Biomarker Responses in Fathead Minnows (Pimephales promelas) during Exposure to Exceptional Quality Biosolids

Constance A. Sullivan
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BIOMARKER RESPONSES IN FATHEAD MINNOWS (*Pimephales promelas*) DURING EXPOSURE TO EXCEPTIONAL QUALITY BIOSOLIDS

A Thesis

Presented to

The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Master of Science

by

Constance A. Sullivan

2007
APPROVAL SHEET

This Thesis is submitted in partial fulfillment of

The requirements for the degree of

Master of Science

Constance A. Sullivan

Approved, May 2007

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ABSTRACT

Adult male fathead minnows (*Pimephales promelas*) were exposed to EQ (exceptional quality) biosolids for 28 days in static renewal aquaria. Treatments were clean water (control), low dose (0.5 g l⁻¹), and high dose (2.5 g l⁻¹). Chemical analysis of biosolids-exposed water revealed nonylphenols to be the dominant contaminant released. Chemical analysis of the biosolids revealed the presence of polycyclic aromatic hydrocarbons, polybrominated diphenyl ethers, and nonylphenols. Cytochrome P4501A (CYP1A) levels were measured in the fish, as was the amount of DNA damage in hepatocytes. Hepatic CYP1A was elevated eight to 21-fold, respectively, in fish maintained in low and high dose biosolids exposures relative to controls. DNA damage measured in hepatocytes using the Comet assay was significantly elevated in low and high dose exposed fish at the majority of time points measured. Our data indicate chemicals associated with biosolids taken up by *P. promelas* have the capability to raise CYP1A levels and induce DNA damage. EQ biosolids are available to the general public. There is no tracking or restrictions on how these are used or restrictions on where EQ biosolids can be applied. As biosolids are broadcast into the environment, their constituents have the potential to migrate to nearby watersheds. They thus have the potential to impact both aquatic and terrestrial ecosystems.
Wastewater Treatment

The goal of wastewater treatment is to remove disease-causing organisms and harmful compounds from the aqueous influent, by either degradation or partitioning to and settling out of solids. The liquid portion discharged is referred to as effluent and the solids left behind as sewage sludge. However, though many compounds are concentrated in sewage sludge as a function of their hydrophobicity, some remain in the water phase.

Previous work has demonstrated negative effects to organisms exposed to effluent in the lab and field. Liney et al. (2006) studied endocrine disruption and DNA damage in roach \( (Rutilus rutilus) \) exposed to wastewater effluent. Induction of vitellogenin, alteration of gonads, and DNA damage in exposed \( R. rutilus \) were measured. DNA damage occurred at concentrations lower that those required to induce significant alterations in the other endpoints. Roy et al. (2003) reported cytochrome P4501A (CYP1A) induction, DNA damage, and vitellogenin induction in hornyhead turbot \( (Pleuronichthys verticalis) \), English sole \( (Pleuronectes vetulus) \), and bigmouth sole \( (Hippoglossina stomata) \) inhabiting an environment receiving effluent from a municipal wastewater plant in Orange County, CA. Hepatocytes were isolated from individuals for use in CYP1A and DNA studies. There was significant induction of CYP1A in organisms at exposed sites versus reference sites. There was no significant DNA damage or vitellogenin induction; however, there was a trend for increased damage and induction at increasingly less pristine sites. In another study, Porter and Janz (2003) reported
significant increases in plasma testosterone, hepatosomatic index, condition factor, and vitellogenin levels in male longear sunfish (*Lepomis megalotis*) between the reference and study sites. At the stream influenced by wastewater, there was an alteration in the species composition. Another study indicated significant increases in plasma vitellogenin levels in male rainbow trout (*Onchorhyncus mykiss*) exposed to sewage treatment plant effluents in the United Kingdom (Harries et al. 1999). Similarly, Jobling et al. (1998) noted increased vitellogenin levels in male fish, as well as a high degree of intersex in the roach (*Rutilus rutilus*). Sumpter and Jobling (1995) reported significant estrogenic effects in roach (*Rutilus tutilus*) from wastewater effluent around the United Kingdom and indicated the need for further study. Pandrangi et al. (1995) assessed DNA damage in bullheads (*Ameiurus nebulosus*) and carp (*Cyprinus carpio*) in locations polluted from a variety of sources, including sewage runoff. Blood cells exhibited significantly increased damage in polluted areas versus reference sites. Following exposure, fish maintained in clean water exhibited a decline in DNA damage that approached control levels after three months.

*Biosolids*

*Treatment/ types of biosolids*

Sewage sludge is generated during the treatment of sewage in wastewater facilities. Biosolids are sewage sludge that has gone through additional treatment, such as alkaline stabilization to reduce pathogens, anaerobic and aerobic digestion, composting, heat drying and pelletization, or dewatering (USEPA 1999). Class A biosolids have undergone treatment by processes that result in an end product that is
presumably virtually pathogen-free. Exceptional Quality (EQ) biosolids are Class A biosolids that meet more stringent concentration limits of metals (USEPA 1999, 1994). Class B biosolids have undergone relatively limited treatment to reduce pathogen levels. They can potentially have higher contaminant loads than Class A and EQ biosolids (USEPA 1999).

Land application practices

The majority (53% in 1998) of biosolids are disposed of over land as an economical option for farmers as fertilizer (USEPA 1999). This activity is expected to increase over time (USEPA 1999). Land application of Class B biosolids is also an economical choice for sewage treatment plants. The more stringent treatment of Class A and EQ increases their production costs. Due to the presumably lower threat posed by EQ biosolids, they are widely distributed to the public for private use and the EPA retains limited records of how they are used (USEPA 1999, 1994). There are no restrictions on where EQ biosolids can be applied (USEPA 1999). Biosolids and associated contaminants thus have the potential to run off into nearby watersheds. Both Class A (excluding EQ) and Class B biosolids have some restrictions on where they can be applied. Limitations include slope of land, proximity to surface and groundwaters, frequency of application, and mode of application (USEPA 1994).

Composition and Fate of Sludge Contaminants

McBride (2003) reviewed metals contained in biosolids and concluded there has not been enough research on their possible toxic impacts. They also noted the potential
for severe effects from the additive effects of metals deposited in varying soil conditions. 

With respect to nutrients, biosolids contain considerable nitrogen and phosphorous levels. Phosphorous does not leach out of soils in significant amounts, even if the soils are sandy and prone to runoff (Elliott et al. 2002). Grey and Henry (2002) also indicated phosphorous is not a major concern with respect to leaching during runoff events because it is not entering the aquatic system with surface runoff. This study also found ammonium to not be a significant concern, as it is not entering nearby waterways. However, these investigators suggested that nitrate has the potential to be a problem during runoff.

Biosolids also contain a variety of organic contaminants, including flame retardants (polybrominated diphenyl ethers (PBDEs)), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane (DDT), dichlorodichloroethylene (DDE), dichlorodiphenyldichloroethane (DDD), tributyltin (TBT), and chlordanes (Hale and La Guardia 2002). Other contaminants found in biosolids may include alkylphenol polyethoxylates and their degradation products, synthetic musk compounds, and triclosan (La Guardia et al. 2004; Hale and La Guardia 2002). Organic contaminants in biosolids are not presently regulated; regulation is restricted to eight metals: As, Cd, Cu, Pb, Hg, Ni, Se, and Zn (USEPA 1999, 1994). Regulation is restricted to metals because organic contaminants are not considered to be in quantities great enough to cause biological harm (Hale and La Guardia 2002).
Biological Effects of Biosolids Contaminants

Relative to the amount of work done involving wastewater effluents, there have been few published studies on the biological effects of biosolids exposure. Paul et al. (2005) reported that sheep raised on pastures fertilized with biosolids exhibited reduced parental and fetal body weights. In addition, these investigators reported reductions in the hormones inhibin A and testosterone and a reduction in the size of testes in male fetuses. Ciparis and Hale (2005) measured bioaccumulation of PBDEs from biosolids in the oligochaete, *Lumbriculus variegatus*. Though this study did not address effects of biosolids on the worms themselves, it demonstrated the bioaccumulation of biosolids-associated contaminants.

