AN ABSTRACT OF THE DISSERTATION OF

Scott A. Hecht for the degree of Doctor of Philosophy in Toxicology presented on August 9, 2002.

Title: Accumulation and Effects of 4-nonylphenol in Chinook Salmon Fry and Their Estuarine Amphipod Prey.

Abstract Approved: Redacted for privacy

Paul C. Jepson

4-nonylphenol (NP), a surfactant degradation product, is an unregulated, ubiquitous aquatic contaminant and endocrine disruptor, for which aquatic life criteria are currently under development by U.S. EPA. The effects of NP on estuarine amphipods and chinook salmon fry were investigated, and this dissertation reports research into the impacts of NP bioaccumulation on the amphipods and resultant endocrine disruption of their juvenile salmon predators. Sensitivity to, and bioaccumulation of, NP by benthic amphipods were quantified. Factors affecting the bioavailability of NP to three species of amphipod (*Eohaustorius estuarius*, *Grandidierella japonica*, and *Corophium salmonis*) were determined in contaminated sediments. Standard bioassay techniques were modified to determine toxicity and bioaccumulation, with varying amounts and differing nutritional qualities of sedimentary organic carbon. ^{14}C-Ring-labeled NP was used as a tracer in the experiments to quantify amphipod exposures. NP was

acutely toxic to *Eohaustorius estuarius* from aqueous exposures, mean (+/-SD) LC50 = 227 µg/L +/- 56, 1 h mean reburial EC50 = 138 +/- 36. The predicted LC50 for NP (202 µg/L) from an amphipod-derived structure-activity relationship was not significantly different (p>0.05) from our empirically derived LC50 (227 µg/L). All three amphipod species accumulated significant NP body burdens. Accumulation was inversely proportional to the total amount of organic carbon, but it did not differ between types of organic matter. Calculated accumulation factors indicated that amphipods could be an important and previously unrecognized source of NP to higher trophic levels. Plasma vitellogenin (Vtg) was quantified in juvenile chinook salmon following dietary exposure to NP contaminated amphipods and aqueous exposure to multiple NP concentrations. Fry that had fed upon contaminated amphipods did not have significantly greater Vtg levels than controls; however, Vtg was detected in 30 percent of fry. NP aqueous concentrations at 60 and 240 µg/L significantly induced Vtg in fry following 5 d exposures. The 240 µg/L aquatic NP treatment fry had comparable levels of Vtg to the positive control treatment in which fry were injected 17*B*-estradiol. These results indicate that amphipods are potential vectors of sediment NP to higher trophic levels within the water column, including juvenile chinook salmon.

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ACCUMULATION AND EFFECTS OF 4-NONYLPHENOL IN CHINOOK SALMON FRY AND THEIR ESTUARINE AMPHIPOD PREY

by
Scott A. Hecht

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Scott A. Hecht, Author

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CONTRIBUTION OF AUTHORS

The following helped with design, execution and analysis of the research reported here, and these contributors appear as authors in the main experimental chapters, or are acknowledged for their contributions within: Dr. Jepson, Dr. Boese, Dr. Gunnarsson, Janet Lamberson, Dr. Giger, Dr. Schaffner, Liz Gooch, and Katie Alayan.

TABLE OF CONTENTS

Page

CHAPTER 1: INTRODUCTION.. 1

DISSERTATION SUMMARY.. 1

GENERAL BACKGROUND.. 2

LABORATORY FOOD CHAIN DESIGN........................ 13

RESEARCH APPROACH.. 16

INTRODUCTION OF CHAPTERS................................ 18

CHAPER 2: SENSITIVITY OF AN INFAUNAL AMPHIPOD, *EOHAUSTORIUS ESTUARIUS*, TO ACUTE WATERBORNE EXPOSURES OF 4-NONYLPHENOL: EVIDENCE OF A TOXIC HANGOVER................................ 21

ABSTRACT.. 22

INTRODUCTION.. 22

MATERIALS AND METHODS.. 23

RESULTS.. 26

DISCUSSION.. 29

ACKNOWLEDGEMENT.. 33

TABLE OF CONTENTS CONTINUED

PAGE

CHAPTER 3: BIOACCUMULATION OF 4-NONYLPHENOL BY ESTUARINE BENTHIC AMPHIPODS: THE ROLE OF SEDIMENTARY ORGANIC CARBON AND TROPHIC TRANSFER.. 35

ABSTRACT.. 35

INTRODUCTION.. 36

MATERIALS AND METHODS.. 40

RESULTS.. 56

DISCUSSION.. 71

ACKNOWLEDGEMENT.. 78

CHAPTER 4: VITELLOGENIN INDUCTION IN CHINOOK SALMON FRY (*ONCORHYNCHUS TSHAWYTSCHA*) FROM DIETARY AND WATERBORNE EXPOSURES TO 4-NONYLPHENOL............. 80

ABSTRACT.. 80

INTRODUCTION.. 81

MATERIALS AND METHODS.. 84

TABLE OF CONTENTS CONTINUED

PAGE

RESULTS.. 95

DISCUSSION.. 99

ACKNOWLEDGEMENT... 105

CHAPTER 5: CONCLUSIONS.. 106

CHAPTER SUMMARIES... 107

IMPLICATIONS.. 112

FUTURE RESEARCH.. 114

BIBLIOGRAPHY.. 116

LIST OF FIGURES

FIGURE		PAGE
1	Degradation of Nonylphenol polyethoxylates (NPEs)............	6
2	Survivorship (96 h) and burial (1, 24, and 48 h) following	30
3	Sediment NP concentration based on radioactivity (^{14}C)	61
4	^{14}C–NP body burdens in *Eohaustorius estuarius* and.............	65
5	^{14}C-NP accumulation by *Eohaustorius estuarius* from spiked...	70
6	Box and whisker plot of vitellogenin [ng/L] in chinook salmon.	96

LIST OF TABLES

TABLE		PAGE
1	Environmental concentrations of nonylphenol in aquatic ecosystems........	7
2	*Eohaustorius estuarius* mean 96-hr lethal concentrations to 50% (LC50s) and burial effective concentrations to 50% (EC50s)[a]........	28
3	Feeding and burrowing characteristics of test amphipod species........	44
4	Initial allocation of exposure beakers (replicates) in experiment 1 and 2 among amphipod species and sediment treatments. Each replicate beaker contained 20 amphipods (C+ = positive control, LA = labile, RE= refractory, and C- = negative control)........	47
5	Time-series sampling scheme for experiment 1 and experiment 2........	50
6	A. TOC and TN content in organic matter (*Ulva sp.* And lignin). B. TOC and TN content in sediment treatments at start and end of experiment. Mean, (SD), n=3. * Denotes significantly difference from C+ (two-way ANOVA, SNK multiple comparison, p = 0.05. NA= Not available........	59
7	Sediment, overlying water, and pore water NP concentrations from day 0 and day 16 based on ^{14}C measurements (1dpm = 0.183 ng NP). n=3, (SD), na = not available........	63

LIST OF TABLES CONTINUED

TABLE		PAGE
8	Mass balance of ^{14}C partitioning on day 0 and day 16. Mass balance was calculated from six matrices: sediment, pore water, overlying water, sediment surface particulate organic matter (POM), sediment surface dissolved organic matter (DOM), and amphipod tissue assuming no loss of ^{14}C. Values are mean percentage of ^{14}C (dpm) recovered in each compartment n=3, (SD)..	64
9	Day 16 NP accumulation factors in *E. estuarius*, *G. japonica*, and *C. salmonis*..	67
10	Uptake and depuration kinetics of ^{14}C-NP by *Eohaustorius estuarius*..	71
11	Predicted NP dose transfer per day to fish feeding on contaminated amphipods..	78
12	Treatment Type and Description....................................	86
13	Mean NP dose to fry from feeding on contaminated amphipods compared with plasma vitellogenin levels. Fry mean NP dose determined from five day feeding rates on contaminated amphipods..	98
14	Mean fry five-day feeding rates of live amphipods from daily 45 min feeding periods. Feeding rates are expressed as percentage amphipods consumed per day (mg WW) divided by fry (mg WW) * 100....................................	99
15	Nominal and mean daily measured aqueous NP concentrations based on radioactivity taken at 6 hr intervals (n=10)...	100

DEDICATION

To Shasta, may you experience fully the awesome power of nature

ACCUMULATION AND EFFECTS OF 4-NONYLPHENOL IN CHINOOK SALMON FRY AND THEIR ESTUARINE AMPHIPOD PREY

CHAPTER 1:

INTRODUCTION

DISSERTATION SUMMARY

This dissertation addresses the timely issue of identifying mechanisms that may underlie salmonid decline in the Pacific Northwest by investigating the potential for endocrine disruption in chinook salmon fry, exposed to 4-nonylphenol (NP) through ecologically realistic dietary pathways. The objectives of this dissertation were to quantify the toxicity to, bioaccumulation by, and estrogenic effects on, amphipods and chinook salmon fry from NP exposures. Additionally, factors affecting the bioavailability of sediment-associated NP including nutritional quality and amphipod feeding strategy were evaluated. Using standardized methods for toxicity and bioaccumulation assessment, we developed a laboratory-based approach to determine effects of NP exposure in a short estuarine grazer/consumer food chain. Chapters 2, 3, and 4, which are drafted in the form of journal articles, report experiments conducted to determine impacts and accumulation from

exposure to NP in amphipods and chinook salmon fry. Chapter five summarizes the conclusions, addresses uncertainties, and examines the relative risk of NP contamination within estuaries.

GENERAL BACKGROUND

Sediment contamination

Approximately one thousand new chemicals are produced annually in the U.S.A. A portion of these are used in high enough volumes and are not contained, such that they ultimately enter aquatic systems, contaminating water and sediments. Sediments can act as a repository for hydrophobic, log K_{ow} >4.5 (octanol:water partition coefficient) compounds, that are resistant to biotic and abiotic degradation (Landrum et al. 1996). As a result of this hydrophobic property, many anthropogenic, organic pollutants, including chlorinated pesticides, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and phenol-based surfactant degradation products, associate with dissolved and particulate organic matter and tend to accumulate in sediments. Freshwater, marine, and estuarine benthic invertebrates accumulate sediment-associated contaminants from direct contact and feeding (Lake et al. 1990; Meador et al. 1997), thereby acting as

one of the major vectors of toxic pollutants to fish and higher trophic levels, including human consumers.

Estuaries are unique ecosystems that receive extensive anthropogenic contamination from point and non-point sources from upstream and near shore activities. As a result of these activities and the estuarine physical environment, sediments become preferential substrate for hydrophobic organic chemicals (Brunk et al. 1997), that not only impact benthic invertbrates (Varanasi et al. 1985), but also fish, such as juvenile salmon (Stein et al. 1995). However, estuarine ecotoxicological assessment development has largely been ignored and focus has been placed on fresh or marine water habitats (Chapman, 2001).

Bioavailability

Contaminated sediments present a wide range of toxicities to aquatic organisms even at similar concentrations (Burton, 1991). This is because only a fraction of sediment-associated toxins are available to the benthic organisms that live and feed within this medium. Association of contaminants with sediment organic carbon is one of the major factors governing the bioavailability of sediment-associated materials (Landrum et al. 1996). Additionally, recent studies have shown that the bioaccumulation of organic contaminants is not only a function

of organic carbon quantity (Di Toro et al. 1991), but also of its amino acid content (Gunnarsson et al. 1999a; 1999b). The bioavailability of organic contaminants is one of the primary considerations in determining uptake by, and therefore toxicity of, organic pollutants to benthic organisms.

Nonylphenol

Nonionic surfactants are receiving increasing attention because of their high rates of use globally and the complexity of their degradation pathway. Their breakdown products are persistent in the environment, acutely toxic, and estrogenically active in aquatic organisms (Swisher, 1987). Alkylphenol polyethoxylates (APE's), a widely used group of surfactants, are used in detergents, pesticides, domestic cleaning products, and in a variety of industrial processes (e.g. pulp and paper mill bleaching, metal processing, textile processing). U.S. production exceeded 242 million kg in 1990 (U.S. International Trade Commission 1990).

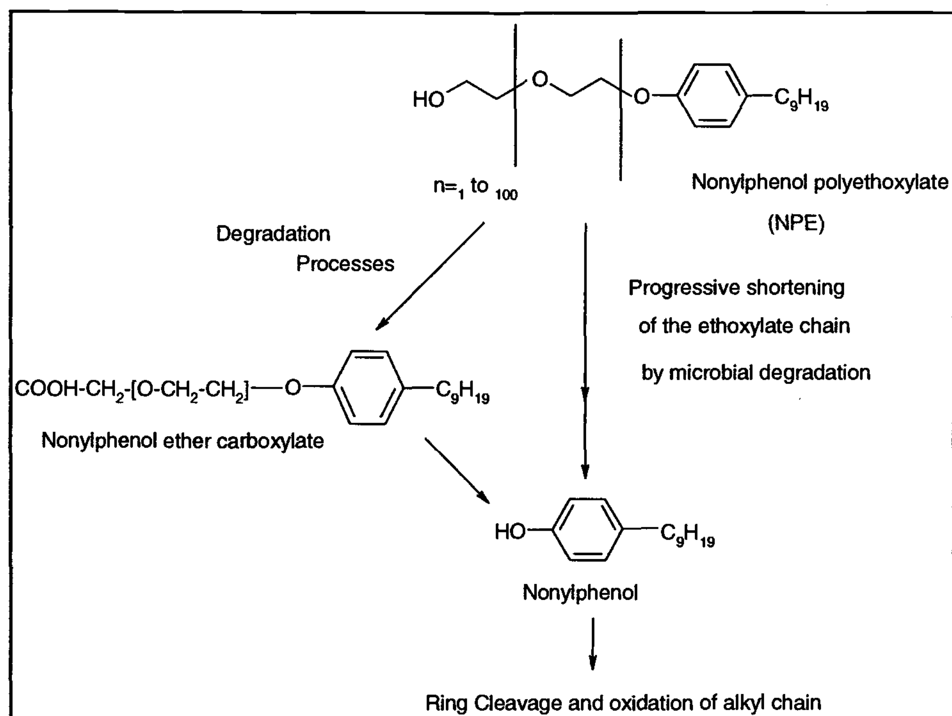
There are two primary types of APEs, nonylphenol polyethoxylates (NPE's) and octyphenol polyethoxylates (OPEs), both of which undergo microbial breakdown during aerobic wastewater treatment to form de-ethoxylated biodegradation products. These products undergo further breakdown in anaerobically stabilized sewage sludge to form nonlyphenol (NP) and octylphenol

(OP), respectively (Giger et al. 1984). Biodegradation of NPE's occurs by step-wise shortening of the ethoxylate chain by oxidation, to yield a complex mixture of compounds including di- and monoethoylates, alkylphenoxy carboxylic acids, and NP (see Figure 1) (Swisher, 1987).

Nonylphenol polyethoxylates make up greater than 80% of APE's produced in the U.S. (Naylor, 1995), and have been detected in water and sediment of U.S. rivers, lakes, streams, and estuaries (Table 1). NP and nonylphenol mono- and diethoxylates (NP1EO, NP2EO), which are more lipophilic and resistant to further microbial degradation, have been detected in wastewater, and in freshwater and estuarine sediments (Kvestak and Ahel, 1994; Bennie et al. 1997).

NPE's are oligomeric mixtures varying in the length of the ethoxylate chain and in their extent of alkyl chain branching. The breakdown products are mixtures as well; even nonylphenols are divided into branched isomers.

Figure 1: Degradation of Nonylphenol polyethoxylates (NPEs)



Distribution

Nonylphenol distribution is widespread in aquatic ecosystems. It has been detected in water and sediments from lentic and lotic environments including oceans and estuaries. Surface water and sediment NP concentrations in lakes, rivers and estuaries are presented in Table 1.

Table 1: Environmental concentrations of nonylphenol in aquatic ecosystems

Ecosystem	[NP] water μg/l	References	[NP] sediment mg/Kg dry wt	References
Lakes	0.1 - 0.8	(Suoanttila, 1996)	0.06 – 37.8	(Bennett and Metcalfe, 2000; Khim et al. 1999; Bennett and Metcalfe, 1998)
Rivers	0.030- 45.00	(Ahel et al. 1981; Ahel et al. 1994; (Ahel and Giger, 1985; Schaffner et al 1987; Ahel et al. 1996; Tsuda et al. 2000a;2000b; Tsuda et al. 2002; Naylor et al. 1992; Blackburn and Waldock, 1995; Sundaram et al. 1980)	0.0029 – 41.1	(Daniels et al. 2000; Ding and Fann, 2000; Sundaram et al. 1980; Hale et al. 2000)
Estuaries	0.0036 - 0.330	(Petrovic et al. 2002; Kvestak and Ahel, 1994; Marcomini et al. 2000; Allen et al. 1999; Blackburn and Waldock 1995)	0.05 – 14.7	(Petrovic et al. 2002; Kvestak and Ahel, 1994; Marcomini et al. 2000; Ferguson et al. 2000; Kannan et al. 2000; Shang et al. 1999)

In U.S. rivers NP was detected at concentrations between 0.11 to 0.64 μg/l, and the average value was 0.12 μg/L (Naylor et al. 1992). Maximum sediment values in urban estuaries were 14 mg/Kg from Jamaica Bay, New York (Ferguson et al. 2001). In a recent study, nonylphenols were detected in 11 of 20 municipal sewage treatment plant riverine sediments, adjacent to the discharge area, 45% of the contaminated sediments contained NP concentrations >0.005 mg /Kg with a mean concentration of 0.365 mg/Kg (Hale et al. 2000).

Regulations

Several European countries have enacted strict regulations on NPE uses and emissions, and the European Union (EU) and Environment Canada have both completed risk assessments of NP. Both groups have concluded that APEs are toxic substances requiring regulation. The EU has set predicted no effect concentrations for aquatic organisms at 0.60 μg/L for surface water and 0.039 mg/Kg NP for sediments. In the United States, NP is currently not regulated as a hazardous substance, but the U.S. EPA Office of Water is drafting aquatic life criteria for NP which may result in future regulations.

