Park Lake in Apex, NC, transported to the lab and sieved with 2 mm sieve. Four and five larvae per vial, which were determined to be the optimal number, were placed onto each type of the sediment and there were 5 to 10 replicates for each treatments, two types of the sediment and different numbers of the larvae (4 vs. 5). There was a control group with each type of sediment and no larvae. Sediment particle size was analyzed by sieve-pipet method [USGS 1979]. Turbidities were measured at 0, 2, 6, 12, 24, 30, 34, 37, and 48 hours. Other methods were the same as previously described in the tests for optimizing larval numbers.

Demonstration of BioTurbTox with Cu-spiked sediment

Based on previous tests, four to five *C. tentans* larvae were determined to be the

optimal numbers for future testing with clay type sediment. Therefore, in the spiked sediment tests, four larvae were placed into each vial. Calculated Cu concentrations in the sediment were 0, 10, 20, 30, and 40 µg/g dry weight of sediment (n=5). After 48 hours of exposure period (e.g., test termination), sediment samples and water samples above the sediment were taken. Water samples (10 mL from each vial) were filtered through 25 mm, 0.45 µm Metricell® membrane filters and combined to form a composite water sample for each Cu test concentration. The remaining water in the vials was filtered in the same manner and used as an individual sample for each Cu concentration. For the Cu analysis, water samples were acidified with concentrated ultrapure nitric acid and sediment samples were digested with hot nitric acid. Digested water and sediment were analyzed by ICP-MS in a contract lab in Raleigh, NC. At the same contract lab, mass of particulate/dissolved organic carbon in water and on the filter papers were measured.

Demonstration of BioTurbTox with fluoranthene-spiked sediment

To ensure solubility for spiking, fluoranthene was dissolved in acetone and was added to the sediment. Excess acetone was evaporated under the hood overnight. The calculated concentrations for the fluoranthene-spiked sediment were, 0, 2.5, 5.0, 7.5, 10.0, and 12.5 µg/g dry weight of sediment (n=3). After 48 hours of exposure period, sediment samples and water samples above the sediment were taken. Water samples (10 mL from each vial) were filtered through pre-weighed and pre-ashed (300 °C) glass fiber filters (0.45 µm GF/C membrane filter) and combined to form a composite water sample for each fluoranthene concentration. The remaining water in the vials was filtered in the same manner and used as an individual sample for each fluoranthene concentration. For the water fluoranthene analysis, liquid-liquid extraction procedure was used. Water samples with the

surrogate internal standard and dichloromethane were placed in a separatory funnel and the organic layer of dichloromethane was drained and dried with anhydrous sodium sulfate. After repeating these steps, the extracts were concentrated under a gentle stream of nitrogen to 1mL. The extracts were analyzed by GC-MS.

Fluoranthene in the sediment was extracted by a Shaker table extraction method and a silica column clean up procedure was used to eliminate any lipid in the samples. Surrogate internal standard (SIS) was spiked into all sediment samples and a matrix spike along with a matrix blank sample composed of clean sediment was analyzed. Sixty mL of 1:1 acetone:dichloromethane (DCM) and 30g of anhydrous sodium sulfate were added to the sediment samples and were shaken on an orbital shaker table overnight. After repeating this process, the extracts were concentrated with a roto-evaporation unit, and then, concentrated under a gentle stream of nitrogen to a final 1 mL volume. The solvent of the samples was exchanged with 95% hexane and the samples were loaded onto a dry pack column consisting of 1cm baked anhydrous sodium sulfate, 3 g activated silica, and 1 cm baked anhydrous sodium sulfate. Twelve mL of hexane and 15 mL of 1:1 95 % hexane:DCM was added serially and the eluted fraction was collected. The collected fraction was evaporated under a gentle stream of nitrogen to ~1mL. The extracted samples were analyzed by using GC-MS in the selected ion monitoring (SIM) mode.

Statistical Analysis

To evaluate the importance of bioturbation on the release of Cu and fluoranthene from sediment into overlying water, all water Cu and fluoranthene data were logarithmically transformed to reduce variance heterogeneity. The fluoranthene concentrations in the control samples were omitted because they were zero. The water concentrations of each chemical in

the presence or absence of larvae were compared by two-way ANOVA testing with interaction using PC-SAS® [SAS Institute 1999]. One-way ANOVA was carried out separately at each time, followed by Least Significant Difference (LSD) to compare the turbidities in different Cu and fluoranthene concentrations with PC-SAS® [SAS Institute 1999]. The analysis was done on turbidity and on log transformed turbidity values. To get the estimate of EC50s (median effective concentration) at 48 hours for both tests, all the turbidities were converted to % proportional to the maximum turbidity at 48 hours at all the treatment levels and it was estimated by Spearman-Karber procedures using TOXSTAT® 3.4 [West Inc. 1994].

RESULTS

Water qualities of the culturing tanks (mean ± S.E.) (pH, 7.40 ± 0.09; temperature, 22.62 ± 0.20 °C; D.O., 6.23 ± 0.35 mg/L; alkalinity, 46.70 ± 0.88 mg/L $CaCO_3$; Hardness, 40.75 ± 1.25 mg/L $CaCO_3$) were maintained constantly. Background Cu concentration in the sediment was 0.655 ± 0.055 μg/g dry sediment (mean ± SE). There was no turbidity change over 48 hours when there were 0, 1, and 2 larvae (data not shown). Turbidity change during 48 hours in the presence of more than 4 larvae in the sediment are shown in Fig. 1. After the larvae were placed onto the sediment, turbidity increased (Fig. 1). When there were 16, and 32 larvae, turbidity was decreased after it reached its maximum point. The testing cells were over-crowded by more than 8 larvae and they did not stay in the sediment. However, turbidity kept increasing in the presence of 4 larvae and the testing cells were not crowded. Therefore, 4 or 5 larvae in each vial (1.2 larvae/cm^2 or 1.5 larvae/cm^2) were chosen for further testing.

That is also relevant number considering natural density of midge larvae in the sediment (up to 10 larvae/cm^2 [Bryce and Hobart 1972]).

Measured concentrations of Cu in the sediment after 48 hours were very close to the nominal concentrations of the Cu (Table 1). However, the measured fluoranthene concentrations in the sediment were somewhat different from the nominal concentrations attributed to some spiking error especially at the lower concentrations and it did not show the apparent concentration gradient (Table 1).

The presence of the larvae increased the turbidity compared to treatment without larvae ($F=86.23$, $p<0.0001$). Since turbidity increase was significantly higher when larvae were placed in clay type sediment than in sand type sediment. ($F=36.33$, $p<0.0001$), clay type sediment was chosen for further testing (Fig. 2). Sand type sediment had slightly higher turbidity than clay type sediment in the absence of larvae from the control test. There was no difference when 4 larvae or 5 larvae were placed onto the vial. Though the turbidities with 5 larvae were slightly higher than with 4 larvae in both types of the sediment, it was not statistically significant ($F=1.06$, $p=0.3237$) (Fig. 2). For the further tests, 5 larvae were used in order to provide extra larvae in case one died.

Turbidity over the 48-h exposure time increased in the presence of larvae at all ranges of Cu concentrations spiked into the sediment and the degree of increase was more apparent at lower concentration of Cu in the sediment (0, 10, 20, and 30 μg/g) (Fig. 3). At 48 hours, the turbidities were divided into three groups 0 / 10, 20, 30 / 40 μg/g and those three groups were significantly different from each other. In the fluoranthene-spiked sediment, turbidity increased in the presence of larvae, but only at the low concentrations (0, 2.5, 5.0 μg/g) during the exposure time, not at the high concentrations (Fig. 4). There were four significantly different groups at the end of the test – 0 / 2.5 / 5.0/ 7.5, 10.0, 12.5 μg/g.

Bioturbation caused a significant release of Cu from the sediment into the overlying water, but not for fluoranthene. Though Cu was also released from sediment into water in the absence of larvae, Cu release with larvae was significantly higher ($F= 16.95$, $p<0.0001$) (Fig. 5). Contrary to Cu treatment, less fluoranthene was released into the overlying water in the presence of larvae compared to the treatment without larvae ($F=2.94$, $p=0.0328$). The EC50 at 48 hours from Cu-spiked sediment was 14.93 μg/L (10.82, 19.04: 95 % confidence interval) and from fluoranthene-spiked sediment was 2.00 μg/L (1.35, 2.66: 95 % CI).

DISCUSSION

Turbidity increase was apparently higher in the presence of larvae ($F=86.23$, $p<0.0001$) because of their remobilizing the sediment, as it was also observed in other studies [Lee 1970; Driscoll 1975; Petr 1977]. The quantity of suspended particles in the overlying water increased linearly with *Corophium volutator* density and over time [Ciarelli 1999]. Clay type sediment was chosen for further testing when toxicant-spiked sediment was used because the pattern for the turbidity increase was more apparent when the larvae were placed into the clay type sediment than in the sand type sediment (Fig.2). Though there was no significant difference in the turbidity increase with four larvae or five larvae, use of five larvae is recommended because it will be safer in case one larva dies. Gradient response of turbidity was shown depending on chemical concentrations spiked in the sediment (Fig. 3, 4). Both at the high concentrations in the Cu-spiked and fluoranthene-spiked sediment, turbidity change was not significant because it is thought that the high concentrations affected behavior of the larvae and the movement decreased significantly though they were still alive.

The LC50 values of Cu in the sediment to *C. tentans* are 4522 and 1905 µg/g from 48-h and 96-h exposures, respectively [Suedel et al., 1996; West et al.,1993]. These values are much higher than the estimated EC50 from Cu-spiked sediment in this study (14.93 µg/L). From Hirthe et al. (2001), high concentrations of lindane prevented borrowing of *C. riparius* by paralysis, delayed emergence times, and made adults wings smaller. They though that paralysis can cause a risk of predation or increased downstream drift.

Significant concentrations of Cu were released from sediment to overlying water by *C. tentans* larvae. *Chironomus* sp. appears to be as important in mixing sediment as deposit feeding conveyor-belt tubificid oligochaetes [Matisoff and Wang 2000]. It was also found in a previous study by Bartsch et al.(1999) that sediment-associated Cd was mobilized into the overlying water by nymphs of a mayfly, through burrowing and respiratory activities. Cu also was released into water as a desorption process though there were no larvae in the sediment. However, more Cu release into water was noticed in the presence of the larvae (Fig. 5). As a possible mechanism of the metal release from sediment into overlying water, Krantzberg and Strokes (1985) found that bioturbation by tubificids, chironomids, and chaoborids altered sediment redox and metal pools, which favors the release of metals to the overlying water. Oxidation of sediments by irrigation not only enhances aerobic metabolism but also alters the mobility of the metals. Since the metals like Cd and Hg are tightly bound by sulfides, reoxidation of sulfides can lead to increased mobility of these metals. Thus, it can cause detrimental effects on the surrounding environment in some cases [Banta and Andersen 2003; King et al. 2000].

More than 90 % of the tested midge larvae survived during the exposure time in the Cu-spiked sediment (less than 40 µg Cu/g dry sediment). Environmental concentrations of Cu are 59 µg/g dry sediment in Tualatin River, Oregon, 18,259 µg/g dry sediment in a creek

downstream from a Cu smelting operation, Mississippi, and 10 μg/g dry sediment in Arkansas River, Arkansas [Cairns et al. 1984; Suedel et al. 1996; Harrahy and Clements 1997]. LC50 values of Cu to organisms are 72.2, 65.6, and 31 μg/L from 48-h, 96 h, and 10-d water only for *H. azteca*, and 2.72 and 1.46 μg/L from 48 h and 96 h water only test for *C. dubia* [Suedel et al.1996; West et al.1993]. Thus, it is possible that the release of Cu by bioturbation into the overlying water poses a risk to aquatic organisms that are sensitive to Cu such as *H. azteca* and *C. dubia*.

In contrast to the Cu release from sediment to overlying water by bioturbation, release of fluoranthene was not affected by bioturbation and it was not dependent on the concentration gradient. *C. tentans* larvae are resistant to fluoranthene in the water and in the sediment relative to other aquatic organisms. For example, the LC50 value in the sediment was incalculable because there was no significant mortality found from the fluoranthene-spiked concentrations studied (range from 0 to 5 μg/g dry wt.) with UV light [Hatch and Burton 1999]. However, decrease of turbidity in the overlying water by *C. tentans* larvae from 2.5 μg/g of fluoranthene-spiked sediment observed in this study and EC50 was estimated at 2.0 μg/L. A 10-day LC50 value of 50 μg/g dry wt. was found for *Corophium volutator*, a marine amphipod [Ciarelli et al. 1999]. LC50 values of fluoranthene to *Hyalella azteca, Daphnia magna*, and *Chironomus riparius* were 5, 10, and 15 μg/g dry sediment, respectively [Verrhiest et al. 2001]. Some possible reasons why there was no difference in the fluoranthene release into the overlying water by bioturbation are thought to be high log Kow of fluoranthene (5.16), which made it strongly sorbed to the sediment and larvae that has high lipid content of 9 - 39 % [Gardner et al. 1985], and to be inactive larvae (no significant changes in the turbidity) at those experimental concentrations tested.

However, there are several ways to lose PAHs within the sediment by metabolism,

depending on the species living there [Banta and Andersen 2003]. *Arenicola marina* bioturbation greatly enhanced flushing of unmetabolized pyrene from the sediment due to its advective irrigation [Christensen et al. 2002a]. However, the main mode of the pyrene removal with *Nereis diversicolor* was in the form of metabolites [Christensen et al. 2002]. In the case of *Capitella* spp., the loss rate of PAHs from sediment was increased by enhancing microbial degradation [Bauer et al. 1988; Hansen et al. 1999]. It was identified that *Capitella* sp. I. was able to metabolize fluoranthene to two tentative metabolites, 3- and 8-hydroxyfluoranthene. And fluoranthene was no longer detected in the worm tissues after 24 h in clean sediment [Forbes et al. 2001]. Release rates of pyrene and its metabolites from sediment into the overlying water was greatly enhanced by the *Arenicola marina* bioturbation compared to the rates in sediment without bioturbation [Christensen et al. 2002]. Typically, PAHs in animals are metabolized by the MFO system and it involves hydroxylation and epoxide formation [Di Giulio et al. 1995]. Pyrene was metabolized by *C. riparius*, but it was species specific [Verrengia Guerrero et al. 2001]. Activity of the MFO system in *C. riparius* was higher than in other aquatic species [Sturm and Hansen 1999]. Only 17% was detected as parent compound in *C. riparius* after 20 h treatment with benzo(a)pyrene [Borchert et al.1997]. There could be some metabolic loss of the contaminants by *C. tentans*, but no study was done on the metabolism of Cu or PAHs by *C. tentans*.

The new method described here is reliable and easy to test the turbidity caused by midge larvae, toxicity of contaminants to larvae, and the analysis of the contaminant concentrations in the overlying water released from sediment. Another advantage is that this method can measure the turbidity throughout any time interval while conducting the toxicity test. Moreover, it can be widely used for testing a suite of chemical classes and sediment particle sizes due to its reliability for bioturbation compared to controls and its ease of

preparation. Thus, bioturbation can be an end-point for sublethal sediment toxicity test.

In conclusion, the new bioturbation toxicity test (BioTurbTox) method is appropriate as a way to measure sediment toxicity and the mobilization of sediment-bound contaminants caused by bioturbation. Bioturbation caused a significant release of Cu from the sediment into the overlying water. Therefore, bioturbation can be used as a method to assess the contaminant risk of sediments in aquatic ecosystems. Depending on the chemical classes, the chemical bound to the sediment can be released in differing quantities. Future tests also include an assessment of the new method's ability to detect toxicity in sediments collected from a natural aquatic ecosystem.

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Table 1. Measured Cu and fluoranthene concentrations in the sediment (dry weight basis) after 48 hours of exposure.

Cu			**Fluoranthene**		
Nominal Concentrations (μg/g)	Measured Concentrations (μg/g)		Nominal Concentrations (μg/g)	Measured Concentrations (μg/g)	
	With Larvae	Without Larvae		With Larvae	Without Larvae
0	0.6	0.71	0	0.13	0.15
10	9.8	10.4	2.5	6.11	5.09
20	20.4	18.9	5	4.21	6.09
30	31.1	32.1	7.5	4.89	5.73
40	38.6	40.8	10	6.16	8.11
			12.5	9.64	15.94

FIGURE LEGENDS

Fig. 1. Turbidity changes over 48 hours in the presence of 4, 8, 16, 32 larvae in each testing cells.

Fig. 2. Turbidity changes over 48 hours with 4 and 5 larvae in clay type sediment and sand type sediment. Error bars represent standard errors.
(CL5: 5 larvae in clay type sediment, CL4: 4 larvae in clay type sediment,
CN: no larvae in clay type sediment, SN: no larvae in sand type sediment,
SL5: 5 larvae in sand type sediment, SL4: 4 larvae in sand type sediment)

Fig. 3. Turbidity increase in the presence of 4 *C. tentans* larvae in the low concentrations of Cu-spiked sediment during the exposure time. Concentrations not accompanied by a common letter were judged to be significantly different at test termination (48 hours)

Fig. 4. Turbidity increase by 4 *C. tentans* larvae in the low concentrations of fluoranthene-spiked sediment over 48 hr exposure time.

Fig. 5. Cu release into the overlying water from the sediment in the presence of larvae and in the absence of larvae. Significantly higher release of Cu in the presence of larvae.

Fig. 6. Fluoranthene concentration in the overlying water in the presence of larvae and the absence of larvae from the fluoranthene-spiked sediment.

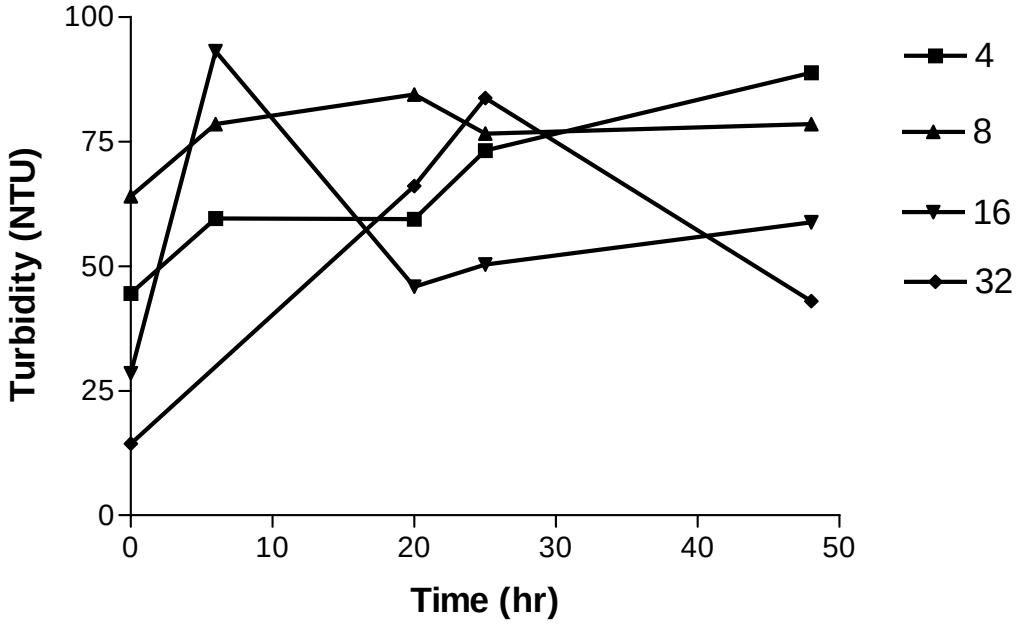

Fig. 1

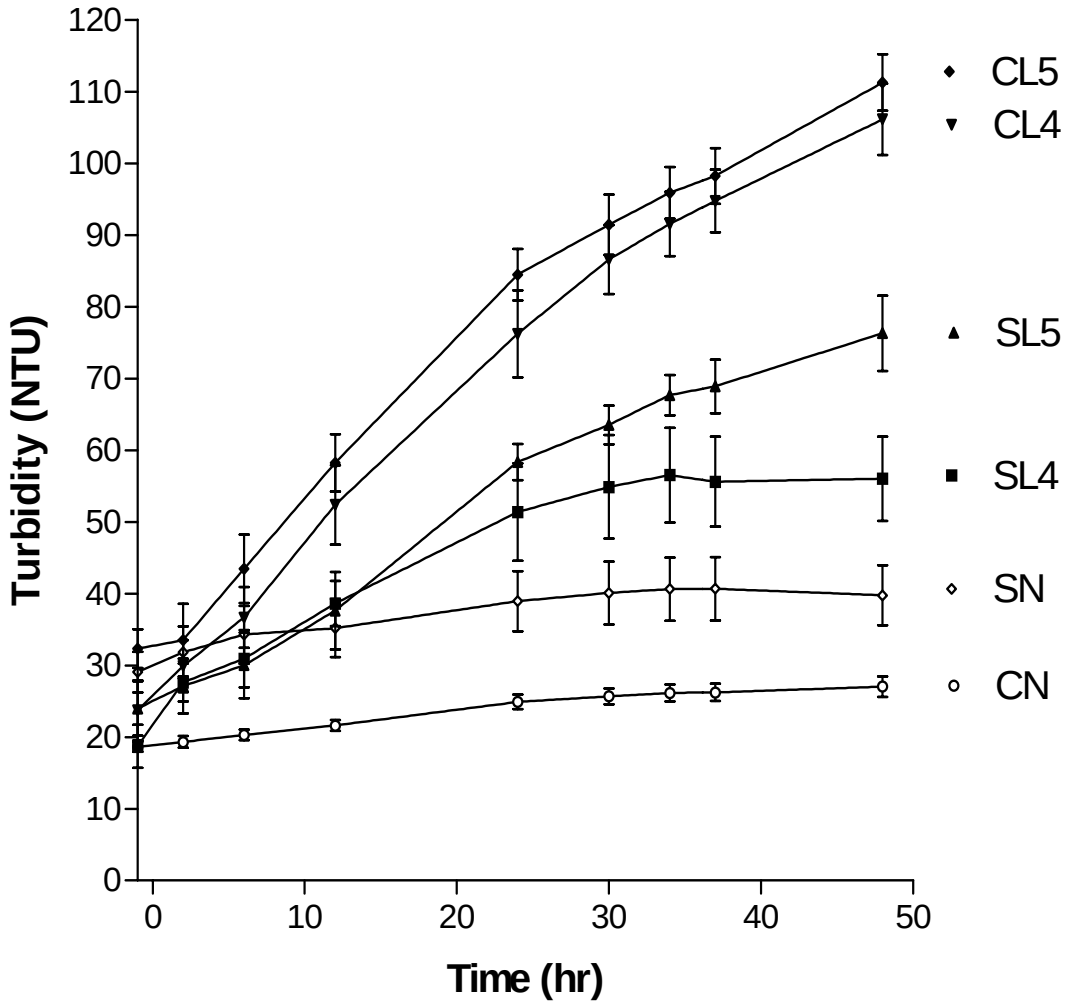
Turbidity (NTU)
Time (hr)
CL5
CL4
SL5
SL4
SN
CN
0
10
20
30
40
50
60
70
80
90
100
110
120