Because of the widespread use of biosolids and the limited amount of biological fate and effect studies, there is the need for work that addresses possible biological effects of biosolids. Studies that address the physiological responses of organisms in laboratory exposures present several advantages relative to field runoff work, including the control of variables and the ability to directly observe effects (i.e., behavioral changes, mortality, etc.) at any given time. Further information detailing the chemical composition of biosolids is also necessary to aid in determining the possible chemicals responsible for inducing effects.

Objectives

The objective of this study was to evaluate the effects of biosolids on *Pimephales promelas* using a biomarker approach. Molecular biomarkers can indicate an effect from exposure to a factor in an environment and are typically measured at sublethal
concentrations. Molecular indicators are advantageous because they are early signs of an organism’s response to an environmental change and thus can be indicators of exposure and/or effect (Stegeman et al. 1992). CYP1A induction and DNA damage were chosen as indicators, as the biotransformation and activation of procarcinogens by CYP1A can lead to detrimental effects to DNA. Biosolids and biosolids-exposed water were analyzed for compounds known to induce these biomarkers.

Hypotheses

H₀: Exposure to EQ biosolids will not cause CYP1A induction or DNA damage in *Pimephales promelas*;

Hₐ: There will be organic contaminants present that are known to induce CYP1A and DNA damage.

*Cytochrome P4501A*

The exposure of an organism to certain environmental toxicants can result in the induction of specific cytochrome P450 forms (Stegeman and Lech 1991). CYP1A is the cytochrome induced when specific PCBs, PAHs, and TCDD bind to the Ah receptor (AhR) (Van Veld et al. 1997; Sanderson et al. 1996; Stegeman et al. 1992). The induction of CYP1A is the most sensitive and best-characterized response to these compounds, and is often used as a biomarker of exposure. A literature search failed to reveal if CYP1A induction has been measured in biosolids-exposed organisms. Some studies utilizing CYP1A as a biomarker to exposure are listed in Table 1.
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<th>Organism</th>
<th>Citation</th>
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<td>Van Veld et al. 1990</td>
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<td>B(a)P*</td>
<td><em>Fundulus heteroclitus</em></td>
<td>Van Veld et al. 1997</td>
</tr>
<tr>
<td>River contaminated with PAHs*</td>
<td><em>Fundulus heteroclitus</em></td>
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Abbreviations used: PAHs: polycyclic aromatic hydrocarbons; B(a)P: benzo(a)pyrene; TCDF: 2,3,7,8-tetrachlorodibenzofuran; PCDF: polychlorinated dibenzofuran
During AhR-mediated induction of CYP1A, a compound moves into a cell and forms a ligand with the aryl hydrocarbon receptor (AhR), displacing two molecules of heat shock protein 90 (Pollenz 2002) (Figure 1). This complex then moves into the nucleus, where it binds to the promoter aryl hydrocarbon receptor nuclear transporter (ARNT) before binding to the xenobiotic responsive element (XRE), a transcription factor on DNA. Once transcription of DNA into mRNA is complete, mRNA coding for CYP1A is translated and inserted into the rough endoplasmic reticulum (ER). CYP1A-mediated biotransformation takes place in the cytosolic side of the smooth ER. Compounds are typically hydroxylated, making them more polar and thus easier to excrete (Di Guilio et al. 1995). In some instances, a harmful epoxide intermediate is formed. For example, when benzo (a) pyrene is bioactivated, its carcinogenic metabolite 7,8-diol-9,10-epoxide is formed (Phillips 1983). These bioactivated products are either rendered harmless in Phase II or can lead to compounds that can form oxyradicals, or adducts with DNA and other cellular macromolecules.

**DNA Damage**

CYP1A-mediated activation of many chemicals leads to the formation of genotoxic products that have the potential to cause damage to DNA (Mitchelmore et al. 1998a). Strand breaks can be produced directly by chemicals or by those that are activated by CYP1A (Mitchelmore et al. 1998a). Strand breaks are a normal occurrence in cells. However, excessive occurrences compared to a baseline are cause for concern (Shugart 2000). Strand breaks can be produced directly as a result of reactive oxygen species attack, or indirectly by alkali-labile sites or via the process of DNA excision
Figure 1. Mechanism of AH receptor mediated induction of CYP1A. The chemical ligand moves into a cell and binds to the Ah receptor, causing a conformational change in the receptor and the release of two molecules of hsp90 (pathway #1). This complex moves into the nucleus, binds to ARNT, and then to the XRE on DNA. DNA is transcribed in the nucleus and translated out to the endoplasmic reticulum where the protein is inserted in the rough endoplasmic reticulum. After the upregulation of CYP1A, other ligands that enter the cell are detoxified or biotransformed by the CYP1A enzyme (pathway #2).
Ligand

A h-receptor

Nucleus

+ ARNT

XRE

Endoplasmic

Reticulum

Transcription

Upregulation

of CYP1A

Translation

Detoxification
(common)

Bioactivation (rare)
repair of chemical adducts (Mitchelmore and Chipman 1998a, b; Mitchelmore et al.
1998a, b). Reactive oxygen species are problematic in that they can oxidize DNA bases,
particularly guanine base sites, by hydroxylation or ring opening (Shugart 2000),
resulting in mutational events. Adducts can be formed when an electrophilic chemical
covalently binds to DNA (Shugart 2000, 1990; Randerath et al. 1985). They are
problematic in that DNA can be modified, triggering mutations (Wu et al. 1999). Table 2
lists some studies detailing compounds inducing DNA damage.

Organism of study: Pimephales promelas

Pimephales promelas is a freshwater oviparous fish in the family Cyprinidae with
a range that encompasses cool to warm habitats in eastern and northern North America.
They are sexually dimorphic and sexually mature by 4 to 5 months of age (Ankley et al.
2001). At the time of sexual maturity, males weigh 4 to 5 grams (Ankley et al. 2001).
Their life history and responses to pollution are well studied and P. promelas are widely
used as toxicity test organisms by the U.S. Environmental Protection Agency (USEPA
2002). Past studies have utilized Pimephales promelas to study CYP1A induction
(Colavecchia et al. 2004; Lindstrom-Seppa et al. 1994) and to measure DNA damage
(Choi and Meier 2000; Choi et al. 2000; Shugart 1988).
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<th><strong>Organism</strong></th>
<th><strong>Citation</strong></th>
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<td>River water known to contain PAHs*, PCBs*, steel mill effluent, refineries,</td>
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<td>Pandrangi et al. 1995</td>
</tr>
<tr>
<td>foundries, mines, plastic processing plants, raw sewage, agricultural runoff</td>
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<td>B(a)P*, 1-NP, NF</td>
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<tr>
<td>oils, suspended solids)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B(a)P*, 1-NP, NF, H2O2, MX</td>
<td><em>Mytilus edulis</em> L.</td>
<td>Mitchelmore et al. 1998b</td>
</tr>
<tr>
<td>B(a)P*, 1-NP, NF, H2O2, MX, MNNG</td>
<td><em>Salmo trutta</em></td>
<td>Mitchelmore and Chipman 1998a</td>
</tr>
<tr>
<td>Metal plating wastewater</td>
<td><em>Pimephales promelas</em></td>
<td>Choi and Meiser 2000</td>
</tr>
</tbody>
</table>