Endocrine disruption

The National Academy Panel on Hormonally Active Agents in the Environment found more than 46 endocrine disrupting compounds (EDCs) present in aquatic environments from industrial, agricultural, and domestic sources (National Research Council, 2000). NP that had leached from plastic Petri dishes was found to induce growth of breast cancer cell lines, which was the first indication that NP could mimic estrogen (Soto et al. 1991). It is now evident that anthropogenic and naturally-derived chemicals can exhibit estrogenic and androgenic activity in aquatic organisms. Questions remain concerning the ultimate effects on wildlife populations from exposure to these hormonally active chemicals. There are but a few examples that establish causation from chemical-mediated endocrine disruption in wild populations and include alligator abnormalities from Lake Apopka in Florida (Guillette and Crain, 1996), invertebrate population declines from exposure to tributyltins (Oehlmann et al. 1996), and correlative evidence of elevated levels of vitellogenin, plasma steroids, and abnormal sex ratios in fish from heavily sewage-impacted United Kingdom rivers (Sumpter, 1995; Purdom et al. 1994).

The vital role that sex hormones play in normal fish reproductive development makes their mimicry/inhibition a serious threat to wildlife populations. Hormonally active chemicals that mimic estrogen, such as NP, have

been shown to disrupt maturation and differentiation of male and female juvenile fish. Intersex, reduced testicular growth, induction of female hormone-mediated processes in male fish, and inhibition of smoltification have all been attributed to EDC exposure.

NP endocrine disruption in fish

APE degradation products, nonylphenols and octylphenols, were implicated in conjunction with synthetic estrogens as putative agents in effects observed in fish. In particular, endocrine disruption by sewage waste water effluent constituents including NP and OP in several species of fish (White et al. 1994; Madsen et al. 1999; Christensen et al. 1999) and in Sprague-Daly rats (Chapin et al. 1999) has been documented. Research has demonstrated a dose-dependent response of vitellogenin expression, mRNA and protein, in fish from aqueous NP laboratory exposures; however the ecologically important dietary exposure pathways remain to be shown (Yadetie et al. 1999; Christensen et al. 1999; Hemmer et al. 2002). Caged rainbow trout in United Kingdom inland waters had significant occurrences of intersex that were attributed to hormonally active agents including NP and synthetic estrogens, from sewage treatment plant discharges (Harries et al. 1996). 4-nonylphenol inhibited sexual differentiation in male carp (Gimeno, 1997). Reduced testicular growth in rainbow trout (Jobling et al. 1996) and impairment of

testicular structure, cytology of germ cells, and appearance of Sertoli cells in juvenile male eelpout resulted from exposures to NP (Christiansen et al. 1998). Early exposure to sex steroids, before the onset of smoltification, can exclude or impair smoltification in salmonids (Langdon and Thorpe, 1985; Lundqvist et al. 1989). A correlation between the presence of NP in an aerially applied pesticide formulation and impairment of Atlantic salmon smolt development has been reported in Canada (Fairchild et al. 1999). The authors demonstrated a positive correlation between declines in salmon returns (based on a 17 year database) and the spraying of pesticide formulations that contain NPEs. As a result of the continued high use of APEs in the U.S., it is imperative that their potential for disrupting juvenile fish life cycles is evaluated.

Vitellogenin

Biomarkers of endocrine disruption are robust and sensitive to detect exposure to hormonally active chemicals, particularly for estrogenic compounds. The role of estrogens in the control of fish reproduction has been well researched in the salmonid, *Oncorhynchus mykiss* (rainbow trout) (van Bohemen and Lambert, 1981; Maitre et al. 1985). The natural hormone 17*B*-estradiol plays a significant role in the sexual development of female salmonids, while in the male salmonid the process is regulated by androgens. Vitellogenin is a high density lipoprotein

expressed by the liver after receptor mediated binding of 17*B*-estradiol. Once transcribed, vitellogenin undergoes post-translational phosphorylation, glycosylation, and lipidation, and is then secreted into the plasma where it enters oocytes of mature females via pinocytotic uptake. Vitellogenin is expressed by hepatocytes following exposure of male and female teleosts to estrogens (natural and synthetic). The oocytes rapid growth prior to ovulation is promoted by the accumulation of vitellogenin within growing oocytes in the ovaries of salmonids. Male fish will express vitellogenin if exposed to estrogens and this has become a very useful and sensitive biomarker to detect exposure to estrogenic acting chemicals (McLachlan 1993; Donohoe and Curtis, 1996; Jobling et al. 1996).

Fish are not the only organisms that may be impacted by endocrine disrupting compounds. Recent research has demonstrated that NP exposure can disrupt endocrine systems in freshwater and marine invertebrates, although in general these organisms have been "...grossly neglected in current routine excotoxicological assessments" (Depledge and Billinghurst, 1999).

Interestingly, in response to NP exposures, invertebrates produced Vtg and Zrp like-proteins, which were considered heretofore to be exclusively vertebrate biomarkers for endocrine disruption (Billinghurst et al. 2000). Evidence of increased egg production and decreased egg viability in the polychaete worm *Dinophilus gyrociliatus* from NP exposures was observed (Price and Depledge,

1998). Exposure in chironomids to NP-contaminated sediment led to significant mouthpart deformities (Meregalli et al. 2001). Decreased settlement of juvenile barnacles exposed to environmentally realistic NP concentrations has also been observed (Billinghurst et al. 1998). These myriad effects from NP exposure speak to the need for continued research on invertebrate populations, given their ecological importance.

LABORATORY FOOD CHAIN DESIGN

Amphipods

Amphipods were selected as the primary accumulator of NP from contaminated sediments for this dissertation research. They are prey items of salmon fry (Shreffler et al. 1992), used extensively in laboratory bioassays (DeWitt et al. 1989), and easily collected. The middle consumer in our constructed estuarine food chain was an amphipod, either *Eohaustorius estuarius*, *Grandiderella japonica*, or *Corophium salmonis*. *G. japonica* is an introduced amphipod found in Yaquina Bay Estuary that burrows and grazes in sediment and feeds on algae. *C. salmonis* is an indigenous amphipod species in Yaquina Bay that resides in intertidal, muddy and sandy substrates. Both species are tube-builders that feed feed on sediment and suspended organic matter (Taghon, 1982). *E. estuarius* is an

indigenous species that digs freely beneath the sediment surface and resides in sandy sediments of Yaquina Bay.

Salmon

The predator in our constructed food chains is the salmon fry *Oncorhynchus tshawytscha* which utilizes estuaries as rearing habitats prior to smoltification and which constitutes an important predator in estuarine food chains (Healey, 1980). Of the multiple species of Pacific salmon, juvenile chinook spend the longest time periods in estuaries, undergoing significant physiological changes that are controlled by hormones. They feed extensively on benthic organisms including amphipods (Healey, 1980; Shreffler et al. 1992).

Field-collected juvenile coho and chinook salmon from estuaries are known to feed on both *C. salmonis* and *G. japonica* (personal communication, Jeff Cordell University of Washington and John Chapman OSU). Chinook fry generally remain in estuaries, feeding and growing until some unknown cue is triggered and they enter into the ocean. Fry undergo several physiological changes during their residence in freshwater and estuarine habitats (Barron, 1986). During this critical developmental stage they are especially vulnerable to hormonally active chemicals, such as NP, from point and non-point sources.

Salmon possess high economic, cultural, and ecological value in the Pacific Northwest, and chinook populations have been listed as endangered in several rivers of the Pacific Northwest. The decline in populations is attributed to a combination of impacts including dams, over harvesting, habitat degradation, and pollution of water and sediments (Kareiva et al. 2000). As sediments act as sources and sinks for heavy metals and persistent organic pollutants contamination may not only be affecting benthic organisms, but may also be promoting accumulation through estuarine food chains.

Nonylphenol bioaccumulation

Few studies have investigated NP bioaccumulation from contaminated sediments. An amphipod, *Ampelisca abdita*, exposed to NP-spiked sediment bioaccumulated significant amounts (Fay et al. 2000). In rainbow trout, NP was found concentrated in fat ($T_{1/2}$ ~ 18 hours), liver ($T_{1/2}$ ~ 5 hours), kidney, and bile (Lewis and Lech, 1996). Lewis and Lech (1996) also demonstrated that NP was metabolized through glucuronic conjugation and oxidation, followed by excretion in bile and urine.

Evidence from field-collected organisms at contaminated sites suggested that there may be considerable bioaccumulation in algae (38 mg/Kg dry weight in

Cladophora glomerata, equivalent to a bioconcentration factor of 10,000) and to a lesser extent in fish tissues from 13-410 (bioconcentration factors) (Ekelund et al. 1990). Detectable concentrations were also found in a wild duck (Ahel et al. 1993). Bioconcentration factors from shrimp (*Crangon crangon*), juvenile Atlantic salmon (*Salmo salar),* mussels (*Mytilus edulis*), and sticklebacks (*Gasterosteus aculeatus*), were 100, 280, 3400, and 1300 respectively from laboratory studies (Ekelund et al. 1990; McLeese et al. 1981).

RESEARCH APPROACH

To assess potential NP impacts on an estuarine food chain, a set of laboratory experiments utilizing ecologically-relevant organisms were designed to quantify NP accumulation using the model food chain; NP contaminated sediment-amphipod-salmon fry. To control environmental variables and to investigate the mechanisms of NP bioaccumulation, all experiments were undertaken in the laboratory, utilizing field-collected or cultured organisms. Amphipods were selected as the primary accumulator of NP from contaminated sediments because they are prey items of salmon fry, used extensively in laboratory bioassays, and easily collected. To identify endocrine disruption in fry exposed to NP via water or NP-contaminated amphipods, a sensitive and robust biomarker, Vtg, was used.

At first, individual components of the food chain were investigated separately to understand the fate of nonylphenol at each trophic level and these were subsequently combined into one experiment to study the pathway of NP transfer into salmon fry and to evaluate the potential for endocrine disruption via induction of vitellogenin. Standard laboratory bioaccumulation and sediment toxicity assays were used and modified to determine bioaccumulation and toxicity values for all levels of the food chain. In the concluding experiment amphipods were exposed to sediments collected from Yaquina Bay that had been amended with ^{14}C-NP and then fed to salmon fry.

Although the entire spectrum of APE compounds are of interest, this dissertation focuses on 4-nonylphenol (NP) because of the high volume use of NPEs in industrial and domestic sectors and the lack of data concerning exposure, toxicology, and transmission through food chains. Combined with the ability to mimic estrogen, NP, is a potentially serious threat to aquatic ecosystems. Little is known about the fate of NP in estuarine benthic systems, particularly, the potential for bioaccumulation by benthic biota via the sediment and the resulting transfer to higher trophic levels.

INTRODUCTION TO CHAPTERS

Chapter 2

The objective of this chapter was to determine the sensitivity of the phoxocephalid amphipod, *Eohaustorius estuarius* to 96-h exposures of water borne NP. Median lethal concentrations to 50% (LC50's) of *E. estuarius* were determined for NP and recovery of surviving amphipods was followed for up to 48-h when placed into uncontaminated sediment and water. This chapter has been published (Hecht and Boese, 2002).

Chapter 3

Using three species of infaunal estuarine amphipods, we tested whether the nutritional quality of organic matter affected NP accumulation by amphipods and whether accumulation was different between species. To investigate the effect of organic matter quality on accumulation of NP by amphipods, we enriched sediments with either labile (dried macrophytic algae) or refractory (dried lignin) materials and non-toxic trace concentrations of NP. This chapter was unique in its

approach, in that NP bioaccumulation was quantified in three Pacific Northwest amphipod species in relation to two types of sedimentary organic enrichment. This chapter is also a journal article that is presently in U.S. EPA internal review (Hecht et al. in preparation)

Chapter 4

The objectives of this chapter were to determine if there was induction of vitellogenin in Chinook salmon fry following daily consumption of NP contaminated amphipods and to determine dose-response induction of vitellogenin in response to NP water concentrations. We investigated the bioaccumulation of NP by amphipods from contaminated water and subsequent transfer of NP via the diet and water to chinook salmon fry. Additionally, this work explored the potential for NP to disrupt endocrine systems of chinook salmon fry quantified by plasma vitellogenin expression, a biomarker for endocrine disruption. Water and dietary laboratory exposures of NP to hatchery reared chinook salmon fry were determined following 5 day exposures. The journal article from this chapter is in review (Hecht et al. in prep).

Chapter 5

The objectives of this chapter were to summarize conclusions, discuss uncertainties of data and methodology, provide future research direction, and to characterize risk to estuaries from 4-nonylphenol contamination.

CHAPTER 2:

SENSITIVITY OF AN INFAUNAL AMPHIPOD, *EOHAUSTORIUS ESTUARIUS*, TO ACUTE WATERBORNE EXPOSURES OF 4-NONYLPHENOL: EVIDENCE OF A TOXIC HANGOVER

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ABSTRACT

Three independent trials were conducted using mortality and burial as endpoints. Amphipod lethal concentration to 50% (LC50) was 227 μg/L. One-hour burial as a sub-lethal endpoint increased the sensitivity of the toxicity test by 40%; however, most amphipods that survived exposure were able to recover within 24 to 48 hours. This toxic hangover was dose-dependent.

INTRODUCTION

This investigation determined the sensitivity of *Eohaustorius estuarius,* an estuarine, infaunal amphipod commonly used in toxicity testing (DeWitt et al. 1989; American Society of Testing and Materials, 1992; Schlekat et al. 1995), to waterborne exposures of 4-nonylphenol (NP). An organic contaminant, NP is found in surface waters and sediments throughout the world (Kvestak and Ahel, 1994; Bennie et al. 1997; Bennett and Metcalfe, 2000; Marcomini et al. 2000), where it is of concern for its acute and chronic toxicity, including endocrine disruption (Soto et al. 1991; Madsen et al. 1999) and bioaccumulation potential (Fay et al. 2000; Ekelund et al. 1990). Because NP is a nonionic organic compound, we hypothesized that it would exhibit a narcosis-like mode of toxicity, which has been described as "a reversible state of arrested activity of protoplasmic structures"

(Veith et al. 1983). Increased lethargy, unconsciousness, and death without any specific mechanisms of toxicity characterize base-line narcosis. If the toxic mode of action for NP is narcosis, then amphipods that survive exposure to NP should eventually be able to recover and rebury in sediment. In addition, we hypothesized that measured NP toxicities should conform to published quantitative structure-activity relationships for other neutral narcotic compounds (Veith and Broderius, 1987; Swartz et al. 1995). The goals of the present study were to determine 96-h lethal concentrations to 50% (LC50s) of the test amphipods and to follow the recovery of surviving amphipods for up to 48-h when placed into uncontaminated sediment and water. If the toxic mode of action for NP was narcosis, we hypothesized that surviving *E. estuarius* exhibiting toxic symptoms should recover over time and rebury in clean sediments.

MATERIALS AND METHODS

Three identical, water-only, static 96-h bioassays were used to determine NP toxicity to *E. estuarius* (trials 1, 2, and 3). Each trial was run within a two-week period of the previous trial during January and February 2001. Two days before trial initiation, *E. estuarius* were collected from a high intertidal site of Yaquina Bay (Newport, OR, USA). Surface sediments (1-10 cm) were sieved (1 mm), and retained adult *E. estuarius* were transported to the laboratory where they were

maintained in culture tubs with sand- filtered seawater (15°C; salinity, 28 ppt) and sediment from the collection site. At trial initiation, amphipods were sieved (1 mm sieve) from their holding sediments and placed into shallow glass trays containing filtered seawater (salinity, 28 ppt). Amphipods were randomly removed from these trays and sequentially placed one at a time into a series of glass culture dishes until 20 amphipods were in each of 30 dishes. Dishes were then allocated randomly among 30 1-L beakers, and amphipods were gently added to each beaker. Exposure beakers containing 750 ml of treatment seawater (salinity, 28 ppt) were covered with a watch glass. Each beaker was gently aerated using a 1-ml glass pipette inserted at the spout that pumped in filtered air. All beakers were placed in a water bath (15°C) that was covered with black plastic to minimize light. In all, six nominal treatments were used, with five replicate beakers per treatment. To determine the amount of NP loss over 96 hr from water, a pilot test was run using ^{14}C ring-labeled NP. This test indicated that 43.7% (SD = 4.73, n=4) of NP was lost from beakers after 96 hrs (S. Hecht unpublished data).

Treatment solutions were prepared using a stock solution of NP (CAS 84852-15-3, Lot 03008LS; Sigma-Aldridge-Fluka, St. Louis, MO, USA.) in acetone (2.5 mg NP/ml acetone) which was prepared fresh for each trial. Aliquots of stock solution were added to 4- L batches of seawater (salinity, 28 ppt) to obtain nominal NP concentrations of 0, 10, 120, 200, 360, and 600 μg/L NP. The lowest NP concentration (10 μg/L) was chosen because it is at the upper end of

environmental concentrations (Lee and Peart, 1995) and has been shown to induce vitellogenin, a biomarker for endocrine disruption, in fish (Nimrod and Benson, 1998). Batches were mixed separately with magnetic stirrers and 750 ml of each treatment were poured into five replicate beakers. The five replicate control beakers contained no NP or acetone; however, in the first trial, five additional carrier control (CC) replicates were prepared that contained the same concentration of acetone (0.025%) found in the highest NP aliquot.

All tests were run for 96-hrs in the dark because pilot tests (96 h) indicated the survival of control *E. estuarius* was reduced when exposed to direct laboratory lighting. Every 24 h, the number of amphipods that were inverted (on their backs) or upright was noted for each beaker. Salinity and temperature were monitored throughout the experiment in controls. After 96 h, amphipods were gently sieved (1 mm) from the water, transferred to Petri dishes, and inspected with a dissecting microscope for 1 minute to determine mortality. Those individuals exhibiting no movement of antennae or limbs following gentle prodding were scored as dead and were discarded. Missing organisms were presumed to have died and decayed over the course of the experiment. Live amphipods were then carefully pipetted into beakers containing 2 cm uncontaminated sediment from their collection site and 750 ml of filtered seawater (salinity, 28 ppt) and placed back into the water bath for a behavioral response test. This test was conducted at 1-, 24-, and 48-hr (trials 2 and 3 only) intervals, with the ability to bury used as an endpoint. After 1 h, the

number of amphipods that had buried was noted. Beakers containing amphipods that had not buried were reassessed after 24 hrs. Amphipods that had not buried after 24 hrs were again inspected with a dissecting scope to assess mortality. In trials 2 and 3, amphipods that had not buried after 24 hrs but were still alive were left for another 24 hrs. Amphipods that had not buried after the second 24 hrs were again inspected with a dissecting scope to assess mortality. Effective concentrations to 50 per cent (EC50s) of the test population were determined from these tests by using the number unable to bury which was added to the number of mortalities in the initial 96-h toxicity test. Thus, the burial values are a cumulative index of toxic effect (number of mortalities + number unable to bury). LC50 and burial EC50 estimates were determined by the Spearman-Karber method, incorporating Abbot's correction for control survival (Hamilton et al. 1977). A two-sample *t*-test was used to test for significant differences between control and acetone carrier control treatments from trial 1 (Sokal and Rohlf, 1981).

