TOXICOLOGICAL PROFILE FOR NONYLPHENOL

September 2009

Integrated Risk Assessment Branch
Office of Environmental Health Hazard Assessment
California Environmental Protection Agency

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Executive Summary

This toxicological profile on **nonylphenol (NP)** describes its effects freshwater and marine life, humans, and laboratory animals. In recent years, NP and related chemical forms of NP have raised concerns because of their effects on the endocrine system, the organ system producing hormones that regulates many of the body's functions. Such concerns have led to many restrictions on the use of NPs within the European Union.

Use and Exposure

NP is a synthetic organic chemical produced in relatively large quantities in the United States. Primary uses include: (1) a building block of nonionic surfactants (i.e., agents that reduce surface tension of liquids) used in lubrication, defoaming agents, scouring fibers, emulsifiers, wetting and de-wetting agents, dyes, and other products; and (2) a component in a stabilizer used in plastics and vulcanized rubber.

NP-surfactants are the principal source of NP release into the environment. Aquatic anf marine life exposure occurs when the substantial quantities of NP-surfactants, discharged into wastewater, biodegrade into several by-products, including NP. While not a significant source of NP in the environment, unreacted NP in plastic may result in direct human exposures when the chemical leaches out of plastic in close contact with foods.

Environmental Occurrence

Due to its physical-chemical characteristics, NP accumulates and persists in sewage sludge, river sediments, and other environmental compartments. The occurrence of NP in the environment is clearly correlated with human activities such as wastewater treatment, land filling and sewage sludge recycling.

NP has been reported at concentrations of up to 0.89 micrograms per liter (µg/L) in freshwater bodies, over 1 µg/L in municipal treatment plant effluents, and up to 2.76 µg/L in coastal waters. Most adverse effects reported in laboratory experiments occur at concentrations above 1 µg/L. More data on environmental concentrations of NP in marine systems will allow a more complete assessment of the impacts of environmental levels of NP on marine organisms.

Effects on Aquatic Life

Laboratory studies indicate that NP can induce a variety of reproductive effects in aquatic life, including fish and shellfish. Reported reproductive effects include:

- Changes in male and female hormone levels in turbot
- Decreased gamete production and fertilization in medaka and zebrafish
- Reduced hatching of rainbow trout embryos
- Altered sex ratios in offspring of NP-exposed oysters
- Development of intersex trout, bream, and frogs (i.e. offspring with characteristics of both sexes)

NP can also induce a variety of non-reproductive effects, such as the inability to maintain fluid and electrolyte balance in sea bream and Atlantic salmon, which could prevent their migration

from fresh water to sea water. Clams and sea urchins exposed to NP have exhibited decreased respiration and increased malformations, respectively.

Health Hazard and Toxicity in Humans and Laboratory Animals

- **Reproductive and Developmental Effects.** NP can act as an estrogen, a group of naturally occurring steroid compounds that function as the primary female sex hormone. Sufficient evidence was found to show that NP causes reproductive effects in laboratory animals. These effects, which are thought to be linked to NP's estrogenic activity, include:
  - Lowered levels of the male sex hormone testosterone
  - Effects on the testes, including decreased sperm production
  - Increased uterine weight, suggesting that NP may affect female reproduction
  - Altered development of the brain region responsible for male and female behavior
  - Hyperactivity in juvenile animals and animals exposed before birth due to effects on the development of regions of the brain.

  In humans, limited information is available on possible reproductive effects. One study reported early onset puberty in children exposed to NP while *in utero*.

- **Cancer.** There is no information on whether NP is carcinogenic in laboratory animals or in humans.

- **Immune and Thyroid Effects.** There is some evidence that NP affects the immune system in laboratory animals and limited evidence that it affects thyroid function and obesity. Many if not all of these effects appear to be related to NP's estrogen-like effect and its ability to disrupt the endocrine system.

- **Nervous System Effects.** Prenatal exposure of laboratory rodents to NP results in neurobiological alterations, including some sexually dimorphic behaviors. Studies conducted with cultured cells and tissues suggest that NP could adversely affect brain development and may cause neurodegeneration.

Summary Table

This table provides some idea of the availability of information on the toxicology of NP for the endpoints and organisms identified. It also provides some sense of the evidence available in that information can be used to determine if the endpoint effect does or does not occur. If there is no information the evidence column will be marked with a "--."

Health Effect	Human		Lab Animal		Aquatic Life	
	Information	Evidence	Information	Evidence	Information	Evidence
Reproductive						
male	L	L	Su	Su	Su	Su
female	L	L	S	S	Su	Su
Developmental	S	S	S	S	Su	Su
Neurological	N	--	S	S		
Cancer	N	--	N	--	N	--
Immunological	N	--	S	S	N	--
Other Chronic effects					Su	Su
Thyroid	N	--	L	L		
Obesity	N	--	L	L		
Acute					Su	Su

N = None S = Some
L = Little Su = Sufficient
These rating categories are qualitative in nature and designed to give the reader a general sense or the availability and strength of the information.

Abbreviations

ADHD	attention-deficit hyperactivity disorder
AGD	anogenital distance
ANOVA	analysis of variance
ATPase	adenosine triphosphatase
AWQC	ambient water quality criteria
BCF	bioconcentration factor
11 β-HSD	β-hydroxysteroid dehydrogenase
cAMP	cyclic adenosine monophosphate
CERHR	Center for the Evaluation of Risks to Human Reproduction
CG	chorionic gonadotropin
Con A	concanavalin A
D	dopamine receptors (D_1 or D_2)
DART	developmental and reproductive toxicities
DNA	deoxyribonucleic acid
dph	days post-hatch
E2	17 beta-estradiol
EC_{50}	median effective concentration
2-EH	2-ethylhexanol
ER	estrogen receptor
ERL	environmental risk limit
FasL	Fas ligand
FSH	follicle-stimulating hormone
hCG	β-human chorionic gonadotropin
hES	human embryonic stem
HPOA	hypothalamic/preoptic area
ICI	ICI 182780 (Faslodex) from AstraZeneca
IFN	interferon
IgE	immunoglobulin E
IL	interleukin
K_{OW}	Octanol-water coefficient
LC_{50}	lethal concentration to 50% of the population
LD_{50}	lethal dose to 50% of the population
LH	luteinizing hormone
LOEC	lowest observed concentration
LOELs	lowest observed effect levels
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinases
MIF	migration inhibitory factor
MIP-1α	macrophage inflammatory protein-1α
μM	micromolar
mRNA	messenger ribonucleic acid
NADPH	β-nicotinamide adenine dinucleotide phosphate
NF	nuclear factor
NGF	nerve growth factor