Abbreviations used: PAHs: polycyclic aromatic hydrocarbons; PCBs: polychlorinated biphenyls; B(a)P: benzo(a)pyrene; 1-NP: 1-nitropyrene; NF: nitrofurantoin; EMS: ethylmethanesulphonate; MX: 3-chloro-4-(dichloromethyl)-5-hydroxy-2[5H]-furanone; MNNG: N'-methyl-N'-nitrosoguanidine
MATERIALS AND METHODS

Exposure

Six-month old male *P. promelas* were obtained from Aquatic Biosystems (Fort Collins, CO). Male fish were used because some species of fish have been shown to have different magnitudes of response of CYP1A based on sex (Williams et al. 1986). Fish were maintained in well water at 21±1°C. Class A EQ biosolids were chosen for this exposure because, though Class B are the most widely used, EQ biosolids have no restrictions on where they are applied (USEPA 1999), and thus have the greatest potential to runoff into nearby waterways. EQ biosolids also contain low or no pathogen content, so risks of handling these materials are lower. *P. promelas* underwent three exposure regimes to EQ biosolids: high dose (2.5 g l⁻¹), low dose (0.5 g l⁻¹), and a control dose (clean water). A biosolids distributing company donated the biosolids. Their origin is Quincy, MA, and they were stabilized by anaerobic digestion and pelletized. Three treatments were prepared in duplicate for a total of six exposure tanks (Figure 2). Twenty-four males were initially placed in each tank with four fish sampled from each tank at each time point. Sampling for CYP1A induction took place at six time points during a 28-day exposure on days 0, 3, 7, 14, 21, and 28. The Comet Assay was used for detection of DNA damage, and was performed on days 0, 3, 7, 14, and 28.

Biosolids-exposure was performed by placing biosolids in 250μm mesh bags to retain the bulk of the particulate matter and to reduce turbidity. This was a static-renewal exposure: water changes (95%) took place once a week on the day after sampling and fresh biosolids were placed in each tank. pH (AZOO water quality test kit), dissolved oxygen (AZOO water quality test kit), and ammonia (NH₃⁺₄ TetraTest kit) were
Figure 2. Diagram of biosolids exposure tank set-up. Twenty-four male *P. promelas* were placed into each tank. Four fish were sampled from each tank at each time point. Duplicate tanks were pooled for a sample size of eight fish per treatment per time point. Doses were: 0 gl$^{-1}$ (control), 0.5 gl$^{-1}$ (low dose), and 2.5 gl$^{-1}$ (high dose). Sampling for CYP1A took place on days 0, 3, 7, 14, 21, and 28. Samples for the Comet Assay were taken on all days sampled for CYP1A, excluding day 21.
Clean Water
Low: 0.5 g l\(^{-1}\)
High: 2.5 g l\(^{-1}\)

Clean Water
Low: 0.5 g l\(^{-1}\)
High: 2.5 g l\(^{-1}\)

28-day exposure:
- Start: 24 males/ tank
- 4 males/ tank/ time point ➔ pool
duplicate tanks ➔ n = 8
- 6 time points for CYP1A
  - (days 0, 3, 7, 14, 21, 28)
- 5 time points for Comet (no day 21)
measured throughout the exposure to monitor tank conditions. Ammonia was measured by subtracting out ammonia from the total ammonia plus ammonium using a conversion based on temperature and pH. To reduce stress, fish were kept in shaded tanks with structures for refuge. Outside interaction was limited to daily feeding, water changes, and dosing.

Chemical Analyses

Biosolids used in the exposure were analyzed to determine several organic contaminant classes of interest (e.g. PAHs, NPs, PBDEs, etc.). Whole biosolids and water from exposure tanks were measured. Unexposed well water was also analyzed to determine the presence of pre-existing contaminants.

Water analyses

The water analyses were performed using EPA Method 625 for Base/ Neutrals and Acids. To obtain representative water for extraction, 2.5 g l\(^{-1}\) of biosolids were placed in a 250\(\mu\)m mesh bags in a tank containing well water. The biosolids were allowed to equilibrate for 24 h before removal of 3 l of water for sampling. No surrogate standard was added. A one liter distilled water blank was run in conjunction with the extractions to assay potential introduction of laboratory contaminants. Samples were processed in triplicate using one liter of exposed water each. The pH of each replicate was adjusted prior to sequential extraction in separatory funnels with three aliquots of dichloromethane (DCM) totaling 200 ml. For the base/ neutral fraction, the pH was increased to > 11 using 6N NaOH. Following DCM extraction, the water was adjusted to pH < 2 using 3N HCl (acid extract) and re-extracted with a total 200 ml of DCM. Emulsions were
collected after the third aliquot of DCM in the acid portion. They were frozen to separate phases and the DCM layer was collected and combined with the previously obtained acid extract. Each extract was concentrated to 500μl under a high purity nitrogen gas stream, solvent exchanged to toluene, and reduced to a volume 200μl. Samples were then spiked with perinaphthenone as the internal standard, and analyzed by gas chromatography/mass spectrometry (GC/MS).

**Whole biosolids analysis**

The extraction procedure for the analysis for whole biosolids was based on La Guardia et al. (2004). The material was not freeze-dried prior to analysis, as it contained minimal water content (2.88%). BDE-166, PCB-30, -65, and -204, perinaphthenone, acenaphthene-d10, chrysene-d12, 1,4-dichlorobenzene-d4, naphthalene-d8, perylene-d12, phenanthrene-d10, and 1,1-binaphthyl were added as surrogate standards. Two grams of biosolids sample were added to 33g NaSO₄. A NaSO₄ blank was run in conjunction with the sample. Solvent extraction using a Dionex ASE 200 was used to remove relatively nonpolar chemicals. Approximately 60ml of the solvent DCM was used. The conditions were: 2 extraction cycles, vessel pressure 1000 psi, temperature at 100°C, heat for 5 minutes, static for 5 minutes. A 60% vessel flush took place, and there was a 180 second nitrogen purge of vessel contents. Sample solvent volumes were reduced to 8 ml under nitrogen gas prior to cleanup on a size exclusion chromatograph (Waters 717+ Autosampler; Envirosep-ABC®, 350 x 21.1 mm column, Phenomenex). The column was eluted with DCM at 5ml/ min. The first 50 ml contained high molecular weight lipids and were discarded. The next 60 ml contained the xenobiotic compounds of major interest. This fraction was collected, solvent exchanged to hexane, reduced to 500μl.
under nitrogen gas, and put on a 2g silica column (Burdick and Jackson) for affinity chromatography. Elution took place as follows: 3.5ml 100% hexane, 6.5ml 60:40 hexane:DCM, 5ml 25:75 acetone:DCM, and 10ml 100% acetone. The first eluent contained aliphatic compounds and was discarded. The second fraction contained moderately nonpolar aromatic compounds (e.g., PBDEs, PCBs, PAHs) and the third contained more polar compounds such as NPs. The fourth fraction contained more polar compounds. The second (S2), third (S3), and fourth (S4) fractions were reduced under a stream of nitrogen gas and examined by GC/MS. An internal standard containing decachlorodiphenyl ether, pentachlorobenzene, and p-terphenyl was added prior to GC/MS.

**Gas Chromatography/ Mass Spectrometry**

Gas chromatography (GC) was used to separate compounds in the extracts. Detection was by mass spectrometry (MS) (Varian Saturn 4D GC/MS). For acquisition segment 1 on the MS, the mass range was 100-500 m/z+ at a rate of 0.670 s/scan for 49.50min. For segment 2, the mass range was 100-650 m/z+ at a rate of 0.770 s/scan for 40.50min. The column used was a 60m DB-5 with a 0.25μm film thickness and 0.32mm inner diameter (J&W Scientific). The carrier gas was helium. The GC temperature program used was: initial column setting 75°C, hold 1 min, ramp at 4°C/min, hold at 350°C for 20.25 min. Total run time was 90 min, injector 320°C, transfer line 315°C, MS manifold 280°C. Compounds of interest were quantified using a five-point linear calibration curve using the internal standard and selected ions for each targeted compound.
Biomarkers

Tissue Sampling

Fish were euthanized by an overdose of tricaine methane-sulfonate (MS-222). Livers were removed from euthanized fish on ice. Approximately two thirds of each liver was used for measuring CYP1A and the other portion was used in the Comet Assay. Liver samples for CYP1A analysis were placed in cryovials in liquid nitrogen until use; liver samples for the Comet Assay were used immediately.