RESULTS

Mean control survival in trials 1, 2, and 3 was 94%, 92%, and 94% respectively, which was within the American Society for Testing and Materials requirement of > 90% (American Society of Testing and Materials, 1992). Survival of *E. estuarius* during trial 1 in controls and in carrier controls was not significantly

different ($p = 0.26$; $df = 8$, two-sample t-test, $\alpha = 0.05$); therefore, the carrier controls were not included in the second and third trials. The LC50 estimates ranged from 189 to 299 µg/L (Table 2). In all trials, clear dose-dependent responses (mortality and burial) to increasing levels of NP were observed (Figure 2). Trial 1 NP LC50s and burial EC50s concentrations were significantly less toxic than trials 2 and 3, although dose-response trends were similar (Table 2). This difference may be due to a small spiking error, but because we did not measure NP concentrations analytically, we are unable to confirm it. All amphipods that survived 96 h exposures to NP were able to bury within 1 h in carrier control and in 0 and 10 µg/L treatments. The surviving amphipods were active and appeared to be unaffected by the lowest NP treatment (10 µg/L), and all immediately buried when placed into uncontaminated sediment. At 120, µg/L amphipods appeared to be less active, and the small percentage that did not bury after 1 h buried after 48 h. At 200

Table 2: *Eohaustorius estuarius* mean 96-hr lethal concentrations to 50% (LC50s) and burial effective concentrations to 50% (EC50s)[a]

Trial	96 hr LC50 (µg/L)	1- h reburial EC50 (µg/L)	increase in sensitivity (%)[b]	24-h reburial EC50 (µg/L)	48-hreburial EC50 (µg/L)
1	299[c] (255-352)	182[c] (157-210)	39	221[c] (189-258)	NA[d]
2	194 (158-238)	109 (87-136)	44	132 (106-165)	139 (110-174)
3	189 (163-219)	123 (107-143)	35	135 (115-158)	137 (117-160)
Mean (2 and 3)	191	116	40	133	138
Mean (1,2,3)	227	138	39	163	NA

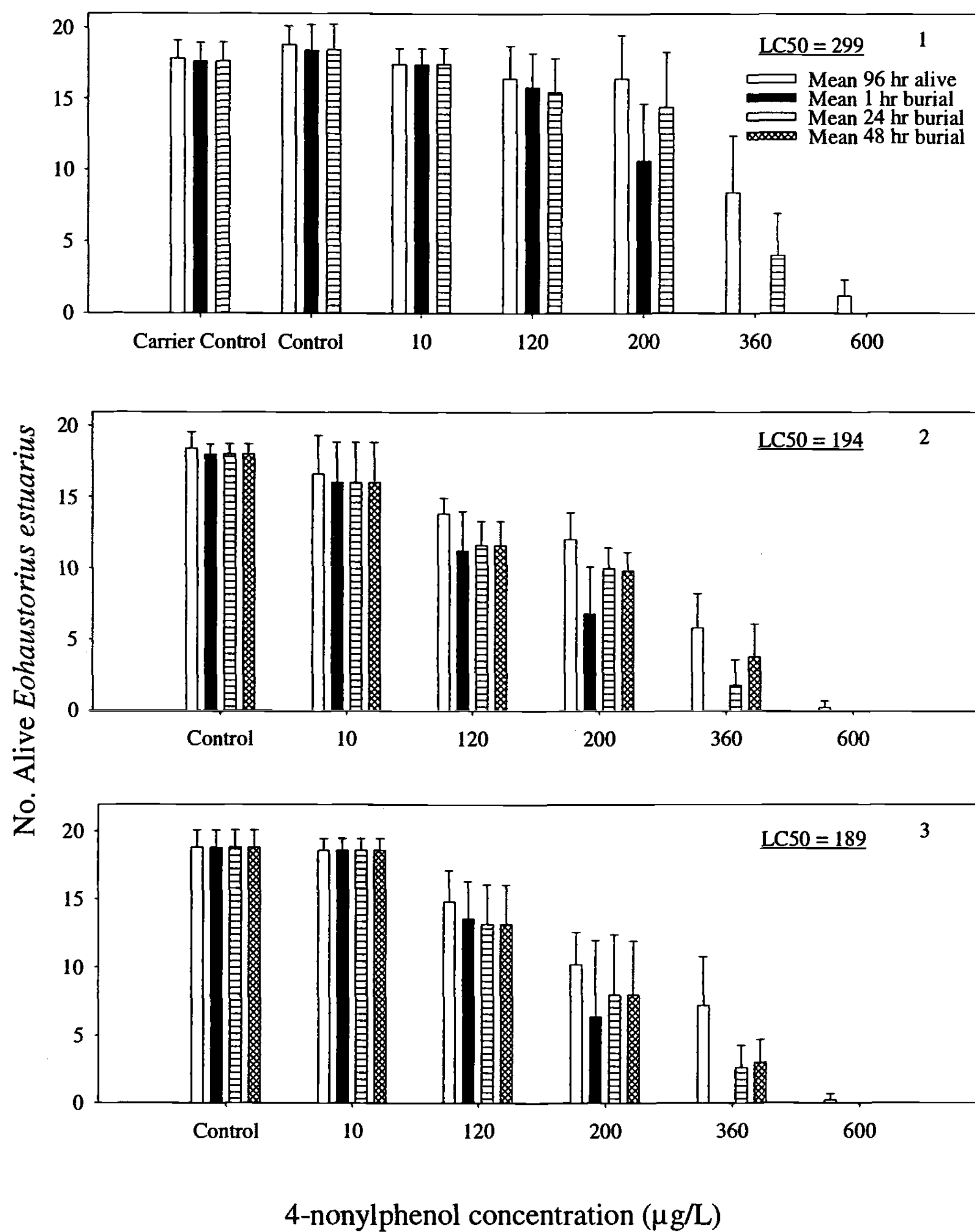
[a] The LC50s of the test population were determined by the spearman-Karber method (Hamilton et al. 1977). The standard deviation is given below each value.
[b] Denotes % increase in sensitivity between 96-h LC50 and 1-h burial EC50 as determined by ([96-h LC50 – 1- hr EC50]/ 96- h LC50)*100.
[c] Denotes significantly different value ($p < 0.05$) between trial 1 and all other trials within columns using the LC50 and EC50 comparison methods of the American Public Health Association (American Public Health Association, Washington, and Federation 1989). Separate means were displayed because of significant difference between trial 1 and trials 2 and 3.
[d] NA = not applicable

and 360 μg/L, amphipods were clearly affected, and most were inverted after 24 h of exposure. The majority of surviving amphipods in these treatments was inverted, lethargic, and immobile. Approximately 57% of amphipods buried at 1 h in 200 μg/L beakers and 0% buried at 1 h in 360 μg/L beakers. Most of the unburied amphipods recovered and buried after 24 h. This recovery period appeared to take longer in amphipods exposed to 360 μg/L, because the number able to bury increased between 24 and 48 hr (Figure 2). The few amphipods that survived exposure at 600 μg/L were unable to recover and were dead within 24 h post exposure.

DISCUSSION

These results demonstrate that NP was toxic to infaunal amphipods over the range of doses tested, and that adding a 1-h burial test increases the sensitivity of the bioassay by about 40%, as indicated by the lower EC50 values compared to the LC50 values shown in Table 2. Although these infaunal amphipods were in an ecologically unnatural environment, survival occurred at all concentrations, and amphipods recovered following acute exposures to NP as measured by their ability to bury. In nature, the delayed ability of an infaunal organism to bury would increase that organism's vulnerability to predation, which has obvious biological ramifications for individuals and populations (Swartz et al. 1985).

Figure 2: Survivorship (96 h) and burial (1, 24, and 48 h) following 96-h water exposures to nominal 4-nonylphenol (NP) concentrations in trials 1, 2, and 3. Bars denote means from five replicates; error bars represent corresponding standard deviations. The LC50 is the NP concentration lethal to 50% of the tested amphipods



In previous studies at our laboratory, surviving infaunal amphipods, including *E. estuarius,* were able to rebury within 1 h following exposure to acutely toxic concentrations of fluoranthene (DeWitt et al. 1989), DDT and dieldrin (Swartz et al. 1994), and a wide range of neutral narcotics (Boese et al. 1998; Boese et al. 1999). In contrast, in the present experiments, NP exposure resulted in a dose-dependent toxic hangover that surviving *E. estuarius* required at least 24 hrs to overcome. This extended time to bury at higher NP concentrations may be a result of a decreased NP depuration rate or of a different mode of narcotic action. Numerous potential mechanisms of narcosis exist, and time lengths of recovery may vary based on inter-and intraspecies differences, including differences in bioassay procedures.

In addition to behavioral differences between amphipods exposed to NP and those exposed to neutral narcotics, NP appears to be an order of magnitude more toxic than compounds exhibiting a narcotic mode of toxic action. Using quantitative structure-activity relationships, Veith and Broderius (1990) demonstrated that narcotic organic chemicals with phenol groups, similar to NP, were more toxic than predicted by type I general narcosis theory, and labeled the toxic mode of action for this group of compounds as type II narcosis.

Using 96-h fathead minnow data, Veith and Broderius (1987) developed a regression model for these type II narcotics based on a chemical's octanol-water partition coefficient (k_{ow}):

$$\text{Log LC50} = -0.65(+/- 0.07) \text{ Log } K_{OW} - 2.29(+/- 0.22)$$

$$(r^2=0.90)$$

Using this model, the predicted LC50 for NP is 1,387 µg/L (lower limit = 410, upper limit = 4700 µg/L), which is one-tenth the observed toxicity in the present study. Our result is supported by Holcombe et al. (Holcombe et al. 1984), who found the LC50 for NP following 96 h of exposure to be 135 µg/L in fathead minnows. Thus, it appears NP does not fit within the type II narcotic family even though the organisms recover if given sufficient time to depurate NP from their systems.

Due to taxa biological differences among amphipods and fathead minnows, we also compared our LC50s to an amphipod quantitative structure-activity relationship.

Using 10-d amphipod data from sediment toxicity assays, Swartz et al. (1995) developed a regression model based on LC50 interstitial water (IW) toxicity of three polyaromatic hydrocarbons:

$$\text{Log LC50}_{IW} = 5.92 - 1.33 \log K_{ow}$$
$$(r^2 = 0.96)$$

The predicted LC50 from this regression equation was 202 µg/L NP, which is not significantly different from the empirically derived LC50 of 227 µg/L. These comparisons indicate that caution should be used when comparing different species to one another using quantitative structure-activity relationships developed from single-species assays. In particular, regression slopes and intercepts may differ significantly among species, leading to invalid predictions.

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CHAPTER 3: BIOACCUMULATION OF 4-NONYLPHENOL BY ESTUARINE BENTHIC AMPHIPODS: THE ROLE OF SEDIMENTARY ORGANIC CARBON AND TROPHIC TRANSFER

by
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ABSTRACT

Nonylphenol (NP) is a moderately persistent, hydrophobic chemical (Log K_{ow} 4.5) with endocrine disrupting and acute narcotic effects in aquatic biota. It is a metabolite of the nonylphenol polyethoxylate class of surfactants, which are widely used in industry and commercial products. Because of their extensive use, there is concern about the ultimate fate of NP in aquatic ecosystems and about the potential for bioaccumulation by benthic biota from the sediment with the potential for further transfer to higher trophic levels. Our goals were (1) to determine if amphipods bioaccumulate significant amounts of NP from sediment (2) to determine if TOC quantity and nutritional quality to amphipods influence NP bioaccumulation and (3) to determine if there were accumulation differences based

on differences in amphipod feeding strategy. In the present study, estuarine sediment was spiked with ^{14}C-NP and enriched with two types of organic carbon of differing nutritional qualities. Bioaccumulation of NP was measured in three amphipod species (*Eohaustorius estuarius*, *Grandidierella japonica*, and *Corophium salmonis*) that have different feeding strategies. Amphipods were exposed for 16 days to equal concentrations of NP in three treatments: "positive control" (no additional TOC), "refractory TOC" (sediment enriched with lignin), "labile TOC" (sediment enriched with algae, *Ulva* sp.). Results indicated that NP bioaccumulation by amphipods was inversely proportional to TOC quantity, but unaffected by TOC nutritional quality. There were significant differences in the accumulation patterns between the 3 amphipod species that were related to their feeding strategies. Mean accumulation factors (BSAF) ranged from 8.1–33.9 in *E. estuarius,* and from 4.6-17.2 in *G. japonica.* Our results show that estuarine amphipods are potential dietary sources of NP to higher trophic levels, such as juvenile salmonids, at doses sufficient, theoretically, to induce endocrine disruption.

INTRODUCTION:

Nonylphenol polyethoxylates (NPEs) are ubiquitous industrial surfactants. In waste water treatment processes NPEs degrade to nonylphenols (NPs), which are

more persistent, toxic, and hydrophobic (Giger et al. 1984). One of these metabolites, 4-nonylphenol (NP), may pose environmental risks because of its toxicity to aquatic organisms, its concentration in the environment (sediments, water and biota), its endocrine-disrupting potential in aquatic fauna, and the high volumes at which it is released. NP is an endocrine disruptor that mimics the female hormone 17*B*–estradiol (Arukwe et al. 1998). NP binds to vertebrate estrogen receptors triggering estrogen-mediated processes including the induction of vitellogenin (Hansen et al. 1998; White et al. 1994) and *zona radiata* proteins; (Arukwe et al. 1997; Celius et al. 1999). These proteins constitute sensitive biomarkers for endocrine disruption in fish and other vertebrates. Most research on the hazards posed by NP to the aquatic biota has focused on NP accumulation in fish, either from water exposure, or following high dose i.p. injection. NP water concentrations in laboratory experiments have tended to be unrealistically high, and other routes of exposure, especially dietary uptake, have not been thoroughly investigated, leaving a gap in our ability to predict potential uptake rates in the field. A potentially significant dietary pathway for NP transfer from contaminated estuarine sediments to fish is via benthic Amphipoda. Data are lacking on the amounts of NP that could be available to fish through this pathway and on the degree to which sediment characteristics affect NP bioavailability to amphipod prey species.

NP is hydrophobic, associating with organic matter and residing in sediments (Ahel et al. 1994; Ferguson et al. 2000). The bioavailability of contaminants in sediment has been defined as, "the fraction of the total quantity of a contaminant in the interstitial water and sorbed to sediment particles that is available to benthic organisms, via respiratory, dietary, and surface absorption processes" (Landrum and Robbins, 1990). The processes that determine the bioavailability of sediment-associated, hydrophobic, organic contaminants to organisms that colonize sediment are not resolved, and remain under investigation (Landrum et al. 1996; Landrum and Robbins, 1990; Lee and Swartz, 1980; Lee, 1992).

One important aspect of bioavailability that may influence the accumulation of sediment-associated contaminants to amphipods is the type and quality of sediment organic matter (Gunnarsson et al. 1999a; 1999b). Types and sources of sediment organic matter vary drastically in sediments and include decaying terrestrial and marine plants, fecal matter, dead animals, and flocculated organic material (humic and fulvic substances). These different organic matter types will have varying degrees of binding strengths for organic contaminants, such as NP (Chiou et al. 1986; Gauthier et al. 1987). The bioavailability of sediment-sorbed contaminants to infaunal prey species has been shown to be dependent upon sediment total organic carbon content (TOC) (Carlberg et al. 1986; DeWitt et al. 1988), organism feeding behavior (Boese et al. 1996; Bott and Standley, 2000), and

the nutritional quality of the organic matter (Gunnarsson et al. 1999a). Selective uptake of contaminant associated particulate organic matter could affect the accuracy of general models of bioaccumulation that are based, in part, on the quantity of sediment organic matter (TOC) which is assumed to act as an unavailable sink for contaminants. Data are lacking on the influence of organic carbon type on accumulation of NP by benthic, estuarine organisms, and the degree to which these species utilize organic carbon to which NP may be sorbed.

By using three species of infaunal estuarine amphipod, we were able to test not only whether the nutritional quality of organic matter affected NP accumulation, but also the degree to which accumulation differs between species. To investigate the effect of organic matter quality on accumulation of NP by amphipods, we enriched sediments with either labile (dried macrophytic algae) or refractory (dried lignin) materials and non-toxic trace concentrations of NP. This is a novel approach for quantifying bioaccumulation of a chemical of current concern (that is presently unregulated in the U.S.A) in three Pacific Northwest amphipod species, in relation to sediment enrichment with two types of organic matter.

MATERIAL AND METHODS

Sediment collection

Surface sediment (0-5 cm) was collected from a high, sandy, inter-tidal site in the Yaquina Bay estuary (Newport, OR), sieved (0.4 mm) with 28 ppt salinity filtered seawater, and frozen for 96 h to remove remaining macrofauna. The resulting base sediment (~16.5 kg) was then stored in 3.8 L glass jars at 4°C in the dark until use. After approximately 4 months in storage, the sediment was sampled to determine TOC and total nitrogen (TN) by combustion (Perkin Elmer 2400 CHN Elemental Analyzer).

Organic enrichment

The aged sediment was divided into four equal portions. Two of the portions were enriched with either refractory (pulp and paper mill lignins) or labile (*Ulva sp.* macrophytic algae) organic matter. The labile organic matter (*Ulva sp.*) was collected at low tide on the mud flat of Yaquina Bay. The algae were rinsed to remove associated epiphytes and epifauna, dried, pulverized and kept at -80° C.