NIS	sodium/iodide symporter
nM	nanomolar
NO	nitric oxide
NOEC	no observed effect concentration
NOELs	no observed effect levels
NP	nonylphenol
NSC	neural stem cells
OEHHA	Office of Environmental Health Hazard Assessment
OPC	California Ocean Protection Council
PCBs	polychlorinated biphenols
PDI	protein disulfide isomerase
p.f.	post-fertilization
PKC	protein kinase C
PND	postnatal day
PPAR-γ	peroxisome proliferators-activated receptor-γ
PPB	parts per billion
Ppm	parts per million
PVC	polyvinyl chloride
qTR LBD	ligand-binding domain of thyroid hormone receptor beta
qTTR	Japanese quail transthyretin
SDN-POA	sexually dimorphic nucleus of the medial preoptic area
SEB	Staphylococcus enterotoxin B
SHBG	sex hormone-binding globulin
T3	triiodothyronine
$^{[125I]}$T3	3,3',5-L-$^{[125I]}$triiodothyronine, iodine isotpe labeled
T4	thyroxine
TH	thyroid hormone
Th	T helper cell
TNF-alpha	tumor necrosis factor-alpha
TSH	thyroid stimulating hormone
VTA	Ventral Tegmental Area
VTG	vitellogenin

Introduction

On February 8, 2007, the California Ocean Protection Council (OPC) passed a resolution, "On Reducing and Preventing Marine Debris." Scientists are investigating whether constituents leach out of plastic products in the marine environment and present a threat to the health of wildlife and humans. The OPC has asked the Office of Environmental Health Hazard Assessment (OEHHA) to prepare toxicity profiles characterizing certain chemical constituents of plastics that are thought to be harmful to marine life and humans. In preparing this profile, OEHHA reviewed reported information on the adverse effects of exposure to NP in aquatic organisms in the laboratory and in the natural environment, humans, and experimental laboratory animals.

Properties and Uses

Nonylphenol (NP) (CAS number, 104-40-05) is a product of industrial synthesis formed during the alkylation process of phenols (ring structure in Figure 1). The addition of ethoxyl groups to the parent compound produces nonylphenol ethoxylates (NPE), which are used to produce industrial surfactants. Alkylphenol ethoxylates are the second largest group of nonionic surfactants in commercial production, of which NPEs account for approximately 80 percent. NP, the predominant environmental biodegradation product of NP ethoxylates, is ubiquitous and moderately persistent. Physical properties and environmental fate have been reviewed by the U.S. Environmental Protection Agency (USEPA, 2005).

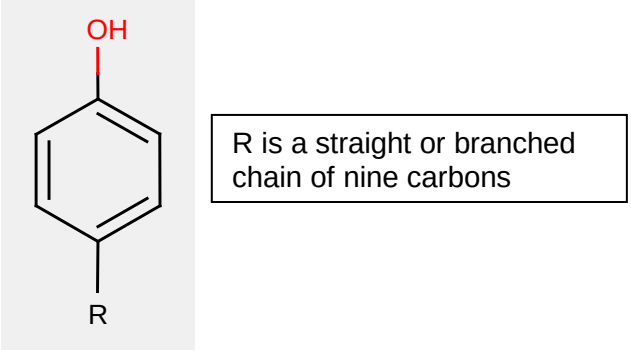

Figure 1: NP structural formula

Table 1: Nonylphenol Properties (based on USEPA (2005)

PROPERTY	VALUE	UNITS
Molecular weight	220	g/mole
Empirical formula	$C_{15}H_{24}O$	
Specific gravity	0.953	g/mL
pKa	10.7±1.0	
Solubility, pH 7	4.6	mg/L
Solubility, pH 9	6.2	mg/L
Solubility, pH 11	11.9	mg/L
Solubility, sea water	3.6	mg/L
Log K_{OW}	3.8 – 4.8 (4.5)	
T ½ in water w/ POTW sludge	8.2	Days
Vapor pressure	0.00455 ± 0.0035	Pa

NPEs are widely used as detergents, emulsifiers, and surfactants (wetting agents) in household and industrial products such as paints, plastics, cosmetics, lubricant oils, construction materials, vulcanized rubber, and paper. They are also used in the processing of fuels, metals, petroleum, textiles, agricultural chemicals, and leather. Substantial quantities of NP-containing compounds reach sewage treatment works, where they biodegrade into several by-products, including NP. Due to its physical-chemical characteristics, such as low water solubility, NP accumulates in environmental compartments like sewage sludge and river sediments, where it is moderately persistant.

Unreacted NP in the stabilizer used in plastic may leach out of the plastic; while this is not a significant source of NP in the environment, it can be a direct route of exposure if the plastic is in close contact with foods or if the plastics are ingested by aquatic organisms. The occurrence of NP in the environment is clearly correlated with human activities such as wastewater treatment, land filling and sewage sludge recycling.

Environmental Criteria and Contamination

USEPA (2005) summarized aquatic toxicity data through early 2003. They established Ambient Water Quality Criteria (AWQC; summarized in Table 2) and concluded that NP is an estrogen agonist (NP can act like the female hormone estrogen in an organism).

Table 2: EPA Ambient Water Quality Criteria*

AWQC, FW (one-hour average)	***28 µg/L***
AWQC, FW (four-day average)	***6.6 µg/L***
AWQC, SW (one-hour average)	***7 µg/L***
AWQC, SW (four-day average)	***1.7 µg/L***

Not to be exceeded more than once every 3 yrs. Criteria are based on whole-animal effects like reproduction; i.e. they do not consider biochemical changes in the absence of whole-animal effects.* *(USEPA, 2005)****. FW=fresh water; SW=salt water.*

Sources and environmental concentrations have been reviewed by USEPA (2005). Sources and environmental concentrations found in various parts of the world are summarized in Table 3.

TABLE 3: Nonylphenol levels in water and sediment

Water (µg/L)	Sediment (µg/kg)	Country	Reference
	<1 - 6760	Korea	(Tanaka and Nakanishi, 2002)
0-2.7 (municipal effluents)		Japan	(Komori et al., 2006)
0.0003 – 0.0025 sea		Japan	(Hashimoto et al., 2005)
0.001 – 0.004 fresh	2 - 12	Japan	(Hashimoto et al., 2005)
0.20 – 2.76 sea		Singapore	(Basheer et al., 2004)
n.d.–0.89; mean 0.031		Austria	(Bursch et al., 2004)
20% >1 µg/l; some > 6 µg/l (POTW effluent)		Canada	(Berryman et al., 2004)
0.5 - 15	20 - 640	Spain	(Petrovic et al., 2002)
2.13 (POTW influent) 0.32 (POTW effluent)		Germany	(Korner et al., 2000)
4.9		Taiwan	(Hong and Li, 2007)
6–7 (municipal effluents)		Canada	(Fernandez et al., 2007)
5	180,000	Laboratory	(Bettinetti et al., 2002)
20	600,000	Laboratory	

Environmental Fate, Transport, and Bio-uptake

Nonylphenol is taken up from water and sediment by aquatic biota. It can accumulate in the tissues of these organisms, but does not accumulate to the degree that NP's K_{OW} (octanol-water coefficient) would suggest based just on its lipid solubility. It can move up the food chain, but does not biomagnify to any great degree.

NP transfer from sediment into benthic worms was inversely related to sediment organic carbon content (F_{OC}). Algae grown in medium containing 100 µg NP/L accumulated up to 917 µg NP/g of algae, indicating that NP has a higher affinity for the algae than water. *Artemia franciscana* (brine shrimp) fed the treated algae grew faster than artemia fed control algae, but accumulated only trace amounts of NP. Zebrafish fed the treated artemia did not show any significant differences in growth, reproduction, cytochrome P450 activity, superoxide dismutase activity and vitellogenin (VTG) levels (Correa-Reyes et al., 2007).