Fig. 2

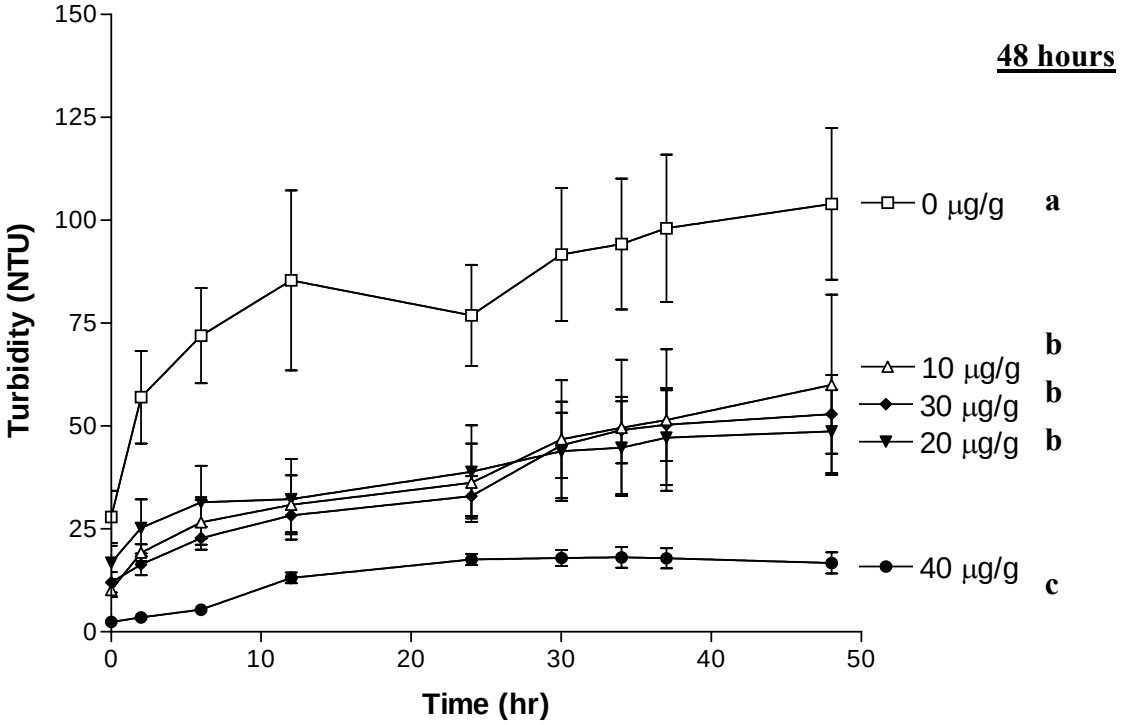
48 hours
Turbidity (NTU)
Time (hr)
0 μg/g
10 μg/g
30 μg/g
20 μg/g
40 μg/g
a
b
b
b
c

Fig. 3

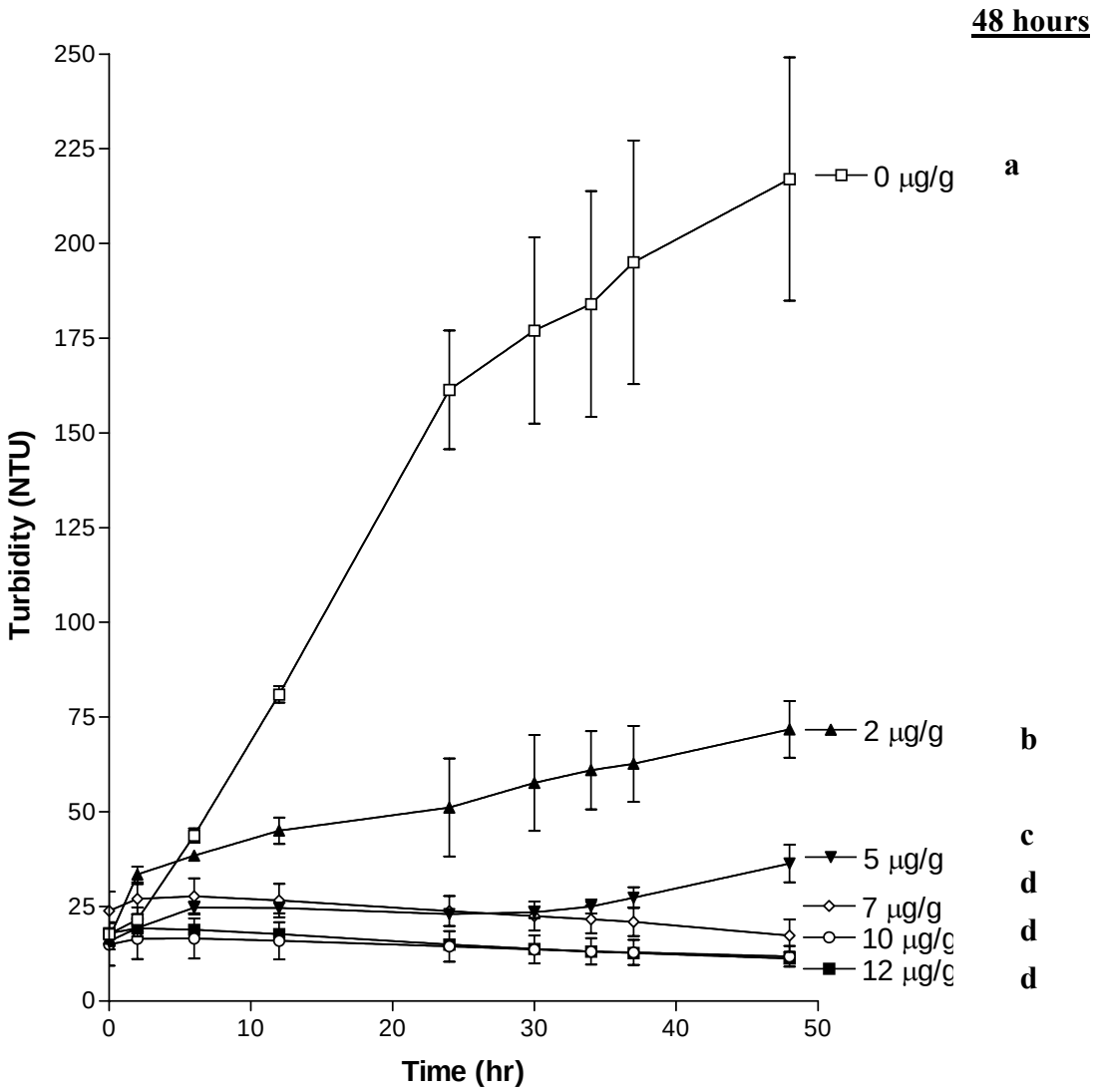
48 hours
Turbidity (NTU)
Time (hr)
0 μg/g
2 μg/g
5 μg/g
7 μg/g
10 μg/g
12 μg/g
a
b
c
d
d
d
0
25
50
75
100
125
150
175
200
225
250
0
10
20
30
40
50

Fig. 4

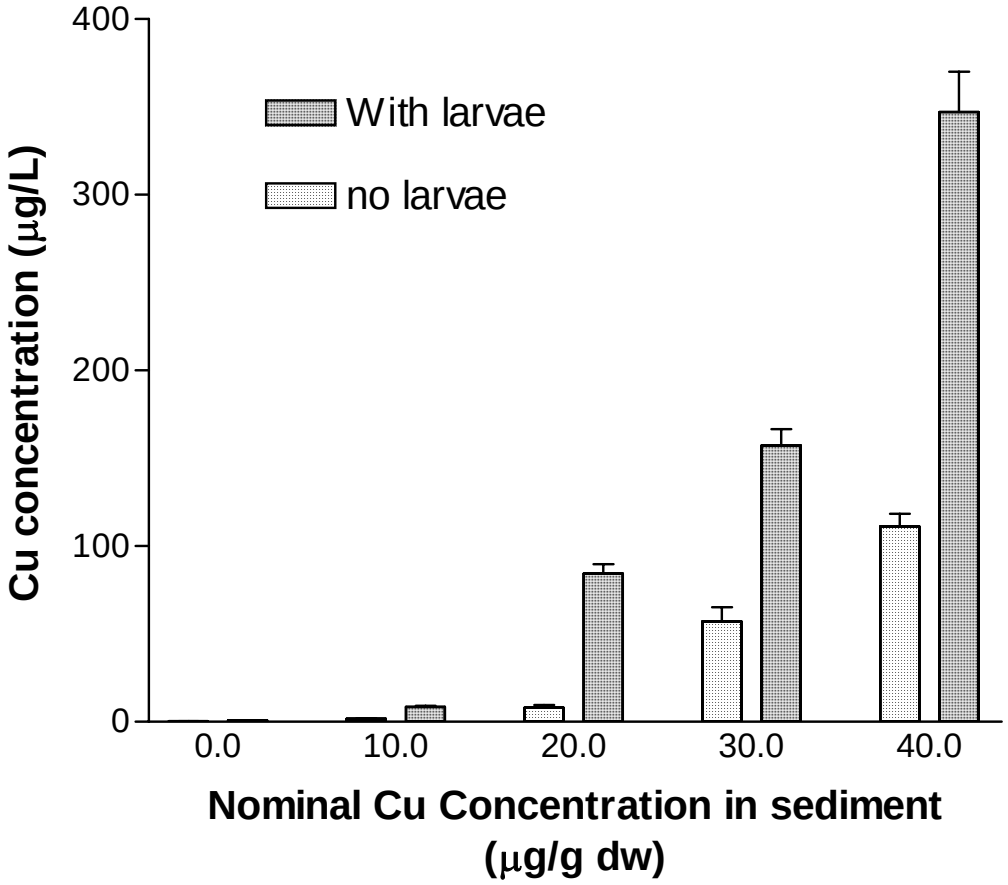
400
300
200
100
0
With larvae
no larvae
Cu concentration (μg/L)
0.0
10.0
20.0
30.0
40.0
Nominal Cu Concentration in sediment
(μg/g dw)

Fig. 5

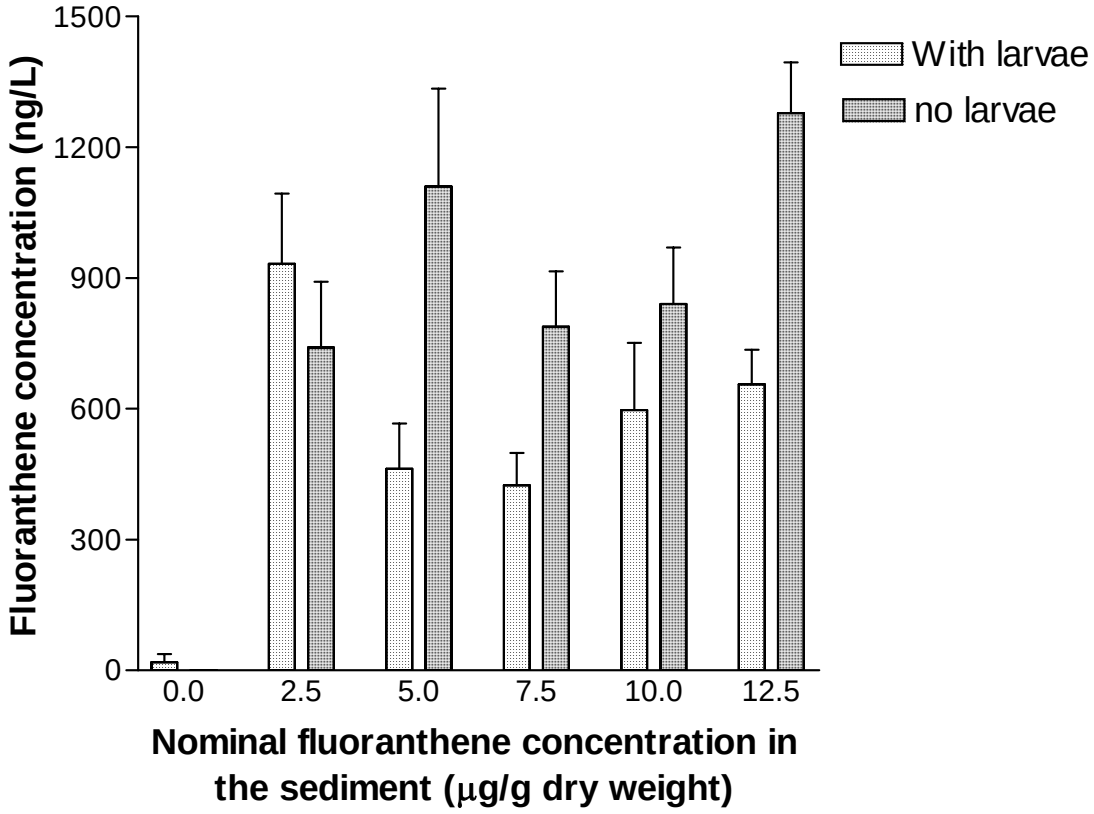
1500
1200
900
600
300
0
Fluoranthene concentration (ng/L)
With larvae
no larvae
0.0
2.5
5.0
7.5
10.0
12.5
Nominal fluoranthene concentration in the sediment (μg/g dry weight)

Fig. 6

CHAPTER III: Assessing the Toxicity of Neuse River, North Carolina Sediments with a Suite of New and Standard Test Methods

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Abstract

Periodic fish kills have been reported from the Neuse River system of North Carolina for many years. Excess nutrients and low dissolved oxygen concentrations associated with algal blooms have been recognized as probable contributing factors. However, few studies have been conducted evaluating other potential causal factors such as waterborne and sediment associated contaminants. Therefore, we used the newly developed sediment toxicity test, BioTurbTox, a chironomid bioturbation-based test, along with other standardized sediment toxicity tests, such as a 10-day *Chironomus tentans* growth test, the BioTox™ Flash assay, and a *Ceriodaphnia dubia* porewater test to evaluate the toxicity of Neuse River sediments during the summer of 2003. We also simultaneously collected water and sediment samples and deployed passive sampling devices (PSDs) at six study sites in the upper-, middle-, and lower-Neuse River and analyzed them for polycyclic aromatic hydrocarbons (PAHs), metals, and pesticides. Atrazine was the most frequently detected pesticide, which was measured at concentrations as high as 120 ng/L in the upper Neuse River watershed. Moreover, the two PAHs, pyrene and fluoranthene, were estimated at concentrations 18.9 and 17.4 ng/L, respectively, based on residues in PSDs deployed in the upper river. Fluoranthene was detected at all site sediments. Concentrations of fluoranthene were correlated with the *C. dubia* porewater toxicity results and BioTurbTox test results. Sediment toxicity test methods were evaluated for similarity of response among assays. We determined that the new BioTurbTox test generated results similar to the standard assays, was useful as a rapid screening method for sediment toxicity information, but required normalization to clay content or to the total organic carbon content of field collected sediments.

1. Introduction

The Neuse River of North Carolina rises in the Piedmont and empties into Pamlico Sound through the Neuse River Estuary. The Neuse River basin (area = 16040 km^2) includes portions of 19 counties in the State of North Carolina. Its clarity and shallow depth generally prevent light limitation of algal production, so the Neuse River has been designated as Nutrient Sensitive Waters (NSW) [NCDEM, 1996]. Eutrophication by excessive nutrients became a water quality concern in the lower Neuse River basin in the late 1970s and early 1980s. After a phosphate detergent ban (a quarterly average phosphorus limit of 2 mg/l from existing facilities with design flows greater than 0.05 MGD) in 1988, phosphorus load and algal blooms were greatly reduced [NCDWQ, 2002].

Several large fish kills in the lower Neuse River during the summer of 1995 prompted the NC General Assembly to pass a package of new environmental laws and hastened the development of new comprehensive nutrient sensitive waters rules for the Neuse River Basin. These rules were intended to reduce nitrogen loading to the Neuse River estuary by thirty percent within five years because it was determined that control of nitrogen was needed to reduce the extent and duration of algal blooms [NCDEM, 1999; NCDWQ, 2002]. Low dissolved oxygen levels associated with algal blooms and the toxic dinoflagellate, *Pfiesteria piscid*a, were also implicated as probable causes of the fish kills [NCDWQ, 2002]. For monitored freshwater streams, there was an apparent decrease in the number of impaired stream miles from 1993 (40 percent) to 1998 (34 percent) and a corresponding increase in the number of fully supporting stream miles from 1993 (60 percent) to 1998 (66 percent). However, within the fully supporting (FS) category, the percentage of streams considered fully supporting but threatened (ST) increased by 19 percent from 1993 to 1998 to 50 percent

of all streams [NCDEM,1999].

Because of the rapid proliferation of intensive livestock operations (ILOs) in eastern North Carolina, concerns with possible negative impacts on water quality from excess nutrient loading by animal feeds and wastes have been rising [Cahoon et al., 1999]. Production of hogs, turkeys, broiler chickens, and cattle increased substantially in North Carolina during 1985-1995 [Cahoon et al., 1999]. Between 1990 and 1998, the hog population in the Neuse River Basin increased by almost 250 percent [NCDEM, 1999]. Nitrogen loading is an important source of nutrients leading to eutrophication in the Neuse River estuary. Internal N loading events, such as sediment ammonium release during N-limited summer and fall months, were of considerable importance in sustaining primary production. Phosphorus played a co-limiting role only during high N discharge and loading events in late-winter and spring, when estuarine N:P ratios exceeded 15:1 [Paerl, 1995].

Aquatic sediment is a source of biologically important materials such as phosphorus, carbon, nitrogen, as well as a source of contaminants, particularly for benthic organisms [Jones and Bowser, 1978; Larsson, 1985]. Ammonia is one of those natural constituents of aquatic sediments. However, it can be toxic to aquatic organisms at concentrations caused by pollution from sewage, industrial effluents, and agricultural runoff [Ankley et al., 1990].

Because many benthic invertebrates cause bioturbation, including the resuspension of sediment into the water column through their burrowing, feeding, locomotive, respiratory, and excremental activities, bioturbation plays an important role in mediating both physical and chemical processes near the sediment-water interface [McCall and Fisher, 1980; Matisoff et al., 1985; Krantzberg and Stokes, 1985]. In the presence of tube-dwelling larvae of *Chironomus plumosus* Linnaeus (2000 m^{-2}), there was a net release of ammonium from a bioturbated eutrophic lake sediment to the overlying water [Svensson, 1997]. This study

indicated that bioturbation by tube-dwelling chironomid larvae can have a major impact on nitrogen turnover in lake sediment, mobilizing the ammonium to the water and stimulating denitrification by reducing the diffusive barrier blocking nitrate from reaching anoxic zones in the sediment [Svensson, 1997].

In assessing the toxicity of freshwater sediment, 10-d tests with the amphipod *Hyallela azteca* (survival) and the chironomid *Chironomus tentans* (survival and growth) are commonly used in the USA [USEPA, 2000]. It is not possible to quantify the toxicity of samples that illicit a 100 % effect (e.g., mortality) by using conventional concentration-response approaches with undiluted, whole sediment. Therefore, DeFoe and Ankley (2003) proposed the use of a time-to-effect approach for quantifying the relative toxicity of test sediment. They used the survival of *H. azteca* and survival and growth of *C. tentans* as test endpoints over the course of a "standard" 10-day assay [DeFoe and Ankley, 2003]. This approach was used in this study with sediment from each site in the Neuse River and the result was compared with the results from pore-water toxicity testing, whole sediment testing with the Biotox™ flash assay and the BioTurbTox test using bioturbation caused by 2nd-instar *C. tentans* larvae as an endpoint [Cho et al., 2005].

The sediment quality assessment generally involves an evaluation of solid-phase sediments. However, pore water is also an important component for the assessment of sediment quality because it represents a major route of exposure to sediment-dwelling organisms and substantially influences the bioavailability of contaminants [Whitema et al., 1996; Carr et al., 1989; Carr et al., 1996; Ankley et al., 1994; Di Toro et al., 1991].

The Biotox™ flash assay utilizes bioluminescent *Vibrio fisheri*. The luminescence from this bacterium is reduced when it is exposed to toxicants and thus is widely used in acute toxicity tests of water, sediment, and complex hazardous wastes [Bulich and Isenberg,

1981; Brouwer et al., 1990; Lappalainen et al., 1999; Symons and Sims, 1988; Wang et al., 1990]. This direct toxicity test can indicate bioavailability of contaminants to organisms [Kwan and Dutka, 1995]. The Flash assay measures the luminescence intensity after the incubation of the samples and bacteria for 15 or 30 minutes and the luminescence intensity is compared to that of the pure bacteria [ISO, 1998].

To monitor the transport and fate of contaminants such as dissolved concentrations of persistent organic pollutants (POPs) in the aquatic environment, passive sampling devices (PSD) have been used because they can provide time-integrated measures of concentrations and estimate the bioavailability of environmental contaminants [Hofelt, 1998; Huckins et al., 1996]. Low density polyethylene (LPDE) strips were used in this study because they have been shown to accumulate significant quantities of contaminants and they are relatively simple and inexpensive to use.

Multiple toxicity tests are commonly applied as a part of sediment quality triad (SQT) studies, which relies upon the analysis of data from chemical analyses, toxicity tests, and infaunal benthic structure assessments [Chapman et al., 1997]. The results from this type of research can be used by sediment assessors to form a weight of evidence to compare and rank the relative quality of sediment samples and study area [Long, 1989; Chapman et al., 1987].

Few, if any, studies assessing the toxicity of Neuse River sediment, especially, in the upper reaches of the river have been conducted. Therefore, the sediment quality triad approach is not yet conducted with Neuse River Sediment. Considering the excessive nutrient input in the Neuse River as a possible cause of the large fish kills along with and the occurrence of potentially toxic algae, the sediment compartment at which many nutrients and contaminants are adsorbed should be studied to understand any potential causes of

impairment in the Neuse River [Cho et al., 2005].

The practical aspects of the newly developed bioturbation test with field samples have not yet been tested. Therefore, the objectives of this study were to characterize the physical and chemical properties of Neuse River sediment and its toxicity to aquatic organisms and to validate the use of newly developed BioTurbTox test with field samples.

2. Materials and methods

2.1 Field Sites

Six sites in the upper, middle, and lower Neuse River were selected for study; site 1: US 401/Louisbug Road, Wake county, site 2: Auburn-Knightdale Road., Johnston county , site 3: Mial Plantation Road (downstream of the City of Raleigh waste water treatment plant, WWTP), Johnston county, site 4: Arrington Bridge Road (downstream of the City of Goldsboro WWTP), Wayne county, site 5: Hwy 55/Old Dover Road (downstream of the City of Kinston's two WWTPs), Lenoir county, site 6: Glenburnie Drive near Glenburnie Park north of New Bern, Craven county. A site on the upper Neuse River immediately downstream of Falls Lake was selected as the reference site. The Falls Lake watershed and the Raleigh metropolitan area were considered as the upper reach of the river, Goldsboro, and the Kinston areas as the middle reach of the river, and the area downstream of Kinston to New Bern as lower reach of the river (Figure 1). The sites were selected based on sites previously studied by the NC Department of Environmental and Natural Resources (DENR) in 1996 and recommendations of DENR biologists and toxicologists.

2.2 Monitoring

At each of the field sites, water quality variables such as turbidity, pH, dissolved oxygen

(DO), temperature, conductivity, phosphorus total as P, total ammonia-nitrogen (TAN), total Kjeldahl nitrogen (TKN) as N, NO_2 + NO_3, and fecal coliform from water samples were measured with standard methods. Water quality data were obtained from the NC DENR, Division of Water Quality near the sites of study. Benthic organisms at each study site were collected, preserved in 80 % EtOH, and later identified. Sediment samples (0-10 cm depth) were collected at each site with a Ponar grab sampler and were characterized for particle size with the sieve pipet method. The amount of total organic carbon (TOC) in the sediment was analyzed with a Perkin-Elmer 2400 CHN Elemental Analyzer (Norwalk, CT). Acid volatile sulfide (AVS) was analyzed from the sediment samples. For the AVS measurement, 20-mL scintillation vial containing 10 mL sulfide antioxidant buffer solution (SAOB) was attached inside a bottle. Five-g wet wt sediment was placed in the bottle with 30 mL 1 N HCl, then, the bottle was capped and placed on magnetic stirrer for 1 hr. The sulfide ion in the trap solution (SAOB) was measured with a sulfide ion selective electrode (ISE) coupled with a double-junction reference electrode and a digital millivolt meter (Accumet pH meter 15) [Brouwer and Murphy, 1994; Leonard et al., 1996]. Low density polyethylene (LDPE) strips as passive sampling devices (PSDs) were attached to plastic mesh cages with cable ties. The cages were deployed at each site for a month, tied with rope and weighted with a brick [Hofelt, 1998]. After PSD retrieval, the strips were wrapped with the baked aluminum foil and stored frozen at -20C. This monitoring was done in the summer of 2003.