CYP1A

Hepatic CYP1A was measured by Western blot using the monoclonal antibody Mab 1-12-3 (a gift from John Stegeman, Woods Hole Oceanographic Institution) as described previously (Van Veld et al. 1997), with some modifications. Microsomal fractions were prepared and frozen in liquid nitrogen for later processing. These fractions were collected by homogenizing livers in 1 ml of stabilization buffer (100mM KP containing 20% glycerol; 1mM dithiothreitol; 1mM EDTA; pH 7.4) and 10 μl of 0.1 mM phenylmethyl sulfonyl fluoride. The homogenate was centrifuged at 12,000 xg for 11 min. Supernatants were transferred to clean tubes and centrifuged at 100,000 xg for 63 min. Microsomes were scraped off the glycogen pellet and added to 50 μl of stabilization buffer. Total protein concentrations were determined by the Bradford total protein assay using BSA to generate a standard curve (Bradford 1976). Proteins (20μg) were separated by electrophoresis through reducing, denaturing SDS-PAGE on 12% polyacrylamide gels and transferred to pure-cast nitrocellulose. Blots were blocked with 5% casein in tris-buffered saline (TBS) and incubated in the primary monoclonal antibody Mab 1-12-3, followed by incubation in IR-linked goat anti-mouse CY5 heavy and light chain specific
secondary antibody (Jackson ImmunoResearch Laboratories, Inc, PA). Before and after each antibody incubation, blots were washed three times for 10 min each in 0.1% Tween-20/ TBS. Images were collected and analyzed using a LiCor Odyssey (LiCor Biosciences). CYP1A concentrations were normalized to microsomal protein concentrations and quantitated against pre-calibrated spot (*Leiostomus xanthurus*) microsomal standards as described previously (Van Veld et al. 1997).

**DNA Damage**

**Preparation of single cell suspensions**

The Comet Assay was performed as a modified version of Mitchelmore and Chipman (1998a). All steps were performed under yellow light to minimize potential damage from UV. Hepatocytes were used to analyze DNA damage, as the liver is the major site of biotransformation and processing of contaminants. To obtain hepatocytes, the liver was placed in 300µl of ice-cold aerated Hepes-buffered HBSS (1 mM Hepes; Ca²⁺ and Mg²⁺ free; pH 7.6) on ice, minced with dissecting scissors, and filtered through a 70µm filter.

Cell viability was verified prior to further analysis on an improved Neubauer haemocytometer using trypan blue exclusion. Liver cell samples from individual fish with viability greater than 85% were used in the assay.

**Comet Assay**

Microscope slides were coated with 1% normal melting point agarose (NMA) in PBS and dried at 37°C (Figure 3). Slides were kept on ice for all steps performed. Ten microliters of the cell suspension were added to 100 µl of 0.6% low melting point agarose (LMPA) in aerated Hepes-buffered HBSS (1 mM Hepes; Ca²⁺ and Mg²⁺ free; pH
Figure 3. Diagram of the Comet Assay procedure. Liver cells are minced and strained to produce a single cell suspension. Cells and low melting point agarose are pipetted onto a slide pre-coated with normal melting point agarose and covered with a layer of low melting point agarose. Slides are put into a lysing solution for up to 24 hours, the DNA is unwound and slides are electrophoresed before neutralization. Slides are then dried in ice-cold methanol until analysis. Immediately prior to analysis, slides are rehydrated in HBSS containing Hepes and ethidium bromide. Slides can also be immediately stained and read after neutralization instead of being dried down.
Remove liver; mince and strain cells

Cells + LMPA onto NMPA-coated slide; cover with LMPA

Lysing solution

Alkaline unwinding solution

Electrophoresis

Neutralization

Stain with Ethidium Bromide

Dry in ice-cold methanol

Rehydrate with ethidium bromide

Read with epifluorescence

Score 50 cells per slide
7.6) at 37°C and layered over the NMA on the microscope slide. After polymerization of the agarose, 100 µl of 0.6% LMPA at 37°C was layered over the cell suspensions. The slides were allowed to solidify before placement in lysing solution (10% DMSO, 1% Triton X-100, 2.5M NaCl, 100 mM Tris, 1% N-laurylsarcosine; pH 10.0) for at least 1 h in the dark at 4°C. The lysed slides were drained, rinsed with milli-Q water and placed into an alkaline buffer (200 mM NaOH, 100 mM EDTA; pH > 12) in a horizontal electrophoresis chamber for 15 min to unwind DNA. Electrophoresis took place at a constant of 25 V, 300mA, for 15 min at which time the slides were removed from the chamber and rinsed with neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min. This step was repeated two times for a total of 15 min of washes. The slides were then drained. Slides were placed in ice-cold methanol for 5 min, allowed to dry in the dark at room temperature (Woods et al. 1999), and placed in a tightly closed container with desiccant.

CYP1A and DNA Damage

Four fish were taken from each tank at each sampling time. Data were tested for significant differences between tanks using the standard deviations; tanks were considered replicates if mean values were within one standard deviation of each other. If no significant difference was found, tanks were pooled for a sample size of eight fish at each time point for each treatment. If data met assumptions for a parametric test, one-way analyses of variance (ANOVAs) were used to determine variation between doses at each time point and doses over time. If data did not meet assumptions of a parametric test, nonparametric tests were used. To test for significant differences over time, a Kruskal-Wallis test was used at an alpha value of <0.05. To test for significant
differences at each time point, a Mann Whitney test was used with an alpha level of \( p < 0.017 \).

Values used in the analysis of CYP1A induction were those extrapolated from densitometric measurement of Western blots using the LiCor Odyssey (LiCor Biosciences).

In the Comet Assay, data were analyzed from digital images using epifluorescence microscopy. Measurements taken were tail % DNA, tail moment, and tail length. Tail % DNA is measured as the amount of DNA found in the tail region of the comet (Figure 4). Tail moment is a product of tail % DNA and tail length. If a tail existed, the tail length was measured in \( \mu m \). Fifty cells per sample were scored.
Figure 4. Diagram of cell appearance in the Comet Assay. The ‘head region’ contains the nucleoid core and the ‘tail region’ contains the negatively charged DNA fragments that moved during electrophoresis. A greater length and fluorescence of the tail region indicates an increased amount of damage in a cell.
RESULTS

Analytical Chemistry

Exposure Water Analysis

No targeted chemical compounds were detected in the well water. Extractions from the base/neutral fraction of exposed, unfiltered water revealed the presence of nonylphenols. The estimated concentration was 2.96 μg/L. As no surrogate standard was used this concentration is likely a minimum value. This number is considered reasonably accurate. A nonylphenol-spiked sample was analyzed and the amount detected was similar to that the water was spiked with: the water was spiked to 10 μg/L and the average for three replicate extractions was 11 μg/L. The acid portion did not contain any detectable levels of targeted contaminants.

Biosolids Chemistry

In the S2 fraction, alkylated and nonalkylated PAHs were present at 43.0 μg/g. BDEs were also in the S2 fraction, totaling 702 ng/g. The S3 and S4 fractions contained mainly nonylphenols, at 1.47 mg/g (Table 4). Mono and diethoxylates of nonylphenol, intermediate degradates of nonylphenol polyethoxylate detergents, totaled 51.2 μg/g. The antibacterial agent Triclosan and the musk compound tonalide were also detected.