Female roach fish exposed to a NP concentration of 4.9 µg/L radio-labelled technical NP over a 4-day period exhibited apparent bioconcentration factors (BCF) of 34,121 and 605, in bile and liver, respectively; in other tissues, apparent BCF values were recorded between 13 and 250 (Smith and Hill, 2004). This suggests that NP is metabolized and excreted in the bile. NP accumulated in the liver, gill, skin, gut, fat, and kidney tissue of trout (Ahel et al., 1993; Coldham et al., 1998; Lewis and Lech, 1996) as well as shrimp, mussels, and stickleback fish (Ekelund et al., 1990). NP has also been found in high levels in seafood from Singapore, especially prawns (Basheer et al., 2004) and at even higher levels in field-collected mussels, clams, and squid from Italy (Ferrara et al., 2001) (Table 4). These data indicate a potential pathway for human exposure through consumption of market seafood items.

The log K_{OW} of nonylphenol ranges from 3.80 to 4.77, indicating that moderate bioaccumulation in aquatic organisms may be expected (USEPA, 2005). NP accumulated in the liver, gill, skin, gut, fat, and kidney tissue of trout (Ahel et al., 1993; Coldham et al., 1998; Lewis and Lech, 1996) as well as shrimp, mussels, and stickleback fish (Ekelund et al., 1990). NP has also been found in high levels in seafood from Singapore, especially prawns (Basheer et al., 2004) and at

even higher levels in field collected mussels, clams, and squid from Italy (Ferrara et al., 2001) (Table 4). These data indicate a potential pathway for human exposure through consumption of market seafood items.

Reported laboratory bioconcentration factors (BCFs) and field-derived bioaccumulation factors (BAFs) do not support the expected accumulations in tissues, indicating that some nonylphenol is metabolized (USEPA, 2005). A single major metabolite of NP was present in liver and bile of the female roach exposed to a NP concentration of 4.9 µg/L over four days. The metabolite was identified as the glucuronide conjugate of 4-(hydroxy-nonyl)-phenol (Smith and Hill, 2004). Similarly, NP was metabolized by hepatic cytochrome P450 enzymes in the rainbow trout (*Oncorhynchus mykiss*) and bile from the fish contained the glucuronic acid conjugates of nonylphenol; thus, bile may be a major route of nonylphenol excretion (USEPA, 2005).

Table 4: Bio-uptake and Bioconcentration in Aquatic Organisms

Species	Water (µg/L)	Tissue (µg/kg)	BCF	Reference
Blue mussel (*Mytilus* sp.)			1.4 – 7.8	(USEPA, 2005)
Atlantic salmon (*Salmo salar*)			75	
Marine amphipods			46 – 185	
Algae			487	
Shrimp (*Crangon crangon*)			90 – 110	(Ekelund et al., 1990)
Mussels (*Mytilus edulis*)			2740 – 4120	
Stickleback fish (*Gasterosterus aculeatus*)			1200 –1300	
periphyton	0.1 – 0.4	8-130	160 – 650	(Takahashi et al., 2003)
benthos	0.1 – 0.4	8-140	63 – 990	
Mussels	<0.0005– 0.21	131 – 211	1000	(Pojana et al., 2007)
Medaka (*Oryzias latipes*) eggs	62	2000-7000	30 – 100	(Ishibashi et al., 2006)
Prawn (*Penaeus monodon*)	0.20 – 2.76	197.0 ± 13.1		(Basheer et al., 2004)
Crab (*Portunus pelagicus*)		103.1 ± 36.0		
Blood cockle (*Anadara granosa*)		54.0 ± 6.1		
White clam (*Meretrix meretrix*)		46.6 ± 11.4		
Squid (*Loligo* sp.)		64.8 ± 13.7		
Indian scad fish (*Decapterus russelli*)		60.5 ± 10.4		
Mussel (*Mytilus galloprovincialis*)		260		(Ferrara et al., 2001)
Clam (*R. decussates* and *C. gallina*, pooled)		248		
Squid (*Loligo vulgaris*)		512		

Species	Water (µg/L)	Tissue (µg/kg)	BCF	Reference
Cuttlefish (*Sepia officinalis*)		240		
Great pond snail (*Lymnaea stagnalis*)	99 – 124	69-266	1 – 2.5	(Lalah et al., 2007)
	104	23548	242	(Lalah et al., 2003)
Blackworm (*Lumbriculus variegates*)			1.8 – 33.6	(Maenpaa and Kukkonen, 2006)
Blue mussels (*Mytilus edulis*)	1985	4.0		(Gunther et al., 2001)
	1995	1.1		
Common carp (*Cyprinus carpio*)		100-200	280	(Mitchelmore and Rice, 2006)
Alga (*Isochrysis galbana*)	100	917,000	9170	(Correa-Reyes et al., 2007)
Rainbow trout (*Oncorhyncus mykiss*)	66	140	2.1	(Uguz et al., 2003)
	220	1280	5.8	
	660	1370	2.1	

Toxicology: Marine and Other Aquatic Organisms

Many of nonylphenol's effects on aquatic organisms are attributed to its estrogenic activity, but it also causes toxic effects that are not obviously related to its estrogenic activity, such as effects on growth, behavior, respiration, and osmoregulation. As noted in the summary table, there is sufficient qualitative information on acute, chronic, reproductive and developmental toxicity of NP to aquatic organisms. There is no information on immunotoxicology or carcinogenicity of NP.

Reproductive and Developmental Toxicity

NP has been found to affect various indicators of reproduction in aquatic organisms at concentrations ranging from 0.13 µg/L up to the milligram/liter range. NP competes with estrogen for binding to the estrogen receptor (Danzo, 1997; Flouriot et al., 1995), thereby affecting reproduction and development (Christiansen et al., 1998; Colborn et al., 1993). The most serious and widespread environmental consequences of exposure to NP are likely to be related to its estrogenic activity rather than direct lethality (Lech et al., 1996). Because steroid hormone balance is thought to affect the development of the brain–pituitary–gonad axis in fish, adverse reproductive effects may potentially arise in juvenile males exposed chronically to such xenobiotics (i.e. exogenous chemicals and in this case with estrogenic activity) (Burkhardt-Holm et al., 2000). NP disrupts steroid hormone balance in juvenile turbot, with males being much more sensitive to this effect than females: NP induced a decrease of the androgen-to-estrogen ratio in testis, plasma, and bile and depressed androgen levels, perhaps as a consequence of 11b-hydroxylase or 11b-hydroxysteroid dehydrogenase activity disturbance, (Labadie and Budzinski, 2006).