2.3 Toxicity Assessment

With sediment samples (around 20 L from each site), pore water toxicity testing with *Ceriodaphnia dubia* and whole sediment toxicity testing with the BioTox™ flash assay, BioTurbTox test with *Chironomus tentans* were conducted. A 10-d *C. tentans* test for survival

and growth was conducted. Sediment from the Elizabeth River, Virginia that was highly contaminated with PAHs was used as a positive control.

Whole sediment was collected with multiple grabs of a Ponar grab sampler, placed into acid and solvent cleaned buckets and transported to the laboratory in coolers with ice. As soon as the sediment was brought to the laboratory, it was homogenized with a stainless steel spoon, and passed through a stainless steel sieve with 2 mm openings (without the use of additional water) to remove large particulate matter and indigenous organisms and stored refrigerated at 4 °C. For the BioTurbTox test, 5 replicates from each site were prepared and turbidity was measured at 0, 2, 6, 12, 24, 30, 35, 38, 48 h with a Hach turbidimeter. For the 10-d survival and growth test, 100 mL of whole sediment and 250 mL of water were prepared in 300 mL beakers. Ten larvae were placed in each beaker and there were 10 replicates from each site. Among the ten replicates, one replicate was sampled every 24-h to measure the length, dry weight, and ash-free dry weight of the larvae and to assess mortality. For the dry weight determination, larvae were dried for 14-20 h in a drying oven at 90-100 °C. For the ash-free dry weight, the dried larvae were placed in a muffle furnace for 2 h at 550 °C and ash weight was measured and subtracted. A portion of the overlying water (~ 100 mL) was changed daily.

For the porewater toxicity test, approximately 640 mL of the sediment from each site was centrifuged at 4°C and 740 rpm to prepare about 200 mL of porewater. Then, it was stored in 500 mL amber glass jars, refrigerated, and taken to the NC DENR Aquatic Toxicology Laboratory for the 48 h pass/fail test with *Ceriodaphnia dubia*. There were 4 replicates for each site in the porewater test and water was changed two times during the 48-h exposure time. Mortality was recorded at 12 h intervals.

For the flash assay, whole sediment was used. *Vibrio fisheri* reagent was mixed by

adding reagent diluent, equilibrated at 4 ºC for at least 30 min, and the reagent was stabilized at 15 ºC for at least 30 min. Samples were stored in a refrigerator before testing and sample diluent (20 % NaCl) was diluted 1:9 with DI water to 2 % NaCl. 0.5 g of sediment sample was mixed with 2 mL of sample diluent (2% NaCl) for 1 min, then the sample was held at room temperature for 5 min and mixed again. pH was adjusted to 6-8 with 1 M NaOH or 1M HCl. The sample was mixed again and pipetted into the cuvette (500 µL). The sample was cooled for 15 min before testing. Sample was loaded and the inhibition of the luminescence was measured for 30 sec with a luminometer.

2.4 Chemical Analysis

All glassware, glass wool, and Na_2SO_4 for the extraction and analysis of samples were baked at 300 °C in a drying oven overnight and cooled. Organic contaminants in the sediment were extracted by the Shaker table extraction method and a silica column clean up procedure was conducted to eliminate any lipid in the samples. Surrogate internal standard (SIS, naphthalene-d8, acenapthene-d10, chrysene-d12 and perylene-d12, Accustandard Inc, New Haven, CT)) was spiked into all the sediment samples and a matrix spike (phenanthrene-d10 and benzo(a)pyrene-d12, Accustandard Inc, New Haven, CT) into the matrix blank sample composed with clean sediment. Sixty mL of 1:1 acetone:dichloromethane and 30g of anhydrous sodium sulfate were added to the sediment samples and shaken on an orbital shaker table overnight. After repeating this process, the extracts were concentrated with a roto-evaporation unit, then, it was concentrated under a gentle stream of nitrogen to a 1 mL volume. The solvent of the samples was exchanged with 95% hexane and the samples were loaded onto a dry pack column consisting of one cm baked anhydrous sodium sulfate, 3 g activated silica, and 1 cm baked anhydrous sodium

sulfate. Twelve mL of hexane and 15 mL of 1:1 95 % hexane:dichloromethane was added serially and the eluted fraction was collected. The collected fraction was evaporated under a gentle stream of nitrogen to ~1mL. The extracted samples were analyzed by GC-MS.

Organic contaminants in water samples were processed by a liquid-liquid extraction method. Water samples with SIS and dichloromethane were placed in separatory funnels and the organic layer of dichloromethane was drained and dried with anhydrous sodium sulfate. After repeating these steps, the extracts were concentrated under a gentle stream of nitrogen to 1mL. The extracts were analyzed by GC-MS. Procedural blanks were run with each sample batch and recovery internal standards (Phenanthrene-d10 and Benzo[a]pyrene-d12, Accustandard Inc, New Haven, CT) were added immediately prior to sample analysis on the GC-MS for analyte peak quantification [Thorsen 2003]..

For the PAHs and pesticides analysis in PSD, LDPE strips were removed from the aluminum foil and placed into a clean stainless pan filled with tap water. Extraneous solid materials were scrubbed off with clean brush and the strips were rinsed with tap water. Then, they were rinsed with acetone followed by isopropanol to remove water and air-dried for 5 minutes on clean aluminum foil. The strips were cut into 1-cm^2 pieces and the pieces, ~ 50 mL of methylene chloride, and SIS were placed into a 250 mL Teflon bottle and the bottles were placed and shaken at 150 rpm for 24 hours. The methylene chloride was decanted into 500 mL boiling flask. More methylene chloride was decanted from the bottle and shaken for 2 hours and added to the boiling flask. The methylene chloride collected in the boiling flask was reduced to 1 mL under a gentle stream of nitrogen. Then, the extract was transferred to HPLC auto-sampler vial for Gel Permeable Chromatography (GPC) cleanup. After the cleanup, the extracts were analyzed by GC/MS for 48 PAHs and 12 current-use pesticides [Hofelt 1998]. To estimate the water concentrations (Cw) of the PAHs and pesticides from

the amount accumulated in the PSD (C_{PSD}), sampling rate (Rs) of 1.875 L/d were used assuming it was still in a linear phase. The equation for the estimation is

$$Cw = C_{PSD}/Rs \cdot t$$

, where t is the time deployed (50 days).

Metal analyses were conducted on dried sediment samples by the Analytical Services Laboratory in the Department of Soil Science at North Carolina State University. Nine mL HNO_3 and two mL HCl were added to a 0.5g dried sediment sample. The mixture was then microwaved at 200 °C for 15 minutes and then diluted with distilled water. The solution was then run via a spike addition method on a Perkin-Elmer Ion Coupled Plasma Spectrometer.

2.5 Statistical Analysis

Growth rates of *C. tentans* larvae exposed to sediment from each site from day 5 to day 10 in 10-day test were calculated by linear regression and it was compared using PC-SAS® [SAS Institute, 1999]. To assess the site-to-site variation in % TOC and % clay content, analysis of variance with linear effects for % TOC and % clay were done with PC-SAS® [SAS Institute, 1999]. Correlations of the toxicity ranks from each tests and sediment PAHs concentrations normalized to % TOC were assessed by Pearson and Spearman tests with PC-SAS® [SAS Institute, 1999].

3. Results

3.1 Toxicity Tests

Water quality of the study sites in the Neuse River (NR) was obtained from NC DENR DWQ. There were some missing data, but all the data in each parameter were in a similar range (Table 1.). Table 2 shows the sediment composition of the NR and Elizabeth

River (ER). From site 1 to site 4, sediment compositions were very similar (high sand content and low silt and clay content), but in site 5, 6, and ER sediment, there was a higher % of clay. TOC percentages increased downstream NR and ER sediment contained the highest TOC. ER sediment contained the highest concentration of AVS and site 6 sediment had the lowest concentration.

Ash-free dry weight (AFDW) of the *C. tentans* larvae did not change much until 6 days. However, after 6 days, it increased except the ones in ER sediment because of early mortality (Figure 2). By estimating the growth rate of larvae from AFDW, the order of sediment toxicity from the highest was ER sediment, site 6, site 5, site 4, site 2, site 1, and site 3. The average growth rate of sites 1, 2, and 3 was significantly higher than that of sites 4, 5, and 6 ($p=0.0497$). Mortality of *C. dubia* with porewater of the sediment showed that ER had the highest toxicity, and then, followed by site 2, site 5, site 3, site 6, and site 1 and 4 (Figure 3). *C. dubia* in site 2 porewater were dead within 24 hours. However, *C. dubia* in site 1 and 4 porewater did not die during 48 hours of exposure (Figure 3). Because there were not enough data points, LT50 could not be calculated from porewater toxicity test. Inhibition of luminescence from flash assay was the highest when the bacteria were incubated with site 6 sediment, and then, it followed ER sediment, site 5, site 1, site 2, site 3, and site 4 sediment (Figure 4).

BioTurbTox test results demonstrated that site 5 and 6 were not as toxic as other sites. All the larvae exposed to ER sediment were dead within 12 hours but turbidity was measured until the experiment was ended (Figure 5 a). There was no turbidity increase in the sediment without larvae (Figure 5 b). The toxicity ranking can be summarized in Table 3. Site effect can be explained by % clay and % TOC ($p=0.6872$). Mean turbidity values from NR sediment were plotted versus % clay and % TOC (Figure 6). Both showed a linear

relationship between % clay and turbidity [(Turbidity) = 5.46 (% clay) – 25.16, r = 0.737, Figure 6 a] and between % TOC and turbidity [(Turbidity) = 60.44 (% TOC) + 12.82, r = 0.77, Figure 6 b]. Because the site 5 and 6 and ER sediment contained high % of clay and % TOC, the turbidity (NTU) values were normalized to the % clay (Figure 7 a) and % TOC (Figure 7 b). When the turbidity was divided by the % clay and % TOC, it showed better separations for each site. The rank of the toxicity was changed for site 1, 3, 5, and 6 (Table 3). Variation of turbidity was explained by % clay or % TOC. % TOC was a slightly better predictor because site effect was not significant if % TOC or % clay was not considered (p=0.3105 and p=0.1761, respectively). Among all the correlations between sediment PAH concentrations normalized to % TOC and the toxicity rank from each test and between each tests, flash assay rank order and 10-day test rank order showed highest correlations by Pearson and Spearman test (correlation coefficient = 0.71429) but it was not statistically significant (p=0.118).

3.2 Chemical Analysis

Recoveries were within acceptable ranges both for PAHs (57-76 %, 62-83 %, and 34-91 % in PSD, water and sediment, respectively) and for pesticides (69 - 132 %, 72 - 122 %, 51 - 162 % in PSD, water, and sediment, respectively). Forty-eight PAHs were analyzed including alkylated PAHs and twelve current-use pesticides were analyzed. PAH concentrations in the river water samples ranged from 1 to 17 ng/L. Naphthalene, 2, 6-dimethylnaphthalene, acenaphthylene, acenaphthene, phenanthrene, fluoranthene, pyrene, C1-fluoranthene/pyrenes, retene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[e]pyrene, benzo[a]pyrene, perylene, indeno[1,2,3-dc] pyrene, and benzo[g,h,I]perylene were detected from the water samples of the NR. The highest

concentrations detected in the water were fluoranthene at site 5 (17ng/L) and pyrene at site 5 (14 ng/L) (Figure 8 a). High concentrations of PAHs in water were detected at all the sites except site 1 (Table 4).

Water concentrations of the pesticides were detected up to 120 ng/L. Simazine, prometon, atrazine, chlorothalonil, prometryn, and metolachlor were detected in the water samples of the NR. Atrazine at site 1 was detected at the highest concentration among 12 current-use pesticides analyzed (117.6 ng/L, Figure 8). Pesticide concentrations in the upstream were higher than those in downstream (Table 4).

Sediment PAHs concentration range was <0.2 to 389 ng/g dry wt. The highest PAHs concentrations found were fluoranthene at site 3 (552 ng/g) and 6 (464 ng/g), perylene at site 6 (412 ng/g), retene at site 4 (389 ng/g), and pyrene at site 3 (385 ng/g) (Figure 9). Sediment PAH concentrations were highly detected except for site 1. Figure 8 shows the 10 PAHs detected at the highest concentrations in the sediment samples from the NR. No current-use pesticides were detected in the sediment except metribuzin (11.09 ng/g) at site 3.

PAHs in PSDs ranged from 0.02 to 21.3 ng/L (Figure 10). Retene at site 4 (21.3 ng/L) was the most abundant, followed by pyrene at site 2 (19.0 ng/L), and fluoranthene at site 2 (17.3 ng/L). High levels of PAHs were detected in the PSDs deployed at site 1, 2, 3, and 4 (Table 4). Pesticides accumulated in PSD ranged from 0 to 12.3 ng/L. Fenamiphos at site 6 was the most abundant current-use pesticide (Figure 11). High levels of current-use pesticides were found at sites 2, 3, 4, and 6 (Table 4).

The trace metal concentrations detected in the NR sediment samples ranged from <1 to 114 μg/g (Table 5). For Cd, Cu, and Ni, there were not many differences in the concentrations detected in each site of NR. However, for Pb and Zn, site 5 and 6 had higher concentrations than other sites in the NR sediment samples. ER sediment samples had very

high concentrations of all kinds of trace metals.

4. Discussion

NCDWQ reported that 43 fish kills (3.6 million cumulative fish mortality) in North Carolina coastal estuarine waters occurred in the year 2003 and it was the highest mortality since systematic reporting began in 1996 [NCDWQ, 2003; Table 5]. Eighty six % (3.1 million) of the 2003 total fish kills were from two large events on the Neuse River [NCDWQ, 2003]. The causes of the fish kills were not identified clearly, but investigators divided the suspected causes into four categories – 1) low dissolved oxygen derived by high inputs of oxygen demanding organic material into the water bodies by severe runoff, flooding of agricultural areas, and flushing of swamps and backwaters, 2) toxic spills of pesticides (chlorpyrifos, fenamiphos, and malathion) and NaOH, 3) other miscellaneous causes such as extremely low water temperatures, and 4) unknown [NCDWQ, 2003]. Toxicity testing with the NR sediment showed that some site sediments (sites 2 and 5) were moderately toxic but we do not believe that the sediment is the direct cause of the massive fish kills in the Neuse River estuary. Since site 5 was located downstream of two waste water treatment plants (WWTPs), it may have received wastewater that caused toxicity to the experimental organisms. This site also had the highest water PAH concentrations among six sites studied. From chemical analysis, site1 had the highest water pesticide concentrations and the lowest water and sediment PAHs and it showed low toxicity. Site 2 had high concentration detected in all the compartments except pesticide in PSD and it was expected to have high toxicity. Due to high PAHs in water, sediment, and PSD in site3, high toxicity was expected, but not observed. Site 5 had high water PAHs, med for others, low water pesticides and it showed

moderate toxicity. Site 6 had medium levels for chemical contaminants but it had high accumulation of pesticides in PSD and it showed low toxicity. From chemical analysis, especially PAHs, it was expected that site 2, 3, and 5 would have relatively high toxicity and the result from porewater toxicity test met this expectation.

Water PAHs concentrations were correlated with porewater toxicity (Figure 12). At site 2 and 5 of NR and at ER (marked as 7), where PAH contamination was high, the porewater toxicity was very high and the *C. dubia* died faster than those in other sites. According to Carr et al. (1989), their major concern in the porewater test from the marine sediment was PAHs though it was site-specific. Porewater toxicity testing has several advantages in that potential artifacts by grain size, water quality parameters, or other physical properties of the sediments can be eliminated and that dilution series by the use of pore water samples can be tested [Carr et al.,1989]. Adams et al. (1985) demonstrated that the key route of exposure for certain nonpolar organic contaminants is from the interstitial (pore) water. However, for the pore water toxicity testing, the samples are manipulated from their natural state to make them testable (i.e., salinity adjustment, increased dissolved oxygen, increased pH) and these adjustments may change the toxicity of certain trace metal contaminants, but it should not substantially affect the bioavailability of most organic contaminants [Carr et al., 1989]. Lahr et al. (2003) said it could be argued that pore water assays overestimate the toxicity of the more soluble compounds in sediments like metals and ammonia compared to the relatively less soluble organic compounds.

From all of the toxicity test results, except for the flash assay, ER sediment was the most toxic among all the tested sediments. The flash assay showed that NR6 sediment was the most toxic and it was very different from the other results. The Microtox® test which uses *V. fischeri*, the same microorganism used in flash assay, is relatively sensitive to organic

substances such as PAHs and mineral oils [Lahr et al., 2003]. However, Lahr et al. (2003) did not see that effect of those organic substances on *V. fischeri*. They suspected other confounding factors such as sulfide [Chapman, 1995; Wang and Chapman, 1999] may have caused the toxicity of the sediment in the Microtox®. Regarding the results from the flash assay with NR sediment, sulfide did not seem to cause the luminescence light inhibition because NR5 had higher AVS concentration and PAHs concentration, but light inhibition was higher with NR6. From Ringwood et al. (1997), Microtox® EC50 was reduced (decreased light production) as the clay composition increased by the adsorption of the bacteria to the silt-clay particles not by toxicity effects. International Organization for Standardization (ISO, 1998) also suggested that insoluble, slightly soluble or volatile substances or substances may react with the dilution water or the test suspension, or it altered their state during the test period, which may affect the result or may impair the reproducibility of the flash assay results. Losses of luminescence can be caused by absorption or scattering of light in the case of strongly colored or turbid wastes. Salt concentrations in the initial sample exceeding 30 g/L NaCl may lead to hyperosmotic effects. ISO (1998) recommends the concentration in the test samples shall not exceed the osmolarity of a 35 g/L NaCl [ISO, 1998]. NR6 had higher salt concentrations than other sites because it is close to Neuse River estuary, but it did not exceed the osmolarity of 35 g/L NaCl that affects the flash assay results.

Considering the contaminant concentration in the sediment and water, site 2 should be the most toxic among the six sites in the NR (Table 4). And that was shown from BioTurbTox test and porewater tests. For other sites, it was difficult to differentiate the toxicity of the sites. Though the same organisms, *C. tentans*, were used in the 10-d test and BioTurbTox test, the two tests did not show the same results. The BioTurbTox test results were more similar to the porewater toxicity test results whereas the 10-d test results were

more related to that from the flash assay. However, it was difficult to assess the toxicity of sediment from 10-d test because the growth of larvae was not significantly different. This can be partially explained because the size of the larvae at the beginning of the test was not similar. Realistically, it was impossible to have all 700 larvae needed for the test the same size at the same time.

Species can have different sensitivities to contaminants due to differences in species behavior, lifestyle and physiology and sensitivity can be contaminant specific. [Hickey and Martin, 1995; Phipps et al., 1995; Borgmann et al., 1998]. Therefore, Day et al. (1995) and Suedel et al. (1996) recommended using several species representing different species habitats as well as different physiological endpoints for sediment toxicity evaluation.

Toward downstream in the Neuse River, sediment contained higher % of clay and % of TOC (Table 3). Estuarine sediments typically have varying amounts of silt-clays from <1 % to >95 % [Summers et al., 1993]. The equilibrium partitioning (EqP) approach for sediment quality criteria (SQC) used organic carbon as a bioavailability normalization factor for nonionic organic compounds in sediment [Chapman, 1989], because this approach assumed that bioavailability is inversely proportional to the organic carbon content of the sediment [Di Toro et al., 1989]. Additionally, the effects of clay particles are also important in determining the availability of chemicals in sediment [Kimerle, 1987]. Because turbidity changes can be related to the clay content and TOC content in the sediment and the downstream sections of the river contained high clay content and TOC content, we normalized the turbidity values both to the % clay and % TOC in the sediment. By normalization, the turbidities at each site were changed and there seemed to be a better differentiation of the toxicity based on the chemical contamination in the sediment. Both normalizations worked out well, but normalization to % TOC was a slightly better predictor

for the test. Therefore, for the BioTurbTox test, we suggest normalizing the turbidity values to % TOC (or % clay) whenever assessing sediment toxicity with field samples.

Upstream water pesticide concentrations were higher than that in downstream water concentrations. Before the analysis, we considered site 1 as a reference site expecting that not much contaminant would be detected from there. However, it had higher pesticide concentrations in the water. Atrazine was the most highly detected pesticide at site 1 (120 ng/L, Figure 7). Atrazine, a pre-emergent herbicide, is frequently detected pesticide in aquatic environments and it was detected in all samples located downstream from the river in Winyah Bay, SC, and it showed seasonality [Kucklick, 1994]. The highest concentration detected was 610 ng/L in May and the lowest concentration detected was 7 ng/L in December because of its application time [Kucklick, 1994]. Atrazine is typically applied in March and April in South Carolina [Pait et al., 1992]. In the upper reaches of the Tar River, NC, metolachlor, atrazine and alachlor were among the most frequently detected pesticides in the year of 2000 and 2001 [Woodside, 2001; Spruill, 1998].

Current-use pesticides were not detected in the sediment except one pesticide (metribuzin) at site 3. As it goes downstream, PAHs contamination gets higher in the water and sediment samples. The order of PAHs concentration in water and sediment were similar except for the order of site 3 and 5 (water PAHs: 5>2>6>3>4>1, sediment PAHs: 3>2>6>5>4>1).

Some individual PAH concentrations in NR sediment exceeded the NOAA Threshold Effects Level (TEL) that is the geometric mean of the 15th percentile of the effects data and the 50th percentile of the no-effects data. The TEL is intended to represent the concentration below which adverse biological effects rarely occurred [Smith et al. 1996]. Phenanthrene, fluoranthene, benz[a]anthracene, and chrysene detected at site 2, 3, 5, and 6 sediment

exceeded TEL of 42, 110, 32, and 57 ng/g. Pyrene at site 2, 3, 4, 5, and 6 sediment exceeded TEL of 53 ng/g. Detected total sediment PAHs concentrations (the highest detected were 3.9 μg/g) did not exceed the reliable dry-weight normalized probable effect concentrations (PEC) of 22.8 μg/g. Consensus-based PECs (above which adverse effects are expected to frequently occur) are calculated as the geometric mean of the existing numerical sediment quality guidelines (SQGs) [MacDonald et al., 2000]. Sediment concentrations of Ni, Pb, and Zn at site 5 slightly exceeded the TEL of 18, 35, and 123 μg/g, respectively. However, no NR sediment samples exceeded consensus-based PECs. Some of trace metal concentrations in ER sediment were close to or exceeded consensus-based PEC. Therefore, we could conclude that the Neuse River sediment contamination is not too serious.

PSD overestimated water concentrations of 48 PAHs in all sites except site 5 (Figure 13 a). The water PAHs concentrations estimated by PSD were more closely related to sediment PAHs concentrations than the actual water concentrations. Hofelt (1998) also found that estimates of water concentrations from LDPE and sediment were in a good agreement and LDPE predicted slightly higher concentrations than sediment. LDPE has about 80 % of organic carbon (OC) fraction, meaning that it has a much greater capacity to sorb contaminants than sediment that has OC fraction less than 3 % [Lefkovitz et al.,1996; Hofelt, 1998]. However, PSD underestimated water concentrations of the 12 current-use pesticides (Figure 13 b) and that estimated water concentrations would be so low. No current-use pesticides were detected in the sediment except metribuzin at site 3. LDPE use as artificial substrates has been demonstrated to be an excellent first screening tool for a large-scale project considering its simplicity, price, and performance [Hofelt, 1998].