Lignin was obtained from a paper mill (Holmen Paper AB, Sweden) as "Curan 100", a fine, brown powder, composed of insoluble, structural lignins remaining after the separation of cellulose during the manufacture of unbleached paper. Before addition, both organic materials were dried in an oven at 60° C for 72 h, pulverized with a mortar and pestle, sieved through 0.25 mm stainless steel sieves and analyzed for TOC and TN. Two of the sediment portions were enriched to 1.5 times their initial TOC content with either *Ulva sp.* ("LA treatment") or lignin ("RE treatment"). The level of TOC enrichment was determined to be high enough to be detectable and low enough not to induce toxic sulfide concentrations based on 10-day pilot dose-response tests (unpublished data). Organic matter (OM) additions were carried out by mixing lignin or *Ulva sp.* with 1.5 kg (wet weight) sediment portions in the 3.8 L glass jars. Following the OM addition, 200 mL of filtered seawater (28 ppt salt) was added to each jar. The jars were shaken and stirred manually for 1 minute and placed on a rolling mill for 16 h in the dark, at 4°C to mix the organic matter and sediment to homogeneity. The remaining two sediment portions served as controls and received no additional organic matter.

Sediment spiking with nonylphenol

Radiolabeled NP (^{14}C-[ring labeled]-4-nonylphenol) (American Radiolabeled Chemicals, Inc., St. Louis, MO; specific activity: 54 Ci mol^{-1}) was

mixed with unlabeled NP (Sigma-Aldridge-Fluka, St Louis, MO) in a 99 to 1 ratio in methanol, to produce a 100 mL spiking mixture containing 0.5 μCi ml^{-1} or 21 μg NP ml^{-1}. This mixture was added to sediments to achieve an initial target sediment concentration of 1400 dpm per g dry wt sediment, i.e. 256 ng NP per g dry wt sediment. The sediment spiking was accomplished following the method of DeWitt et al. (1989). Briefly, 0.081 mL of the NP mixture was dissolved in 15 mL of analytical grade acetone, applied to the walls of the 3.8 L glass jars and the solvent was evaporated. Wet sediment (1.5 kg) was then added to each jar. The jars were sealed and rolled on a rolling mill for 15 h in the dark at 4°C. After rolling, sediments were stored in the jars for 28 days (4°C, dark). The control sediment portions were stored in the same manner. This sediment spiking procedure yielded two experimental and two control sediment treatments. These treatments are subsequently referred to as follows:

RE = Refractory:	^{14}C-NP-amended base sediment that was enriched with TOC (lignin);
LA = Labile:	^{14}C-NP-amended base sediment that was enriched with TOC (*Ulva sp.*);
C+ = Positive Control:	^{14}C-NP-amended base sediment without TOC enrichment;
C- = Negative Control:	base sediment without ^{14}C-NP or TOC additions and no experimental manipulation

Amphipods

Infaunal amphipods were selected as test organisms in these experiments because of their high densities in the tidal and inter-tidal regions of estuaries, (DeWitt et al. 1989) their importance as prey items for fish and birds (Healey 1980; McCabe et al. 1983; Shreffler et al. 1992), and their extensive use in standardized testing for toxicity and bioaccumulation (DeWitt et al. 1989; American Society of Testing and Materials, 1992; Hecht and Boese, 2002). Three infaunal amphipod species were tested, *Eohaustorius estuarius* [Amphipoda, Haustoriidae], *Grandidierella japonica* [Amphipoda, Corophiidae], and *Corophium salmonis* [Amphipoda, Corophiidae] which differed in feeding behavior and lipid content (Table 3). *E. estuarius* is a free-burrowing, infaunal species that typically remains below the sediment surface and is abundant in Yaquina Bay (OR), in high intertidal sandy habitats. G. japonica and C. salmonis are tubicolus species inhabiting mid to low intertidal sediments in PNW estuaries, including Yaquina Bay (OR).

Table 3: Feeding and burrowing characteristics of test amphipod species

Species	Indigenous/ Non-indigenous (Pacific NW estuaries)	Standard Test Species	Feeding behavior	Burrowing Behavior Preference
Eohaustorius estuarius	Indigenous	Yes[b]	Unknown, Probable deposit feeder [a]	Digs freely within sediment
Grandidierella japonica	Non- indigenous, from Japan	Yes[b]	Suspension and deposit feeder [b]	Constructs burrows
Corophium salmonis	Indigenous	No	Suspension and deposit feeder [c]	Constructs burrows

a = (DeWitt et al. 1989)
b = (American Society of Testing and Materials, 1992)
c = (Miller, 1984)

Adult *E. estuarius* were collected two days prior to test initiation from a high intertidal site in Yaquina Bay using a 1.4 mm sieve near the same location as the sediment collection. Amphipods were transported to the laboratory and placed in filtered seawater (28 ppt salt) at 20°C until use. *G. japonica* and *C. salmonis* are infaunal species that construct shallow, U-shaped burrows from fine sandy substrates that are open to overlying water. They are often found at the same

locations in Yaquina Bay, and are common prey items of juvenile salmonids (Shreffler et al. 1992). *G. japonica* and *C. salmonis* were collected immediately before the experiment from laboratory cultures at the U.S. EPA Laboratory, Newport, OR. Juvenile *G. japonica* were used in the experiments because adults had been found to be cannibalistic in our previous experiments. Male and female *C. salmonis* are easily discernable from one another and were analyzed separately to test potential accumulation differences based on sex.

Experimental start

One day prior to addition of amphipods, 290 g wet weight of the appropriate sediment treatment was placed in 1L beakers and 775 mL of seawater (28 ppt salt) was added carefully to minimize sediment resuspension. Beakers were then assigned to three vented exposure chambers using a randomized block design to assure that an equal number of beakers of each experimental and control treatment were placed in each chamber. Each beaker was covered with a watch glass and gently aerated using a 1 mL glass pipette. Each chamber was held at a constant temperature (20° C) under continuous lighting. After 24 h the overlying water of each beaker was changed and 20 amphipods were added to each beaker. In the experiments with *C. salmonis* ten males and ten females of this species were

assigned to each beaker. Two separate experiments were conducted (Table 4) due to space limitations, availability of organisms, and limited amount of ^{14}C-NP.

Experiment 1

This experiment determined whether organic carbon nutritional quality in sediment affected NP accumulation by *E. estuarius.* We hypothesized that significant differences in accumulation would occur based on the nutritional quality of amended sediments. Three replicate beakers from each treatment (C+, LA, RE) were removed on each sampling occasion on days 0, 1, 3, 6, 10, and 16. Steady-state accumulation factors and uptake and elimination kinetics were determined for *E. estuarius* and compared among treatments. Additionally, *G. japonica* and *C. salmonis* were exposed to unamended (i.e. without organic enrichment) sediments to compare accumulation factors between the three species. Male and female *C. salmonis* were analyzed separately to determine gender based difference in bioaccumulation (See Table 4). The effect of TOC quality and quantity on accumulation by *G. japonica* and *C. salmonis* was not tested in Experiment 1.

Table 4: Initial allocation of exposure beakers (replicates) in experiment 1 and 2 among amphipod species and sediment treatments. Each replicate beaker contained 20 amphipods (C+ = positive control, LA = labile, RE= refractory, and C- = negative control)

	Experiment 1			**Experiment 2**
Species Treatment	*E. estuarius*	*G. japonica*	*C. salmonis*	*G. japonica*
C+	15	3	3	3
RE	15			3
LA	15			3
C-	3			

Sediments

Sediments were sampled according to the time-series presented in Table 5. Bulk sediment samples were taken in duplicate from each beaker using 5 mL

plastic disposable syringes (tips removed) as corers. The collected sediment was immediately placed in labeled test tubes and frozen (−80° C), then freeze-dried for 24 h, and stored in a desiccator until analyzed for TOC, NP, or ^{14}C (described below). Overlying water (5 ml) was sampled from each beaker and immediately analyzed for ^{14}C . Sediment pore water was sampled by centrifuging (3000 *g*, 30 min) ~100 g of bulk sediment sample and removing the supernatant for analysis. Surficial sediment POM was sampled on day 0, before test sediments were transferred to exposure beakers, by removing 5 ml of the organic matter layer from the sediment surface using a 10ml pipette. The sediment was placed in test tubes that were centrifuged (3000 *g*, 30 min), the aqueous layer removed for DOM measurements, and the pellet (sediment POM) extracted for ^{14}C.

Amphipods

Amphipods were collected by sieving (0.5 mm) test sediments from the exposure beakers. Live amphipods were counted and mortality assessed for each beaker. Surviving amphipods were placed in a Petri dish containing distilled water to remove salt and sediment particles, then transferred to a test tube (10 ml), frozen (-80° C), freeze-dried for 24 hours, then stored under desiccation until analysis for ^{14}C-NP, dry weight, and lipid content (described below). At day 0, three samples of each species (20 organisms/sample, including separate samples for male and female

C. salmonis), were taken immediately before animals were placed into experimental beakers to determine initial dry weight (dry wt), lipid content, and background levels of ^{14}C.

Experiment 2

A second experiment was undertaken to test the effects of TOC quality and quantity on NP accumulation by *G. japonica*. This experiment utilized four sediment treatments (C+, C-, LA, RE). Sediments collected on day 6 in Experiment 1, were saved and re-used in the *G. japonica* exposures. This was necessary in order to be able to make direct comparisons of NP accumulation between *G. japonica* and *E. estuarius*, given the limited supplies of ^{14}C-NP. We assumed that the bioavailable fraction of NP would be similar in both experiments. In order to assure this, we compared day-16 sediment concentrations of NP and *G. japonica* NP body burdens from experiments 1 and 2. Twenty juvenile *G. japonica* were added to each following the procedures used in exp 1. Sediments and amphipods were sampled after 16 d of exposure using the same techniques used in experiment 1 (Table 5).

Table 5: Time-series sampling scheme for experiment 1 and experiment 2

Sampling Day	-28	-1	0,1,3,6,10	16	0* Exp2	16 Exp2
Chemistry						
Bulk Sed.	^{14}C	^{14}C	^{14}C	^{14}C	^{14}C	^{14}C
	T_{OC}/T_N	T_{OC}/T_N	T_{OC}/T_N	T_{OC}/T_N	T_{OC}/T_N	T_{OC}/T_N
	NP	NP		NP	NP	NP
PW		^{14}C	^{14}C	^{14}C	^{14}C	^{14}C
OW			^{14}C	^{14}C	^{14}C	^{14}C
POM			^{14}C	^{14}C	^{14}C	^{14}C
DOM			^{14}C	^{14}C	^{14}C	^{14}C
Amphipod						
E.estuarius			^{14}C	^{14}C	^{14}C	^{14}C
			lipid	lipid	lipid	lipid
			drywt	drywt	drywt	drywt
G.japonica			^{14}C	^{14}C	^{14}C	^{14}C
			lipid	lipid	lipid	lipid
			drywt	drywt	drywt	drywt
C.salmonis			^{14}C	^{14}C	^{14}C	^{14}C
			lipid	lipid	lipid	lipid
			drywt	drywt	drywt	drywt

* exp 2 used salvaged sediment from exp 1 following sampling on day 6. ^{14}C = carbon 14 radioactivity, T_{OC}/T_N= Total Organic Carbon and Total Nitrogen, NP =nonylphenol, Lipid and dry weight determined from amphipod tissues. PW=pore water; OW = overlying water; POM=particulate organic matter; DOM= dissolved organic matter.

Chemical analyses

Radioactivity of sediment, water, and tissue samples were determined by liquid scintillation counting (LSC) on a Packard TRI-CARB 2000CA Liquid Scintillation Analyzer. Samples were counted for 10 min, with corrections made for quenching and background. Freeze-dried tissue and sediment samples were weighed (± 0.001 mg) and then pulverized using a glass mortar and pestle. The resulting powder was extracted twice in 5 mL of acetone/hexane 1:1. Solvent was added to each sample, vortexed (1 min) and sonicated (1 min) (Fisher Sonic-Dismembrator Model 300). The sonicate was then centrifuged (2000 rpm for 10 min) and the supernatant poured into a scintillation vial. The pellet was extracted a second time following the same procedure. The supernatants from each extraction were pooled in a scintillation vial and mixed with a scintillation cocktail (Ultima Gold, Packard). Sediment sample extraction efficiency was 93.3% +/- 3.97% (mean +/- SD). Radioactive analyses of overlying water and pore water samples were undertaken by mixing the samples (5ml) with Ultima Gold XR. DOM fractions (5 mls) were added to 15 mls Ultima Gold XR and counted. Radioactivity was expressed as DPM g dry wt (sediment and tissue) $^{-1}$ and DPM ml^{-1} (H_2O). Radioactivity was converted to NP µg g dry wt $tissue^{-1}$ or $sediment^{-1}$, using the relationship 1 DPM = 0.183 ng NP, based on the specific activity of ^{14}C-NP in the original spike solution.

Actual NP concentration was determined using a modified normal-phase HPLC method (Ahel et al. 2000). Freeze-dried samples were extracted in a Soxhlet apparatus with 50 ml *n*-hexane. Known amounts of internal standard (4-*tert*-butylphenol and 1,3,5-trimethyl- phenol) were added into the extract and the extract was evaporated to a small volume (1-2 ml) using rotary evaporation. The extracts were analyzed on an HPLC apparatus with a fluorescence detector using excitation and emission wavelengths of 277 nm and 300 nm, respectively. The analytes were separated on an aminosilica column and eluted with a hexane/2-propanol mixture (98/2) at a flow rate of 0.6 ml/min. The detection limit was 0.01 mg/kg dry sediment, and the standard deviation was less than 10%. Lipid content of freeze-dried amphipod tissue samples were tested in duplicate using the micro-gravimetric method of Gardner et al. (1985).

Calculations

Bioconcentration factors (BCF)s were calculated as:

$$BCF = C_t / C_{ow,pw}$$

Where

C_t = NP tissue concentration (ug NP g wet wt^{-1})

$C_{ow,pw}$ = NP overlying or pore water concentration (ul NP L^{-1})

Bioaccumulation factors (BAF)s were calculated as:

$BAF = C_t/C_s$

Where:

C_t = NP tissue concentration (ug NP g dry wt^{-1})

C_s = NP sediment concentration (ug NP g dry wt^{-1})

Biota-sediment accumulation factors (BSAF)s were calculated by:

$BSAF = (C_t/L_t)/(C_s/TOC)$

Where:

C_t = NP tissue concentration (g NP g tissue dry wt^{-1})

L_t = tissue lipid concentration (g lipid g tissue dry wt^{-1})

C_s = NP sediment concentration (ug NP g sediment dry wt^{-1})

TOC = Total organic carbon concentration (ug TOC g sediment dry wt^{-1})

Mass balance determination

Sediment samples were taken from jars with 5ml syringe corers and sediment NP mass balance calculations were based on total sediment jar^{-1} (C+ = 1.3, RE = 1.24, LA = 1.24 kg dry wt) or $beaker^{-1}$ (233 g dry wt). Pore water

samples were not replicated. NP mass balance pore water calculations were based on total pore water jar^{-1} (300 ml) and $beaker^{-1}$ (57 ml). Overlying water samples were taken in 5ml aliquots. Mass balance calculations were based on total overlying water jar^{-1} (200 ml) and $beaker^{-1}$ (775 ml). POM was sampled from the sediment surface in 5ml aliquots. Total POM and DOM were determined by calculating the area of the POM or DOM on the sediment surface as a cylinder ($\prod r^2h$), where h was the height of the organic matter layer; thus POM jar^{-1} was 16.5 g and POM $beaker^{-1}$ was 2.4 g. DOM jar^{-1} was 33.0 ml and DOM $beaker^{-1}$ was 4.75 ml. Amphipods had no detectable ^{14}C in jars. Total amphipod dry wt was determined using the equation: 20 amphipods x average mass of one animal (0.000753 g).

Statistical design

Hypotheses:

1. H0. There are no differences in NP bioaccumulation by amphipods among the three TOC quality treatments, labile, refractory, and non-enriched. This hypothesis was tested for the two species, *G. japonica* and *E. estuarius*, exposed to all 3 TOC quality treatments. If the hypothesis is rejected, then either organic carbon amount (H1.) or quality (H2.) explains the bioavailability of NP.

H1. NP bioaccumulation is directly affected by TOC content of sediments. If this hypothesis is not rejected then TOC quantity explains differences in NP accumulation; but if it is rejected, then TOC quality, explains differences in NP bioaccumulation by amphipods.

H2. Type of organic matter affects bioaccumulation by amphipods. If not rejected, then the type of TOC determines NP bioaccumulation differences among treatments.

2. H0. There are no differences in NP bioaccumulation among species. If this hypothesis is rejected, then organism characteristics (life stage, particle selection, burrowing ability and habitat preference) mediate bioaccumulation.

3. H0. There are no differences in NP uptake rates of *E. estuarius* exposed to the three treatments. This hypothesis was tested by determining whether NP uptake rates varied significantly among the 3 treatments. If rejected, then accumulation kinetics are significantly different among TOC treatments and either the quality or quantity of organic matter affects NP uptake.

Statistics

Statistical comparisons among treatment differences were carried out using a two-factor ANOVA with treatment (LA, RE, and C+) and day (0 and 16) as fixed factors and mean TOC g dry wt^{-1} or %TN g dry wt^{-1} sediment as dependent variables. Significant differences between treatments were tested using a *post hoc* multiple comparisons test (Student-Newman Keul's test, SNK, $p<0.05$). Treatment effects on amphipod dry wt, and tissue lipid concentrations were tested with one factor ANOVAs. ^{14}C-NP concentrations in amphipods were tested with a one-factor nested-ANOVA, with sub-samples nested within replicates and replicates nested within treatments. *G. japonica* body burdens from experiment 1 were compared to body burdens from experiment 2 using Students *t*-test.