Vitellogenin (VTG) Assay in Fish

A useful way to determine the estrogenicity of a xenobiotic in fish is through a specific assay for vitellogenin (VTG). The VTG assay is a frequently used *in vivo* biomarker for estrogenicity in egg laying vertebrates (Heppell et al., 1995; Lattier et al., 2001). VTG is a large, complex,

calcium-binding phospholipoglycoprotein that is required for normal oocyte (prefertilized egg cell) maturation in developing female fish (Matozzo et al., 2007). It is produced in the liver in response to estrogen stimulation, secreted in the blood, and transported to the oocyte, where it is incorporated as constituents of the yolk. Being estrogen-dependent, VTG production is normally restricted to females; little if any VTG can be detected in males or sexually immature females. However, males do carry the VTG gene and exposure to estrogens can trigger its expression (Sumpter and Jobling, 1995). VTG can be measured in the liver, blood, and mucus from male and female fish as well as in primary hepatocyte cultures (Navas and Segner, 2006).

Increased VTG synthesis resulting from NP exposure has been reported in flounder (Kirby et al., 2007), cod (Larsen et al., 2006), rainbow trout (Ackermann et al., 2002), fathead minnow (Brian et al., 2005), atlantic salmon (Meucci and Arukwe, 2006), killifish (Garcia-Reyero et al., 2004), medaka (Ishibashi et al., 2006; Lee et al., 2003), sheepshead minnow (Hemmer et al., 2002), and mysid shrimp (Ghekiere et al., 2006). Male carp in the Cuyahoga River near the outfall of the city of Akron's wastewater treatment plant had higher tissue NP levels and about twice the serum VTG levels compared to carp from up-river sites. Serum VTG was correlated with tissue NP levels ($r = 0.30 – 0.97$) (Mitchelmore and Rice, 2006). Pickford et al. (2003) estimated that NP absorbed from the water was 10 times more potent in inducing VTG in male fathead minnows than NP administered in the diet.

VTG induction is a marker of estrogenic exposure, but it has also been correlated with other endpoints. Turbot exposed to 29 µg/L NP exhibited increased VTG and zona radiata protein (protein subunits of the inner eggshell) (Larsen et al., 2006) as well as decreased plasma, testicular, and biliary testosterone, androstenedione, and β estradiol (Labadie and Budzinski, 2006; Martin-Skilton et al., 2006). Dose-dependant plasma VTG production was the most sensitive biomarker of exposure to NP in male killifish (*Fundulus heteroclitus*) (Pait and Nelson, 2003). Medaka exposed to NP concentrations >11.6 µg/L had increased hepatic VTG levels and their offspring showed altered sex ratios and formation of testis-ova (Seki et al., 2003; Yokota et al., 2001). High concentrations of plasma VTG and an increased prevalence of ovotestes (gonads with both testicular and ovarian aspects) occurred in wild male bream in a small river receiving effluent from a large sewage treatment plant. After employing *in vitro* and *in vivo* bioassays the authors concluded that hormones (especially 17a-ethynylestradiol) and possibly also NP ethoxylates are primarily responsible for these effects (Vethaak et al., 2005).

Four months prior to spawning, adult rainbow trout of both sexes were exposed intermittently to NP concentrations of 1 and 10 µg/L. VTG levels in the plasma of adult male rainbow trout were significantly increased compared to the control group. Embryo mortality was increased in both treatment groups, while hatching rates were significantly reduced in the 10 µg NP/L group. VTG levels were significantly higher in female offspring than in the controls, but there was no alteration in sex ratios. Occasionally, intersex occurred in both male and female offspring of exposed fish and estradiol was increased by two-fold in the plasma of male offspring and testosterone by 13-fold in the plasma of female progeny. These hormonal imbalances in the offspring of exposed fish indicate a transgenerational endocrine disruption (Schwaiger et al., 2002).

Other Reproductive Endpoints in Fish

NP-exposed cod had higher metabolism rates of estradiol in their livers (Martin-Skilton et al., 2006). NP-exposed turbot had lower ovarian P450 aromatase (an enzyme that converts

testosterone to estradiol), lower levels of testosterone and estradiol in plasma, and lower metabolism rates of sex steroids than those from the control group (Martin-Skilton et al., 2006). In medaka, decreased fecundity (ability to reproduce) and fertility (Ishibashi et al., 2006; Kang et al., 2003), increased ratio of females to males and mixed sex characteristics (Balch and Metcalfe, 2006; Kang et al., 2003), and decreased ratio of motile spermatozoa and decreased spermatozoa swimming speed (Hara et al., 2007) have been reported. Semen volume was reduced in rainbow trout exposed to a NP concentration of 0.13 µg/L, and embryo survival and development were reduced at concentrations of 0.28 to 0.75 µg/L (Lahnsteiner et al., 2005).

Zebrafish (*Danio rerio*) exposed to ≥100 µg/L NP from 2 to 60 days post-hatch (dph) had concentration-dependent suppression of gametogenesis (formation of the mature egg and sperm) in both males and females. NP concentrations of 10 µg/ml and higher caused enlargement of Sertoli cells and significantly stimulated DNA replication and mitosis of type A spermatogonia in cultured Japanese eel (*Anguilla japonica*) testicular cells. However, these spermatogonia did not further develop unless 11-ketotestosterone was added to the culture medium (Miura et al., 2005).

Exposure to NP ≥100 µg/L (nominal) from 2 to 60 days post hatch caused concentration-dependent suppression of gametogenesis in both male and female zebrafish. Some recovery was indicated by histologically normal testes after fish were placed in clean water from 60 to 300 dph. In females, however, recovery was incomplete at 300 dph (Weber et al., 2003). Brian et al. (2005) demonstrated the potential for estrogenic chemicals to act additively at environmentally relevant concentrations. Using VTG induction in male fathead minnows as an endpoint, they showed that the combined effects of a mixture of five estrogenic chemicals, estradiol, ethynylestradiol, NP, octylphenol, and bisphenol A—each chemical at one-fifth of its median effective concentration (EC_{50})—were consistent with the effects predicted by concentration addition.

NP exposure was associated with increased intersex frogs, altered sex ratios, and increased gonadal development (Mackenzie et al., 2003). African clawed frog (Xenopus laevis) embryos were exposed to eight different concentrations of NP, from 3 to 96 hours post-fertilization (p.f.). Short body length, microcephaly, flexure, edema, and abnormal gut coiling were induced by 20µM NP by 96 hours p.f. (Sone et al., 2004). Development was delayed in *Rana catesbeiana* tadpoles (Christensen et al., 2005).

Reproductive Effects on Invertebrates

There are many reports of reproductive impairment in invertebrates exposed to NP concentrations >50 µg/L; several studies report reproductive effects at concentrations between 1 and 50 µg/L, and there are a few reports of reproductive effects at concentrations <1 µg/L. It was lethal to some tubifex worms

at concentrations of 600 µg/g sediment. Those that survived this concentration had empty spermatheca (a female tubifex worm organ that receives sperm from the male). If present, spermatic sacs had few germinal elements and no spermatozons. Ovaries were present but ovocytes were not developed (Bettinetti and Provini, 2002). *Lymnaea* sp. snails exhibited decreased fecundity at 100 µg/L (Czech et al., 2001), along with decreased egg masses, increased embryo mortality, and delayed development (Lalah et al., 2007). A NP concentration of 0.2 µg/L was toxic to sea urchin sperm, resulting in reduced fertilization (Ghirardini et al., 2001). Development was delayed in copepods at concentrations as low as 0.1 µg/L (Marcial et

al., 2003). NP exposure also resulted in fewer mudsnail embryos (Duft et al., 2003). Altered sex ratios were reported in chironomids exposed to 1 µg/L (Lee and Choi, 2006) and decreased fecundity in daphnids at concentrations between 25 and 50 µg/L (Brennan et al., 2006). The 96-hr LC_{10} for *Hydra attenuata* was 20 µg/L, making it one of the most sensitive freshwater invertebrate species behind the amphipod *Hyalella azteca* (Pachura-Bouchet et al., 2006; Pachura et al., 2005).