Molecular Endpoints

CYP1A

Hepatic CYP1A was measured in fish exposed to EQ biosolids for 28 days (Figure 5). CYP1A was induced in low and high dose fish after day 7 and after day 3 in
Table 3: S2 fraction of biosolids extraction

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>112</td>
</tr>
<tr>
<td>2-methyl naphthalene</td>
<td>267</td>
</tr>
<tr>
<td>1-methyl naphthalene</td>
<td>286</td>
</tr>
<tr>
<td>biphenyl</td>
<td>262</td>
</tr>
<tr>
<td>diphenyl ether</td>
<td>38.4</td>
</tr>
<tr>
<td>2,6 and 2, 7 dimethyl naphtalene</td>
<td>520</td>
</tr>
<tr>
<td>1,3 dimethyl naphtalene</td>
<td>599</td>
</tr>
<tr>
<td>1,6 dimethyl naphtalene</td>
<td>463</td>
</tr>
<tr>
<td>1,4 and 2,3 dimethyl naphtalene</td>
<td>275</td>
</tr>
<tr>
<td>1,5 dimethyl naphtalene</td>
<td>156</td>
</tr>
<tr>
<td>acenaphthylene</td>
<td>26.9</td>
</tr>
<tr>
<td>1,2 dimethyl naphtalene</td>
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</tr>
<tr>
<td>1,8 dimethyl naphtalene</td>
<td>554</td>
</tr>
<tr>
<td>acenaphthene</td>
<td>151</td>
</tr>
<tr>
<td>dibenzofuran</td>
<td>176</td>
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<tr>
<td>2,3,5-trimethyl naphtalene</td>
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<td>fluorene</td>
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<tr>
<td>dibenzothiophene</td>
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<td>phenanthrene</td>
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<td>anthracene</td>
<td>662</td>
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<tr>
<td>2-methylphenanthrene</td>
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<tr>
<td>1-methylphenanthrene</td>
<td>2,530</td>
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<tr>
<td>perylene</td>
<td>549</td>
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<tr>
<td>indenone (1,2,3-cd) pyrene</td>
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<td>BDE 99</td>
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<tr>
<td>BDE 154</td>
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<tr>
<td>BDE 153</td>
<td>21.6</td>
</tr>
<tr>
<td>Compound</td>
<td>Total (ng/g)</td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>nonylphenols</td>
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</tr>
<tr>
<td>nonylphenol 1 ethoxylate</td>
<td>48,100</td>
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<tr>
<td>nonylphenol 2 ethoxylate</td>
<td>3,040</td>
</tr>
<tr>
<td>triclosan</td>
<td>4,450</td>
</tr>
<tr>
<td>tonalide</td>
<td>1,890</td>
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</table>
Figure 5. Western Blot of the concentration of CYP1A in *P. promelas* from Day 21 of the exposure to EQ biosolids. Lanes 1 and 2 are from the control dose; lanes 3 - 6 are *Leiostomus xanthurus* microsomal standards ranging from 0.05 - 0.70 pmol CYP1A/mg protein; lanes 7 and 8 are from the low dose; lanes 9 and 10 are from the high dose. Image is from the Li Cor Odyssey IR Imaging System.
the high dose relative to controls (Figure 6). CYP1A levels peaked in the high dose on
day 21 with a 21-fold induction compared to the control. The low dose also reached peak
induction on day 21, with an eight-fold difference between dosed and control fish.

Data for analyzing significance in values of CYP1A induction did not meet the
assumptions of equal variance and a normal distribution for using a parametric statistical
test so data were tested using the Kruskal-Wallis and Mann-Whitney tests. Weights of
fish were tested to determine if they varied significantly over time and would thus have to
be used as a covariate using a 2-way ANOVA on log-transformed data. There was no
significant variation over time (p = 0.072). There was no significant difference over time
in the control dose (Kruskal-Wallis, p = 0.127). Values between the treatments at time
zero were compared and there was no difference (Kruskal-Wallis, p = 0.175), so data
were pooled for a common starting point. Differences between doses were tested
pairwise at each time point using the Mann-Whitney test. To correct for the data not
being independent of each other, the alpha value was adjusted to 0.017. On day 3, there
was no difference between the control and low doses (p = 0.127). There were significant
differences between the control and high doses (p = 0.0014) and low and high doses (p =
0.0015). Days 7 and 14 exhibited the same trend, with no difference between the control
and low doses (p = 0.0428 and p = 0.3253, respectively), and significant differences
between controls and the high dose (p = 0.0015 and p = 0.0024, respectively) and the low
and high doses (p = 0.0022 and p = 0.0034, respectively). On day 21, significant
differences were found when pairing all three doses: control versus low, p = 0.0024;
control versus high, p = 0.0024; low versus high, p = 0.0009. The same trend was found
on day 28: control versus low, p = 0.0055; control versus high, p = 0.0051; low versus
Figure 6. Hepatic CYP1A in *P. promelas* exposed to 0, 0.5, or 2.5 g l⁻¹ EQ biosolids for 28 days. Values shown are means +/- standard deviation.
high, $p = 0.0024$. The control and low dose treatments did not differ significantly until day 21, whereas the high dose treatment showed a significant increase by day 3 when compared to the mean control value.

**DNA Damage**

Exposure of fish for 28 days to EQ biosolids resulted in significant DNA damage in low and high dose hepatocytes (Figure 7). Tail % DNA was statistically analyzed for this study (Figure 8). Tail moment and tail length are shown graphically but statistical tests were not used in this analysis (Figures 9 and 10, respectively).

Weights of fish were tested to determine if they varied significantly over time and would thus have to be used as a covariate using a 2-way ANOVA on log-transformed data. There was no significant variation over time ($p = 0.072$). Prior to analysis, data were log-transformed to allow data to meet requirements for parametric analyses, so one-way ANOVAs were used to analyze tail % DNA. There was no significant difference ($p = 0.232$) between the doses on day zero, allowing these points to be pooled for a single value on day zero. On day three, no significant difference was found between doses ($p = 0.057$); however there was a trend for increased strand breakage at the low and high doses compared to the control. No significant difference was found on day seven ($p = 0.127$). On day 14, there were significant differences between the doses ($p = 0.000$). A Tukey’s post-hoc test showed the low and high doses were significantly higher than the control; however, the low and high doses were not significantly different from each other. Day 28 also had significant differences between the doses ($p = 0.011$). A Tukey’s post-hoc test showed the control was significantly lower than the low and high doses, though there was no difference between the low and high doses.
Figure 7. *P. promelas* hepatocytes analyzed by the Comet Assay. (a) Control-dose hepatocytes from *P. promelas* after exposure to EQ biosolids. Note there is an absence of a tail, indicating little damage. (b) *P. promelas* hepatocytes after exposure to EQ biosolids. Note the increase of fluorescence in the tail region, indicating increased damage compared with the control cells.
Figure 8. Percent of DNA that has migrated into the tail region of the comets in hepatocytes of *P. promelas* exposed to 0, 0.5, or 2.5 gl⁻¹ EQ biosolids for 28 days. Values shown are means +/- standard error of the mean.
Figure 9. Tail moment of hepatocytes in *P. promelas* exposed to 0, 0.5, or 2.5 gl⁻¹ EQ biosolids for 28 days. Values shown are means +/- standard error of the mean.
Figure 10. Tail length in micrometers of hepatocytes in *P. promelas* exposed to 0, 0.5, or 2.5 gl⁻¹ EQ biosolids for 28 days. Values shown are means +/- standard error of the mean.
A one-way ANOVA was used to test for variations over time within each treatment. A Tukey’s post-hoc test was used to determine where, if any, significant differences occurred. Again, log-transformed data were used. The control dose had significant variance over time ($p = 0.001$): day 28 was significantly lower than days 0, 3, and 7. The low dose also varied significantly over time, at a $p$-value of 0.002. Days 3 and 14 were significantly higher than day 0, but not significantly different from the other time points. The high dose also varied significantly over time ($p = 0.002$). Days 3 and 14 were significantly elevated compared to days 0, 7, and 28.