RESULTS

Amphipod behavior

E. estuarius remained buried within the sediment throughout the experiment, while *G. japonica* built tubes which were clearly observed on the side of beakers in the upper layer of the sediment and constructed these burrows in the

organic matter layer on the sediment's surface in both labile and refractory treatments. *C. salmonis* males were consistently present in the overlying water while females remained in burrows throughout the exposure period. Mean (+/- SD) amphipod survivorship in the organic matter treatments was 95 +/- 12% in experiment 1 and 83 +/- 4% in experiment 2. The negative control treatment, C-, had a mean (+/- SD) amphipod survivorship of 98.5 +/- 3%. One beaker from LA on day 3 had 100% mortality; the glass pipette delivering air to the beaker had fallen out after the day 1 sampling, presumably leading to lethally low oxygen content in the water and was removed from all analyses. Temperature and salinity were monitored on days 0, 10 and 16 in all beakers and ranged from 16.5-17.3 °C and 27-34 ppt salt in experiment 1, and from 13.9-14.8°C and 28-34 ppt salt in experiment 2. *E. estuarius* progeny were detected in all treatments on day 10 and 16. Progeny were not detected in beakers containing *C. salmonis* or *G. japonica*. A thin layer (1-2 mm) of organic material was observed on sediment surfaces in the LA and RE treatments at experimental initiation and *G. japonica* and *C. salmonis* (female) constructed burrows within this layer. It was not possible to determine the location of *E. estuarius* within the sediments as they did not build burrows, but freely foraged within the sediment.

TOC and TN enrichment

TOC-enriched treatments (RE and LA) resulted in a 1.5 fold greater TOC concentration than measured in C+. There was no statistically significant difference in TOC between LA and RE from day 0 and day 16 (two-way ANOVA, $F_{2,18}$=7.6, p=0.47) Table 6). *Ulva sp.* thad half as much TOC (based on dry wt) compared with the lignin, yet *Ulva sp* had 40 times more TN than the lignin. This was consistant with previous characterizations of *Ulva sp* and lignin (Gunnarsson et al. 1999b). TOC concentrations did not decline significantly over the course of the experiment (Table 6). Day 0 and day 16 TN concentrations varied significantly between treatments (Table 6). Day 0 sedimentary-TN concentration was significantly less than day 16 (two-way ANOVA, $F_{2,18}$=45.6, p<0.001) (experiment 1), but was not significantly different in experiment 2. Day and treatment did not interact significantly (p=0.156).

Table 6: A. TOC and TN content in organic matter (*Ulva sp.* and lignin).
B. TOC and TN content in sediment treatments at start and end of experiment.
Mean, (SD), n=3. * Denotes significantly difference from C+ (two-way ANOVA, SNK multiple comparison, $p = 0.05$. NA= Not available

A.

Organic matter	**% TOC g dry wt^{-1}**	**% TN g dry wt^{-1}**
Labile (*Ulva* sp.)	32.58 (1.22)	4.831 (0.47)
Refractory (Lignins)	57.75 (0.11)	0.122 (0.12)

B.

Treatments	**% TOC g dry wt^{-1}**		**% TN g dry wt^{-1}**	
Experiment 1	Day 0	Day 16	Day 0	Day 16
C+	0.17 (0.01)	0.20 (0.02)	0.016 (0.00)	0.089 (0.01)
RE	0.25 (0.05)*	0.24 (0.06)	0.016 (0.00)	0.050 (0.02)*
LA	0.27 (0.02)*	0.19 (0.02)	0.028 (0.00)*	0.097 (0.03)
Experiment 2	Day 0	Day 16	Day 0	Day 16
C+	NA	0.19 (0.01)	NA	0.011 (0.02)
RE	NA	0.25 (0.05)*	NA	NA
LA	NA	0.22 (0.05)	NA	0.014 (0.01)

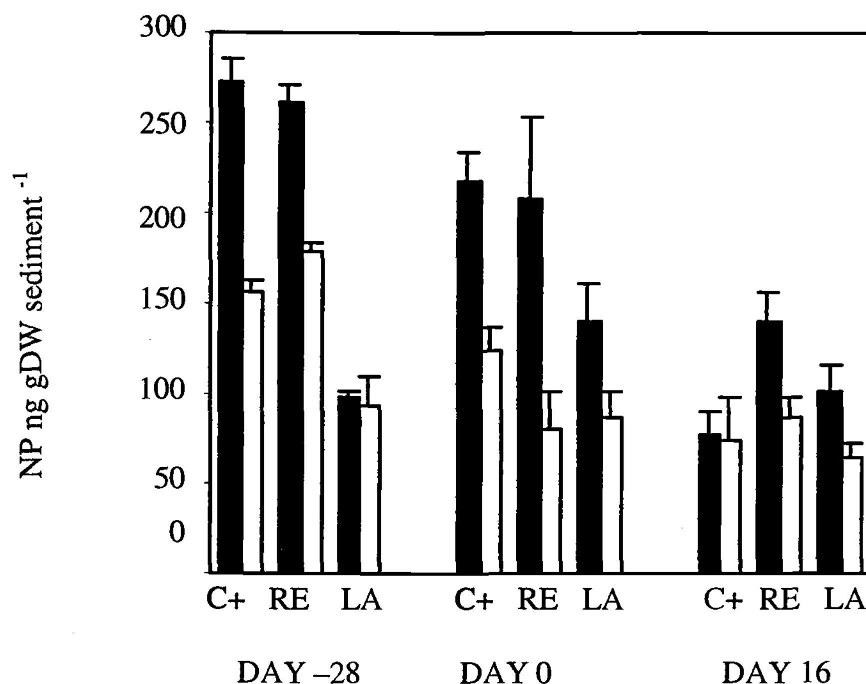
Dry weight and lipid content of amphipods

E. estuarius dry weights and lipid contents did not change significantly from day 0 to day 16. In contrast, the day 16 *G. japonica* dry wt was reduced by 54% in the C+ and by 41% in the RE treatments, but remained unchanged in LA. Similarly, tissue lipid content overtime tended to decrease in all treatments but these decreases were only significantly different from C+ in the RE treatment, where a 47% reduction in tissue lipid was measured. Both male and female *C. salmonis* decreased in dry weight during the experiment, however, the only significant difference in lipids (Students *t*-test, $p = 0.037$) was among *C. salmonis* females which exhibited a 56% increase.

NP partitioning

NP concentration in sediment (both radioactivity and chemistry measurements) declined over the 16 days of experiment 1 (Figure 3). Sediments were spiked on day –28 to achieve a nominal NP concentration of 257 ng NP g dry wt sediment $^{-1}$ in all treatments except C-. Corresponding mean analytical chemistry derived NP concentrations were 157 (C+), 179 (RE), and 94 ng NP g dry wt sediment^{-1} (LA) (Figure 3). Sediment concentration of NP at day -28 averaged 65% of nominal target concentration, which was in agreement with other similar

Figure 3: Sediment NP concentration based on radioactivity (^{14}C) compared to sediment NP concentrations based on analytical chemistry from days –28, 0, and 16. First bar of each pair is ^{14}C based NP concentration (black) and the second bar of each pair is NP concentration based on chemistry (white). Error bars denote standard deviations (n=3). Treatments are C+, Control positive; RE, refractory organic matter (lignins); and LA, labile organic matter (*Ulva sp.*).



sediment spiking experiments (Fay et al. 2000). A mean of 63.5% of LSC derived sedimentary NP was attributed to parent NP from this relationship and was used to adjust pore water, overlying water and tissue concentrations that were derived from ^{14}C detection. NP sediment concentrations based on ^{14}C measurements were consistently larger (36.5%) than chemical analysis based concentrations.

To estimate NP concentrations from ^{14}C measurements a simple linear regression model was fitted to the sediment data:

$$[^{14}C\text{-NP ng g}^{-1}] = 7.043 + (1.512*[\text{NP ng g}^{-1}] \qquad r^2 = 0.66$$

NP pore and overlying water concentrations were greatest in C+ at Day 0 (Table 7). Mass balances based on ^{14}C distribution and measurements were derived for day 0 and day 16 (Table 8). Most radioactivity was detected in the sediment compartment for treatments C+, RE, and in LA. Day 16 sediment % NP declined slightly from day 0 to day 16, except in the LA treatment. C+ pore water % ^{14}C increased, but did not change appreciably in RE and LA treatments.

Accumulation of ^{14}C-NP in amphipods

The accumulation of NP followed the same pattern in both experiments (Figure 4). *E. estuarius* and *G. japonica* accumulated significantly greater concentrations of ^{14}C-NP in C+ than in the RE and LA treatments. Uptake of ^{14}C-NP by *E. estuarius* and *G. japonica* increased significantly in the absence of organic matter enrichment, regardless of TOC quality. The first null hypothesis, of no significant differences in NP accumulation by amphipods based on organic matter addition was rejected.

Table 7: Sediment, overlying water, and pore water NP concentrations from day 0 and day 16 based on ^{14}C measurements (1dpm = 0.183 ng NP). n=3, (SD), na = not available.

Treatment	**Sediment (ng NP gdrywt^{-1})**		**Overlying water (ng NP ml^{-1})**		**Pore water (ng NP ml^{-1})**	
	Day 0	**Day 16**	**Day 0**	**Day 16**	**Day 0**	**Day 16**
C+	123.67* (13.32)	73.67** (24.50)	1.77 (0.28)	2.37 (0.34)	21.75 (na)	11.54 (2.87)
RE	81.33 (19.86)	87.33 (11.02)	1.01* (0.07)	2.24** (0.25)	10.13 (na)	7.73 (1.36)
LA	87.67 (14.64)	64.33 (8.50)	0.94* (0.12)	2.88** (0.59)	15.78 (na)	9.73 (2.57)

* is significantly different than ** ($p<.05$)

Table 8: Mass balance of ^{14}C partitioning on day 0 and day 16. Mass balance was calculated from six matrices: sediment, pore water, overlying water, sediment surface particulate organic matter (POM), sediment surface dissolved organic matter (DOM), and amphipod tissue assuming no loss of ^{14}C. Values are mean percentage of ^{14}C (dpm) recovered in each compartment n=3, (SD)

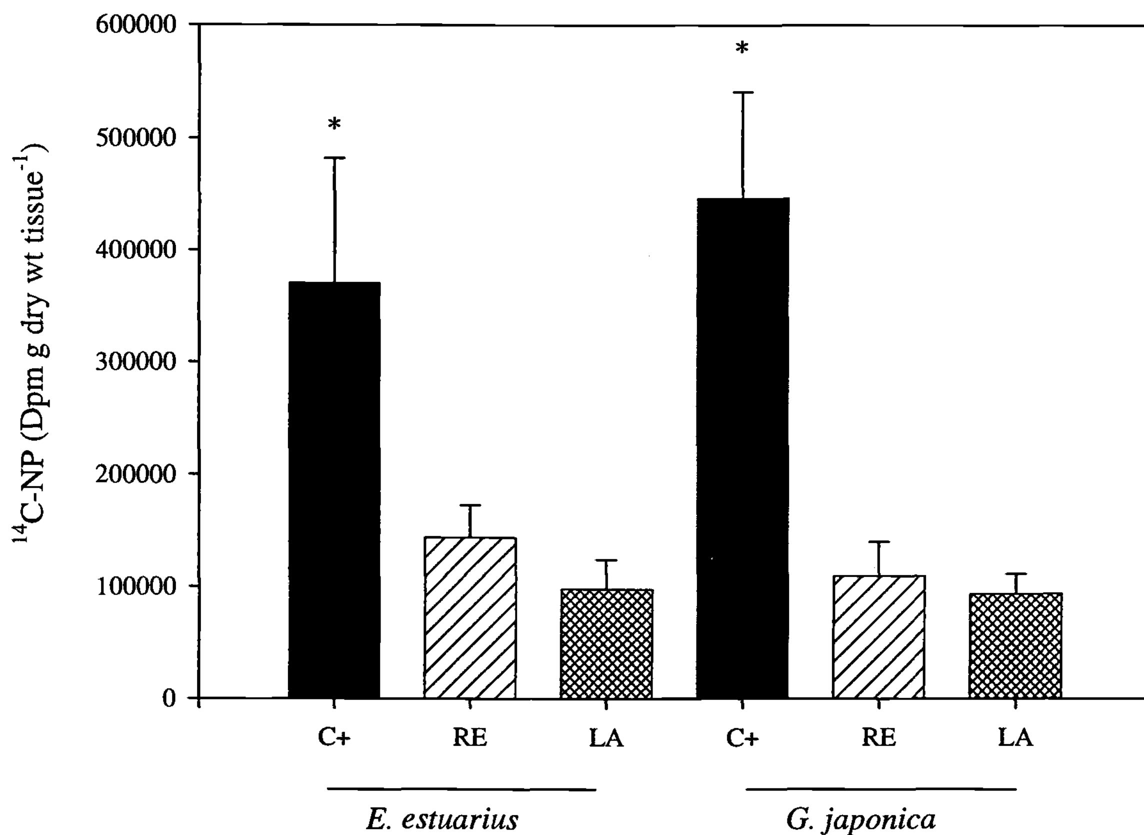
Day 0: jars

Treatment	Sediment	Pore water	Overlying water	Sed Surface POM	Sed Surface DOM
C+	94.1 (5.6)	3.3	2.4 (0.3)	0.4 (<0.1)	2.2 (0.3)
RE	96.8 (21.2)	2.1	0.6 (<0.1)	0.9 (<0.1)	0.4 (<0.1)
LA	72.3 (13.7)	3.0	1.6 (0.1)	21.9 (<0.1)	1.2 (0.1)

Day 16: beakers

Treatment	Sediment	Pore water	Overlying water	Sed Surface POM	Sed Surface DOM	Amphipods
C+	58.1 (9.9)	7.0 (1.2)	19.7 (2.8)	8.2 (6.4)	0.15 (0.03)	6.8 (1.9)
RE	83.6 (1.0)	1.8 (0.3)	7.2 (1.5)	6.2 (1.1)	0.06 (0.02)	1.0 (0.2)
LA	77.4 (2.7)	2.9 (0.7)	11.5 (1.1)	7.2 (3.1)	0.09 (0.01)	0.9 (0.2)

Figure 4: ^{14}C–NP body burdens in *Eohaustorius estuarius* and *Grandidierella japonica* at day 16. Error bars denote standard deviations (n=3). Treatments are C+, Control positive; RE, refractory organic matter (lignins); and LA, labile organic matter (*Ulva sp.*).* = significantly different than other treatments



Because there was no statistically significant difference between organically enriched treatments, RE and LA, in respect to NP accumulation, these results provided a conclusive test for the alternative null hypotheses, i.e., type of organic matter (labile or refractory) enrichment had no effect on accumulation, but amount of organic matter (TOC content) had a significant effect. NP accumulation by

amphipods decreased compared to un-amended sediments, in response to a 1.5-fold increase in TOC. *G. japonica* (juveniles) and *C. salmonis* (adult males and females) from experiment 1 were exposed to the C+ treatment only, and sampled at day 16. There was no statistically significant difference in NP body burdens among *G. japonica* from experiments 1 and 2 (t-test, 4_{df}, $p = 0.086$). *C. salmonis* females had significantly greater body burdens of NP than *C. salmonis* males ($F_{1,6} = 20.475$, $p = 0.004$,1 factor nested ANOVA). In experiment 2, the same pattern of accumulation was observed as in experiment 1, *G. japonica* body burdens of ^{14}C-NP increased significantly in the absence of organic matter enrichment. The greatest NP body burdens in *G. japonica* were from the C+ treatment. Accumulation factors (BCFs, BAFs, and BSAFs) were determined for each species and indicated differences between species (Table 9). The second null hypothesis of no significant differences in NP accumulation between species was rejected i.e., feeding strategy and behavior differences between species potentially influenced accumulation of NP. *E. estuarius* accumulated the greatest amount of ^{14}C-NP compared with *G. japonica* and *C. salmonis*. BSAFs in LA treatments were not significantly different than RE treatments within species. Mean *G. japonica* BSAFs from enriched treatments were approximately half of *E. estuarius* BSAFs. Female *C. salmonis* accumulated greater concentrations of ^{14}C-NP than males.

Table 9: Day 16 NP accumulation factors in *E. estuarius*, *G. japonica*, and *C. salmonis*.

Species	**%Lipid[a] mean (std)**	**[NP] tissue[b] mean (std)**	**BCF ow[c] mean (std)**	**BCF pw[c] mean (std)**	**BCF ow[d] lipid mean (std)**	**BCF pw[d] lipid mean (std)**	**BAF[e] mean (std)**	**BSAF[f] mean (std)**
E. estuarius								
C+	4.65(0.86)	67.9(20.3)	3744(883)	770(182)	73.8(4.8)	15.2(1.0)	895(324)	33.9(4.9)
RE	5.59(0.76)	26.3(5.3)	1542(258)	448(75)	27.6(4.1)	8.0(1.2)	200(32)	8.7(2.9)
LA	5.96(2.11)	17.9 (4.8)	827 (241)	246(71)	15.9(9.6)	4.7(2.8)	225 (124)	8.5(7.2)
G. japonica								
C+	11.23(0.92)	81.7(17.3)	4505(841)	926(173)	38.8(5.4)	8.0(1.1)	1074(308)	17.3(4.2)
RE	7.23 (2.86)	20.0 (5.7)	1170(310)	340(90)	17.8(8.1)	5.2(2.4)	149(25)	5.8(2.5)
LA	9.49 (0.57)	17.1 (3.3)	791 (59)	235(18)	7.9(1.5)	2.3(0.4)	208(73)	4.7(2.1)

Table 9: (Continued)

C. salmonis								
Male C+	6.57 (2.37)	20.5(13.4)	1129(648)	232 (133)	15.6(7.8)	3.2(1.6)	253 (107)	7.1(3.7)
Female C+	11.81(2.78)	58.5(18.9)	3226(808)	663 (166)	35.1(20.3	7.2(4.2)	743 (112)	16.0(9.7)

a % lipid gdry wt tissue^{-1} day 16
b ug NP gdry wt tissue^{-1};determined from radioactive measurements day 16
c BCF= $C_t/C_{ow,pw}$; C_t=ng NP gw.w. tissue^{-1}; $C_{ow,pw}$= ug NP L^{-1}
d BCF= (C_t/%lipid) /$C_{ow,pw}$; %lipid = g lipid gdry wt tissue^{-1}
e BAF= C_t/C_s;C_t = ng NP gdry wt tissue^{-1}; C_s=ng NP g dry wt sediment^{-1}
f BSAF=(C_t/lipid)/(C_s/TOC); lipid = g lipid gdry wt tissue^{-1}; TOC= g TOC gdry wt sediment^{-1}

^{14}C-NP accumulation rate

Kinetic uptake data was obtained from sampling *E. estuarius* from experiment 1 on days 0, 1, 3, 6, 10, and 16 (Figure 5, Table 10). *E. estuarius* ^{14}C-NP body residues reached steady state during the experiment and followed classic first-order kinetics. The rate of ^{14}C-NP uptake was significantly greater in the C+ treatment compared with the RE and LA treatments (Table 8). The null hypothesis of no significant difference between treatments was rejected and TOC quantity, but TOC quality did have a significant effect on accumulation rate. Elimination rates were modeled from uptake curves and did not differ significantly between treatments (Table 10).