Toxicity of heavily contaminated sediments could partially be explained by comparing chemical analysis results of the priority pollutants with available toxicity data of

the standard test species used. At present, to assess the true toxicity of sediments, bioassays with environmental samples as well as chemical analysis are still needed until more research has been conducted [Lahr et al., 2003]. From this chemical analysis and toxicological bioassays with NR sediment, the assessment of NR toxicity was performed properly. Site 2 was found to be the most toxic site in the NR because it is located downstream of the Raleigh WWTP.

5. Conclusions

The BioTurbTox test was a suitable method for measuring sediment toxicity, especially highly toxic sediment and bioturbation-inferred toxicity of field sediment with that test requires normalization to % TOC (or % clay). The results from the BioTurbTox tests were similar to those from the porewater toxicity tests. Toxicities from both tests were related to the PAH contamination. The use of LDPE PSDs was appropriate for this study, especially for the estimation of water concentrations of PAHs, which were correlated with sediment PAHs concentrations. Among the Neuse River sediment sampling sites, site 2 was the most toxic. However, Neuse River sediment is probably not overtly toxic and does not likely contribute to the massive fish kills in the Neuse River basin.

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Table 1. Water quality data at near the six study sites of the Neuse River at the end of July 2003 obtained from NCDENR DWQ.

	At SR 2000	At NC42	At SR 1915	At NC11 Bypass	At SR147	At US 17
Location	Near Falls	Near Clayton	Near Goldsboro	At Kinston	Nr Fort Barnwell	At New Bern
NCDENR site	J189	J417	J597	J615	J785	J857
Date	7/25/03	7/28/03	7/28/03	7/31/03	7/31/03	7/23/03
Water temperature (°C)	27.9	27.5	27.1	27.8	27.8	28.1
Conductivity field (µmho/cm @25°C)	77	152	101	99	105	109
Dissolved oxygen (mg/L)	6.6	5.7	5.5	6.3	4.9	5.9
pH (SU)	6.9	7.1	6.6	6.5	6.5	6.5
Ammonia nitrogen	0.03		0.04	0.05	0.06	
Total Kjeldahl Nitrogen (TKN) As N (mg/L)	0.48		0.59	0.54	0.56	
Nitrate/nitrite NO_2 + NO_3 as nitrogen (mg/L)	0.02		0.37	0.57	0.51	
Phosphorus total as P (mg/L)	0.03		0.13	0.16	0.15	
Fecal coliform MF method (colonies/ 100 mL)	7	73	42			
Turbidity lab	5.2	20	25			
Salinity (ppt)				0.05	0.05	0.06

Table 2. Sediment composition of each study sites in the Neuse River and the Elizabeth River (ER).

Sites	% sand	% silt	% clay	AVS (µg/g)	% TOC
1	82	5	13	0.28	0.45
2	84	3	13	2.95	0.63
3	84	1	15	3.04	0.75
4	84	0	16	2.82	0.78
5	25	19	56	27.90	2.97
6	58	1	40	0.03	4.56
ER	43	13	44	312.90	6.56

Table 3. Toxicity Ranking from each toxicity tests.

Toxicity Assays	Toxicity Rank
10-day test (Based on slope, but it was not significantly different.)	ER > 6 > 5 > 4 > 2 > 1 > 3
Bioturbation test	ER > **2** > 4 > 1 > 3 > 5 > 6
(After normalization,	ER > **2** > 4 > 6 > 5 > 3 > 1)
Porewater test	ER > **2** > 5 > 3 > 6 > 1 = 4
Flash assay	6 > ER > 5 > 1 > 2 > 3 > 4

Table 4. Sum of 48 PAHs and sum of 12 current-use pesticides concentrations in water, PSD, and sediment of the Neuse River.

Neuse River sites	**1**	**2**	**3**	**4**	**5**	**6**
PAHs						
Water (ng/L)	7	31	19	18	104	27
PSD (ng/L)	86.1	140.0	112.0	85.1	22.9	35.5
Sediment (ng/g)	72.1	2703.4	3943.1	1071.2	1379.3	2478.0
Sediment normalized to %TOC (μg/g)	16.0	429.1	525.8	137.3	46.4	54.3
Pesticides						
Water (ng/L)	184	131	122	78	53	74
PSD (ng/L)	0.7	3.3	6.2	0	5.1	12.3
Sediment (ng/g)	0	0	11.1	0	0	0

Table 5. Trace metal concentrations in sediment samples of the Neuse River and the Elizabeth River.

µg/g	1	2	3	4	5	6	ER
Cd	<1	<1	<1	<1	<1	<1	2.3
Cu	18	13	20	10	31	21	183
Ni	17	9	12	9	20	14	25
Pb	7	11	18	14	35	28	239
Zn	35	49	72	36	130	114	489
Total	77	83	123	69	216	178	936

Table 6. Fish kill data at the lower Neuse River (near sites 5 and 6) around the time of this study from NCDENR.

Date	County	Species	Mortality
7/11/2003	Pamlico	Menhaden	800
7/15/2003	Craven	Menhaden	288
7/17/2003	Craven	Menhaden	400
7/30/2003	Craven	Spot, pinfish, flounder, croaker, mullet, menhaden	3500
7/30/2003	Craven	Spot, flounder, pinfish, eel, menhaden	200
8/18/2003	Craven	Spot, croaker, pinfish, silver perch	74500
8/31/2003	Craven	Spot, croaker, menhaden, pinfish, flounder, mullet, silver perch, catfish, shad	1300000
9/5/2003	Craven	Menhaden, striped mullet, pinfish	1800000

FIGURE LEGENDS

Figure. 1. Map of the Neuse River Basin in the State of North Carolina.

Figure. 2. Ash-free dry weight of *C. tentans* larvae exposed to NR and ER sediment for 10 days. Mortality of larvae in ER sediment reached 100% within 12 hours.

Figure 3. Mortality of *C. dubia* in the porewater of NR and ER sediment for 48 hours.

Figure 4. Flash assay results with the NR and ER sediment.

Figure 5. Turbidity change in BioTurbTox test over 48 hours (a) in the presence of *C. tentans* larvae and (b) in the absence of larvae with NR sediment and ER sediment.

Figure 6. Mean turbidity value at the end of the exposure versus % clay (a) and % TOC (b) in the NR sediment. (a) Turbidity vs. % clay, (Turbidity) = 5.46 (% clay) – 25.16, r = 0.737, (b) Turbidity vs. % TOC, (Turbidity) = 60.44 (% TOC) + 12.82, r = 0.77

Figure 7. Normalization of the bioturbation test results to clay content.
(a) Normalization to % clay content. (b) Normalization to % total organic carbon (TOC).

Figure 8. PAHs (a) and current-use pesticides (b) concentrations in the water samples of the Neuse River.

Figure 9. 10 PAHs the most highly detected in the sediment samples of the Neuse River.

Figure 10. Estimated water concentrations of 10 PAHs the most highly detected in the PSD deployed at the Neuse River.

Figure 11. Estimated water concentrations of current-use pesticides in the PSD deployed at the Neuse River.

Figure 12. The relationship of the porewater toxicity and the sum of 48 PAHs (tPAHs) in the water samples of the Neuse River.

Figure 13. The sum of 48 PAHs in water, sediment, and PSDs (a) and the sum of 12 current-use pesticides in each compartments (b). (a) Sum of 48 PAHs detected.
(b) Sum of 12 current use pesticides detected.

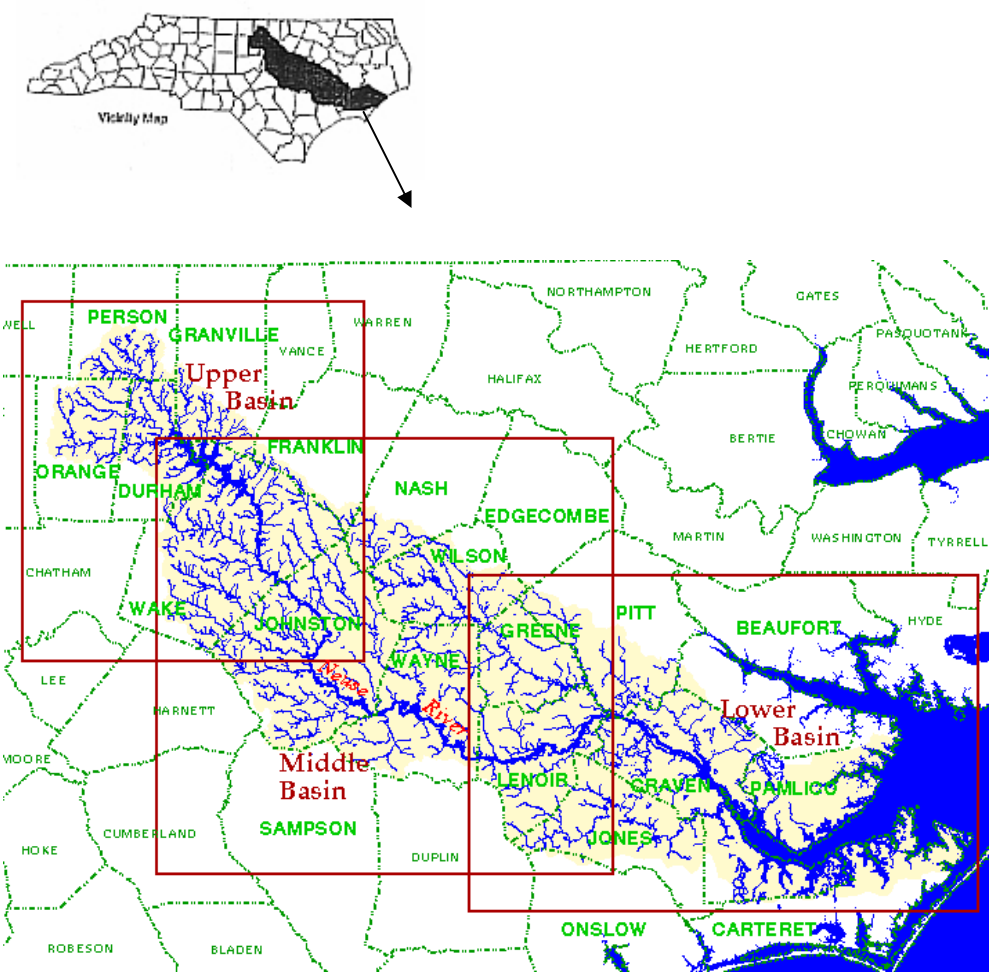

Vicinity Map
PERSON
GRANVILLE
VANCE
WARREN
Upper
Basin
FRANKLIN
ORANGE
DURHAM
NASH
EDGECOMBE
WILSON
CHATHAM
WAKE
JOHNSTON
GREENE
PITT
WAYNE
LEE
HARNETT
Middle
Basin
LENOIR
CRAVEN
PAMLICO
Lower
Basin
MOORE
SAMPSON
CUMBERLAND
DUPLIN
JONES
HOKE
ONSLOW
CARTERET
ROBESON
BLADEN
NORTHAMPTON
HALIFAX
HERTFORD
GATES
PASQUOTANK
PERQUIMANS
CHOWAN
BERTIE
MARTIN
WASHINGTON
TYRRELL
BEAUFORT
HYDE

Figure 1.

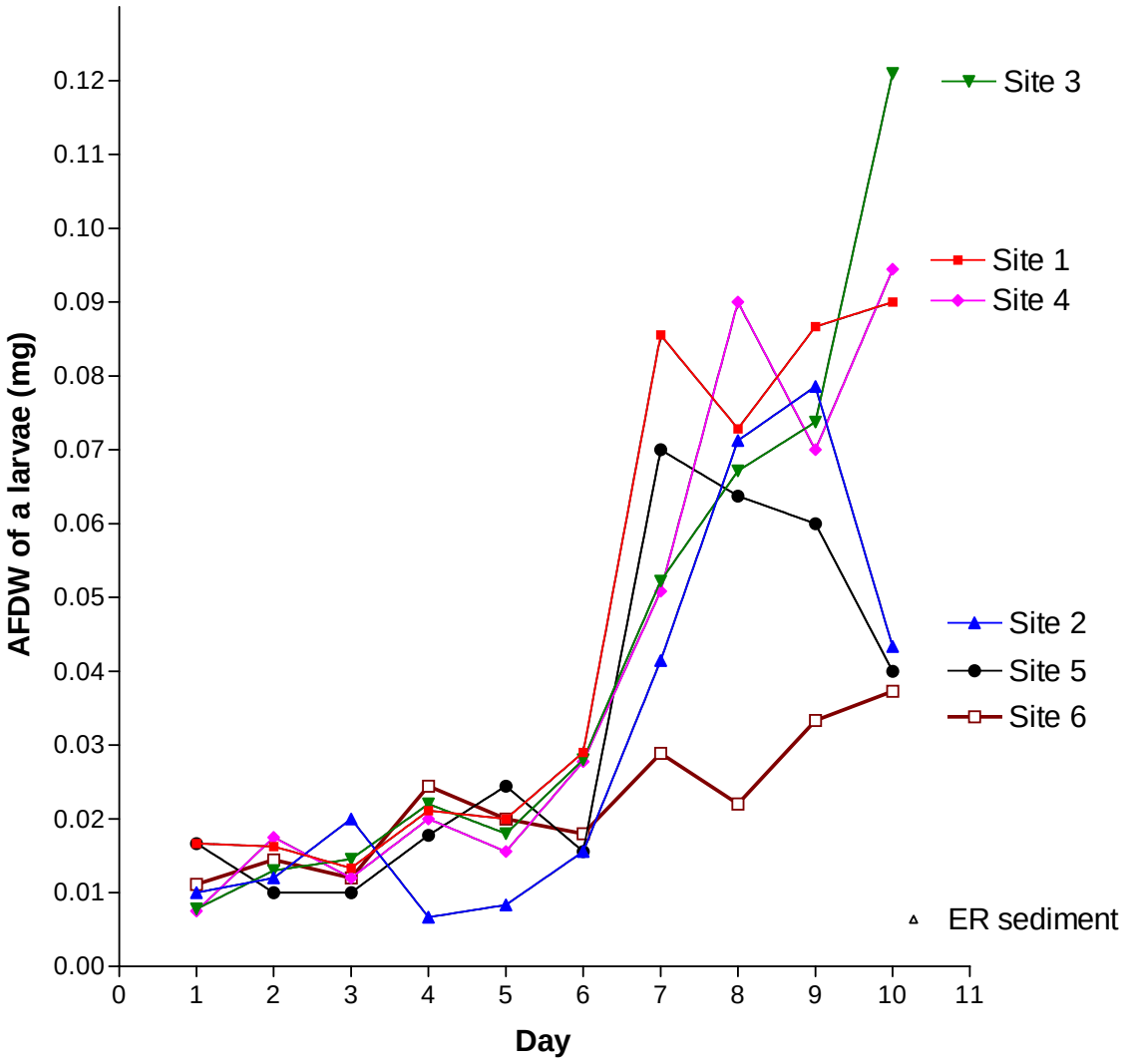

AFDW of a larvae (mg)
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0
1
2
3
4
5
6
7
8
9
10
11
Day
Site 3
Site 1
Site 4
Site 2
Site 5
Site 6
ER sediment

Figure 2.

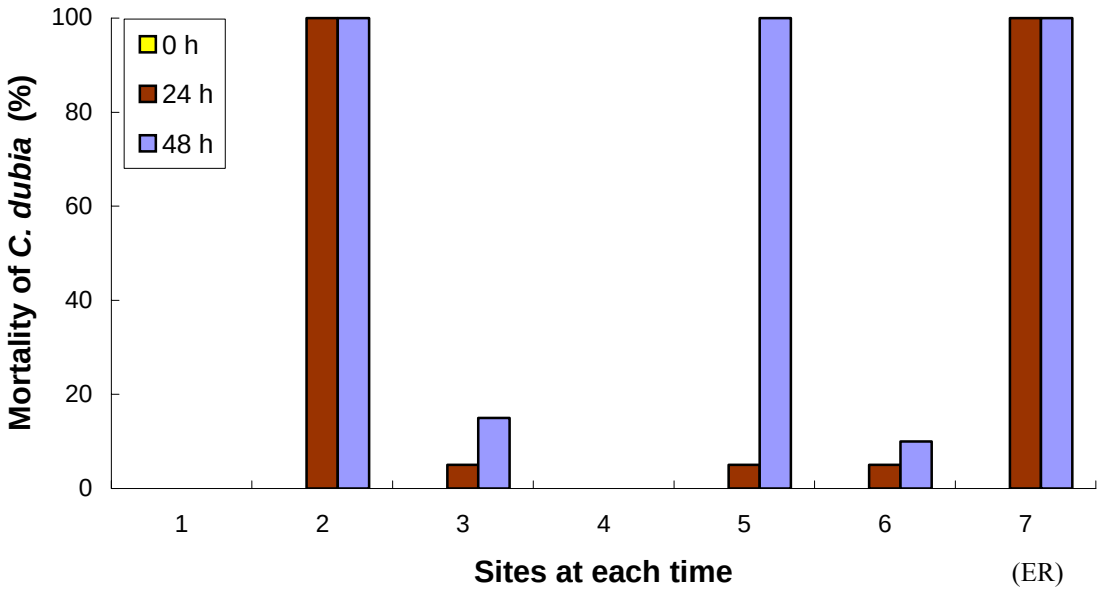

100
80
60
40
20
0
0 h
24 h
48 h
Mortality of C. dubia (%)
1
2
3
4
5
6
7
Sites at each time
(ER)

Figure 3.

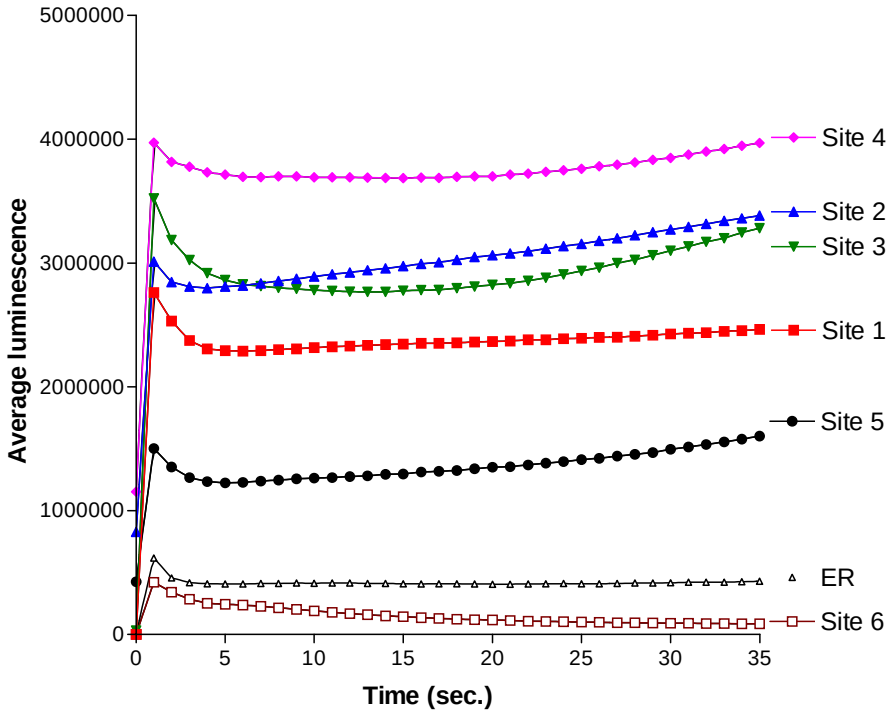

5000000
4000000
3000000
2000000
1000000
0
Average luminescence
0
5
10
15
20
25
30
35
Time (sec.)
Site 4
Site 2
Site 3
Site 1
Site 5
ER
Site 6

Figure 4.

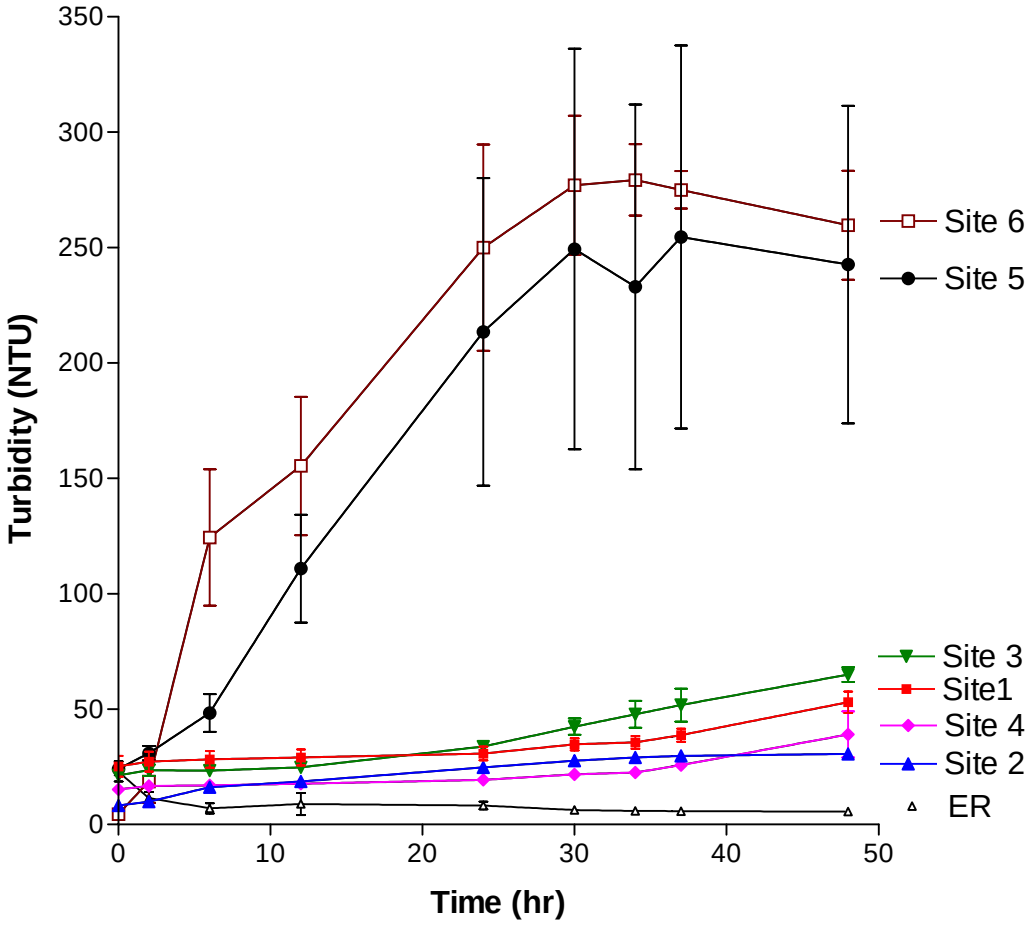

Turbidity (NTU)
Time (hr)
0
50
100
150
200
250
300
350
0
10
20
30
40
50
Site 6
Site 5
Site 3
Site1
Site 4
Site 2
ER

Figure 5 (a)