A 48- hour exposure of oyster (*Crassostrea gigas)* larvae to NP at postfertilization days 7–8 resulted in significant alterations to the sex ratio towards females and a 30 percent incidence of fully functional hermaphroditism. Transgenerational effects included poor offspring survival rates, delayed larval growth rates, and inhibition of settlement and metamorphosis. Gamete viability was also affected, resulting in poor embryonic and larval development (up to 100 percent mortality) of the subsequent generation. Three days' exposure to 1 or 100 µg/L NP resulted in a 70 percent or 90 percent reduction in oysters with motile sperm, respectively (Nice, 2005).

Transgenerational effects were not detected in the freshwater water flea (*Daphnia galeata)*. The population-level EC_{50}, the concentration of NP that reduced the intrinsic rate of natural population increase by 50 percent, was estimated as 65.2 µg/L for the first generation and 81.5 µg/L for the second generation. The 48-h LC_{50}, 60.8 µg/L, is a good indicator of the chronic population-level effects of this chemical to this species (Tanaka and Nakanishi, 2002).

Other Types of Toxicity

Other Toxicity in Fish

NP can interfere with osmoregulation. Sea bream had decreased renal sodium-potassium dependent ATPase and increased plasma osmolality (Carrera et al., 2007). Atlantic salmon smolts showed decreased sodium-potassium dependent ATPase in the gills, decreased ability to adapt to sea water, increased plasma cortisol, lower plasma insulin-like growth factor, decreased T_3, and, at higher concentrations, complete loss of osmoregulatory control and death (Arsenault et al., 2004; Lerner et al., 2007a; Lerner et al., 2007b). Zebrafish (*Danio rerio*) exposed to $\geq$10 µg/L NP from 2 to 60 dph had renal lesions including pyknotic nuclei in tubular and interstitial (hematopoietic) cells. Some recovery was indicated by histologically normal kidneys after fish were placed in clean water from 60 to 300 dph (Weber et al., 2003).

Juvenile rainbow trout exposed to 220 mg NP/L for up to 28 days appeared healthy, but had histopathological changes in their livers, which also showed an increase in the activity of glutathione-S-transferase. All juvenile rainbow trout exposure to 660 mg NP/L died after 4 days (Uguz et al., 2003). Other effects in fish include increased micronuclei in turbot (Barsiene et al., 2006), vacuolation of rainbow trout epidermal cells (Burkhardt-Holm et al., 2000), increased mortality in medaka (Ishibashi et al., 2006), increased mortality and decreased weight and length in platyfish (Magliulo et al., 2002), increased cytochrome P_{450} (CYP19A2) transcription (Kazeto et al., 2004), and decreased plasma insulin-like growth factor and growth of chinook salmon (Fernandez et al., 2007). Fifteen days' exposure to 30 ppb NP caused a decrease in 7-ethoxyresorufin O-deethylation and cytochrome P_{450} (CYP1A) activity and a decrease in glutathione in juvenile Atlantic cod (Sturve et al., 2006).

Other Toxicity in Invertebrates

Midge larvae exposed to NP exhibited increased heat-shock protein mRNA, increased glutathione-S- transferase, (Lee and Choi, 2006; Lee et al., 2006), DNA strand breaks at NP concentrations of ≥0.045 µM (9.9 µg/L), with a marginal effect at 0.005 µM (1.1 µg/L) (Park and Choi, 2007) and increased hemoglobin mRNA (Lee and Choi, 2006). LeBlanc (2000) observed abnormal water flea embryos. Development was delayed in copepods (Forget-Leray et al., 2005). Aquatic microcosms and mesocosms demonstrated changes in zooplankton and phytoplankton species composition (Hense et al., 2003).

Decreased respiration, decreased absorption efficiency, decreased superoxide mutase activity, decreased re-burrowing, and destabilation of hemocyte lysosomal membranes have been reported in bivalves (Canesi et al., 2007; Matozzo and Marin, 2005). Malformed sea urchins have been reported (Cakal Arslan and Parlak, 2007).

Summary and Aquatic Hazard Assessment

NP is toxic to a wide variety of marine and freshwater vertebrate and invertebrate species in laboratory settings. Toxic effects include reproductive and endocrine effects as well as general and systemic toxicity. Most effects are associated with concentrations ranging from 1 to 1000 µg/L, but there are some reports of effects at environmental concentrations less than 1 µg/L. Since most environmental concentrations are less than 1 µg/L, it appears that only the most vulnerable species are likely to be affected and only at the upper range of environmental concentrations. A summary of aquatic toxicity can be found in Appendix 1.

Unfortunately, most of the environmental concentration data are from fresh water systems. It would be useful to gather data on levels in marine environments, especially near municipal and industrial outfalls, landfills, and other possible point sources of NP. Although extrapolating the results of laboratory studies to environmental settings is common practice, it would be preferable to have data based on free-living marine organisms. Unfortunately, there is a paucity of these more-difficult studies.

Animal and Human Studies

Reproductive Toxicity

Introduction

Reproductive toxicity encompases the adverse effects of a substance on the reproductive ability of male and female organisms. Developmental toxicity, discussed in the section following below, is a subset of reproductive toxicity. Reproductive toxicity studies on laboratory animals and *in vitro* studies on human cell lines may be helpful in understanding the mechanism by which NP can alter reproduction in marine organisms, particularly marine mammals. Laboratory animal studies and *in vitro* studies are controlled studies—dose, routes of exposure and length of exposure are all known—which allows for postulation on what minimum levels of exposure exert effects and possibly postulation on the mechanism by which NP might alter reproductive function.

Because the bulk of the literature available is toxicological studies in laboratory rodents, some consideration should be given to the relevance of the findings to marine life. NP is "estrogenic" and may act through the estrogen receptor (ER). A number of ERs have been identified in mammals. This family of nuclear receptors is present in all known vertebrates (Thornton, 2001).

Invertebrates have a variant ER (based on DNA sequencing), which does not, however, bind estrogen.

Laboratory Animal Studies

Males

The varied effects of NP—an exogenous (i.e. originating outside the organism) estrogenic compound—has been extensively examined in laboratory rodents. The effects of NP on numerous endpoints such as reproductive organ characteristics and weight, characteristics of spermatozoa, and hormone profiles have been studied at various dose levels.