Figure 5: ^{14}C-NP accumulation by *Eohaustorius estuarius* from spiked sediments. Modeled line of best fit determined using an iterative, nonlinear, lest-squares curve fitting technique (Sigmastat®, SPSS Inc.). N=3, error bars denote standard deviation.

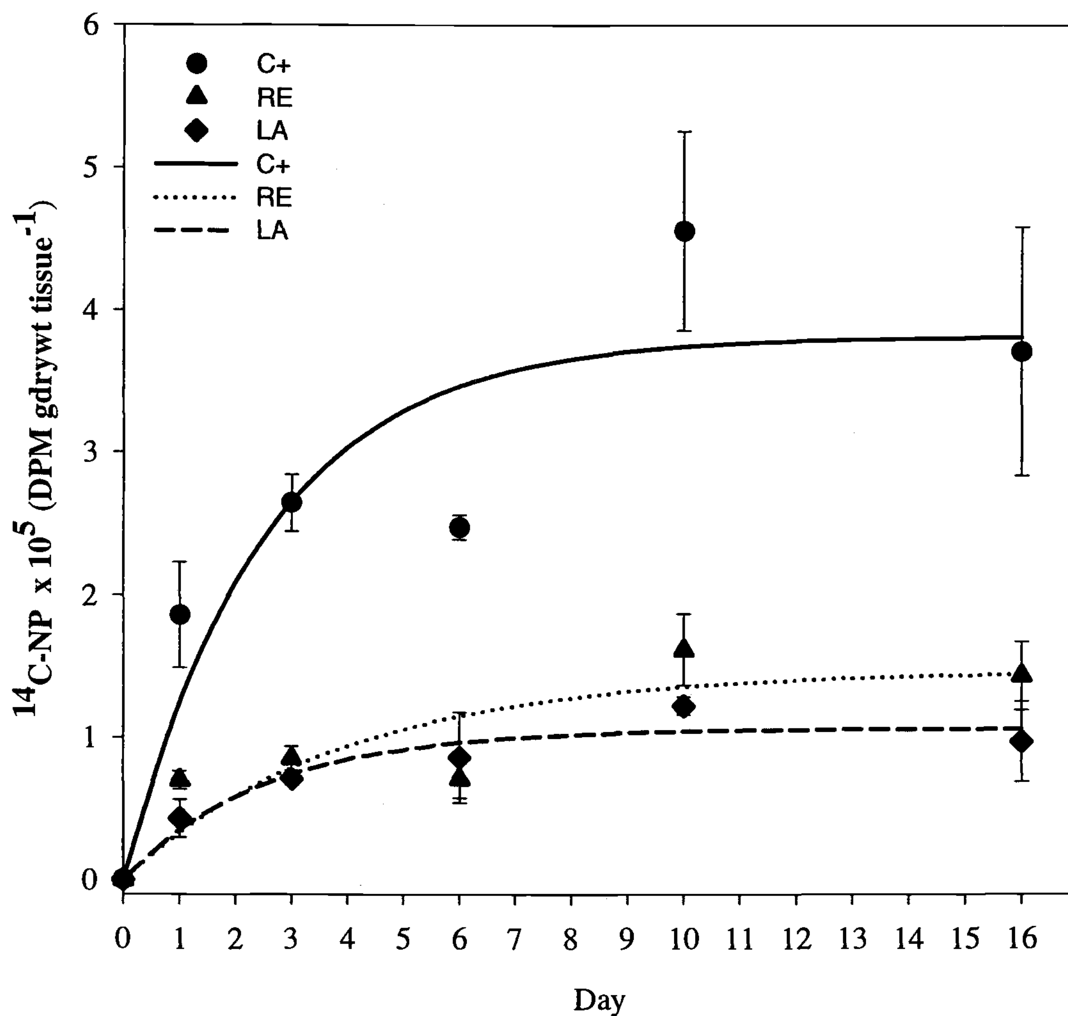


Table 10: Uptake and depuration kinetics of ^{14}C-NP by *Eohaustorius estuarius*

Treatment	Uptake rate[1] K_s (SE)	Depuration rate[1] K_2 (SE)
C+	355.91 (147.00)	0.40 (0.20)
RE	48.96 (23.05) *	0.25 (0.16)
LA	76.06 (19.42)*	0.40 (0.30)

[1] dpm g DW tissue (dpm g DW sediment)$^{-1}$ day^{-1}, determined from line of best fit using the iterative, nonlinear, least squares curve fitting technique (Sigmastat®, SPSS Inc.). * significant difference from C+ (Student's *t*-test, $p<0.05$)

DISCUSSION

Organic matter enrichment and accumulation of ^{14}C-NP

The significantly lower NP concentrations in *E. estuarius* and *G. japonica* from organically-enriched sediment compared to non-enriched sediment was in accordance with equilibrium partitioning theory, which predicts that bioaccumulation is inversely proportional to sediment TOC concentration(Lake et al. 1990). Amending sediment by 1.5 times its original TOC content increased sorption of NP to sediment and concurrently decreased NP bioavailability to amphipods. This is in accordance with our mass balance measurements which show

that a greater percentage of ^{14}C-NP partitioned from sediment to water in the treatment C+ compared with the LA and RE treatments (Table 8).

Surprisingly, in the TOC-amended sediment, amphipod tissue residues were equal in the labile and refractory treatments. We originally hypothesized that feeding preferences by amphipods for labile, nutritious *Ulva sp.* would cause an increase in NP accumulation compared to refractory (lignin) associated NP. *Eohaustorius estuarius* and *G. japonica* preferentially ingested labile material, as is evident from their greater day 16 dry weights and lipid contents, and from qualitative observations of algal material in their intestinal tracts. We conclude that dietary uptake of NP is of minor importance compared with aqueous routes of uptake because no differences in tissue residues were observed in labile treatments.

This result is different to that observed by (Gunnarsson et al. 1999a) where TOC quality accounted for differences in accumulation of a PCB, 3,3'4,4'-^{14}C-tetrachorobiphenyl, by infaunal brittle stars, *Amphiura filiformis*. In that study, brittle stars accumulated 15 times the sediment concentration of PCB in *Ulva lactuca* amended sediment, while brittle stars in lignin-amended sediments accumulated 2.7 times the sediment concentration of PCB. Based on the high K_{ow}s (~6.5) of PCBs compared with NP (K_{ow} ~ 4.48), it is likely that a considerably greater proportion of the total PCB was associated with sediments compared to the proportion of NP associated with the sediment in our experiments.

Differences between amphipod species

We detected species-specific differences in NP accumulation (Table 9) even when tissue concentrations were normalized to lipid content and sediment was normalized to TOC content. *Eohaustorius estuarius* accumulated the greatest NP concentrations. This species does not construct burrows but remains beneath the sediment surface, essentially swimming in pore water. In contrast, *G. japonica* and *C. salmonis* construct permanent burrows and ventilate them using overlying water. Thus *E. estuarius* was more likely exposed to pore water concentrations and therefore accumulated higher NP concentrations than *G. japonica* and *C. salmonis*. *C. salmonis* were exposed to the C+ treatment only and were analyzed separately based on sex. Sex differences in accumulation by *C. salmonis* have not previously been examined.

Differences were likely a result of organism location in the beakers due to sex-specific behaviors. Because females remained in their burrows, while males remained on the sediment surface during the experiment, females were potentially exposed to pore water to a greater extent than males.

BCFs, BAFs and BSAFs

BCFs and BSAFs are often used to predict the bioaccumulation potential of a contaminant for water (BCF) or sediment (BAFs and BSAFs) into aquatic biota. In

the present study pore water BCFs ranged from 232 to 926, BAFs from 149 to 895, and BSAFs from 4.7 to 33.9 (Table 9). Lipid normalized BCFs did not reduce variability between treatments or species suggesting that factors other than partitioning are important in accumulation of NP. These unusually large BSAFs for NP are much greater than a previously reported BSAF of 0.93 for *Ampelisca abdita*, an East Coast benthic amphipod (Fay et al. 2000) and are some of the largest BSAFs values ever reported for organic contaminants. However, high BSAFs for amphipods are not without precedent. In 10-day spiked sediment bioassays at our laboratory the polyaromatic hydrocarbon, fluoranthene exhibited BSAFs up to 15.19 for *Rhepoxinius abronius*, an estuarine amphipod (Boese et al. 1999). These authors speculated that the uncommonly large BSAFs of *R. abronius* were in part due to their selective feeding behavior and probable lack of ability to metabolize contaminants. Species differences in feeding strategy, burrowing behavior, and metabolic activity are likely factors influencing accumulation of NP. Using sandy sediments that have very low total organic carbon content may also explain in part the large accumulation factors. Much of the spiked NP may have been more available for uptake in sandy sediments than in muddier sediments. Additionally, spiked sediment bioaccumulation assays often show greater accumulation factors than bioassays using field-collected sediments, which may be a result of aging and of the complex sediment matrix in undisturbed sediments (Landrum and Eadie. 1992). The high NP accumulation ability of amphipods indicates that the potential for food web transfer to higher trophic levels exists.

Implications of food chain transfer

Amphipod accumulation data can provide a rough estimate of the potential NP dose transferred to fish. Food chain models of aquatic ecosystems have demonstrated that predation on aquatic invertebrates can lead to significant food chain transfer of sediment associated hydrophobic organic contaminants to higher trophic levels (Thomann et al. 1992). Because amphipod populations are significant prey items to predators in estuaries, it is not only important to determine direct effects on populations; but in addition, to determine the extent to which amphipods could serve as vectors of sediment-associated contaminants to predators. *Grandidierella japonica* and especially *C. salmonis* are important food items for foraging juvenile salmonids in Pacific Northwest estuaries (Shreffler et al. 1992; McCabe et al. 1983). Using NP contaminated sediment concentrations from the largest values reported for a U.S. estuary (14 μg g dry wt sediment^{-1}) (Ferguson et al. 2001), our experimentally derived amphipod BSAF, lipid, and TOC values, estimated fish feeding rates (5% of its body weight d^{-1}) and assuming no metabolism of NP by amphipods, rough dose estimates were generated for each amphipod species according to treatment (Table 9). According to these calculations amphipods could serve as NP vectors to higher trophic levels at doses sufficient to induce biomarkers of endocrine disruption (Table 11). Further research is required to elucidate the impacts of NP to estuarine food chains through trophic transfer.

Summary

These experiments demonstrated that estuarine, infaunal amphipods accumulated significant NP concentrations, particularly, in unamended sediments, suggesting that aqueous routes of exposure represented the dominant mode of uptake, compared with feeding. The non burrow-constructing amphipod, *E. estuarius*, had the largest BSAFs, which was probably a result of their greater contact with pore-water.

Table 11: Predicted NP dose transfer per day to fish feeding on contaminated amphipods

Species	Treatment	Dose* ug NP/ g fish w.w. /day
E. estuarius	C+	1250
	labile	351
	refractory	298
G. japonica	C+	1480
	labile	363
	refractory	257
C. salmonis		
Male	C+	392
Female	C+	1290

* NP dose calculated from BSAF = $[(C_{tissue}/\%lipid)/C_{sediment}/\%TOC]$, [NP] sediment = 14 ug/g (highest [NP] detected in U.S. estuaries reference), assuming fish feeding rate for a 10 g wet weight fish of 5% of their body weight day^{-1}. w.w. = wet weight.

Sex differences in accumulation were also noted for *C. salmonis*. Further investigations are needed to determine if there are differences in the rate of accumulation of organic contaminants between the sexes of amphipod species. The high BSAFs reported in this paper need to be validated, but if accurate, they suggest a strong potential for amphipods to bioaccumulate NP and therefore serve as potential vectors to fish and birds. Field investigations at highly contaminated sites are recommended to determine benthic biota body burdens of NP. Continued non-regulated use of NPEs by the private and public sector in the USA could pose a potential threat to U.S. aquatic ecosystems.

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CHAPTER 4:

VITELLOGENIN INDUCTION IN CHINOOK SALMON FRY (*ONCORHYNCHUS TSHAWYTSCHA*) FROM DIETARY AND WATERBORNE EXPOSURES TO 4-NONYLPHENOL

by
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ABSTRACT

It remains unclear whether or not NP-contaminated amphipods could serve as vectors of NP to higher trophic levels. Amphipods are important prey items for endangered populations of juvenile chinook salmon in Pacific Northwest estuaries. Direct comparisons were made between chinook fry exposed to NP via the diet and fry exposed to a range of NP concentrations via water. Amphipods (*Eohaustorius estuarius*), were exposed to NP (60 μg/L) for 96 h, then fed (~7 g wet weight per day) to hatchery-raised salmon fry for 5 days. In parallel, fish were exposed to one of 4 concentrations of unlabeled NP or [^{14}C]-NP in the water at concentrations ranging from 0-240 μg/l for 5 days. NP was quantified using liquid scintillation counting of [^{14}C]-NP. Following exposure, salmon fry plasma vitellogenin (Vtg), a

standard biomarker of endocrine disruption, was measured using Enzyme Linked Immunosorbent Assay (ELISA) techniques. Results showed that water treatments of 60 and 240 µg/L NP induced high levels of Vtg in salmon fry. Thirtythree-percent of fry fed contaminated amphipods on a daily basis, had detectable Vtg, while control fry had no detectable levels of Vtg. Contaminated estuarine amphipods are potential vectors of NP to chinook salmon fry.

INTRODUCTION

Non-ionic surfactants, alkylphenol polyethoxylates (APEs), are used ubiquitously in domestic and industrial products. They are relatively non toxic, yet degrade to more persistent, toxic compounds via biotic and abiotic processes (Giger et al. 1984). One of these degradation products, 4-nonlphenol (NP) is toxic to fish and invertebrates in the part per billion range. To understand the potential for uptake by, and effects on fish, the pathways of exposure need to be identified and the level of transfer and possible accumulation estimated. NPs are hydrophobic (Log $K_{ow} = 4.48$) (Ahel et al. 1993), Log $K_{oc} = 5.39$ (Ferguson et al. 2001), and therefore they associate with particulate organic matter in aquatic systems, ultimately residing in sediments. Previous research has demonstrated that NP bioaccumulates in invertebrates from spiked sediments (Fay et al. 2000; Hecht et al. in review), yet there are no data presently available concerning the potential for

NP to transfer from invertebrates to fish. This is an important gap in knowledge to fill, particularly because other hydrophobic organic compounds (PCBs, DDT, DDE and PAHs) have been shown to accumulate through aquatic food chains (Froese et al. 1998; Leatherland and Sonstegard, 1982; Wu et al. 2001).

It is now evident that anthropogenic and naturally derived chemicals can exhibit estrogenic and androgenic activity in aquatic organisms. Questions however remain concerning the ultimate effects on wildlife populations of exposure to these hormonally active chemicals. Fish reproductive systems are complex and mediated by several hormones, and they are susceptible to disruption at many stages (Kime, 1999). The female sex hormone, estrogen or 17*B*-estradiol (E_2), modulates female reproductive and developmental processes including sexual differentiation and sexual maturation. Nonylphenol, in particular, mimics estrogen-mediated processes within vertebrates and invertebrates including induction of the egg yolk protein, vitellogenin (Vtg), and the egg shell *zona radiata* proteins (Zrp) (Arukwe et al. 1997). Nonylphenol binding to, and activation of, the estrogen receptor mirrored that of the natural ligand, 17B-estradiol (E_2) (White et al. 1994; Shelby et al. 1996). Male fish exposed to estrogenic compounds, such as NP, produce the estrogen-mediated proteins, Vtg and Zrp. Both types of proteins have shown promise as biomarkers, and Vtg in particular has been extensively used to detect endocrine disruption in juvenile and adult male fish.

Salmonids have the capability to metabolize NP (Thibaut et al. 1999; 1998; Coldham et al. 1998) and thus bioaccumulation by fishes may be less important in the mediation of internal exposure at the site of action than the immediate impacts following receipt of a dose of NP from water or the diet. For this reason, we did not quantify NP in fish tissue, but chose to concentrate on a standard biomarker of exposure to endocrine disruption, salmon fry plasma vitellogenin induction (Vtg). An immediate concern is the possibility that NP could ascend benthic-pelagic food chains and ultimately impact the endocrine systems of fish.

Our previous research has demonstrated that benthic estuarine amphipods accumulated significant levels of NP from spiked sediments (Hecht et al. in review [Chapter 3]). The potential of NP to transfer to higher trophic levels however, remains unclear. This is of particular interest in Pacific Northwest estuaries because amphipods are important prey items for juvenile chinook salmon (McCabe et al. 1983; Shreffler et al. 1992). Chinook salmon fry may be affected by NP found in estuarine sediments because of their use of estuaries as rearing grounds, prior to entering the ocean. The transfer of NP from sediments to benthic amphipods to their salmon fry predators remains an ecologically relevant exposure pathway that has not been previously investigated.

Here, we investigate the trophic transfer of NP to chinook salmon fry via a diet of contaminated amphipods. Additionally, we explore the potential for NP to

disrupt the endocrine systems of chinook salmon fry, quantified by plasma vitellogenin expression. Water and dietary exposures of chinook salmon hatchery fry to NP were determined following 5 day exposures. The objectives of this study were to determine if there was induction of vitellogenin in Chinook salmon fry following this daily consumption of NP contaminated amphipods and to determine dose-response induction of vitellogenin in response to the NP water concentrations.