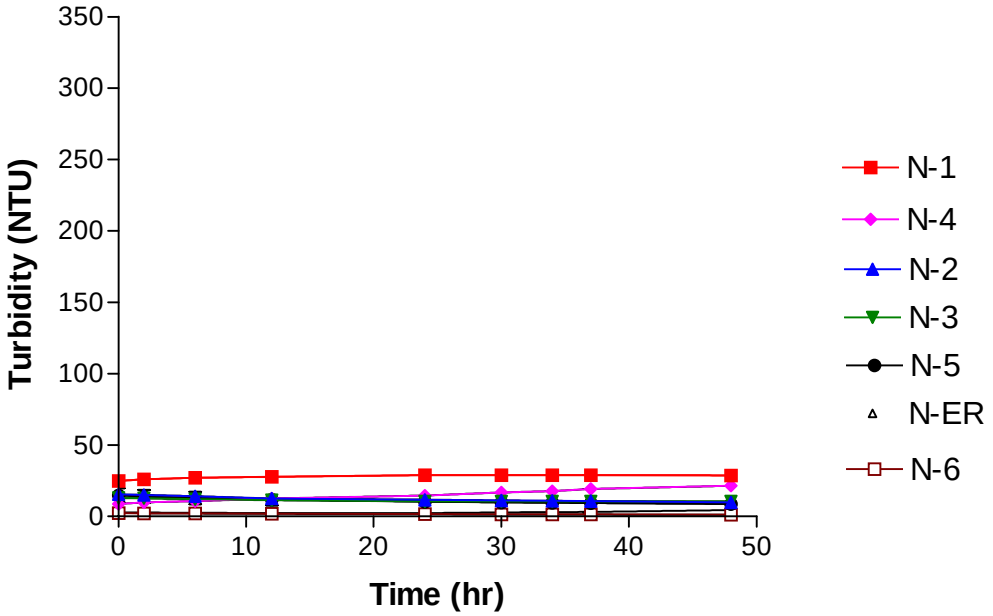

Turbidity (NTU)
350
300
250
200
150
100
50
0
0
10
20
30
40
50
Time (hr)
N-1
N-4
N-2
N-3
N-5
N-ER
N-6

Figure 5 (b)

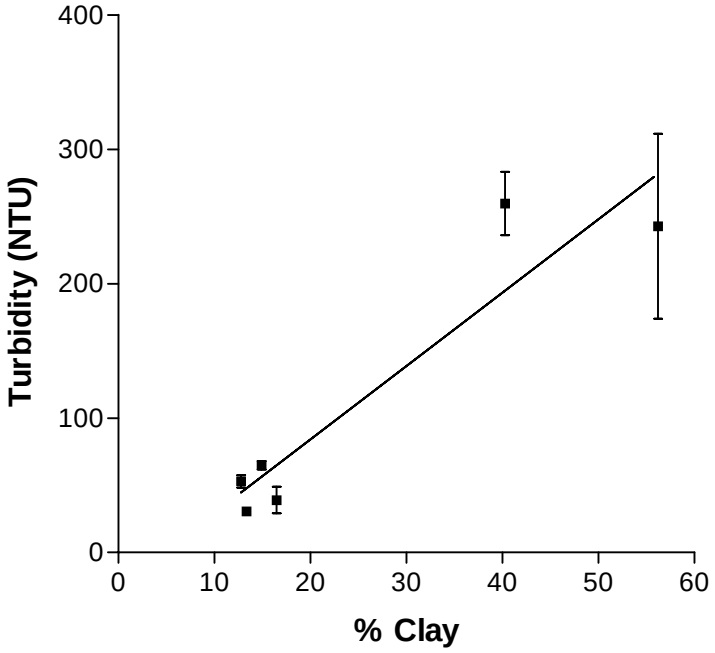

400
300
200
100
0
Turbidity (NTU)
0
10
20
30
40
50
60
% Clay

Figure 6 (a)

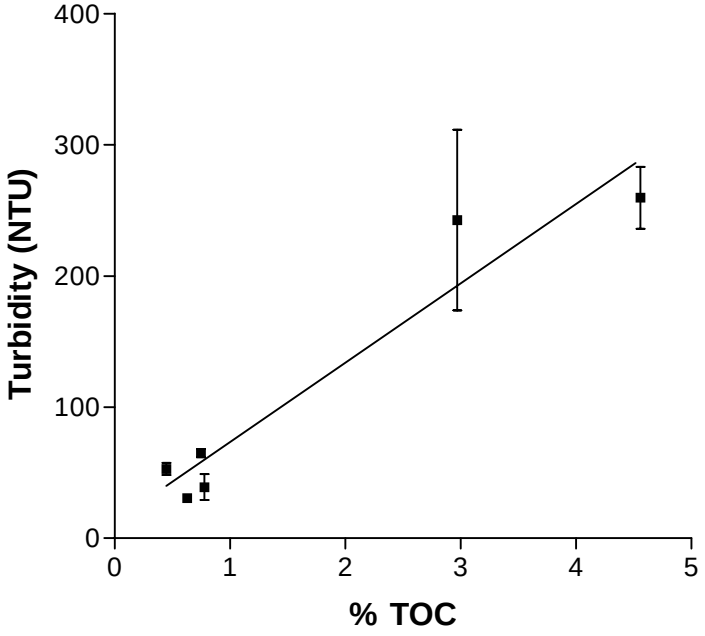

400
300
200
100
0
Turbidity (NTU)
0
1
2
3
4
5
% TOC

Figure 6 (b)

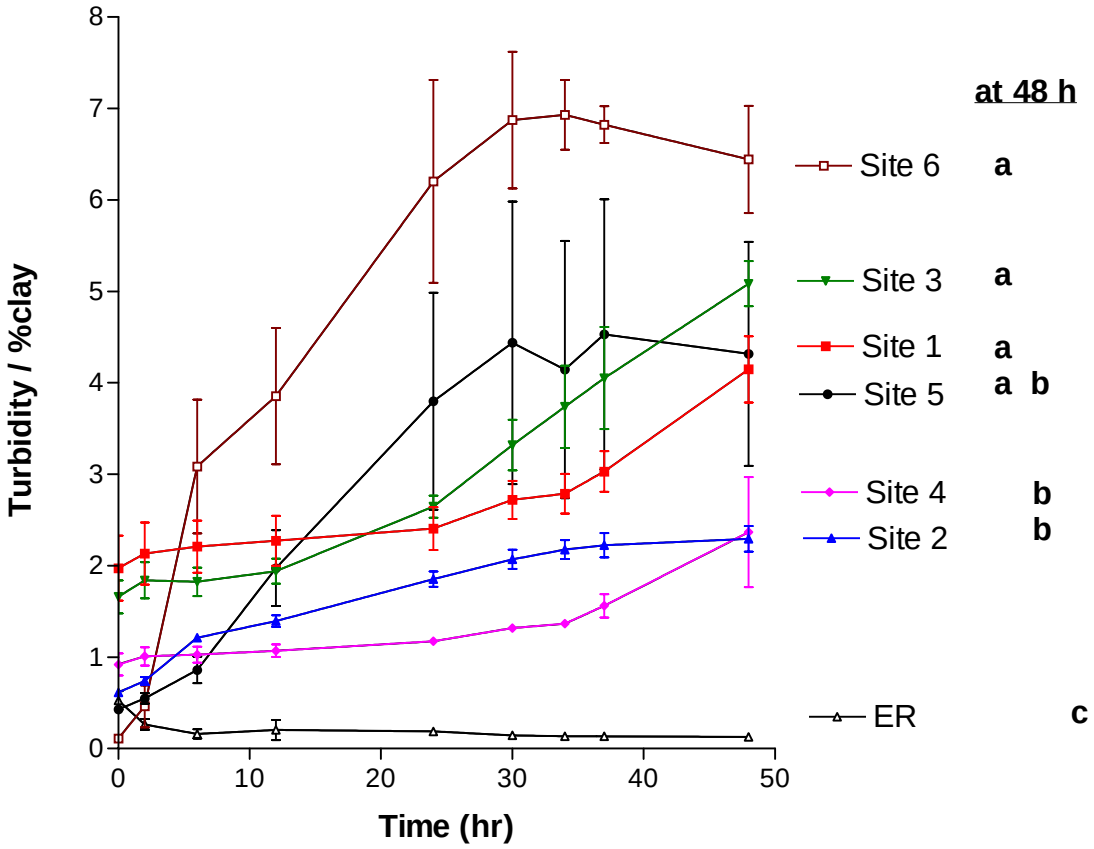


Figure 7 (a)

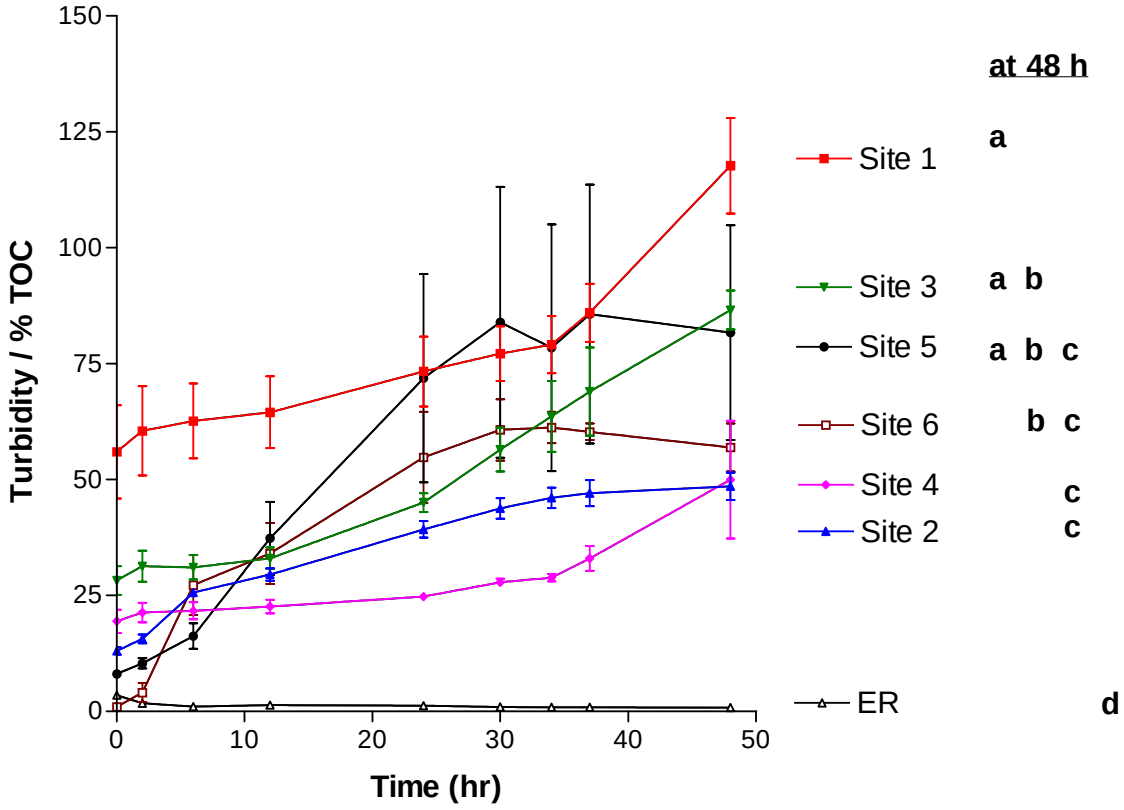
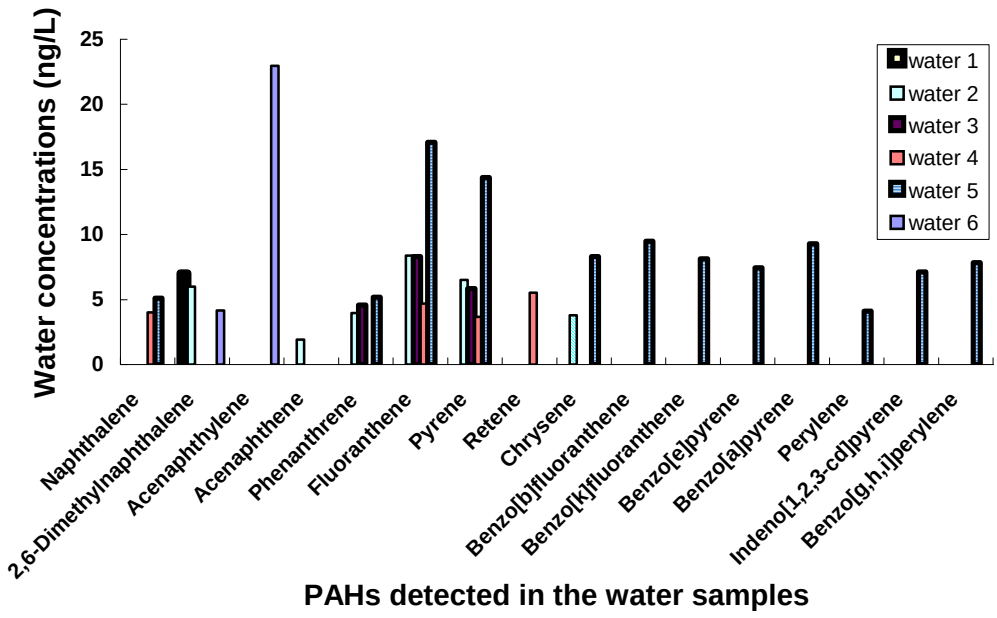

150
125
100
75
50
25
0
Turbidity / % TOC
0
10
20
30
40
50
Time (hr)
at 48 h
Site 1
a
Site 3
a b
Site 5
a b c
Site 6
b c
Site 4
c
Site 2
c
ER
d

Figure 7 (b)


Water concentrations (ng/L)
25
20
15
10
5
0
water 1
water 2
water 3
water 4
water 5
water 6
Naphthalene
2,6-Dimethylnaphthalene
Acenaphthylene
Acenaphthene
Phenanthrene
Fluoranthene
Pyrene
Retene
Chrysene
Benzo[b]fluoranthene
Benzo[k]fluoranthene
Benzo[e]pyrene
Benzo[a]pyrene
Perylene
Indeno[1,2,3-cd]pyrene
Benzo[g,h,i]perylene
PAHs detected in the water samples

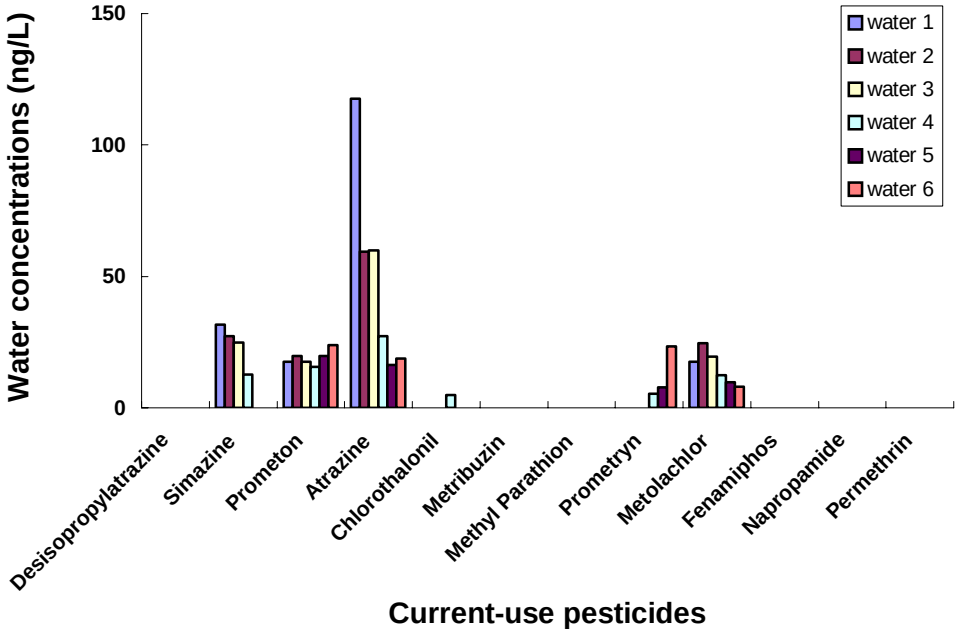

Water concentrations (ng/L)
150
100
50
0
water 1
water 2
water 3
water 4
water 5
water 6
Desisopropylatrazine
Simazine
Prometon
Atrazine
Chlorothalonil
Metribuzin
Methyl Parathion
Prometryn
Metolachlor
Fenamiphos
Napropamide
Permethrin
Current-use pesticides

Figure 8 (a), (b)

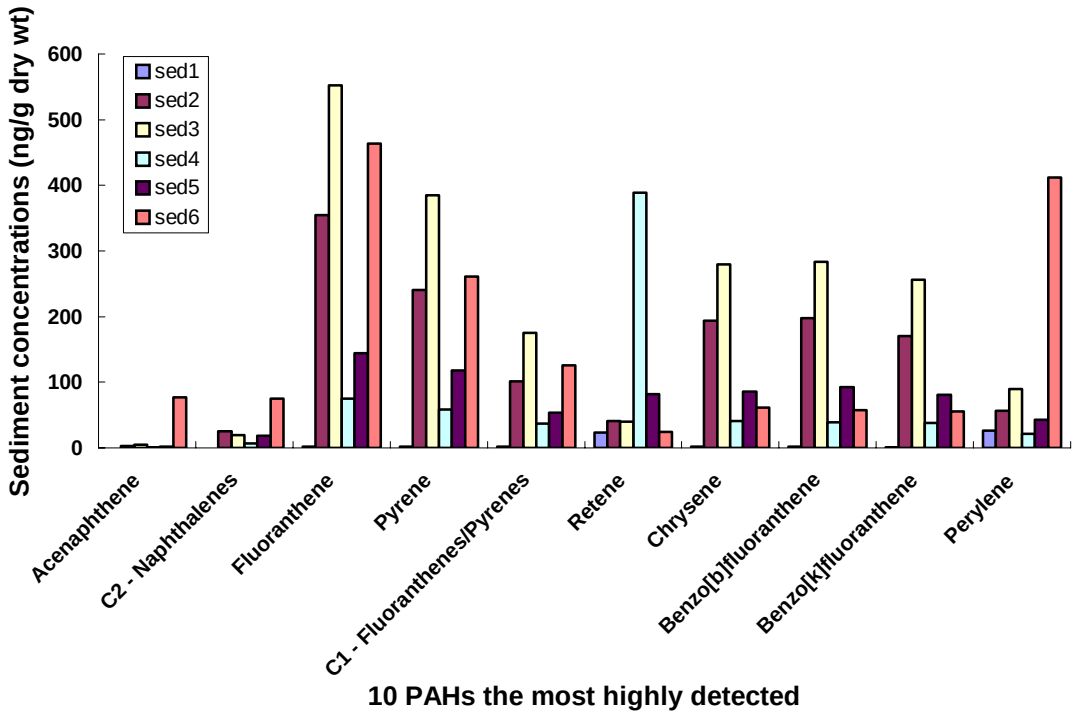

Sediment concentrations (ng/g dry wt)
600
500
400
300
200
100
0
sed1
sed2
sed3
sed4
sed5
sed6
Acenaphthene
C2 - Naphthalenes
Fluoranthene
Pyrene
C1 - Fluoranthenes/Pyrenes
Retene
Chrysene
Benzo[b]fluoranthene
Benzo[k]fluoranthene
Perylene
10 PAHs the most highly detected

Figure 9.

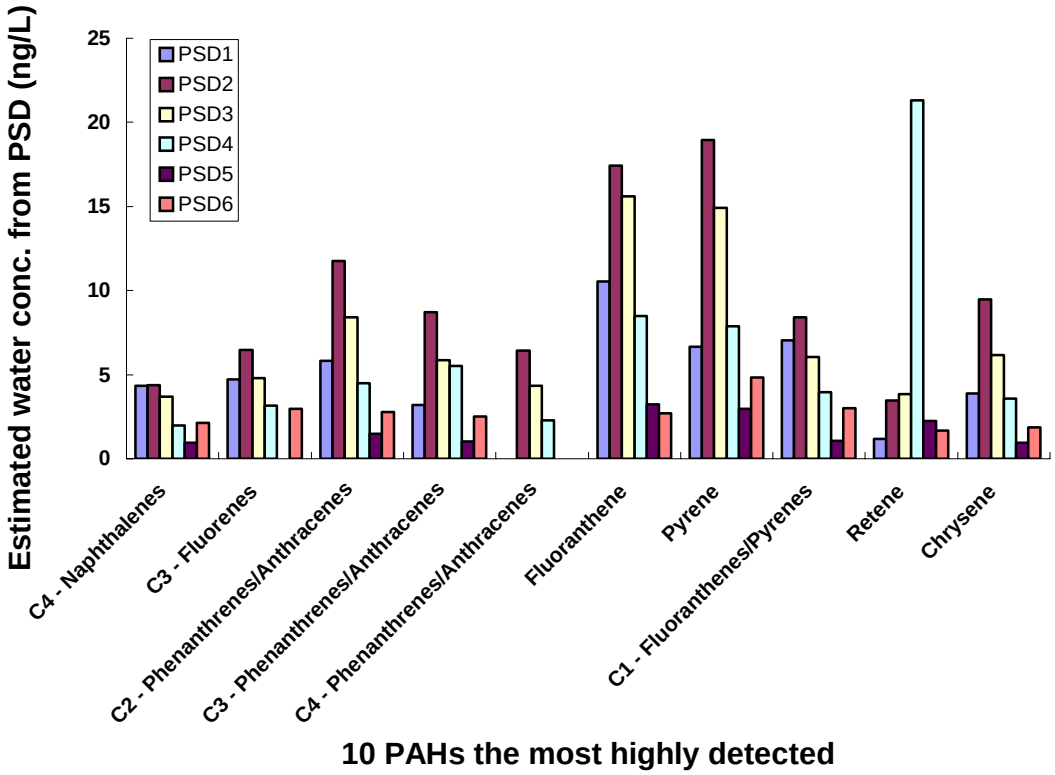

Estimated water conc. from PSD (ng/L)
25
20
15
10
5
0
PSD1
PSD2
PSD3
PSD4
PSD5
PSD6
C4 - Naphthalenes
C3 - Fluorenes
C2 - Phenanthrenes/Anthracenes
C3 - Phenanthrenes/Anthracenes
C4 - Phenanthrenes/Anthracenes
Fluoranthene
Pyrene
C1 - Fluoranthenes/Pyrenes
Retene
Chrysene
10 PAHs the most highly detected

Figure 10.

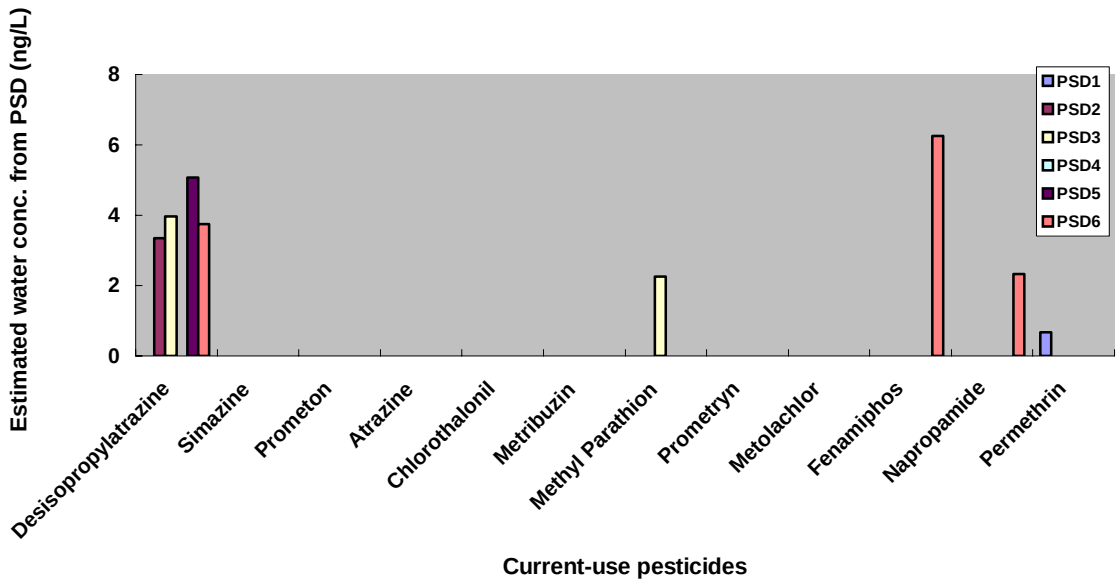

Estimated water conc. from PSD (ng/L)
8
6
4
2
0
PSD1
PSD2
PSD3
PSD4
PSD5
PSD6
Desisopropylatrazine
Simazine
Prometon
Atrazine
Chlorothalonil
Metribuzin
Methyl Parathion
Prometryn
Metolachlor
Fenamiphos
Napropamide
Permethrin
Current-use pesticides

Figure 11.