**Pearson Correlations**

A Pearson correlation index was used to test for correlations of CYP1A induction and DNA damage at an alpha value of 0.05 (Table 5). A significant correlation was found between CYP1A induction and DNA damage. CYP1A induction was also significantly correlated with time ($p = 0.000$) and treatment. No significant correlation was found when comparing tail % DNA over time; however there was a significant correlation of tail % DNA and treatment. All significant correlations found were positively correlated, indicating variables tended to increase together.

**Tank Conditions**

Tanks were maintained at $21\pm1$ °C for the duration of the exposure. pH, dissolved oxygen, and ammonia were measured after water changes and dosing to monitor changing conditions. Between water changes, the pH in the control tank remained at 7.5, dissolved oxygen ranged from 10 to 9 mg/l, and ammonia was non-
Table 5: Correlation of [CYP1A] (pmol/mg protein), Tail % DNA, Time Point, and Treatment

<table>
<thead>
<tr>
<th></th>
<th>[CYP1A]</th>
<th>Tail % DNA</th>
<th>Time Point</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tail % DNA</td>
<td>0.192</td>
<td>0.038</td>
<td></td>
</tr>
<tr>
<td>Time Point</td>
<td>0.384</td>
<td>0.024</td>
<td>0.011</td>
</tr>
<tr>
<td>Treatment</td>
<td>0.579</td>
<td>0.278</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>0.000</td>
<td>0.002</td>
<td>0.907</td>
</tr>
</tbody>
</table>

Cell Contents: Pearson Correlation
p-value
detectable for the duration. The pH in the low dose tank ranged from 7.5 to 7.0,
dissolved oxygen remained at 9 mg/l, and ammonia ranged from 0.0186 to 0.0009 mg/l.
Conditions in the high dose tanks were: pH 7.5 – 6.5; dissolved oxygen 8.5 – 6 mg/l;
ammonia 0.0495 – 0.006 mg/l. Water clarity decreased over time between exposures as
more biosolids particles were released from the mesh bags.
DISCUSSION

The present study indicates there are contaminants in water-accommodated EQ biosolids capable of inducing CYP1A and DNA damage relative to control fish. The complex chemical matrix contained in biosolids complicates the task of elucidating which compounds are responsible for the observed effects. However, based on the compounds detected in the biosolids, it is possible to speculate regarding the cause of the responses of the biomarkers in this study.

The major class of compounds responsible for the induction of CYP1A is most likely the PAHs. Not all PAHs induce CYP1A. The most potent are the higher molecular weight forms (Chaloupka et al. 1995). Many of these were present in the biosolids used in this exposure, including: acenanthylene, acenaphthene, dibenzofuran, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz (a) anthracene, chrysene, benzo (b) fluoranthene, benzo (k) fluoranthene, and benzo (a) pyrene (Chaloupka et al. 1995; Goksoyr and Forlin 1992). The model CYP1A-inducing compound is benzo (a) pyrene (BaP), and it is used in many induction studies (Chaloupka et al. 1995; Van Veld et al. 1997; Stegeman et al. 1981). The fact that these compounds were not detected in the water leads to the possibility that the majority of these compounds were taken up directly by the fish through interaction with biosolids particles via the gills or orally. A large amount of particles of biosolids were in the water, indicating a strong possibility of direct uptake of these particles for an exposure route. Future studies should consider extracting larger volumes of water to improve method quantitation limits.
In the present study mesh bags were used to expose *P. promelas* to biosolids. As a result, they received sediment- and aqueous-borne doses. Fish in receiving waters of wastewater treatment plants also receive exposure by both routes as effluents contain modest levels of suspended solids. BaP has been used as a model contaminant to determine how an organism takes up PAHs. Van Veld et al. (1997) were able to induce CYP1A in fish after dietary and aqueous exposures. They demonstrated that the primary sites of CYP1A induction from an aqueous exposure were the gill pillar cells, heart endothelium, and vascular elements in all tissues. The primary site of CYP1A induction from the dietary exposure was the gut. In each type of exposure, all fish in that study had CYP1A induction in hepatocytes by 456 h. Though determining the route(s) of uptake by which the fish in the present study took up contaminants was not part of this study, it is thought the induction is from both dietary (i.e., sorption of compounds to food) and aqueous exposure via desorption of contaminants from the biosolids. The study organisms also may have taken up contaminants from direct contact of sediment with the gills.

PBDEs have been postulated to induce CYP1A due to their similarities to dioxin-like compounds. Certain congeners (BDE-66, -85, -153, -183) induce CYP1A. However, the dominant congeners found in this study (BDE-47 and BDE-99) can act as inhibitors to other Ah-receptor agonists (Chen and Bunce 2003). Other studies confirm the results that PBDEs contained in the biosolids used in the present study do not induce CYP1A (Timme-Laragy 2006; Tomy et al. 2004; Boon et al. 2002; Darnerud et al. 2001). Tomy et al. (2004) suggest PBDEs may be biotransformed in a manner similar to thyroid hormones and thus do not induce CYP1A activity.
As with CYP1A induction, PAHs are most likely responsible for the increase in tail % DNA. DNA damage has been observed in a variety of PAH-contaminated environments (Anderson et al. 1999; Winter et al. 2004; Roy et al. 2003; Brown and Steinert 2003). BaP is a specific PAH known to induce DNA damage (Mitchelmore and Chipman 1998a; Nacci et al. 1996; Mitchelmore et al. 1998a, b; Shugart 1988). The damage to DNA arises through a variety of mechanisms. PAHs are procarcinogens; that is, they require metabolic activation to a reactive state. This commonly results from the biotransformation of PAHs by CYP1A to harmful metabolites that subsequently bind to DNA and form adducts (Mitchelmore et al. 1998a; Buhler and Williams 1988). These metabolites can also form reactive oxygen species that induce damage (Mitchelmore et al. 1998a; Anderson et al. 1994; Buhler and Williams 1988). PBDEs are another class of compounds detected in these biosolids that have contributed to DNA damage. BDE-47, an abundant constituent of the commercial Penta-BDE mixture, was reported to produce a significantly increased number of erythrocytes in *Scophthalmus maximus* with micronuclei after aqueous exposure (Barioene et al. 2005). Increased tail % DNA could also have arisen from exposure to the endocrine disrupting compounds contained in these biosolids, such as nonylphenols. Hagger et al. (2006) demonstrated the possibility that endocrine disruptors may contribute to DNA damage, as they found a correlation between TBT-induced imposex and DNA damage in *Nucella lapillus*. TBT has been detected in other biosolids (Hale, personal communication). As its detection requires a separate sample workup, its analysis was not pursued in the present study.

There was a significant correlation between CYP1A levels and tail % DNA. This result may suggest that damage to DNA is occurring from products of CYP1A induction.
One method for testing this hypothesis is to perform experiments similar to those done by Mitchelmore et al. (1998a) in which different inhibitors were utilized to determine whether damage occurred from the biotransformation of BaP and nitroaromatics or directly by these chemicals. BaP was found to induce DNA damage via its biotransformation. Also, each of the biomarkers chosen demonstrated a marked increase in effect at the later time points.

In this study, CYP1A demonstrated a dose-related response. The trends in each dose mirrored each other; the amount of CYP1A present did not. Goksoyr et al. (1988) noted a dose-dependent response of CYP1A from exposure to water-soluble compounds from crude oil in the North Sea. An exposure of differing doses of PAHs also resulted in a dose-dependent induction of CYP1A in mice (Chaloupka et al. 1995). Dose dependency was noted by Sanderson et al. (1996) when studying CYP1A induction in the rat hepatoma cell line H4IIE. In scup (Stenotomus chrysops), Schlezinger and Stegeman (2001) demonstrated a dose-dependent induction of CYP1A by 3,3',4,4',5-pentachlorobiphenyl.