MATERIALS AND METHODS

Experimental design

A randomized block design with eight treatments (10 replicates per treatment) was used to determine the induction of vitellogenin from dietary and waterborne exposures of NP to juvenile chinook salmon during 5 day of exposure (Table 12). Water was replenished in each replicate at 12 h intervals (static renewal) by siphoning (manual glass pipette suction device). Following 5 days of dietary or waterborne exposures, fry were weighed, sexed, and plasma extracted for determination of vitellogenin and total protein.

Salmon fry

Five month old chinook salmon, *Oncorhynchus tshawytscha,* fry (mean wet weight (WW) = 7 grams) were obtained from the Oregon Department of Fish and Wildlife's Salmon River Hatchery (Otis, Oregon, U.S.A). Fry were transported in 15° C hatchery water to the Oregon State University Hatfield Marine Science Center (Newport, Oregon) in two 60 gallon drums that were placed in large plastic bags filled with ice to maintain temperature and under constant aeration. In the laboratory, fry were transferred immediately to two 15 gallon (metric) glass aquaria that were located in a 15° C temperature bath under constant aeration. Filtered marine water was dripped into each aquarium at 5 ml/ h until 15 ppt salinity was attained. Aquaria were covered with black plastic to minimize fluorescent light penetration, and temperature, salinity, and dissolved oxygen were monitored daily. Fish were acclimated to these conditions for 1 week and fed live amphipods , *Eohaustorius estuarius* (~200 mg W.W.)/ fish , once per day until test initiation.

Table 12: Treatment Type and Description

Treatment	Exposure type	Description
NP Contaminated Amphipods	Diet	Fry fed NP contaminated amphipods once daily
Amphipods	Diet	Fry fed uncontaminated amphipods once daily
Control	Aqueous	No NP, No carrier, negative control
NP water	Aqueous	Fish exposed to 5, 20, 60, or 240 μg/L
17*B*-estradiol (E2)	IP injection	Positive control; fry injected with 5 mg/kg E2 in peanut oil at experimental initiation and 48 h later.

Fry pre-experimental feeding rates

Prior to test initiation, twenty fry were selected and transferred from holding aquaria to glass jars (1 fry/ jar), placed in exposure tables equipped with constant temperature water baths, covered with black plastic sheets, and fed aliquots of uncontaminated, live amphipods daily for five days. Previous pilot tests indicated that fry could consume a maximum of 400 mg WW amphipod tissue in a 45 min feeding period. Daily feeding rates were determined for each fry by blotting dry, live amphipods with paper towels, weighing them (+/- 0.1 mg WW) into 500

mg WW aliquots before feeding, then sieving (0.25 mm sieve) and weighing the amphipods that were not eaten after a 45 minute time period. After 45 minutes, fry were transferred to jars containing clean water (filtered 15 ppt salinity sea water), and remaining uneaten amphipods were sieved (0.25 mm sieve) from the chambers, weighed, and discarded. Mean feeding rates (%) were calculated by dividing amphipod weight consumed (mg WW) by fry weight (mg WW) times 100.

Amphipods

E. estuarius were collected from a high intertidal, sandy site of Yaquina Bay (Newport, Oregon). Surface sediments (1-10 cm) were sieved (1.4 mm) and retained adult *E. estuarius* were transported to the laboratory where they were maintained for 48 h in culture tubs with sediment (sieved 0.25 mm) from the collection site in filtered seawater (15°C, and 15 ppt salinity) prior to experimental use. At experimental initiation amphipods were sieved (1 mm sieve) from their holding sediments and placed into shallow glass trays containing filtered seawater (15 ppt salinity, 15°C) to remove any sediment. Amphipods were then gently sieved and brushed onto paper towels with a small paint brush and weighed into 3000 mg WW aliquots.

Treatment water preparation

A stock solution of 4-nonylphenol (technical grade, Sigma-Aldridge-Fluka, St. Louis, MO, USA) which was used as unlabeled spiking material was prepared by dilution with acetone (reagent grade) to attain a concentration of 720 mg/L. 4-nonylphenol- [ring U-^{14}C] (Biodynamics Radiochemicals, Manor Key, UK; 95.7% 4-nonylphenol, 3.96% o-nonylphenol [determined by radio HPLC], specific activity of 76.6 Ci/mol) was diluted with methanol (reagent grade). These two stock solutions were combined to prepare corresponding water treatments (0, 5, 20, 60, and 240 µg/L total NP) for amphipod and fish exposures. Within each treatment, unlabeled batches were prepared by micro-pipetting the prescribed quantity of unlabeled NP stock solution into a beaker of clean, filtered sea water (15° C, 15 ppt salinity). These solutions were mixed on a magnetic stirrer for 10 minutes, and poured into 20-50 L volumes (depending on treatment) of clean, filtered sea water (15° C, 15 ppt salinity) in 80 L polycarbonate containers. These were stirred for 10 minutes with a large plastic spatula. Jars were then filled to the 3 L mark, the fish were replaced (see below) and returned to their designated place on the exposure table.

After five replicates were renewed with unlabeled NP concentrations, ^{14}C-NP was added to the remaining treatment water to achieve a concentration of 100 DPM/ml and mixed for 10 min with a designated plastic spatula. The labeled

treatment water was then added to the 5 corresponding replicates in the same procedure as used for the unlabeled renewal. During renewals, fry were netted (1 mm mesh), placed into a separate jar containing 3 L corresponding treatment water, 2.4 L of old treatment water was carefully siphoned out (0.6 L remained) and replaced with 2.4 L of fresh treatment water. Fry were then placed back into jars and covered with a glass lid and aeration pipettes added through the glass lid. The highest nominal carrier solvent concentrations in replicates (jars with one fry) were 83.3 ppm (0.0083%), acetone, and 3.8 ppm (0.00038%), methanol.

The use of radiolabeled NP allowed for the quantification of NP in water and amphipod tissues using liquid scintillation counting. Within each NP treatment, five replicates were exposed to unlabeled NP and five replicates were exposed to a mixture, containing unlabeled NP and radio-labeled ^{14}C-NP.

Water exposures

Fry were randomly selected from holding aquaria, weighed (mg WW) following 2 min incubation periods in tricane methanesulfonate (MS222) to immobilize them, and placed into 3.78 L glass jars (1 fry/jar), that contained 3 L treatment water (15 ppt salt, 15° C) and a glass pipette that delivered filtered air through a hole in the glass lid. ^{14}C-NP aqueous concentrations were measured using

liquid scintillation counting in treatment batches before renewal, six hours following renewal, and immediately before exchange of water. Fry were exposed for 5 days.

Amphipod dosing

NP contaminated amphipods were prepared by exposing them at the 96h LC_{15} concentration of 60 µg/L (Hecht and Boese, 2002) for 96 hrs prior to feeding fry. Pilot tests indicated a 96 hr exposure was sufficient to reach steady-state for NP in amphipods from aqueous exposures. Amphipods to be used for NP dosing were sieved from tubs, divided in 3000 mg WW aliquots following a brief drying period (2 mins) on towel paper, weighed, and gently brushed into 3.78 L jars containing 3 L filtered marine water (20 ppt salinity, 15° C) at 60 µg/L NP. The jars were placed on exposure tables and aerated. Treatment water (60 µg/L NP) was replenished every 12 h in these chambers, following the spiking procedure above. Radioactivity was measured in 5 ml aliquots before treatment water was renewed, and at 6 h following each renewal. Amphipod chambers were set up each day for five days to insure equal NP-dosing of amphipods before being fed to fry. On each of these days, 4 chambers were prepared. One of these was dosed with ^{14}C-NP and unlabeled NP, one with unlabeled NP, and the other two chambers containing no

NP, were used as controls. After 96 h, amphipods were sieved from chambers and divided into 500 mg WW aliquots which were immediately fed to fry.

Dietary Exposures

Fry were randomly assigned to the contaminated amphipod treatment group or the to the uncontaminated amphipod treatment group (Control). As with the water exposures individual fry were exposed in 3.78 L glass jars. Prior to feeding on amphipods, water was completely exchanged in each chamber. At the same time (~10:00 am) each day for 5 days, amphipods were fed to each of the 20 fry in 500 mg WW aliquots for 45 minutes. Following the 45 minute feeding period, water was immediately exchanged to limit the potential for aqueous exposure to NP that may have desorbed from contaminated amphipods. In the contaminated amphipod treatment, five fish were exposed to a mixture of ^{14}C-NP and unlabeled NP, and the other five were exposed to unlabeled NP only. ^{14}C was measured in those jars that had fry being fed ^{14}C-NP contaminated amphipods (n=5) from 5 ml aliquots, using liquid scintillation counting.

Positive controls

To determine maximal Vtg induction, positive control fish were injected with 17*B*-estradiol (E_2) following the method of Donohoe and Curtis (1996). At experiment initiation, ten fry were randomly selected from holding aquaria, and transferred to a glass jar containing the fish anesthizing agent MS-222 (100 µg/L) for 1 minute. Anesthetized fry were then weighed, and injected with 5 mg kg^{-1} E_2 in peanut oil. Each fry received a second E_2 injection 48 h later, following the same procedure.

Chemical analyses

Radioactivity of water and amphipod tissue samples were determined by liquid scintillation counting (LSC) using a Packard Instruments Analyzer (2000 CA Tri-Carb) with corrections made for quenching and background. Freeze-dried amphipod tissue samples were weighed (± 0.001 mg) and then pulverized using a glass mortar and pestle. The resulting powder was extracted with 5 ml each of acetone and hexane. The solvent mixture was pipetted into each sample, mixed with a vortex mixer (1 min) then sonicated (1 min) using a Fisher Sonic-Dismembrator (Model 300). The sonicate was centrifuged (2000 rpm, 10 min), and the supernatant poured into a scintillation vial and counted (10 min) using 10 mls

Ultima Gold (Packard Instruments) as the scintillation cocktail. Radioactive analysis of water samples was accomplished by adding samples (5ml) directly to LSC vials containing 15 mls of Ulima Gold XR which were counted for10 minutes. Radioactivity was expressed as DPM/g dry wt tissue and DPM/ml (H_2O). Radioactivity was converted to NP µg/g dry wt tissue, based on the specific activity of ^{14}C-NP in the original mixture of unlabeled and ^{14}C-NP solution.

At experiment termination, one fry at a time was rinsed with filtered, 15 ppt salt water and placed into a glass chamber containing 300 µg/L MS-222 for two minutes to euthanize them, and weighed (+/- 0.001 g). Blood was then extracted from the caudal vein/artery with a 1 cc syringe and blood was immediately placed into a 1.5 ml microfuge tube containing 1 ml EDTA, 50 K.I.U. aprotinin /ml (trypsin inhibitory unit), and 45 units sodium heparin, to prevent proteolytic breakdown of vitellogenin. Microfuge tubes were placed immediately on ice and centrifuged (16 tubes at a time) at 2000 *g* for 10 minutes. The plasma fraction was removed and stored at -80 ˚C. Fry were sexed by examination of gonads at necropsy, and carcasses were frozen at -80 ˚C.

Calculation of bioconcentration factors (BCF) in amphipods:

$BCF = C_t/C_w$ where:

C_t = [NP] µg/g WW tissue based on radioactivity

C_w = [NP] µg/ml water based on radioactivity

Vitellogenin (Vtg) quantification (ELISA method)

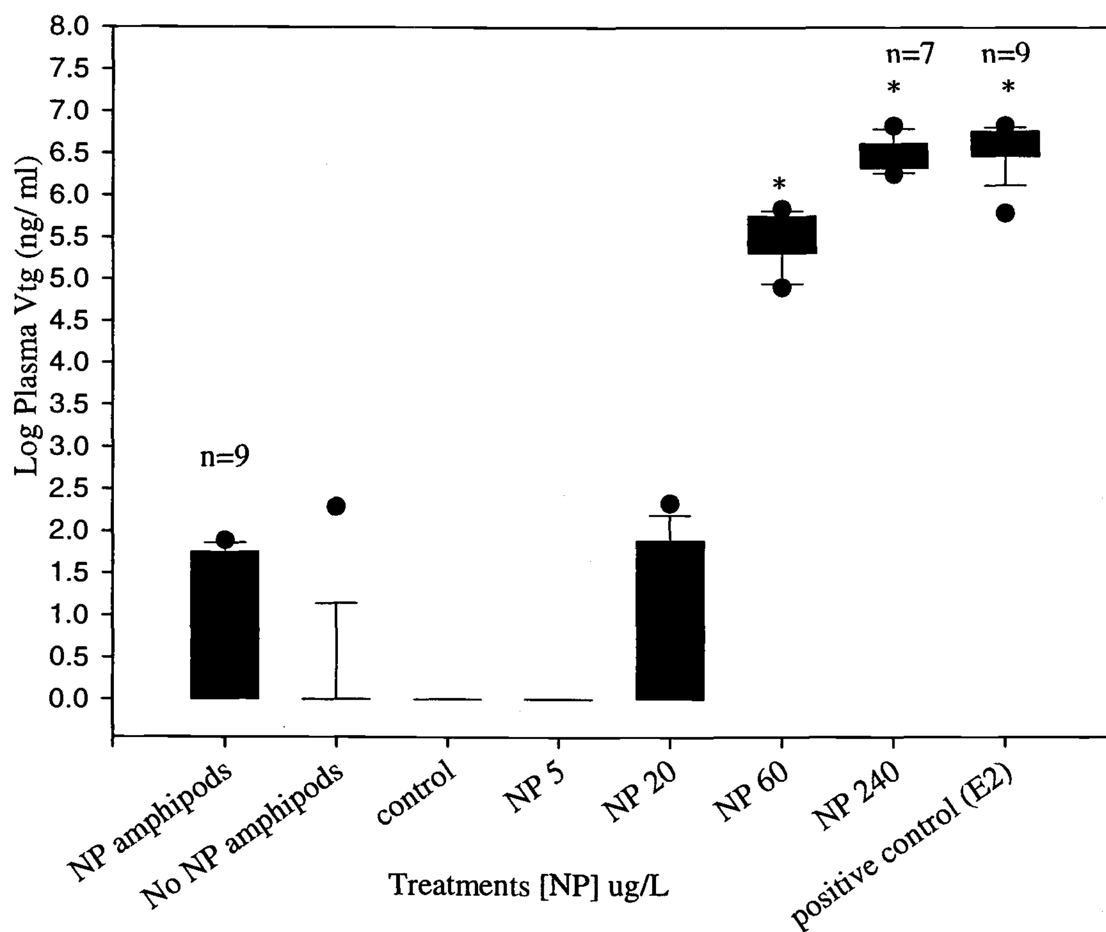
Vitellogenin quantification in plasma was undertaken as described by Donohoe and Curtis (1996) and modified by Shilling and Williams (2000). We used purified polyclonal immunoglobulin G antibodies (427 ng/ml) raised in rabbits against Chum salmon Vtg (1:1500 dilution) as our primary antibody (D. Buhler, Oregon State University, originally from A. Hara, Hokkaido University, Japan). The limit of detection was determined to be 40 ng Vtg/ml sample with intra-assay and inter-assay variability of less than 20%. Plasma Vtg was normalized to protein concentration determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard. Five replicates representing five individual fry per treatment were analyzed for protein content. Significant induction of Vtg between treatment groups was determined by one-way ANOVA based on ranks followed by a Student-Newman Kuel (SNK) multiple comparison test.

RESULTS

Vitellogenin induction

Vitellogenin induction in chinook salmon fry followed a dose-response relationship to aqueous NP exposures (Figure 6.) Significant induction occurred in the 60 and 240 µg / L NP water treatments and in the positive control fish injected with 17*B*-estradiol (E_2). The positive control and 240 µg /L NP treatment levels were not statistically different (SNK $p< 0.05$). No detectable Vtg was observed in the negative control or within the 5 µg /L water treatments. While not statistically significant, Vtg induction was detected in 3 of the 10 fry fed NP- contaminated amphipods (Table 13). No Vtg was detected in any control fry with the exception of one fish fed uncontaminated amphipods which exhibited an unusually high plasma Vtg level of 195 ng/ml. There was no apparent correlation between sex, dose, or size of fry in relation to Vtg levels (Table 13). Normalization of plasma vitellogenin to plasma protein content did not alter trends (data not shown). Insufficient blood volume from poor extraction caused a loss of two samples for plasma Vtg analysis (one fry fed contaminated amphipods and one fry exposed to 60 µg/L NP).

Figure 6. Box and whisker plot of vitellogenin [ng/L] in chinook salmon fry plasma following five day nonylphenol dietary and aqueous exposures. Lower boundary of each box indicates 25th percentile, a line within the box marks the median, boundary of the box farthest from 0 indicates the 75th percentile, whiskers indicate 10th and 90th percentiles, dots indicate outlier values. * indicate significant difference between control Kruskis-Wallis One-Way ANOVA on Ranks, Dunn's multiple comparison test ($p < 0.05$). n = 10



Amphipod NP accumulation:

E. estuarius accumulated NP from aqueous 96 h exposures to a concentration of 60 μg /L, giving a mean BCF = 1616 (SD = 318). Amphipod batches fed to fish on separate days did not differ significantly in body burdens (n=5, one-way ANOVA, F = 0.318, $p = 0.864$).

Fry behavior

No mortalities occurred in the two controls. Three fry died during Day 4 and 5 in the highest NP water treatment, 240 μg/L, and remaining fry appeared sluggish compared to fry in other treatments. To determine if feeding rates were affected by consuming amphipods, we measured amount of amphipods consumed (WW mg) at each 45 min feeding period for the two amphipod treatments. There was no significant difference in feeding rates between fry that fed on contaminated amphipods, compared with fry that had fed on uncontaminated amphipods (Table 14). However, there was a greater variance in feeding rate in the fry that fed upon contaminated amphipods compared to fry that fed on uncontaminated amphipods.

Table 13: Mean NP dose to fry from feeding on contaminated amphipods compared with plasma vitellogenin levels. Fry mean NP dose determined from five day feeding rates on contaminated amphipods.