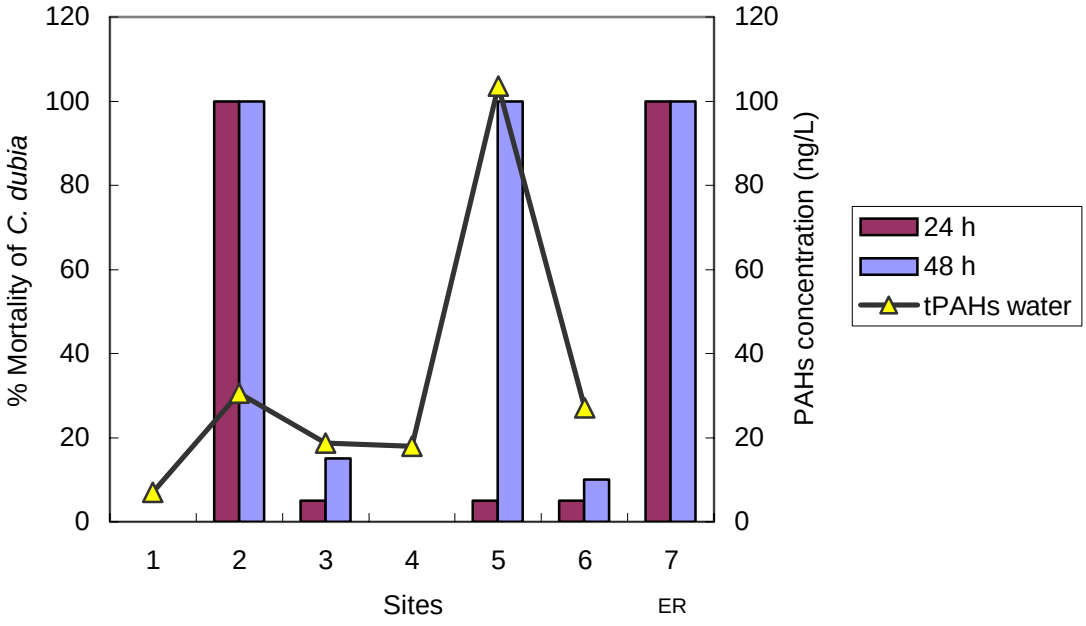
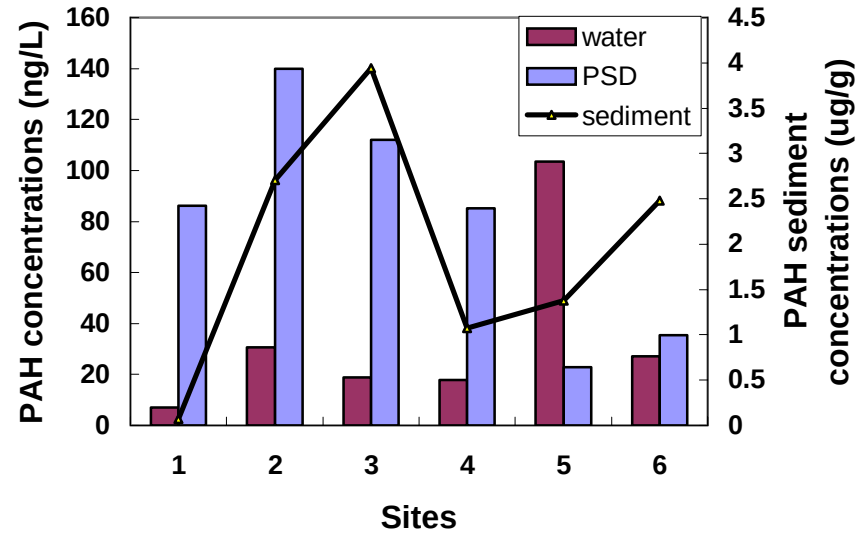

120
100
80
60
40
20
0
% Mortality of C. dubia
PAHs concentration (ng/L)
1
2
3
4
5
6
7
Sites
ER
24 h
48 h
tPAHs water

Figure 12.


PAH concentrations (ng/L)
PAH sediment concentrations (ug/g)
water
PSD
sediment
Sites

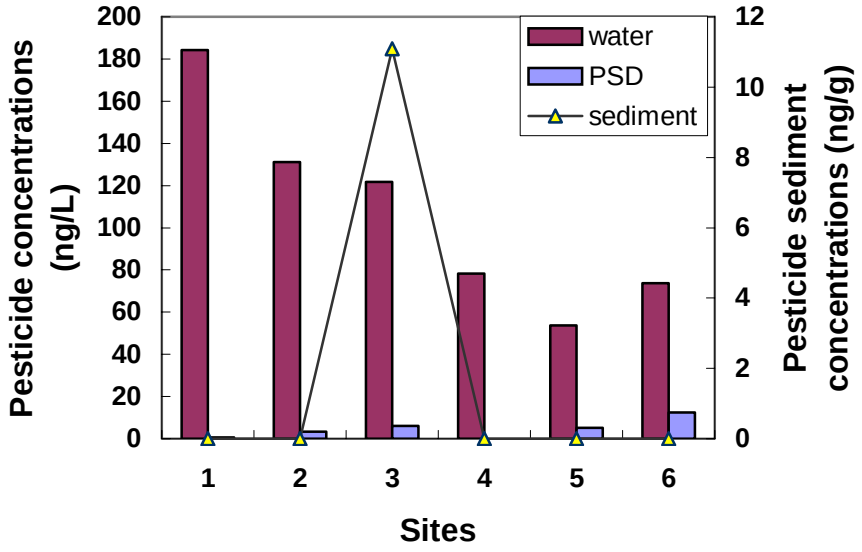

Pesticide concentrations (ng/L)
Pesticide sediment concentrations (ng/g)
water
PSD
sediment
Sites

Figure 13.

CHAPTER IV: Assessing the Toxicity of Selected Pharmaceuticals and Personal Care Products (PPCPs) with *Ceriodaphnia dubia* and BioTurbTox

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ABSTRACT

Studies of the transport, fate, and effects of pharmaceuticals and personal care products (PPCPs) found in the environment are needed for the holistic assessment of human and ecological risk. PPCPs are continually introduced to the aquatic environment as complex mixtures via both untreated and treated sewage effluent and terrestrial run-off. The toxicological implications of PPCPs in aquatic environment are largely unknown. Considering the physico-chemical properties of some PPCPs, they may partition into soils and sediment. Because the effects of PPCPs on nontarget organisms, especially invertebrates, are mostly unknown, seven PPCPs – fluoxetine, bisphenol A, caffeine, triclosan, carbamazepine, 17-α-ethynyl estradiol, and naproxen - were chosen and tested with the newly developed BioTurbTox test using *Chironomus tentans* and three - 17 α-ethylnyl estradiol, bisphenol A, and triclosan – with the *Ceriodaphnia dubia* reproduction test. Fluoxetine and bisphenol A significantly affected bioturbation caused by *C. tentans* especially at high concentrations (1 and 2 mg/L, p=0.002 and 0.004, respectively) and the turbidity change induced by caffeine, fluoxetine, and bisphenol A showed a concentration-response relationship. Triclosan affected reproduction of *C. dubia* at lower concentrations (IC50: 85.4 μg/L). Though the effect of PPCPs was not significant at environmentally relevant concentrations, additional tests with mixtures of PPCPs and chronic tests are warranted.

1. Introduction

Environmental contaminants research during last three decades has largely focused on the priority pollutants that are acutely toxic, carcinogenic, and persistent [Daughton and Ternes 1999]. Recently, however, the study of environmental pharmaceuticals and personal care products (PPCPs) is gaining attention for the holistic risk assessment. PPCPs can originate from human usage and excretion, disinfectants used for industrial, domestic, agricultural and livestock practices, and disposal of unused/unwanted drugs and those that have expired, both directly into the domestic sewage system and via burial in landfills. Therefore, they are continually introduced to the aquatic environment as complex mixtures via both untreated and treated sewage effluent and terrestrial run-off. [Bosch 1998, Daughton and Ternes 1999]. Many pharmaceuticals have relatively short environmental half-lives. However, they may be regarded as highly persistent pollutants and may impact ecosystem health and potentially affect drinking water supplies because of their continual introduction into the environment [Daughton and Ternes 1999, Kolpin et al. 2002, Roefer et al. 2000, Trussell 2001].

The toxicological implications of PPCPs in the aquatic environment are largely unknown [Jones et al. 2001]. The combined concentrations of PPCPs in current use could prove toxicologically significant to aquatic organisms, though individual concentrations of pharmaceuticals in natural waters are low [Halling-Sorensen et al. 1998, Jones et al. 2001]. The total mean antibiotic concentration in municipal wastewater approached 50 μg/L [Kummerer 2001]. About 70 % of the total antimicrobial usage came from agriculture [Mellon et al. 2001]. Naproxen (ND - 145 ng/L), ibuprofen (ND – 674 ng/L), triclosan (ND – 29 ng/L), and bisphenol A (1.9 – 158 ng/L) were detected from the stormwater canals in New Orleans, Louisiana, USA [Boyd et al. 2004]. In Louisiana sewage treatment plant effluent,

naproxen (81 – 106 ng/L) and triclosan (10 – 21 ng/L) were detected. From Ontario surface water samples, naproxen was detected at 22 – 107 ng/L. Clofibric acid was detected in surface water samples from the Detroit River [Boyd et al. 2003]. Boyd et al. (2003) found that conventional drinking-water treatment processes (coagulation, flocculation, and sedimentation), in addition to continuous addition of powdered activated carbon at a dosage of 2 mg/L, did not completely remove naproxen from Mississippi River waters. Clofibric acid, diclofenac, ibuprofen, propyphenazone, primidone, and carbamazepine were detected individually up to the μg/L-level in influent and effluent samples from sewage treatment plants (STPs) and in all surface water samples collected downstream of the STPs in Berlin, Germany [Heberer 2002 a].

Considering the physico-chemical properties of some PPCPs, they may partition into soils and sediment [Diaz-Cruz et al. 2003]. Oxytetracycline was highly persistent in marine sediments (≥ 10 months) [Capone et al. 1996]. Synthetic estrogens, widely administered as contraceptives and for treatment of certain cancers and hormonal disorders like menopause, are likely to adsorb onto marine and river sediment on the basis of their octanol-water partition coefficient [Jurgens et al. 1999]. Their sorption onto sludge particles and persistence indicates their potential to accumulate in the sediment [López de Alda et al., 2002].

Still, there are no drinking water guidelines or aquatic life criteria for PPCPs [Kolpin 2002]. Because the effect of PPCPs on nontarget organisms, especially, invertebrates, are mostly unknown [Daughton and Ternes 1999]. In this study, seven PPCPs – fluoxetine, bisphenol A, caffeine, triclosan, carbamazepine, 17-α-ethynyl estradiol, and naproxen - were chosen for use in toxicity tests with *Chironomus tentans* and three of those - 17 α-ethylnyl estradiol, bisphenol A, and triclosan - for *Ceriodaphnia dubia* based on the frequency of detection in the aquatic environment and sediment, usage, and potential to bioaccumulate.

Because many PPCPs are not expected to cause acute toxicity at environmentally relevant concentrations, sublethal endpoints such as bioturbation by *C. tentans* and reproduction of *C. dubia* may be more sensitive measures of toxicity for the PPCPs. The properties and toxicities of the seven PPCPs tested in this study are summarized in Table 1.

2. Materials and methods

2.1 Chironomus tentans *bioturbation test*

A newly developed bioturbation method was used to test the acute toxicities of seven pharmaceuticals –fluoxetine, bisphenol A, caffeine, triclosan, carbamazepine, 17-α-ethynyl estradiol, and naproxen, individually. Because of the potential for rapid degradation of the PPCPs, we spiked the compounds directly into the overlying water rather than to the sediment compartment--a more environmentally realistic exposure pathway.

All test chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The concentrations used were 0, 10, 100, 1000, and 2000 μg/L for fluoxetine, 0, 1, 10, 100, and 1000 μg/L for bisphenol A, caffeine, 17-α-ethynyl estradiol, and naproxen, 0, 2, 100, 500, 1000 μg/L for carbamazepine, and 0, 1, 10, 100, 500, 1000 μg/L for triclosan. Due to the hydrophobicity of bisphenol A, triclosan, carbamazepine, naproxen, and 17 α-ethynyl estradiol, these chemicals were dissolved in ethyl alcohol and the same volume of the ethyl alcohol was injected to the control.

A relatively uncontaminated sediment (~15 g wet wt, clay type) collected from the Apex Community Park Lake (ACPL), a former drinking water reservoir in Apex, North Carolina, was used in the tests. The sediment was placed into turbidimeter vials and 20 mL of de-chlorinated tap water was added. After 24 hours of settling, the pharmaceuticals were

spiked into the water and five larvae were placed onto the sediment of each vial. The relative turbidity and mortality were measured in each vial (n=4-6) at –1, 6, 12, 24, 30, 36, and 48 h. To determine if the turbidity change was linear over the exposure time and related to the concentrations tested (on log scale), and to the interactions of the time and the concentration (on log scale), a response surface model assuming repeated measures over time was done with PC-SAS® [SAS Institute 1999].

2.2 Ceriodaphnia dubia *test*

Three pharmaceuticals - 17 α-ethylnyl estradiol, bisphenol A, and triclosan – were used for the 7-day *Ceriodaphnia dubia* survival and reproduction test. The *C. dubia* tests were conducted in collaboration with the NC Department of Environmental and Natural Resources (DENR) Aquatic Toxicology Laboratory. *C. dubia* were fed with *Selenastrum* suspension and ground Trout chow. Target concentrations of the pharmaceuticals were 0, 1, 10, 100, 1000, and 2000 μg/L. The solutions and a organism were held in a 30 mL cup and each cup contained 15 mL of the test solution (n=5). The solution was changed 2 times during the 7-d test period. On days 3, 6, and 7, reproduction was measured and neonates were counted. Mortality was recorded daily. The tests were terminated when the organisms in the control produced their 3rd brood. After the test, EC50 values were calculated using ToxCalc v5.0.23.

3. Results

3.1 C. tentans *results*

Water quality variables were measured before and after the exposure (temperature, 22.1 ± 0.34˚C; D.O., 3.8 ± 0.77 mg/L, mean ± SD). Turbidity showed a concentration-

response relationship in the water spiked with fluoxetine, bisphenol A, and caffeine. At the highest concentrations of those PPCPs, larvae became inactive and little turbidity change was observed. Responses to other PPCPs were not statistically significant. With 17 α-ethynyl estradiol exposure, the turbidity reached its maximum at time 6 h. After this time, turbidity decreased and then remained at similar turbidity at all concentrations, but the turbidity change was linear over time ($p<0.0001$, Fig. 1a). In bisphenol A spiked water, turbidity increased linearly over time ($p<0.0001$) in a similar pattern at all the concentrations ($p=0.004$) and it showed a concentration-response relation at 48 h (e.g., high turbidity at lower concentrations and low turbidity at higher concentrations (Fig.1b). Caffeine spiked water also showed a linear increase in turbidity over 48 h ($p<0.0001$). At the second highest concentration of caffeine (100 μg/L), turbidity was reduced the most (Fig. 1c). Carbamazepine inhibited turbidity at all concentrations and turbidity increases were linear over time ($p<0.0001$), but not in a concentration-dependent manner ($p=0.7549$) (Fig. 1d). At the lowest concentration of carbamazepine, turbidity was the most reduced. A linear increase of turbidity over time ($p<0.0001$) was observed in fluoxetine-spiked water and it also showed a gradient response ($p=0.002$). At the highest concentration (2 mg/L), larvae became very inactive and they did not come up to the sediment-water interface (Fig. 1e). At 2 mg/L, turbidity suppression was the most notable. For fluoxetine, the turbidity increase was linear over time ($p<0.0001$) in a concentration-dependent manner ($p=0.002$) and its interactions of time and concentrations were also significant ($p=0.0002$). In naproxen and triclosan spiked water, individually, there was no apparent pattern of reaction to concentrations (Fig. 1f, g), just a linear increase in turbidity over time ($p<0.0001$).

3.2 Ceriodaphnia dubia results

Water quality variables (temperature, 25.7 ± 0.9 °C; D.O., 8.2 ± 0.1 mg O_2/L; pH, 7.4 ± 0.2) were measured before the initiation and termination of the test and between water changes. Only these three PPCPs that were lacking toxicity information from the literature were tested for *C. dubia* tests. IC50 data are shown in Table 2. Triclosan was the most toxic (IC 50, 85.48 μg/L) among the three PPCPs tested. 17-α-ethynyl estradiol affected reproduction of *C. dubia* at the highest concentration tested. Bisphenol A did not seem to affect the reproduction of *C. dubia* at concentrations lower than 2 mg/L.

4. Discussion

PPCPs are distinguished from the persistent organic pollutants (POPs) for its higher polarity of the parent PPCPs [Daughton and Ternes 1999]. Though most PPCPs are not known to be acutely toxic at environmental concentrations, their continual introduction into the aquatic environment may cause undesirable effects on aquatic organisms and their effects could be cumulative and gradual. It is possible that the cumulative level of these effects may lead to irreversible change in populations [Daughton and Ternes 1999]. The draft of European Union regulatory guidance which was going to be implemented in early 2004 required new pharmaceuticals to go through phase II risk assessment based on standard acute toxicity tests (algae, *Daphnia magna* and fish) when the PEC (predicted environmental concentration) of the active ingredients is >0.01 μg/L. In the United States, it was set at >1 μg/L [CDER 1998]. Further chronic testing is needed when there is a potential of the compound to bioaccumulate (log Kow>3.5) [EU 2001, EMEA 2003].

The range of the log Kow of the selected seven PPCPs was medium to high (2.25-4.76) (Table 1). This means that those PPCPs may possibly adsorb onto sediment [Heberer 2002, Peck et al. 2004]. Synthetic steroids are frequently detected organic wastewater

contaminants and they are accumulated in the sediment phase [Peck et al. 2004]. In turn, PPCPs with higher log Kow values can be removed by activated sludge treatment. The efficiency of removal for 17-α-ethynyl estradiol was 85 % [Baronti et al. 2000]. Because the PPCPs used in this study may possibly adsorb onto sediment, that may adversely affect the *C. tentans* inhabiting the sediment. Because we previously found that bioturbation was demonstrated to be a sensitive indicator of sediment toxicity (BioTurbTox test) [Cho et al. 2005. unpublished data], it was used to test the toxicity of PPCPs. In this study, bioturbation was a suitable endpoint for some PPCPs, but not to all, at the tested range of concentrations.

17-α-ethynyl estradiol is the most potent synthetic estrogen known [Daughton and Ternes 1999]. It was detected at low concentrations (ng/L) in effluent from publicly owned wastewater treatment plants (POWTs) [Henschel et al. 1997] because its removal of this by waste water treatment was efficient. It affected reproductive organ development and induced high plasma vitellogenin [Jobling et al. 1998] and it provoked feminization in wild male fish at low concentrations [Purdom et al. 1994]. However, it did not significantly affect bioturbation. Because there was no toxicity data available for 17-α-ethynyl estradiol and *C. dubia*, we conducted a *C. dubia* reproduction test. The IC50 value determined was 935.5 μg/L, and it did not seem to affect *C. dubia* reproduction at low concentrations.

Bisphenol A is produced to make polycarbonate plastics. From the bioturbation test with *C. tentans*, the presence of bisphenol A reduced turbidity at concentrations between 100 – 1000 μg/L and did so in a concentrations-dependent manner ($p=0.004$). The mechanism for this changed behavior is unknown. However, it did not affect *C. dubia* reproduction at low concentrations (IC 50: >2 mg/L). Bisphenol A was reported to delay emergence time of *C. riparius* under chronic sediment exposure [Watts et al. 2002], and at concentrations over 640 μg/L of bisphenol A, somatic growth of adult male fathead minnow was inhibited and

vitellogenin synthesis was induced in a multi-generation study [Sohoni et al. 2001].

Caffeine was expected to stimulate *C. tentans* at all concentrations, but it did not. At all concentrations, turbidity increases were less than the control but were not significantly different at 48 h (p=0.4783). Maximum concentrations of caffeine detected were 1.39 μg/L from streams in Iowa [Kolpin et al. 2004] and 1.3-2.4 μg/L from surface water as far as 13.5 km downstream from a municipal wastewater discharge point in Texas [Buszka et al. 1994].

Carbamazepine is an analgesic and antiepileptic drug. It is eliminated from wastewater treatment plants (WWTPs) at a low rate (7 %) [Ternes 1998]. Therefore, it produces a high daily load and is persistent (POTW max. effluent: 6.3 μg/L) [Ternes 1998]. It has been detected in surface water samples in Berlin, Germany, up to 1.1 μg/L [Heberer et al. in press]. It also seemed that the presence of carbamazepine in the overlying water reduced the activity of *C. tentans* larvae and turbidity was decreased, but not in a concentration dependent manner (p=0.7549). The effect was apparent at low concentrations that are in the range of the environmental concentrations. However, at higher concentrations it was not significantly different from the control.

Fluoxetine HCl is anti-depressant that inhibits serotonin reuptake from the pre-synaptic nerve cleft [Ranganathan 2001]. It induced *C. dubia* fecundity at 56 μg/L [Brooks et al. 2003] and elicited significant spawning in male mussels at around 150 μg/L [Fong 1998]. Weston et al (2001) estimated that fluoxetine concentration from effluent might reach up to 0.54 μg/L. It may bind to sediment considering its log Kow value. Atta-Politou et al. (2001) reported that 95 % of fluoxetine tested was adsorbed onto activated charcoal within 5 minutes. Fluoxetine may affect pelagic organisms as well as benthic organisms due to its binding to sediment [Fong 2001]. Fluoxetine reduced turbidity over 48 h in a concentration-dependent manner (p=0.002). The most affecting concentration (2mg/L) was a high and non-

environmentally relevant concentration. However, others reported that growth of *C. tentans* and *H. azteca* were significantly reduced and LOECs were determined at 1.3 mg/kg and 5.6 mg/kg, respectively [Brooks et al. 2003]. At low concentrations (ng/L level), it affected plasma estradiol level and caused developmental abnormalities at all exposure in Japanese medaka [Foran et al. 2004].

Antibiotics are efficiently removed by WWTPs [Heberer, in press]. Triclosan has been widely used as an antiseptic for 30 yr [Daughton and Ternes 1999] and it is incorporated in variety of personal care products [van Wezel 2000]. 10-20 μg/L of triclosan in effluent and 80-100 μg/g of triclosan in sediment near the outfall of a wastewater treatment plant were detected from samples in Rhode Island [Lopez-Avila and Hites 1980]. It is known as a weak endocrine disruptor [Foran et al. 2000] and it affected *C. dubia* reproduction (IC50: 85.48 μg/L), but not to *C. tentans* ($p=0.7323$). Triclosan is known to act either as a reactive chemical or as a non-polar narcotic chemical [Verhaar et al. 1992]. All nonstreroidal anti-inflammatiory pharmaceuticals Cleuvers (2004) studied act by nonpolar narcosis. Narcosis is a type of toxicity considered to be caused by an absolutely non-specific disturbance of membrane integrity and function, because hydrophobicity determines the potency of a chemical to induce narcosis. Consequently, a chemical will always be as toxic as its log Kow indicates within certain boundaries apart from any other specific mechanism of toxicity [Escher and Hermans 2002, Verhaar et al. 1992].