There was no significant difference in DNA damage between the low and high doses over time. However, there was a trend for an increased effect in the low dose treatment compared to the high dose. These results imply there was not a dose-related effect of biosolids on the amount of DNA damage. Typically, there is a dose-dependent response of DNA damage to an exposure from compounds requiring biotransformation up to a certain concentration (Mitchelmore and Chipman 1998a; Mitchelmore et al. 1998b). However, once a chemical reaches saturation kinetics, the DNA damage from it may decrease (Mitchelmore et al. 1998b). The results of this study may indicate the
levels of the compounds in the EQ biosolids-accommodate water may have been high
eough to reach saturation. A relevant future study is to use a serial dilution of the
concentration of biosolids to determine the concentration at which a maximal response is
induced.

Tail % DNA was the only parameter to undergo statistical analysis because the
other parameters (especially tail moment) though very informative, are more susceptible
to differences between gels than tail % DNA and do not always give a linear dose
response curve (Mitchelmore and Chipman 1998a; McKelvey-Martin et al. 1993).
Anderson et al. (1994) reported that tail % DNA could be a more accurate way to report
damage to cells. Tail length has been reported to be a less informative measure of DNA
damage than other parameters (Collins 1992). In this study, tail % DNA and tail moment
showed similar trends, while tail length differed from these measurements.

Of particular note is the statistically significant decline over time of tail % DNA
in the control doses. One possible explanation is that depuration of the fish is still
occurring. They were allowed to depurate after arrival for approximately three weeks
before the exposure commenced. The fish were purchased through a breeder known to
supply fish for research; however, they may have been exposed to a chemical that
induces DNA damage and were still depurating at the start of the exposure. Another
possibility is stress to the fish lessened as the study progressed. For example, as fish
were sub-sampled, this allowed more space per fish in each tank. However, though there
was a significant variation over time, error bars remained modest throughout the
exposure, indicating little individual variation.
Previous studies have mainly focused on wastewater treatment plant effluent outfalls rather than on water-accommodated biosolids. Most were aimed at determining endocrine disruption as a result of exposure (Jobling et al. 1995; Sumpter and Jobling 1995). However, some incorporated alternative endpoints. In Orange County, CA, a study by Roy et al. (2003) showed a significant induction of CYP1A, but only trends indicating slightly elevated DNA damage and vitellogenin. Livingstone et al. (2000) studied CYP1A and vitellogenin simultaneously in the Thames River.

Endocrine disruption may be a valuable endpoint in assessing effects of biosolids. Nonylphenols were at high concentrations in the biosolids used in this study, and were dominant contaminants in the exposure water. Detection of a substantial concentration of nonylphenol in the EQ biosolids was expected, as the biosolids were stabilized by anaerobic digestion. Nonylphenols are degradation products of alkylphenol ethoxylates and are produced under anaerobic conditions (Hale and La Guardia 2002; La Guardia et al. 2001). We performed an assay for detection of vitellogenin (Van Veld et al. 2005), but the results were inconclusive, as the controls showed low amounts of induction (see Appendix A). The exposed fish showed a variable response; some fish had mild induction of vitellogenin, while others had no detectable levels. The variable response may be due to anti-estrogenicity of compounds that act as Ah receptor agonists, such as the PAHs found in these biosolids (Smeets et al. 1999; Navas and Segner 2000). A future study focusing on endocrine disruption would determine if it occurs, and if so, pinpoint the effects of endocrine disruptors contained in these biosolids.
CONCLUSIONS

Chemicals associated with EQ biosolids have the capability to enter aquatic organisms and raise CYP1A levels and induce DNA damage. Thus, the unrestricted application of EQ biosolids to land has the potential to impact both aquatic and terrestrial ecosystems.

There are currently no regulations on the distribution of EQ biosolids. The assumption is that their composition is benign enough that there would be no adverse effect on either terrestrial or aquatic organisms and can thus be spread anywhere by anyone. At present there are no restrictions on organic contaminants in any biosolids in the U.S., including the less well-treated and more widely-applied Class B biosolids. The additional treatment steps employed to render EQ and Class A biosolids are primarily intended to reduce pathogen content. The sale of EQ biosolids is also not currently regulated, so no information exists on the amount purchased or applied. In addition, there is no restriction on the time between application and use of the land.

These data show the potential detrimental effects onto an aquatic organisms and the need for further evaluation of possible ecosystem impacts. The evaluation of more effective biosolids treatment techniques to reduce concentrations of organic contaminants therein may also be prudent.
FUTURE RESEARCH

This was the first study of the molecular effects of EQ biosolids on aquatic organisms. There are many other studies that could be done to further assess the impact of biosolids on aquatic organisms. Endocrine disruptors such as nonylphenol are typically associated with biosolids (La Guardia et al. 2001, 2004; Hale and La Guardia 2002). Further studies evaluating the induction of vitellogenin or thyroid level alterations would show if there is cause for concern about reproductive effects on organisms.

*Pimephales promelas* (Ankley et al. 2001, 2003; Nichols et al. 1999) and *Gambusia holbrooki* (Ogino et al. 2004; Toft et al. 2004; Batty and Lim 1999) have been used test organisms in testing morphological alterations from exposure to contaminants. Male *P. promelas* have fatty patches on their faces called tubercles that in the presence of estrogenic compounds become reduced in size and/or number. Female *G. holbrooki* have a splayed caudal fin while males have a rod-shaped caudal fin. In the presence of androgenic compounds, the female caudal fin will morph to become similar to that of males.

Though field experiments are vital to determining what actually occurs in the environment, further laboratory studies are also required, as techniques for studying the exposure and effect of biosolids need improvement. A longer exposure would show how both CYP1A levels and DNA damage act over time. Determining Phase II biotransformation products such as glutathione-s-transferase is one avenue to determine other long-term effects of these chemicals.

Characterization of contaminants in the tissues of fish would further our knowledge of the bioavailability of unmetabolized biosolids constituents. Fish may not
take up all of the compounds they are exposed to, and though present both in water and biosolids, the effects of these chemicals may be diminished as a result.
REFERENCES


APPENDIX

Introduction

Vitellogenin is a phosphoprotein precursor to egg yolk that is typically only expressed in females; however, the gene controlling this expression is contained in both male and female organisms (Copeland et al. 1986; Denslow et al. 1999). Males show induction when exposed to estrogens and compounds that behave like estrogens (Sumpter and Jobling 1995; Sumpter 1995). When an estrogen or estrogen mimic enters an organism, vitellogenin is induced in the liver. Though the vitellogenin gene exists in all cells, only the vitellogenin gene in liver cells of organisms becomes upregulated (Larkin et al. 2003). In females, vitellogenin travels through the bloodstream to be sequestered in oocytes (Copeland et al. 1986; Denslow et al. 1999), where it is cleaved into the yolk proteins, lipovitellin and phosvitin (Copeland et al. 1986). In males, vitellogenin circulates in the blood until it is degraded or until the kidneys clear it (Denslow et al. 1999). The ER-mediated pathway synthesizes vitellogenin, and its elevated levels result from both a direct and a delayed synthesis (Bowman et al. 2002), showing that this induction lasts for a long time in an organism. The exposure of an organism to estrogenic compounds also increases the amount of estrogen receptors (Larkin et al. 2003; Bowman et al. 2002), which can increase vitellogenin expression during the second wave of transcription and translation (Bowman et al. 2002).