Gender	Fry WW g	Mean NP dose NP µg g dry wt pods^{-1}day^{-1} (std) n=5	Plasma Vtg ng ml^{-1}
male	5.8	8.9 (7.8)	<LLD*
male	7.5	41.1 (10.6)	65
male	7.6	35.4 (10.2)	<LLD
female	7.1	51.1 (12.6)	NA
female	6.2	47.2 (14.9)	<LLD
female	9.7	49.6 (13.4)	54
female	7.1	19.04 (11.3)	<LLD
female	5.4	27.0 (11.77)	<LLD
female	5.8	29.2 (16.1)	<LLD
female	5.8	42.5 (9.3)	78

<LLD* = Vtg below lower limit of detection, detection limit = 40 ng ml^{-1};
NA= not available

Chemistry

^{14}C-NP aqueous concentrations were measured in all treatments six hours after water renewal (Table 15) and in all batches prior to renewal (data not shown). Concentrations in NP treatments increased gradually during the experiment. Measured mean concentrations from radioactive tracers (^{14}C-NP) were ~12% greater than nominal concentrations.

Table 14: Mean fry five-day feeding rates of live amphipods from daily 45 min feeding periods. Feeding rates are expressed as percentage amphipods consumed per day (mg WW) divided by fry (mg WW) * 100

Treatment	Mean Feeding Rate Day^{-1} N=10 (SD)
NP Pods- Fry fed NP contaminated live amphipods	2.21 (1.13)
Pods- Fry fed uncontaminated live amphipods	2.76 (0.20)

Table 15. Nominal and mean daily measured aqueous NP concentrations based on radioactivity taken at 6 hr intervals (n=10).

Treatments	Nominal $\mu g\ L^{-1}$	Measured (std)
NP Pods	0	3.26 (2.03)
Pods	0	<LLD*
NP 0	0	<LLD
NP 5	5	6.07 (1.13)
NP 20	20	22.80 (3.32)
NP 60	60	67.64 (11.75)
NP 240	240	268.06 (35.20)
E2	0	<LLD

<LLD* = Below lower limit of detection
* = Below lower limit of detection for ^{14}C derived NP concentrations, <10 ng/L

DISCUSSION

We evaluated Vtg levels following five-day exposures to NP from aqueous and dietary routes in chinook salmon fry. It is evident that NP water concentrations greater than 20 µg/L were sufficient to elicit a significant vitellogenin response in fry. Salmon fry feeding on NP-contaminated amphipods elicited a much weaker response, in that only three of ten fry had detectable Vtg. NP water concentrations

in treatments were 12% greater than nominal values. The lack of a significant difference of feeding rates between fry feeding on NP contaminated amphipods and fry feeding on uncontaminated amphipods was not wholly unexpected because acute toxicity from ingestion of contaminated amphipods is unlikely.

Water exposures

Plasma Vtg levels followed a dose-dependent relationship to NP concentrations and were similar to other laboratory-obtained results. The highest NP water concentration, 240 μg/L, was toxic to fry with three fish deaths during the final day of exposure. Although acute toxicity to juvenile chinook salmon from NP is not known, the results of a 96 h pilot test we conducted (12 h static renewal) indicated an approximate LC50 of 350 μg/L, which is higher than reported for rainbow trout (mature) and Atlantic salmon (mature) with reported LC50s of 221 μg/L (Brooke, 1993) and 145 μg/L (McLeese et al. 1981) respectively. Aqueous NP exposures have been conducted extensively in laboratory experiments with a variety of fish species including Atlantic salmon (Yadetie and Male, 2002; Arukwe et al. 2000; Madsen et al. 1999), rainbow trout (Thorpe et al. 2000; White et al. 1994; Harris et al. 2001), catfish (Nimrod and Benson, 1996), flounder (Christensen et al. 1999), sheepshead minnow (Hemmer et al. 2001; 2002), Japanese Medaka (Metcalfe et al. 2001), fathead minnows (Giesy et al. 2000;

Nichols et al. 2001), and swordtail fish (Kwak et al. 2001). Vitellogenin induction (> 40 mg/ml plasma) occurred at exposures as low as 5.6 μg /L NP in sheepshead minnows using 12 d exposures, with a corresponding increase in variability (Hemmer et al. 2002). In the present study, no Vtg was detected at 5 μg /L, but there was large variability of Vtg induction by fry exposed to 20 μg/L (Figure 6).

The lowest variation in induction within treatments, that in turn also elicited the highest Vtg levels was detected in the 240 μg/L (aqueous exposure) and 5 mg/Kg E_2 (i.p. administration). This supports the hypothesis that maximal Vtg expression is a result of saturated estrogen receptors and resultant Vtg mRNA transcription (Flouriot et al. 1996).

Dietary exposures

We detected a minimal Vtg response from fry that had fed upon NP contaminated amphipods; 3 of 9 fry had detectable plasma vitellogenin levels that were just above detection limits of the ELISA method (Table 13). The level of Vtg in fry that had consumed contaminated amphipods was 6 orders of magnitude below the positive control treatment, E_2, and the highest NP aqueous treatment, 240 μg /L. It is possible that increasing the feeding time course may have resulted in a greater Vtg response in fry. This is quite likely, given that the optimal Vtg

expression in rainbow trout from aqueous NP exposures was observed at 14 days by Thorpe et al. (2000), and Vtg response was greatest at 12 days following i.p. administration of 5 mg/Kg E_2 on days 1 and 3 to juvenile rainbow trout (Donohoe and Curtis, 1996).

Although we believe it to be an outlier, we can not explain the Vtg value 195 ng/L detected in one male fry from the uncontaminated amphipod treatment. This is the only control replicate in which Vtg was detected. Male fry are not expected to have the basal level of Vtg expression observed in juvenile female fish (Carlson and Williams, 1999). It is unlikely that ^{14}C-NP contaminated the treatment, as samples taken for radiotracer analysis contained only background amounts. Contamination might have occurred from an unlabeled NP misallocation during water renewal; however, it is unlikely that such a misallocation occurred more than once.

The maximal Vtg levels observed in the present experiments mirrored the results of similar experiments with rainbow trout. Carlson and Williams (1999) fed juvenile rainbow trout (12 months old) for seven days with E_2 spiked food ranging from 0.05 – 2.5 mg/ Kg and analyzed for Vtg using the same ELISA techniques that were used in this work. These fish exhibited a mean (SD) plasma Vtg of 4.19 (0.24) mg/ml when exposed to 2.5 mg/kg E_2 compared to mean (SD) plasma Vtg of 4.34 (1.83) in fry administered 5 mg/kg E_2 in the present experiments. Donohoe

and Curtis (1996) using a similar i.p. administration of E_2 (5 mg/Kg) showed that rainbow trout juveniles (12 mo old) produced more than 0.60 mg Vtg /ml.

Our experimental-design model could easily be adapted to other chemicals of concern and used to quantify additional endpoints including steroid levels, histology of affected cells, and fertility. DiPinto and Coull (1997) used a similar experimental approach, exposing copepods to PCB-contaminated sediment, feeding the contaminated copepods to bottom-feeding fish, and quantifying trophic transfer. Results indicated significant transfer of PCBs from sediment to copepod and to fish, unfortunately biological effects from accumulation were not measured in either species.

Potential risk to salmon populations

Our results suggest that estuarine amphipods could serve as potential vectors of NP to chinook salmon fry, although we can make no definitive statements concerning the chronic dietary impacts of NP on fry endocrine systems. Amphipods are a major prey item of chinook fry in estuaries where fry may remain for up to 6 months) (Myers and Horton, 1982). Thus there is a potential for amphipods to serve as vectors of sediment-associated contaminants to salmon. It would be of considerable ecological significance if a link between bioaccumulation

to prey organisms, leading to individual or population level effects at higher trophic levels could be established. We recommend that additional laboratory-based research, exploring the dietary route be carried out for longer time courses, and that field-based research be undertaken to quantify potential impairment of salmon fry from water-borne and dietary exposures to NP.

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CHAPTER 5:

CONCLUSIONS

The overall objectives of this research were to quantify the potential for trophic transfer of 4-nonylphenol (NP) in a simple estuarine food chain, and to quantify vitellogenin induction in juvenile chinook salmon fry as a result of dietary and aqueous exposures. An ecologically, relevant estuarine food chain was developed in a laboratory system, to mimic typical Pacific Northwest estuarine food chains that would be at risk from NP contaminated sediments. Sediments were chosen as the primary source of NP for organism exposure because of the high concentrations found in sediments and the physical properties of NP. The organisms forming this chain were tested in a series of experiments to determine sensitivity to NP, and accumulation from sediment, water, diet, or some combination of these, dependent on trophic level. Amphipods were chosen as the primary accumulator of NP from contaminated sediments because of their intimate association with sediments and their role as primary prey items for juvenile salmonids. Juvenile chinook salmon were selected as the predatory organism within the chain because they are endangered, and susceptible to anthropogenic stressors; they are also a cultural and economic icon of the Pacific Northwest and a high value is placed upon their continued survival. Juvenile salmon develop in estuaries

coincident with their amphipod prey and with sources of NP. They are also susceptible to endocrine disrupting compounds. Laboratory exposure studies determined the sensitivity of amphipods to NP (Chapter 2); the bioavailability, uptake, and accumulation of NP by three species of amphipods (Chapter 3); and the induction of vitellogenin in juvenile chinook salmon from aqueous and dietary NP exposures (Chapter 4).

CHAPTER SUMMARIES

Chapter 2

Three 96 h aqueous, independent, trials were conducted with the benthic amphipod, *Eohaustorius estuarius*, using mortality and burial as endpoints. Amphipod mean lethal concentration to 50% (LC50) was 227 µg/L. One-hour burial, selected as a sub-lethal endpoint, increased the sensitivity of the toxicity test by ~ 40% however, most amphipods that survived exposure were able to recover within 24 to 48 hours. This toxic hangover was dose-dependent. Narcosis has been suggested as the mode of action for NP on aquatic organisms. Our experimentally derived LC50s were an order of magnitude more toxic than predicted values generated from quantitative structure-activity relationship models based on fish acute toxicity to other neutral narcotics. When we compared our LC50s to an

amphipod derived model they were not significantly different. This data suggest that NP is acutely toxic to *E. estuarius* in the ppb range (LC50 227 ug/L) and that models derived from other species should be used with caution for species to species comparisons. NP concentrations in the water column are very unlikely to reach 227 ug/L, however sub-lethal effects occur at much lower concentrations (Jones et al. 1998; Christensen et al. 1999; Shurin and Dodson, 1997; Brown et al. 1999).

Uncertainty

There is great uncertainty when applying laboratory-derived toxicity values such as LC50s to predict toxicity in field exposed populations (Kimball and Levin, 1985). We caution against the use of this data to predict field impacts on amphipod populations. This approach was part of a broader program to determine toxicity and accumulation of NP to benthic estuarine amphipods. The bioassay we used was based on U.S. EPA standardized methods developed at this laboratory (American Society of Testing and Materials, 1992); however, it was surprising that toxicity was higher in one of the three trials. We believe this difference was a result of operator error in the spiking formulation. We did not measure NP using analytical methods, and therefore can not verify the actual NP water concentration.

Chapter 3

Modified U.S. EPA standardized test protocols were utilized to determine accumulation of NP by three species of benthic amphipods and the affects on bioavailability of NP by varying the type of sedimentary organic carbon. Results indicated that NP bioaccumulation by amphipods was inversely proportional to TOC quantity, but unaffected by TOC nutritional quality. There were significant differences in the accumulation patterns of the 3 amphipod species that can be related to their feeding strategies. Our results show that estuarine amphipods are potential dietary sources of NP to higher trophic levels, such as juvenile salmonids, at doses sufficient to induce endocrine disruption.

Uncertainty

NP concentrations in water and amphipod tissue were based on radioactivity detection of ^{14}C ring-labeled 4-nonylphenol. Metabolism of NP by amphipods is unknown and remains a source of uncertainty in our data. If NP was metabolized and incorporated by amphipods our accumulation factors would represent overestimates. There is also evidence that hydrophobic organic compounds in sediment which are "spiked" exhibits greater bioavailability than

sediments collected from contaminated sites and used in the same bioassay. We followed a U.S. EPA standardized protocol for aging sediments to allow hydrophobic organic contaminants to reach equilibration after mixing. There is also uncertainty in assuming that juvenile salmonids feed solely on benthic amphipods during their estuarine residence. Chinook salmon fry are opportunistic feeders and feed on a variety of insects in the water column and on the surface (Toft 2000). There is also minimal information in the diet of amphipods and seasonal trends in accumulation of contaminants from sediments. If amphipods do seek out high TOC areas in sediment that preferentially bind organic contaminants than amphipods would have larger than expected body burdens. This selective feeding would bias accumulation factors based on equilibrium partitioning.

Chapter 4

Direct comparisons were made between chinook fry exposed to NP via the diet and fry exposed to a range of NP concentrations via water. Amphipods (*Eohaustorius estuarius*), were exposed to NP (60 µg/L) for 96 h, then fed to hatchery-raised salmon fry for 5 days. In parallel, fish were exposed to one of 4 concentrations of NP in the water at concentrations ranging from 0-240 µg/l for 5 days. Following exposure, salmon fry plasma vitellogenin (Vtg), a standard biomarker of endocrine disruption, was measured using Enzyme-Linked Immunosorbent Assay (ELISA)

techniques. Results showed that water treatments of 60 and 240 μg/L NP induced high levels of Vtg in salmon fry. Thirty-percent of fry fed contaminated amphipods on a daily basis, had detectable Vtg, while control fry had no detectable levels of Vtg. These experiments demonstrated that NP-contaminated estuarine amphipods are potential vectors of NP to chinook salmon fry, but it did not provide definitive results in the form of a significant treatment effect. In these experiments salmon fry were at greater risk from NP water-borne exposures than dietary exposures.

Uncertainty

Although five days of dietary exposure via NP-contaminated amphipods were sufficient to detect Vtg in plasma of some fry, longer exposure periods have been employed in other studies (e.g. 14 days for rainbow trout) (Thorpe et al. 2000). Therefore, a greater vitellogenic response by fry may have been observed if fry were fed contaminated amphipods for longer periods of time.

Because we used ^{14}C radio-labeled NP, the actual NP concentration in amphipods is unclear. As explained above, this could lead to an over estimation of accumulation factors in amphipods. Although juvenile salmonids, and specifically chinook fry, feed on amphipods, their foraging activities while in estuaries are not well understood in regard to amphipod population distribution/densities. There is

little information on the link between NP contaminated sediments, amphipod NP accumulation and subsequent predation by juvenile salmon fry at contaminated sites. To assess the risk of NP sediment contamination on an estuarine ecosystem, amount/rate of NP discharge, location of discharge, seasonal abundance of amphipods, accumulation of NP by amphipods, salmon residence time, fry feeding rates on amphipods, and information on accumulation of NP by other potential prey items are required.

IMPLICATIONS

This dissertation provides evidence that 4-nonylphenol, a degradation product of widely used alkylphenol polyethoxylates, is acutely toxic in the ppb range to amphipods, bioaccumulates in amphipods, and can trigger induction of Vtg in juvenile chinook salmon fry from water and dietary exposures. With the continued high volume use of NPEs in the U.S., it is likely that aquatic organisms are being exposed to NP. The U.S. Environmental Protection Agency is currently reviewing the toxicity of NP and its parent compounds to develop aquatic life criteria. Predicted no effect concentrations (PNEC's), have not been generated for U.S. sediments or surface waters; however, the European Union has calculated surface water and sediment PNECs of 0.60 ug/L and 0.039 mg/Kg, respectively. U.S. sediment concentrations exceed these values, in some locations by an order of

magnitude (Ferguson et al. 2001; Hale et al. 2000). Our work fills an important gap in the understanding of the toxicity and bioaccumulation of 4-nonylphenol.

There is a potential ecological threat to estuarine, aquatic organisms from present day exposures to 4-nonylphenol, particularly near point source discharges of industrial and municipal sewage treatment plants. Benthic amphipod exposure to NP, based on our results, is primarily from pore water, but can also occur through dietary intake of contaminated organic matter. NP concentrations in pore water are governed by complex abiotic and biotic interactions. Sediment resuspension, desorption of NP, and physical characteristics (grain size, TOC content, and type) determine NP bioavailability.

Amphipod populations may be at risk from chronic exposure to highly contaminated NP sediments; however, it is difficult to separate NP effects from other prevalent sediment contaminants (PCBs, chlorinated pesticides, etc.). However, in highly contaminated sediments, NP is acutely toxic to amphipods. Questions remain concerning chronic toxicity to invertebrates from endocrine disrupting effects of NP.

It is likely that amphipods could serve as vectors of NP to higher trophic levels based on their sensitivity to NP, concentrations found in sediments, and the large NP accumulation factors (BAFs, BCFs, and BSAFs) generated from our

laboratory experiments. NP accumulation by amphipods in highly contaminated areas may be a significant dietary source to predator fish (Table 9 and 10). The relative risk to juvenile salmon from NP exposure that causes low level Vtg induction remains unknown and needs further investigation. Also, there are numerous other organisms in estuarine food chains that may be exposed, and therefore potentially affected by NP-contaminated sediments including shell fish, dimersal fish, estuarine birds, polychaetes, and other amphipod species.

FUTURE RESEARCH

This dissertation has established a starting point for further NP investigations both in the field and the laboratory. A field based study at NP-contaminated sediment sites is recommended to assess accumulation by benthic invertebrates and vitellogenin induction in salmon fry for comparison to the present data. One field approach that would identify potential exposures and effects of NP to salmon fry would be to utilize caged- juvenile salmonid experiments. Cages would be placed in locations near sewage waste water effluent inputs and likely agricultural runoff areas. Following exposures, Vtg induction, effects on smoltification, and accumulation of NP could then be assessed. These experiments would provide information on exposure and effects from field locations. Field sampling of estuarine amphipods and fish for NP tissue analysis is also

recommended to identify populations potentially at risk. We recommend that additional laboratory-based research, exploring the dietary route be carried out for longer time courses to quantify Vtg induction in salmon fry. Additional laboratory experiments are also required to quantify endocrine disrupting effects not only from NP exposures, but also from those of chemical mixtures. Vitellogenin induction is a sensitive biomarker, but actual effects on growth, fecundity, behavior, and imprinting are necessary to assess the ultimate effect of NP on salmonid populations.

Discontinuation of the use of nonylphenol polyethoxylates would preclude the above research because NP is much less resistant to degradation than PCBs, DDTs, and other chlorinated pesticides, and is expected to degrade over time.

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