Naproxen inhibits the cyclooxygenases, catalyzing the biosynthesis of prostaglandins that are responsible for pain and inflammation [Vane and Botting 1998]. Its POTW removal efficiency is 66 % and POTW max. effluent concentration was 0.52 μg/L [Ternes 1998]. In WWTP effluents in Lake Greifensee (Switzerland), naproxen concentration was 2.6 μg/L [Tixier et al. 2003]. In the present study, naproxen did not affect bioturbation by *C. tentans*

and no concentrations tested were different from the control (p=0.9247).

The IC50 value from the *C. dubia* reproduction test and the concentrations which reduced turbidity caused by *C. tentans* were higher than the detected environmental concentrations. However, the possibility of mixtures and continuous exposure can lead to toxicological effects to aquatic organisms [Halling-Sørensen et al. 1998, Jones et al. 2001]. Mixture tests with various PPCPs showed stronger effects than single PPCP effects. Both the combinations of clofibrinic acid and carbamazepine and combinations of diclofenac and ibuprofen have been found to act by a non-specific mode of action (non-polar narcosis) and increased mortality [Cleuvers 2003]. If the joint action of estrogenic chemicals is ignored, risk can be underestimated [Silva et al. 2002]. Therefore, additional research on mixtures of PPCPs and chronic toxicity of PPCPs should be done for the holistic risk assessment of environmental PPCPs.

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Table 1. Properties and toxicity of selected PPCPs.

Chemicals	**Classification**	**log Kow**	**$t_{1/2}$**	**LC50**
17 α-ethinylestradiol	synthetic estrogen	3.67		none
Bisphenol A	platicizer synthetic estrogen	3.32		Oral rat LD50: 3250mg/kg
Triclosan	antibiotic	4.76	No degradation in water in 28 d	*Daphnia*: 0.39 mg/L fish:0.25-2mg/L
Carbamazepine	analgesic/ antiepileptic	2.25	63-93 d	*Daphnia* EC50:>100 mg/L *Lemna* EC50:25.5 mg/L *C. dubia* EC50 77.7 mg/L
Naproxen	analgesic/ anti-inflammatory	3.18	14 d	*Daphnia* EC50:174 mg/L *Lemna* EC50:24.2 mg/L
Fluoxetine HCl	anti-depressant	4.3 at pH 11		*C. dubia*: 234 *D. magna*: 820 *P. promelas*: 705 μg/L *C. tentans*: 15.2 μg/g (EC50,reduced growth)

Table 2. IC50 values from *Ceriodaphnia dubia* 7-day reproduction tests.

PPCPs	IC50 (µg/L)	SD
Triclosan	85.48	46.43
17-α-ethynyl estradiol	935.5	118.33
Bisphenol A	>2000	

FIGURE LEGENDS

Fig. 1. BioTurbTox test results with 7 pharmaceuticals

(a) 17-α-ethynyl estradiol, (b) Bisphenol A, (c) Caffeine, (d) Carbamazepine,

(e) Fluoxetine, (f) Naproxen, (g) Triclosan.

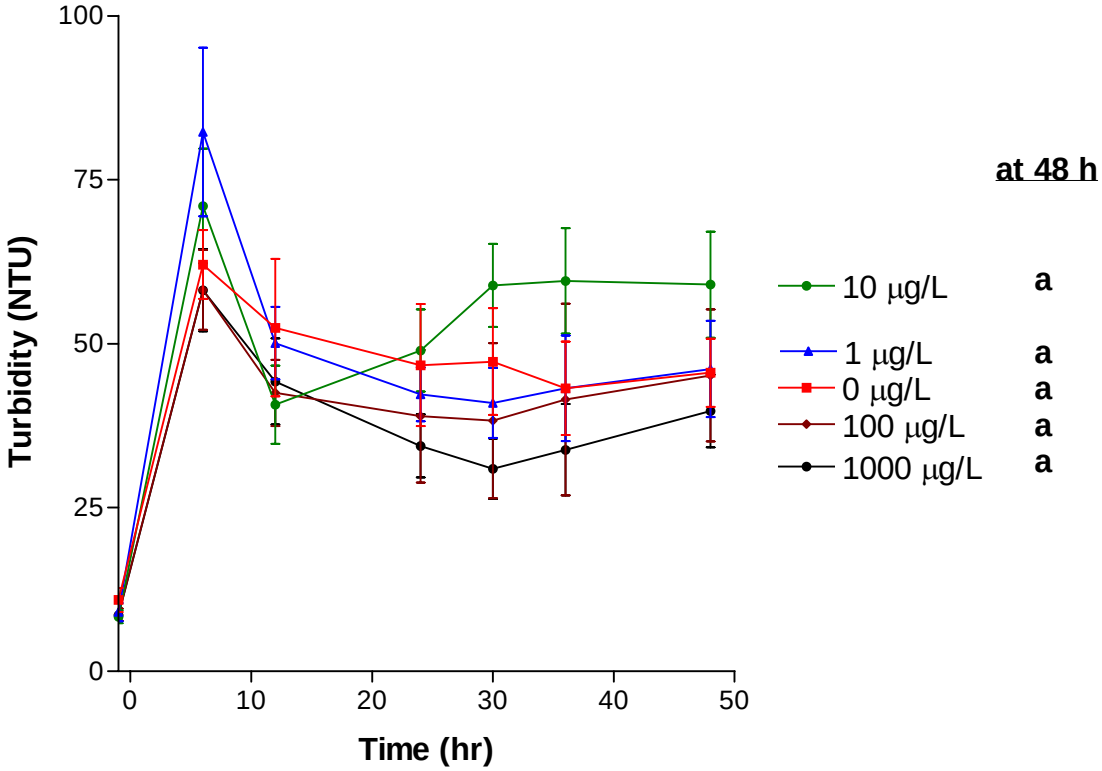
100
75
50
25
0
Turbidity (NTU)
0
10
20
30
40
50
Time (hr)
at 48 h
10 µg/L a
1 µg/L a
0 µg/L a
100 µg/L a
1000 µg/L a

Fig. 1 (a)

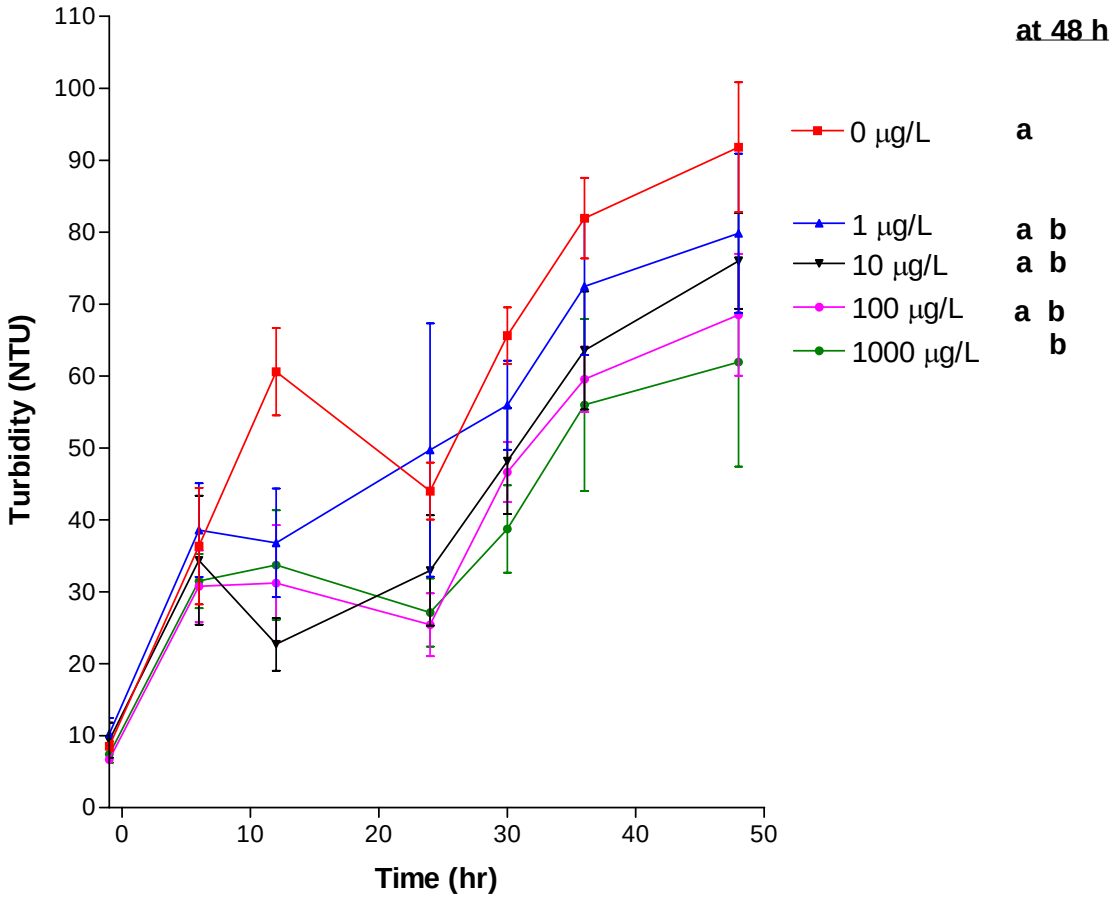
Turbidity (NTU)
Time (hr)
0
10
20
30
40
50
60
70
80
90
100
110
0
10
20
30
40
50
at 48 h
0 µg/L
a
1 µg/L
a b
10 µg/L
a b
100 µg/L
a b
1000 µg/L
b

Fig. 1 (b)

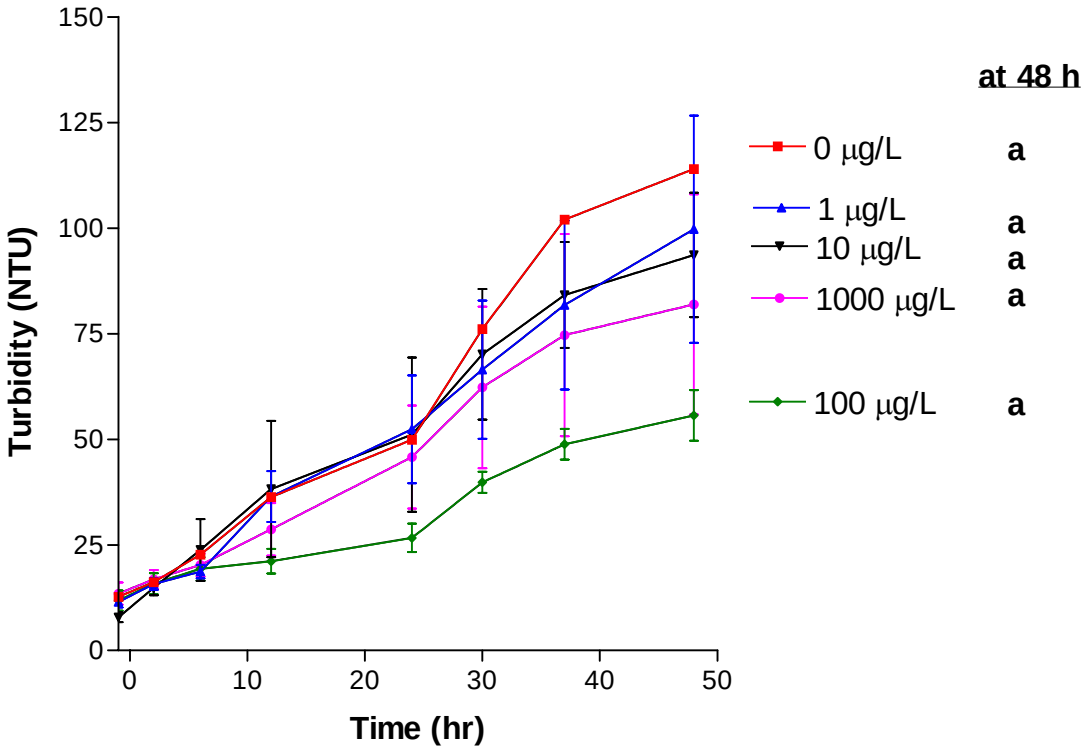
150
125
100
75
50
25
0
Turbidity (NTU)
0
10
20
30
40
50
Time (hr)
at 48 h
0 μg/L
a
1 μg/L
a
10 μg/L
a
1000 μg/L
a
100 μg/L
a

Fig. 1 (c)

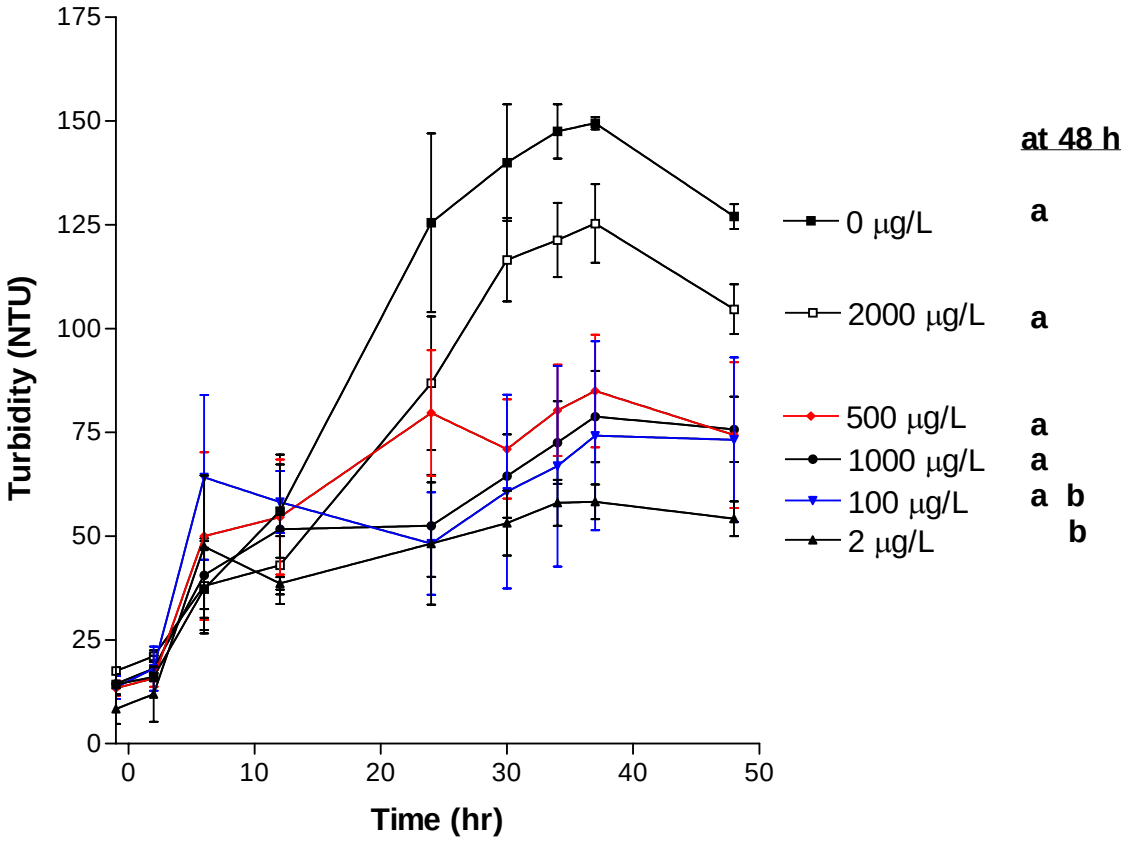
Turbidity (NTU)
175
150
125
100
75
50
25
0
0
10
20
30
40
50
Time (hr)
at 48 h
0 µg/L
a
2000 µg/L
a
500 µg/L
a
1000 µg/L
a
100 µg/L
a b
2 µg/L
b

Fig. 1 (d)

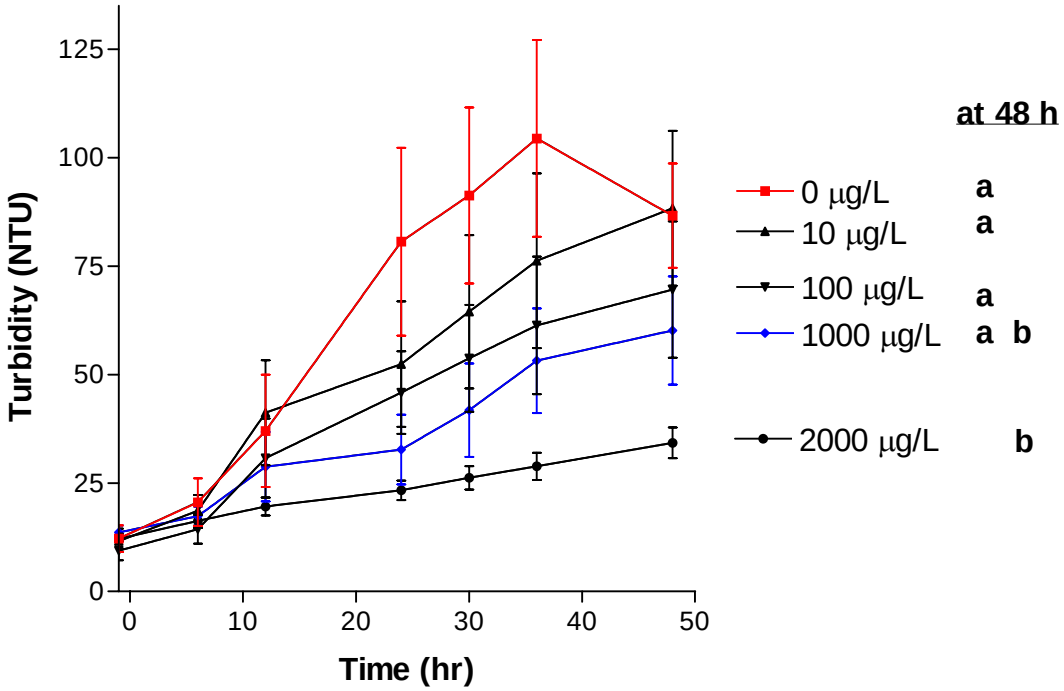
Turbidity (NTU)
125
100
75
50
25
0
0
10
20
30
40
50
Time (hr)
at 48 h
0 µg/L a
10 µg/L a
100 µg/L a
1000 µg/L a b
2000 µg/L b

Fig. 1 (e)

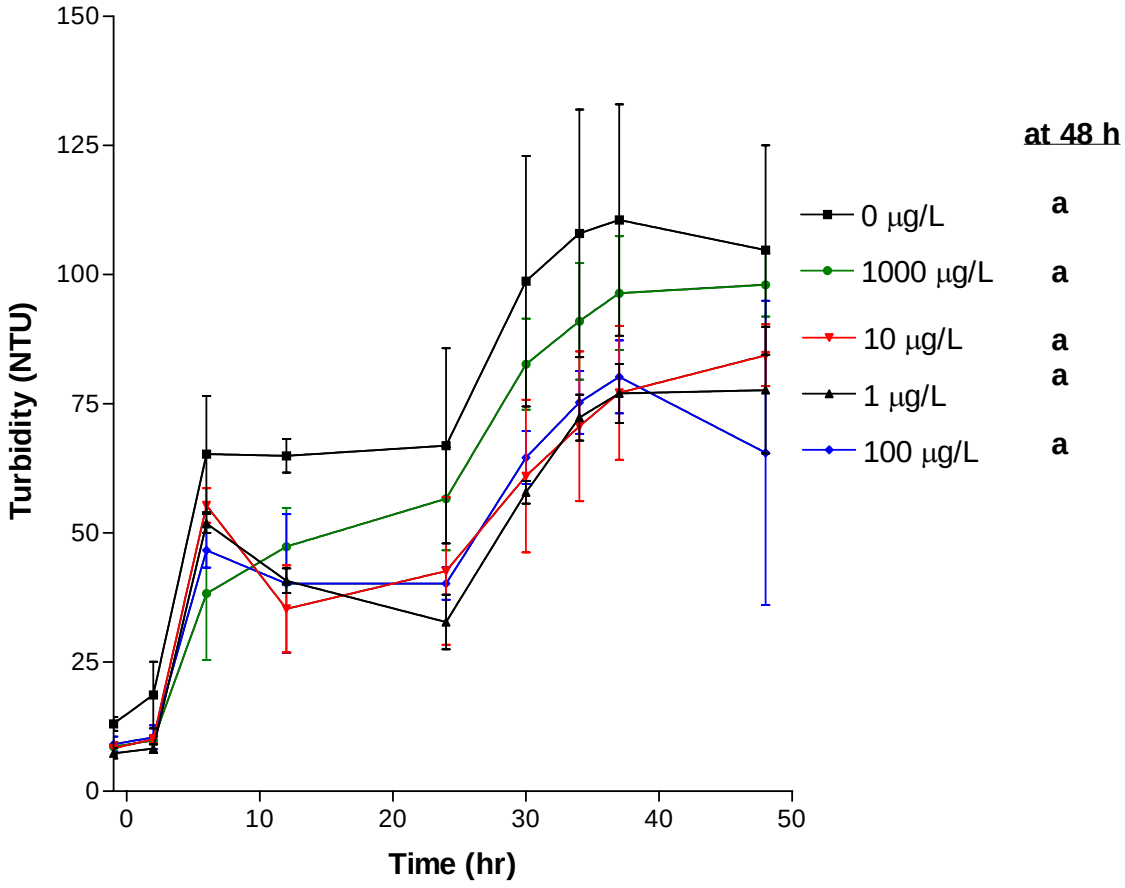
Turbidity (NTU)
Time (hr)
0
25
50
75
100
125
150
0
10
20
30
40
50
at 48 h
0 μg/L
a
1000 μg/L
a
10 μg/L
a
1 μg/L
a
100 μg/L
a

Fig. 1 (f)

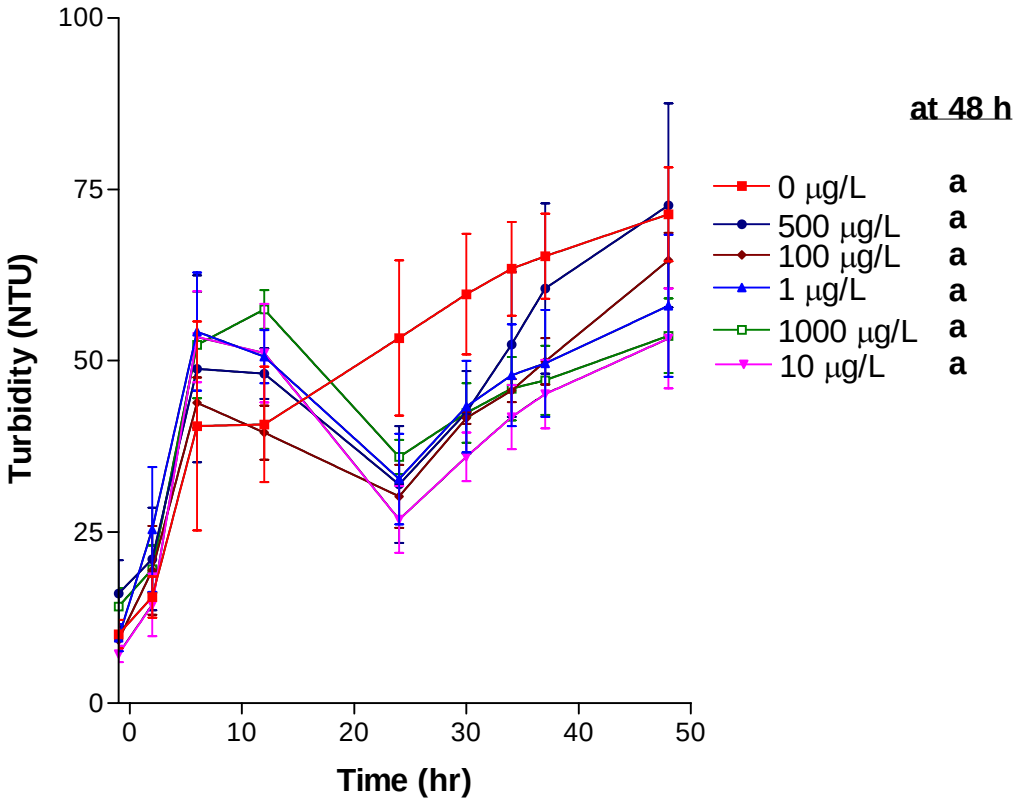
100
75
50
25
0
Turbidity (NTU)
0
10
20
30
40
50
Time (hr)
at 48 h
0 μg/L a
500 μg/L a
100 μg/L a
1 μg/L a
1000 μg/L a
10 μg/L a

Fig. 1 (g)

CHAPTER V: Comparison of two spiking methods for assessing the toxicity of Elizabeth River, Virginia Sediments

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ABSTRACT

Although there have been several methods published for spiking aquatic sediments, a standardized spiking method is not yet available. In an effort to extend the information database on sediment spiking methods, we compared an extract mixing method to a whole sediment dilution method. The sediment spiking methods were evaluated by measuring the relative toxicity with the bioturbation-based BioTurbTox test, which uses the turbidity generated by 2nd-instar *Chironomus tentans* larvae as the endpoint. An organic solvent extract of sediment from the Elizabeth River (ER), Virginia (primarily contaminated by polycyclic aromatic hydrocarbons (PAHs)), was spiked into a relatively uncontaminated sediment obtained from the Apex Community Park Lake (ACPL; a former drinking water reservoir) in Apex, North Carolina. Based on chemical analysis of the extract, the estimated sediment concentrations after spiking were 0, 8.8, 39.5, 134.4, and 574.7 μg/g. For the whole sediment dilution method, ER sediment was diluted with ACPL sediment. The percentages of ER sediment were 0, 1.6, 3.1, 6.3, 25, 50, and 100, which corresponded to PAH concentrations of 0, 5.4, 10.8, 21.5, 85, 167.9, and 327.4 μg/g, respectively. *C. tentans* larvae responded to both of the spiked sediments in an inverse concentration-response manner, with high turbidity generated at low concentrations, and low turbidity generated at high concentrations. The 48-h EC50 (median effective concentration) for the extract mixing method was 31.397 μg/g (95 % confidence interval, 22.977-85.772) and was 35.896 μg/g (95 % CI, 15.871, 55.920) for the whole sediment dilution method. Based on the similarity of our toxicity test results, we conclude that both of the spiking methods are appropriate for estimating the toxicity of aquatic sediments in screening level assessments.