Oral exposure of male Sprague-Dawley rats from postnatal day (PND) 23 to PND 52 - 53 with 100 mg NP/kg body weight resulted in testicular damage. Testicular tube diameter was significantly decreased, and 5 out of 12 rats from the NP-treated group did not show any form of spermatogenic cycle (Tan et al., 2003). A number of *in vivo* studies examined the testicular effects of NP, although there was no apparent apoptosis (ie: cell death) in the Sertoli cells. However, several studies using *in vitro* methods showed NP can cause dramatic changes in rat Sertoli cells. An *in vitro* study demonstrated NP induced apoptosis of rat Sertoli cells. Sertoli cells from 20 day old Sprague-Dawley rats were cultured at a density of 5.0×10^4 cells/90 μl; 10 μl of medium containing NP was added such that NP concentrations in 100 μl medium were 0, 200, 1,000, 3,000, or 5,000 parts per billion (ppb) (Wang et al., 2003). NP exposure induced a concentration- and time-dependent decrease in Sertoli cell proliferation. At 3,000 ppb, proliferation was significantly decreased after 72 hours of exposure (Wang et al., 2003). At 5,000 ppb, Sertoli cell proliferation was significantly decreased as early as 24 hours, and was further inhibited at 48 and 72 hours (Wang et al., 2003). Lee et al. demonstrated treatment of neonatal Sprague-Dawley male pups with 8.0 mg NP/kg body weight for 15 days after birth caused changes in the histology (increased intertubular spaces) of the testes when examined on 31 days of age and at approximately 8 months of age (Lee et al., 1999).

Neonatal exposure of Alpk:APfSD (Wistar derived) male rat pups to 8 mg NP/kg/day on PND 1 to PND 10 via intraperitoneal injection produced no significant effect on the male reproductive tract (Odum and Ashby, 2000). NP did not appear to affect other body systems, nor was the growth rate different between treated and control males. Weights of the reproductive organs (testes, epididymides, seminal vesicles, and ventral prostate) were comparable between treated and control animals (Odum and Ashby, 2000).

In a study examining the effects of NP on sperm in mice, sperm capacitation (next to the last step in spermatozoa maturation) and the acrosome reaction were altered; these events are necessary for sperm to be capable of fertilization (Fraser et al., 2006). Cauda epididymal sperm were collected from mature mice, and then incubated in 100 nmol NP/L (22 μg/L). The production of cyclic adenosine monophosphate (cAMP) was significantly stimulated in NP-treated sperm compared with untreated control suspensions (Fraser et al., 2006). Numerous studies have demonstrated that cAMP plays a pivotal role in sperm physiology, with many treatments that accelerate sperm capacitation causing an increase in cAMP. In another study demonstrating NP effects on rats, male Wistar rats were orally given 1, 10, and 100 μg NP/kg/day for 45 days (Chitra et al., 2002). Consequently, the weights of the testes and epididymides significantly decreased, and epididymal sperm counts decreased in a dose-dependent manner while the

activities of antioxidant enzymes (superoxide dismutase, catalase and glutathione reductase) also significantly decreased (Chitra et al., 2002).

In a multi-generation study, Sprague-Dawley rats were exposed by diet to 200, 650 and 2000 parts per million (ppm) NP (which correspond to dietary intakes of 9-35, 30-100, and 100-350 mg/kg/day). The effect in males in the F_2 generation was a reduced epididymal sperm density (8 percent and 13 percent reduced in the 650 ppm and 2000 ppm treatment groups, respectively), and testicular spermatid count was reduced by 13 percent in the 2000 ppm treatment group (Chapin et al., 1999). In a study by Han et al., Sprague-Dawley rats were treated by gavage with 0, 125, and 250 mg NP/kg/day. Rats treated with 250 mg NP/kg/day had a decreased absolute weight of the epididymis, and sperm number in the head of the epididymis was also dramatically decreased (Han et al., 2004). Han et al. also demonstrated the level of testosterone significantly declined in the 250 mg NP/kg/day group, follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels in serum were higher in both NP treatment groups compared with the control group, and the histology within the seminiferous tubules was different within treatment groups. The 125 mg NP/kg/day group had less compact cells, and the 250 mg NP/kg/day group had irregular and disordered arrangement with shedding of cellular material from the seminiferous epithelium compared with the compact and regular arrangement of cells in the control group (Han et al., 2004). The high doses of NP used by Han et al. for a 50 day treatment period also resulted in increases of the relative weights of the kidney and liver of treated animals, which may suggest chronic toxicity.

Laurenzana et al. examined the effect of NP on serum testosterone levels and testicular steroidogenic enzyme activity. Male rats in the F_1 and F_2 generations were exposed through dams or directly through dietary doses of 0, 25, 200 and 750 ppm throughout gestation until sacrifice (PND 2, 50, or 140) (Laurenzana et al., 2002). At PND 2, serum testosterone levels were significantly decreased in the F_1 generation (Laurenzana et al., 2002). The activity of 17α–hydroxylase/C17, 20 lyase (P450c17) was significantly decreased in testicular microsomes of the F_2 generation on PND 50 and PND 140, and testicular β-nicotinamide adenine dinucleotide phosphate (NADPH) P450 reductase activity was also reduced at PND 50 and 140 in the F_1 and F_2 generations (Laurenzana et al., 2002). The activity of P450c17 and NADPH P450 reductase enzymes are necessary for testosterone synthesis. Results from Laurenzana et al. suggest NP can inhibit the activity of enzymes involved in testosterone synthesis.

Gong et al. examined the effect of NP on steroidogenesis in rat Leydig cells using both *in vivo* and *in vitro* exposures. Serum testosterone and LH levels were measured after males were treated with 1, 125, and 250 mg/kg/day for 50 days by gavage. Leydig cells were cultured for 48 hours in low concentrations (0 to 0.022 mg/L) and higher concentrations (0.11 to 5.5 mg/L) (Gong and Han, 2006). *In vivo* exposure to NP resulted in a dramatic decline in testosterone levels at the dose of 250 mg/kg/day, while the LH level increased at the 125 and 250 mg/kg/day dose. The response of Leydig cells to *in vitro* NP exposure was biphasic; at low NP concentrations there was an increase in testosterone levels, while at higher concentrations there was a decrease in testosterone levels (Gong and Han, 2006).

Females

The reproductive effects of NP on female laboratory rodents are less well-examined: endpoints examined include uterotrophic effects, and age at vaginal opening. Administration of increasing doses of NP (1.0, 2.0, and 4.0 mg) to immature female Sprague-Dawley rats 24 hours before

sacrifice resulted in significant increases in the following uterine parameters: weight, protein content, DNA content (in the 4.0 mg group only), and uterine peroxidase activity (Lee and Lee, 1996). Uterine peroxidase activity is known to be sensitive to modulation by substances which modify the uterine responses to estrogen (King et al., 1981). Pre-pubertal Long Evans rats given oral doses of NP (50-100 mg/kg) on PND 21 to 35 had a significant increase in uterine weight, and the age at vaginal opening of treated rats was younger compared with the controls (Laws et al., 2000). Oral exposure to 50, 100, and 200 mg NP/kg resulted in a significant increase in uterine weight compared with exposure to 50, 100, and 200 mg NP/kg by subcutaneous injection (Laws et al., 2000).