Vitellogenin is a widely used and sensitive biomarker of estrogenic compounds in different species of organisms (Guillette et al. 1994; Jobling et al. 1995; Sumpter and Jobling 1995; Denslow et al. 1999; Cheek et al. 2001). There have been many assays
developed to measure vitellogenin induction including enzyme-linked immunosorbent assays (ELISA) (Denslow et al. 1999; Parks et al. 1999; Tyler et al. 1999), radioimmunoassays (Copeland et al. 1986), western blots (Heppell et al. 1995; Parks et al. 1999), and the utilization of the Pro-Q Diamond Phosphoprotein stain (Van Veld et al. 2005). \textit{P. promelas} have been the species of choice in many studies for the detection of vitellogenin induction (Parks et al. 1999; Tyler et al. 1999; Korte et al. 2000; Zerulla et al. 2002; Mylchreest et al. 2003; Leino et al. 2005; Van Veld et al. 2005).

Biosolids contain a number of known endocrine disrupting compounds, including nonylphenols. Many studies have shown nonylphenol to be a reliable inducer of vitellogenin (Matozzo and Marin 2005; Smith and Hill 2004; Christiansen et al. 1998). There have also been studies regarding vitellogenin induction in fish from sewage treatment plant effluent (Sumpter and Jobling 1995; Harries et al. 1999; Porter and Janz 2003), leading us to believe there may be a similar induction from exposure an aqueous to biosolids.

\textit{Materials and Methods}

\textit{Biosolids Exposure}

Male \textit{P. promelas} were obtained from Aquatic Biosystems (CO) and were approximately six months old at the start of the exposure. Fish were maintained in well water at 20 – 22°C. Class A EQ biosolids were chosen for this exposure because, though Class B are the most widely used, EQ biosolids have no restrictions on where they are applied (USEPA 1999), and thus have the greatest potential to runoff into nearby waterways. \textit{P. promelas} underwent three exposure regimes to EQ biosolids: high dose
(2.5 g l⁻¹), low dose (0.5 g l⁻¹), and a control dose (clean water). A biosolids distributing company donated the biosolids. Their origin is Quincy, MA and they were stabilized by anaerobic digestion. Three treatments were prepared in duplicate for a total of six exposure tanks (Figure 2). Twenty-four males were initially placed in each tank with four fish sampled from each tank at each time point. Sampling for CYP1A induction took place at six time points during a 28-day exposure on days 0, 3, 7, 14, 21, and 28. The Comet Assay was used for detection of DNA damage, and was performed on days 0, 3, 7, 14, and 28.

Biosolids were placed in 250μm mesh bags to retain the bulk of the particulate matter and to reduce turbidity. This was a static-renewal exposure: 95% water changes took place once a week on the day after sampling. pH (AZOO water quality test kit), dissolved oxygen (AZOO water quality test kit), and ammonia (NH₃,₄ TetraTest kit) were measured throughout the exposure to monitor tank conditions. Ammonia was measured by subtracting out ammonia from the total ammonia plus ammonium using a conversion based on temperature and pH. To reduce stress, fish were kept in shaded tanks with structures for refuge. Outside interaction was limited to daily feeding, water changes, and dosing.

Nonylphenol Exposure

Experimental Design

The positive control for vitellogenin was a separate tank exposure of *P. promelas* to nonylphenol and ran for 14 days in a separate study. The positive control served to demonstrate the inducibility of vitellogenin in male fish and thus did need to be run for the duration of each experiment. Two treatments were tested: 10 μg/L nonylphenol and
an acetone control. Treatments were run in duplicate and four fish were placed in each tank. Acetone was used as the carrier solvent (Kinnberg et al. 2000). Ninety-five percent water changes took place every other day. To reduce stress, fish were kept in shaded tanks. Outside interaction was limited to daily feeding, water changes, and dosing.

**Chemistry**

The water analyses were performed by liquid-liquid extract. Sampling occurred twice during the exposure: 1 h after a fresh dose was added and 24 h after dosing the tanks. Two liters were collected for analysis. Samples were run in duplicate using one liter of exposed water each. The stock solution was also analyzed by adding 10 μg/L each to three replicates of 1-L DI water and analyzed. The pH of each replicate was adjusted to < 2 using 3N HCl prior to sequential extraction in separatory funnels with three aliquots of dichloromethane (DCM) totaling 200 ml. If emulsions existed, they were collected after the third aliquot of DCM. They were frozen to separate phases and the DCM layer was collected and combined with the previously obtained acid extract. Each replicate was concentrated to 500μl under a high purity nitrogen gas stream, solvent exchanged to toluene, and reduced to a volume 200μl. Samples were then spiked with p-terphenyl as the internal standard, and analyzed by gas chromatography/ mass spectrometry (GC/MS).

Gas chromatography (GC) was used to separate compounds in the extracts. Detection was by mass spectrometry (MS) (Varian Saturn 4D GC/MS). For acquisition segment 1 on the MS, the mass range was 100-500 m/z⁺ at a rate of 0.670 s/scan for 49.50min. For segment 2, the mass range was 100-650 m/z⁺ at a rate of 0.770 s/scan for 40.50min. The column used was a 60m DB-5 with a 0.25μm film thickness and 0.32mm inner diameter (J&W Scientific). The carrier gas was helium. The GC temperature
program used was: initial column setting 75°C, hold 1 min, ramp at 4°C/ min, hold at 350°C for 20.25 min. Total run time was 90 min, injector 320°C, transfer line 315°C, MS manifold 280°C. Compounds of interest were quantified using a five-point linear calibration curve using the internal standard and selected ions for each targeted compound.

Vitellogenin Measurement

Vitellogenin measurements from plasma of male fish were performed using a recently developed technique (Van Veld et al. 2005). Blood was collected by nicking the lateral line and collection via heparinized capillary tubes. After separating serum from plasma by centrifugation, a minimum of 5 μl serum was mixed with aprotinin, a protease inhibitor. A total of 8 μg of protein was loaded in each lane of reducing, denaturing 8% SDS-PAGE gels. Total protein concentrations were determined using the Bradford total protein assay using BSA to generate a standard curve (Bradford 1976). After electrophoresis, gels were fixed overnight in a solution of 50% methanol and 10% acetic acid. Gels were washed three times, 10 min each, in ultra pure water and placed in Pro-Q Diamond Phosphoprotein Gel Stain (Invitrogen) for 90 min in the dark under gentle agitation. Two 1-hour destains using the Pro-Q Diamond Destain took place in the dark, after which time gels were visualized using the Alpha-Innotech imaging system.
Results and Discussion

The stock solution used for spiking contained 11.3 µg/L nonylphenol. The measured concentration of nonylphenol in exposure tanks an hour after spiking was 7.46 µg/L. After 24 h, nonylphenol concentrations dropped to 2.40 µg/L in exposure tanks.

Vitellogenin in control fish appeared to be induced at Day 0 of the exposure (Figure 1). Previous investigators have observed that male fathead minnow maintained in close proximity with females exhibited Vtg in plasma (Nancy Denslow (personal communication). Induction presumably occurs from exposure of males to estradiol excreted by females. No apparent difference was observed in exposed fish relative to controls at any time during this study (Figure 2). Apparent induction of Vtg in all fish at time ) confounds interpretation of these observations. However, it appears that fish were not exposed to insufficient levels of estrogenic compounds (e.g. nonylphenols) to induce a measurable increase in exposed fish relative to that of controls.

In the future, male fish need to be separated from female fish prior to testing, with frequent water renewal periods to ensure a lack of estrogen in the water. Another exposure using clean males needs to be performed to determine whether biosolids and nonylphenols induce vitellogenin in P. promelas. Also, other fish species should be tested for their responses to biosolids, as species are known to upregulate vitellogenin in the presence of nonylphenol and estrogens (Kinnberg 2000; Hemmer et al. 2001; Todorov et al. 2002; Smith and Hill 2004).
Figure 1: Day 0 of the biosolids exposure.

Figure 2: Day 28 of the biosolids exposure.
Figure 3: Day 14 of the nonylphenol exposure.
References


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