INTRODUCTION

Sediment spiking is a commonly used tool for determining cause and effect of sediment toxicity. However, the methods of sediment spiking have varied considerably among studies [Murdoch et al, 1997]. The simplest spiking method involves direct mixing of the compounds into the sediment, most often used for water-soluble compounds such as metals [Swartz et al., 1988; Jepsen et al., 1995; Bartsch et al., 1999], but also for low water-soluble compounds [Bridges et al., 1994]. Sediment spiking with low water-soluble compounds usually involve a carrier solvent at a minimum volume so that test organisms are not affected [ASTM 1998]. Acetone is a suitable solvent for polar compounds but not suitable for many nonpolar compounds. Dichloromethane is a popular solvent for spiking dry soil or sediment [Northcott and Jones, 2000]. The carrier solvent may be added directly to wet sediment [Swartz et al., 1999; Harrahy and Clements 1997], or added to dry sediment [Cho et al., 2003], and the carrier evaporated before mixing with the bulk wet sediment [Suedel et al., 1993], or evaporated onto a glass surface then scraped off by wet sediment [Booij K, 1993; DeWitt et al., 1992]. Murdoch et al. (1997) demonstrated that both methods, evaporation of the carrier before sediment addition and addition of carrier containing the spike directly to the sediment, were successful in achieving target concentrations from spiked concentrations. Dilution mixing is a spiking method where a compound/carrier solvent solution is added to a portion of wet or dry sediment and thoroughly mixed. It is subsequently mixed and diluted with the bulk of remaining sediment [Northcott and Jones, 2000].

In this study, we compared two sediment spiking methods; an extract mixing method to a whole sediment dilution mixing method. The sediment spiking methods were evaluated by measuring the relative toxicity with the bioturbation-based BioTurbTox test, which uses

the turbidity generated by 2nd-instar *Chironomus tentans* larvae as the endpoint.

For the PAHs spiking, Elizabeth River (ER) sediment in Virginia were used. The Elizabeth River is the most southern tributary of the Chesapeake Bay and it is designated as a "toxic hot spot" because of heavy loads of organic contaminants and metals in the bed sediments from industries such as pesticide plants, creosote and cement factories, coal loading facilities, *etc*. along the shoreline of the river [Conrad and Chisholm-Brause, 2004].

Materials and Methods

Extract mixing method.

The first method was to spike the relatively uncontaminated Apex Community Park Lake (ACPL) sediment (%sand:%silt:%clay = 42:34:24, wet/dry ratio = 3.0723) with the extract from the ER sediment (%sand:%silt:%clay = 43:13:44, wet/dry ratio = 2.3185). ER sediment was extracted by the shaker table extraction procedure and PAHs concentrations were determined using GC/MS. Two hundred g dry wt ER sediment (463.7 g wet wt) was extracted with 1:1 acetone:dichloromethane (CH_2Cl_2, DCM) and 30 g of anhydrous sodium sulfate overnight on an orbital shaker table. It was collected in 6 boiling flasks, filtered with GF/B glass fiber filter paper, and concentrated to the total volume of 75 mL. Total PAHs concentration was summed from each individual 48 PAH concentrations (327 μg/L) and the estimated sediment concentrations after spiking – 0, 8.8, 39.5, 134.4, and 574.7 μg/g - were based on the analytical data in Table 1.

The extract was spiked into 120 g dry wt (369 g wet wt.) ACPL sediment and hand-mixed for 3 minutes every day for a 2-week equilibrium period. Because mixing the sediment with extract on an orbital shaker table generated heat and microbial degradation was

enhanced in the sediment, hand mixing and storage of the sediment at the refrigerator at 4°C was chosen. And the volume of the DCM was reduced up to 200 μL because it was highly toxic to the midge larvae when 10 mL of DCM was used.

Using the spiked sediment with ER sediment extract, BioTurbTox test was conducted. Approximately 19 g wet wt. of the spiked sediment were placed into the turbidimeter vials and 20 mL of de-chlorinated tap water was added. After 24 hours of settling, five 2nd in-star *C. tentans* larvae were placed onto the sediment in each vial. Turbidity and mortality were checked at –1, 6, 12, 24, 30, 36, and 48 hours. There were 5 replicates in each treatment. Behavior of the larvae during the exposure was also observed.

Whole sediment dilution mixing method.

As a second method of spiking, sediment-to-sediment dilution mixing was made. ER sediment was diluted with ACPL sediment. Percentages of ER sediment were 0, 1.6, 3.1, 6.3, 25, 50, and 100, which corresponded to PAH concentrations of 0, 5.4, 10.8, 21.5, 85, 167.9, and 327.4 μg/g, respectively. The diluted sediments were hand mixed for 5 minutes once a week and equilibrated for 2 weeks. BioTurbTox test was conducted with these sediment for 48 hours (n=5). One-way ANOVA was carried out separately at each time, followed by Least Significant Difference (LSD) to compare the turbidities in different concentrations of the sediment in both methods with PC-SAS® [SAS Institute 1999]. The analysis was done on turbidity and on log transformed turbidity values. To get the estimate of EC50s (median effective concentration) at 48 hours for both method, all the turbidities were converted to % proportional to the maximum turbidity at 48 hours at all the treatment levels and it was estimated by Spearman-Karber procedures using TOXSTAT® 3.4 [West Inc. 1994].

Results

Water quality was analyzed before and after the exposure (temperature, 22.1 ± 0.68 ˚C; D.O., 3.57 ± 0.54 mg/L; pH, 7.1 ± 0.32; mean ± SD). Table 1 shows the 48 PAH concentrations in the ER sediment. It was highly contaminated with PAHs. Fluoranthene, benzo[b]fluoranthene, C1-fluoranthenes/pyrenes were detected at the highest concentrations. Recovery range for surrogate internal standards was 24.70 – 74.32 %. The sum of 48 PAHs concentrations was 327 μg/g.

Turbidity change by *C. tentans* in the sediment spiked with ER sediment extract is shown in Figure 1. There were three groups depending on the degree of the turbidity increase at low concentrations (0 and 8.8 μg/g), mid-concentration (39.5 μg/g), and high concentrations (134.4 and 574.7 μg/g) of PAHs (Fig. 1). The turbidities for each group were significantly different at 48 hours ($p<0.0001$). Though there was not much increase in the turbidity at the two highest concentrations (134.4 and 574.7 μg/g), there was no mortality. It was thought that the concentrations were not acutely toxic to larvae during 48 hours, but it seemed to inhibit the activity of the larvae in the sediment.

Turbidity change by *C. tentans* larvae in sediment spiked by dilution mixing shows that the larvae at the high PAHs concentrations (85, 168, and 327 μg/g) were very active at the initiation of the test, and then they died (Fig. 2). The mortality of the larvae at 48 hours in the sediment concentrations of 85, 168, and 327 μg/g were 52, 60, and 100 %, respectively. Mortality in the sediment concentration at 21.5 μg/g was 8 %. Final turbidity decreased as the mortality increased. The larvae at the low PAH concentrations (5.4, 10.8, and 21.5 μg/g) were also active, but they seemed to avoid the contact with the sediment, staying in the water and active. Because they stayed in the water, water turbidity was decreased though it reached

higher value than the control at the first time except for the sediment concentration of 21. 5 μg/g. The larvae in the control were burrowing into the sediment and they did not come up to the water, and the turbidity kept increasing.

The EC50 at 48 hours for the extract mixing method was 31.4 μg/g (-23.0, 85.8: 95 % confidence interval) and was 35.9 μg/g (15.9, 55.9: 95 % CI) for the whole sediment dilution method. Thus, there was no significant difference in the turbidity response generated by the larvae to both spiking methods.

Discussion

When organic compounds are spiked into experimental systems, subsequent compound behavior can be affected. If the test medium is sediment, the difficulties involved in spiking into experimental systems are even greater [Northcott and Jones, 2000]. Carrier solvent used when spiking contaminants into sediment has the potential to have a significant effect on sediment organic carbon (OC). Excessive solvent can alter natural organic matter distributions and/or extract labile components from the sediment [Northcott and Jones, 2000]. However, the use of spiked sediment to elucidate cause and effect of sediment toxicity is widely accepted [Reichert et al., 1985; Lamberson et al., 1992; Luoma and Ho, 1993], though the spiking methods have not been standardized [Chapman, 1995].

In an effort to compare the two spiking methods to sediment, bioturbation response of *C. tentans* to spiked sediment were examined. Considering the properties of PAHs, whole sediment prepared by the dilution mixing method would take more time to equilibrate than that by the extract mixing method, which might make the sediment from the extract mixing method more toxic. However, the sediment by dilution mixing method seemed to be more

toxic judging from the higher mortality of the larvae and the turbidity decrease during the exposure time. Considering the EC50 at 48 hours, sediment prepared by extract mixing was slightly more toxic than sediment from dilution mixing, though it was not significantly different. While turbidity in the sediment concentration at 8.8 μg/g from extract mixing was higher than the control at 48 hour (though not significantly different between 0 and 8.8 μg/g sediment, $p<0.0001$), turbidity in the 10.8 μg/g sediment by dilution mixing was significantly lower than that of the control ($p<0.0001$). Thus, it is thought that, in the sediment prepared by dilution mixing, PAHs were more bioavailable than in the sediment prepared by extract mixing, which might lead to higher mortality of larvae exposed to the sediment by dilution mixing. The use of DCM at 200 μL did not seem to affect the mortality of the larvae significantly at 48-hour exposure.

It was found from other spiking experiments that mixing contaminated whole sediments with clean materials disrupted chemical equilibrium and partitioning and it could take weeks to months for the mixed samples to be fully equilibrated [Karickhoff and Morris, 1985; Landrum, 1989; Landrum et al., 1992; Coel et al., 2000]. Experiments using whole sediments by dilution procedures sometimes showed monotonic concentration-response relationships [Ciarelli et al., 1998], but also often resulted in U-shaped concentration-response relationships [DeWitt et al., 1989; Giesy et al., 1990; Nelson et al., 1993]. In this present study, it showed monotonic concentration-response relationships; survival and turbidity decreased more in higher concentrations of the contaminants in the sediment.

When turbidity was measured during the exposure time, the behavior of the *C. tentans* larvae were monitored. There were several different behaviors of the larvae in the test vials with the sediment. First, the larvae in the control vials preferred to stay in the sediment and actively moved in the sediment, increasing the turbidity. Secondly, larvae came up to the

water when the sediment condition was hostile to them. In this case, turbidity increased significantly at the initial exposure, but then decreased over the exposure time when they just stayed up in the water. Thirdly, larvae came up to the water when the toxicity of the sediment was too high. They were dying off as the exposure time progressed. Similar behaviors were found from other studies. When *C. tentans* were exposed to low concentration of metals, they burrowed 2-4 cm into the sediment within a few hours and was not visible on the surface throughout the test period. But when they were exposed to higher concentrations, they remained on the surface of the sediment or did not burrow as deep [Harrahy and Clements, 1997]. The presence of larvae in the water column was attributed to unsuitable substratum, poor food quality, or positive phototaxis [Wright and Mattice, 1981; MacLachlan, 1969; Davies, 1976]. However, *C. tentans* larvae stayed in the sediment in water exposure of fluoxetine and did not come up to the overlying water because the water had higher concentrations of test chemicals than sediment and avoided contact with overlying water. Because they were not active in the sediment, turbidity did not increase [Cho et al. 2005].

Accurate matching of test sediments with appropriate diluent materials is not simple because of complex sediment physico-chemical characteristics interacting to control contaminant bioavailability [Chapman, 1987; Giesy and Hoke, 1990]. However, organic extract spiking method has an advantage in that it can eliminate confounding factors such as ammonia and sulfides [Côté et al., 1998]. Vigano et al. (2003) indicated that the toxicities of sediment extracts were substantially consistent with both longitudinal and seasonal patterns of sediment chemistry. Further, they confirmed both the covariance of chemicals and the seasonal independence of most of them.

When sediment substrate is spiked, it should be minimally manipulated such that physico-chemical nature of the test substrate and the stability of the test compound should be

maintained and sediment flora and/or fauna should not be affected by carrier solvent used for the spiked sediment. However, since it is impossible to meet all these conditions, any spiking method with organic contaminants to sediment will be a compromise between experimental requirements and the practicalities of changes to the sediment [Northcott and Jones, 2000]. The two spiking methods examined here were apparently appropriate, because sediment spiking method and the response of the *C. tentans* to the spiked sediment followed a concentration-response relationship. There can be some toxicity change if the equilibration time of the sediment is extended. Moreover, use of the extract from the Elizabeth River was a good way of mixture test because it contained the natural mixtures of the PAHs (though some could be lost during the extract procedure), which were environmentally realistic. Whole sediment mixing method is more close to real-world mixtures of sediment. By extract mixing method, more focused study with PAHs mixtures are feasible.

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Table 1. 48 PAHs concentrations and the sum of those in the ER sediment.

Parameter	Concentrations (ng/g)
Naphthalene	215.58
2-methylnaphthalene	102.19
1-methylnaphthalene	73.55
Biphenyl	40.21
2,6-dimethylnaphthalene	76.29
Acenaphthylene	1964.80
Acenaphthene	515.31
Dibenzofuran	426.33
2,3,5-trimethylnaphthalene	167.86
C1-naphthalenes	217.94
C2-naphthalenes	376.74
C3-naphthalenes	1144.65
C4-naphthalenes	1121.67
Fluorine	583.86
1-methylfluorine	604.41
C1-fluorenes	1436.13
C2-fluorenes	2638.98
C3-fluorenes	3475.86
Dibenzothiophene	328.36
C1- dibenzothiophene	789.60
C2- dibenzothiophene	1522.69
C3- dibenzothiophene	1422.10
Phenanthrene	4118.94
Anthracene	4442.59
1-methylphenanthrene	630.69
C1-phenanthrenes/anthracenes	7138.98
C2-phenanthrenes/anthracenes	11340.82
C3-phenanthrenes/anthracenes	6184.25
C4-phenanthrenes/anthracenes	1555.72
Fluoranthene	35047.00
Pyrene	27972.01

C1-fluoranthenes/pyrenes	31609.31
Retene	296.70
Benz[a]anthracene	25943.28
Chrysene	10935.70
C1-chrysenes	10935.70
C2-chrysenes	5004.11
C3-chrysenes	2467.30
C4-chrysenes	bdl
benzo[b]fluoranthene	32706.23
benzo[e]pyrene	15652.40
benzo[a]pyrene	17583.91
Perylene	3725.02
indeno[1,2,3-c,d]pyrene	10270.66
dibenz[a,h]anthracene	2876.13
Benzo[g.h.i]perylene	7896.58
Coronene	679.83
Total	**327383.86**

FIGURE LEGENDS

Figure 1. Turbidity change in the overlying water over 48 hours with *C. tentans* larvae in the sediment spiked with PAHs extract from ER sediment to ACPL sediment. Concentrations not accompanied by a common letter were judged to be significantly different at test termination (48 hours, $p<0.0001$).

Figure 2. Turbidity change over 48 hours by *C. tentans* larvae in the sediment mixture of ER and ACPL sediment. Concentrations not accompanied by a common letter were judged to be significantly different at test termination (48 hours, $p<0.0001$).

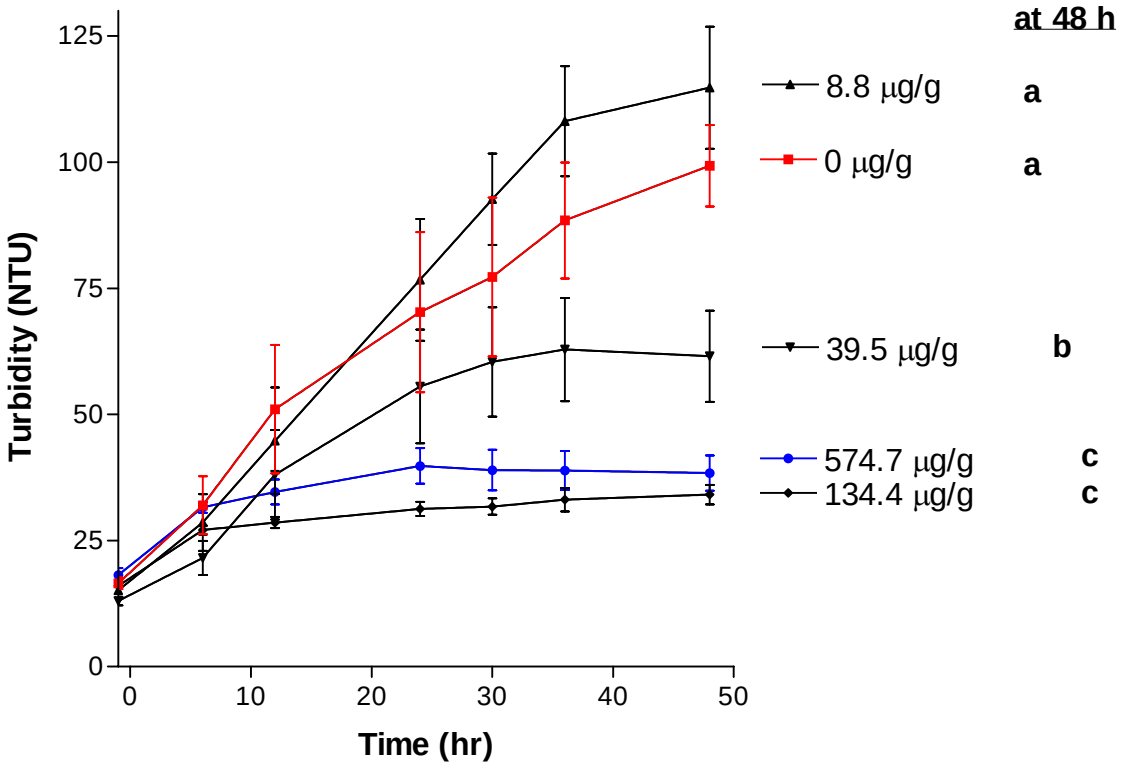
at 48 h
8.8 μg/g
a
0 μg/g
a
39.5 μg/g
b
574.7 μg/g
c
134.4 μg/g
c
Turbidity (NTU)
Time (hr)
0
25
50
75
100
125
0
10
20
30
40
50

Figure 1.

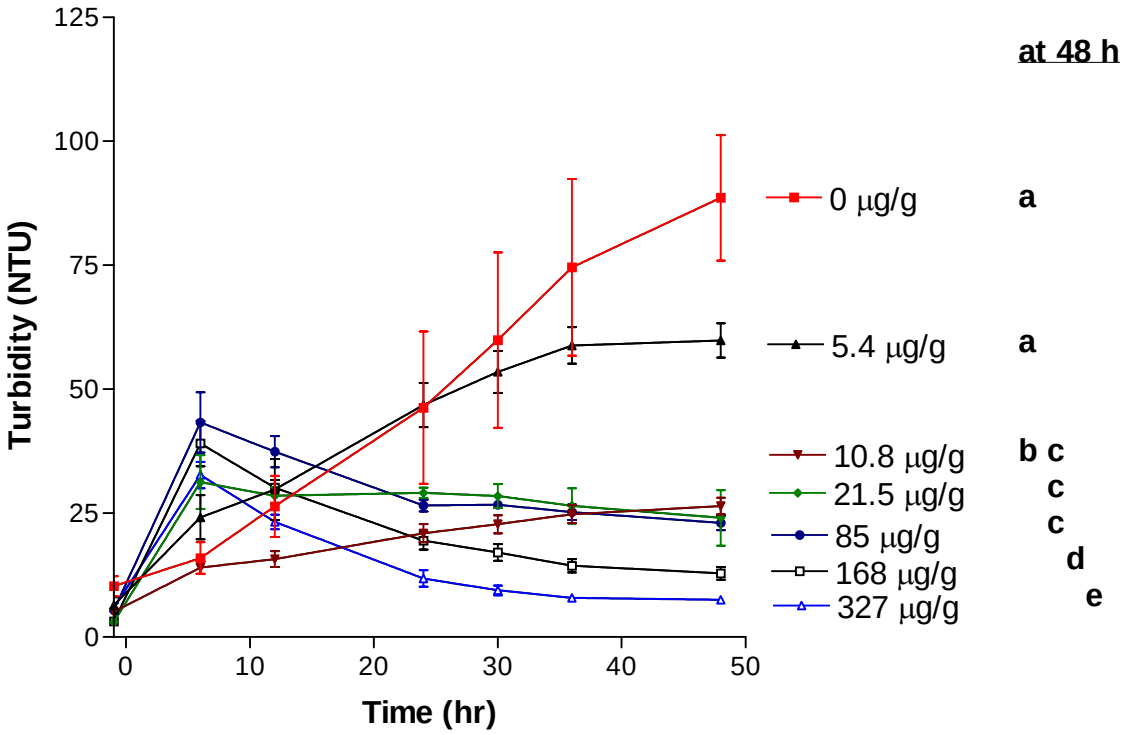
at 48 h
Turbidity (NTU)
Time (hr)
0
25
50
75
100
125
0
10
20
30
40
50
0 µg/g
a
5.4 µg/g
a
10.8 µg/g
b c
21.5 µg/g
c
85 µg/g
c
168 µg/g
d
327 µg/g
e

Figure 2.