In a multi-generation study conducted by Chapin et al., Sprague-Dawley rats were exposed by diet to 200, 650 and 2000 ppm NP. Uterine weight at PND 21 was increased in F_1 females treated with 650 and 2000 ppm NP. Vaginal opening was accelerated by approximately 2 days in the 650 ppm exposure group, and by approximately 6 days in the 2000 ppm exposure group (Chapin et al., 1999).

In Vitro Human Cell-Line Study

Males & Females

The reproductive effects of NP on humans are not well known. Ohshima et al. examined the effects of NP on the enzyme 11 β-hydroxysteroid dehydrogenase (11 β-HSD type 1) using an *in vitro* approach. The 11 β-HSD type 1 enzyme regulates the bioavailability of glucocorticoids by inter-converting physiologically active glucocorticoids to their inactive metabolites. Human liver microsomal 11 β-HSD type 1 and type 2 activities were inhibited by NP (Ohshima et al., 2005). Ohshima et al. also assessed the gonads and accessory genital glands. Expression of an 11 β-HSD isozyme in a reproductive organ suggests the organ may be adversely affected by NP exposure. The porcine uterus is known to express 11 β-HSD type 1 (Yang et al., 1996). The human testis, ovary, and prostate expressed 11 β-HSD type 1 (Ohshima et al., 2005). Except for the prostate, only small amounts of the 11 β-HSD type 2 isozyme were detected in these human tissues compared to kidney (Ohshima et al., 2005).

Summary

Overall, the exposure of males and females to NP results in effects consistent with estrogenic activity of NP. Testicular tube diameter and uteri primarily exhibit seemingly minor alterations in size and weight. Alterations in reproductive organ weight do not necessarily indicate toxicity, but indicate estrogenic activity by NP. The hormonal profiles of male laboratory animals showed reductions in testosterone, increases in FSH, and increases in LH. In females, the earlier average age of vaginal opening suggests puberty occurred at a younger age.

Developmental Toxicity

Introduction

Developmental toxicity is generally considered to include any adverse effects induced by exposure to a toxic chemical during the developmental period (e.g., *in utero*, *in ovo*, during larval development, or postnatally until sexual maturation). Exposure of the parents prior to conception can also contribute to developmental toxicity. Adverse developmental effects can be manifested at any point in the life span of the organism. Developmental toxicity studies on

laboratory animals and humans may provide data that are helpful for deducing the mechanism by which NP can alter development in marine organisms.

Laboratory Rodent Studies

Generally, maternal exposure to hormonally active substances during pregnancy (particularly the period of sexual differentiation) produces adverse effect(s) in the reproductive organs of offspring. Pregnant Long Evans rats gavaged with 100 mg NP/kg body weight on gestation days 15 to 19 had female offspring with advanced lobular development of their mammary gland on PND 22, an increase in uterine weight compared with controls, and a markedly lower staining intensity of progesterone receptors in the mammary gland epithelium (Moon et al., 2007). In contrast, pregnant Donyru rats gavaged with 0, 0.1, 10, or 100 mg NP/kg daily from gestation day 2 up to the day before weaning (PND 21) produced female offspring who had no significant effects on the reproductive system. Exposure of rats to 0.1 – 100 mg NP/kg did not affect uterine growth and development, vaginal opening, hormonal secretion, estrous cyclicity, and morphology of the reproductive organs compared with controls (Yoshida et al., 2003).

A study by Kimura et al. examined the effect of gestational exposure to NP on the development and fertility of female and male ICR mouse offspring. On day 5 to 20 of gestation, pregnant ICR mice were dosed with subcutaneous injections of 1/1000, 1/100 and 1/10 the LD_{50} of NP (1231 mg/kg) (Kimura et al., 2006). There were no significant differences in litter size, sex ratio, or gestational length. Treatment with 1/100 the LD_{50} of NP significantly increased ovarian weight of the offspring, and the uterine weights tended to increase in a dose-dependent manner with large variations (Kimura et al., 2006). The absolute testis weight of males was dose-dependently reduced by gestational exposure to NP; there were no significant differences in testis weights relative to body weight (Kimura et al., 2006). The weights of the testis and epididymis of the 1/10 the LD_{50} of NP group were the most significantly reduced (Kimura et al., 2006). In another study, prenatal exposure of Wistar rats to 75 mg NP/kg body weight from gestational day 11 to 18 resulted in no differences on PND 11 in testis weight, histopathology, or length or diameter of the seminiferous tubules compared with the control group (Dalgaard et al., 2002). The number of Sertoli cells was also comparable between NP-treated and control rats (Dalgaard et al., 2002).

Prenatal exposure of laboratory rodents to NP also results in neurobiological alterations, including some sexually dimorphic behaviors. Masculinization of the sexually dimorphic nucleus of the medial preoptic area (SDN-POA) of the brain depends on perinatal estrogenic exposure during a critical period. Pregnant rats and their pups were continuously exposed to NP through their feed from gestational day 7 until sacrifice at PND 50 (Scallet et al., 1999). NP-treated males had a smaller SDN-POA on average compared with controls (Scallet et al., 1999). The SDN-POA of females were unaltered (Scallet et al., 2001).

Pregnant Sprague-Dawley rats and their offspring were fed diets containing 0, 25, 500, or 2000 ppm NP beginning on gestational day 7 (offspring continued on the same maternal diet until PND 77), and the offspring were evaluated for behavioral alterations. A significant effect of sex and intake of flavored solutions was noted in the offspring (Ferguson et al., 2000). Females consumed nearly twice as much regular water as males over a 3-day period, and females consumed approximately 1.5 times the amount of salt solution as males from the same treatment groups (with the exception of the offspring from the 2000 ppm treatment group) on PND 73 to PND 75 (Ferguson et al., 2000). Pregnant F344/N rats orally dosed with 0.1, or 10 mg NP/kg/day beginning on gestation day 3 until PND 20 had male offspring that displayed altered

behavior in a passive avoidance test (Negishi et al., 2004). NP-treated male offspring tended to delay entry into a dark compartment, and exhibit slightly fewer avoidance responses compared with controls (Negishi et al., 2004).

Exposure to NP also affects synaptogenesis in primary cultures of fetal hypothalamic cells. Fetal rat hypothalamic cells were cultured in 1, 10, 100, and 1000 nM NP; the synaptic density (synapsin 1-positive area / MAP 2-positive area) was significantly increased by 10 nM NP treatment and decreased by 100 nM and 1 µM NP treatments (Ohtani-Kaneko et al., 2002).

Avian Studies

The effects of NP have been examined in the development of immune and endocrine organs of Japanese quail embryos. Japanese quail embryos were injected with a volume of 20 µL containing 1, 10, or 100 µg NP/g egg. Injection of NP into embryonated yolks increased the disappearance of the lymphoid cells from the lymphoid of the bursa at 10 µg NP/g egg, decreased the height of the simple cuboidal epithelial cells surrounding the thyroid follicle at 100 µg NP/g egg, and increased the follicle-like structure in the thymus on the male embryo at 100 µg NP/g egg (Razia et al., 2006).

Human Studies

Disturbances in hormonal regulation during fetal or postnatal development in humans may induce adverse effects on the reproductive system of male and female offspring. There are numerous estrogen responsive tissues which could be affected by exposure to NP such as the testes in males, and mammary glands and placentas in females. Estrogens play an important role in regulating functional differentiation of the placental villous trophoblast. In a longitudinal study of fetal exposures to endocrine disrupting chemicals in Japan, NP was detected in umbilical cords, and evidence showed puberty in prenatally exposed boys and girls occurred at an earlier age (Mori, 2000). The effect of NP on human placental development was also examined using an *in vitro* model of chorionic villous explants. Estrogen receptor (ER) expression was unaffected, but hormone and cytokine secretion were significantly modulated. A gradual increase of β human chorionic gonadotropin (hCG) and a decrease in migration inhibitory factor (MIF) production was observed in NP-treated versus control cultures (Bechi et al., 2006b). Similarly, treatment of placental explant cultures with NP significantly increased β-hCG secretion and trophoblast cell apoptosis, but did not modify ER expression (Bechi et al., 2006a).

Summary

The effects of NP on development in laboratory animals, Japanese quail, and humans are less conclusive compared with reproductive effects. Prenatal exposure to NP appears to have effects consistent with those of other estrogenic compounds (e.g., bisphenol-A) such as early mammary gland development in female offspring. Neurobiological alterations—such as sexually dimorphic behaviors—were also noted as a result of exposure to NP.

Cancer

Introduction

While there are no NP lifetime carcinogenicity assays using rats or mice, there are studies showing that exposure to NP causes effects that have been associated with cancer.

Laboratory Rodent Studies

F344 rats were given NP in the diet at concentrations of 25 or 250 ppm for 28 weeks. The exposed rats had a higher incidence of adenomas and carcinomas, combined, than did rats given a diet without NP. DNA from lung tissue of rats given 25 or 250 ppm NP had an increased amount of 8-hydroxy-2-deoxyguanosine, suggesting the formation of reactive oxygen species during metabolism of NP.

In a 28-day toxicity test in Sprague-Dawley rats, animals given a daily dose of NP (250 mg per kilogram body weight) by oral gavage had enlarged livers and kidneys (Woo et al., 2007). Histological examination of livers of immature male Sprague-Dawley rats given 60 mg/kg body weight NP by i.p. injection found increased mitotic index and abnormal mitoses (Zumbado et al., 2002).

Effects on Cultured Cells

At concentrations of 5 mg/L or 10 mg/L NP, increased the DNA content of cultures of 3T3-L1 cells by 32 percent or 68 percent, respectively, above the DNA content of cells cultured in the absence of NP (Masuno et al., 2003). In a 2-stage initiation-promotion transformation assay using BALB/3T3 cells, NP acted as a promoter (Sakai, 2001).

HeLa cells cultured in the presence of NP had more breaks in DNA than did cells cultured without NP (Park and Choi, 2007). At a concentration of 10 µmoles/L (2.2 mg/L), NP killed 55 percent of cultured MG63 human osteosarcoma cells (Wang et al., 2005). Jumket cells cultured for 24 hours in the presence of 10 µmoles/L NP, were killed.

Summary

There is very little information on the carcinogenicity of NP in the literature. The information available gives some reason for concern, but significantly more information is needed for a determination on the carcinogenicity of NP.

Obesity

Introduction

Stemp-Morlock (2007) observed that the obesity rate has greatly increased over the past 20 years. An estimated one-third of U.S. adults are overweight and more than one-third of U.S. children are overweight or at risk for becoming overweight. There is a strong association between obesity and a number of health issues such as diabetes, coronary heart disease, hypertension, and gall bladder disease. Traditionally obesity has been viewed as a result of reduced physical activities and increased caloric intake. Data from recent studies, however, suggest that exposure to chemicals that perturb the critical pathways in lipid formation, lipid metabolism or energy balance could also initiate or exacerbate obesity. While data are limited, they seem to suggest that NP may possess certain obesogenic properties.

Laboratory Studies

The study of environmental obesogen is an outgrowth of endocrine disruptor research. Hormones are key players in the development and maintenance of adipose tissues. In adults, sex steroids together with growth hormone have fat mobilizing properties (anti-adipogenic), whereas cortisol and insulin have lipogenic effects (Grun and Blumberg, 2007). Adachi et al. (2005)

investigated and found that NP promoted insulin secretion in rat pancreatic islets. NP was shown to bind estrogen receptors (ERα and ERβ) (Shelby et al., 1996; Waller et al., 1996) and these receptors were expressed in rat pancreatic islets (Adachi et al., 2005). ICI 182780 (ICI), an estrogen receptor blocker, suppressed the increase in insulin secretion in NP stimulated pancreatic islets. This led Adachi et al. to conclude that estrogen receptor binding by NP is required for an increase of insulin secretion. The authors also pointed out that the insulin inducing effect could potentially cause hyperinsulinemia, resulting in obesity, exhaustion of pancreatic β-cells, and diabetes.

Treatment with NP significantly stimulated the accumulation of triacylglycerol in mature adipocytes differentiated from 3T3-L1 preadipocytes (Wada et al., 2007). The lipid accumulation was time- and dose-dependent. Increased adipocyte size was noted. Upregulation of expression of genes involved in lipid metabolism, adipocyte differentiation, and inflammation were also observed. Specifically, an increase in the levels of phospholipase A_2 and phospholipase C that are involved in lipid metabolism and TNF-α, an adipocytokine involved in insulin resistance, were seen.

Masuno et al. (2005; 2003), on the other hand, provided data to indicate that NP caused cell proliferation but not lipid accumulation in differentiated 3T3-L1 cells. The presence of NP in the cell cultures caused a dose-dependent increase in DNA contents. Use of bromodeoxyuridine to label DNA during synthesis confirmed that NP enhanced 3T3-L1 cell proliferation. However, triglyceride levels and lipoprotein lipase activity were down, suggesting that NP may interfere with terminal differentiation of adipocytes. ICI suppressed NP stimulated cell proliferation but had no effect on NP induced reduction in lipid accumulation. Using ICI, Masuno demonstrated that the NP stimulated cell proliferation was mediated by the estrogen receptor; whereas NP's interference with terminal differentiation was mediated by a mechanism other than the estrogen receptor.

Summary

Existing data do not provide conclusive evidence that NP is an environmental obesogen. However, several laboratory models suggest that NP may possess obesogenic properties.

Thyroid

Introduction

Thyroid hormones, thyroxine (T4) and triiodothyronine (T3), have diverse functions. They are essential to brain development, influence growth via stimulation of growth hormone, and regulate basal metabolic rates, as well as lipid and carbohydrate metabolism (Greenspan and Gardner, 2003). Environmental chemicals can disrupt thyroid hormone (TH) functions by preventing the biosynthesis via the inhibition of iodide uptake or thyroid peroxidase activity, interfering with the activity of transthyretin that transports THs to target tissues, increasing the metabolism via deiodinases and uridine diphosphate glucuronyltransferase enzymes, or perturbing the binding to thyroid hormone receptors (Zoeller, 2007). The resulting hypothyroidism or thyroid hormone dysregulations in adults may lead to fatigue, weight gain, weak pulse, cold intolerance, mental sluggishness, and depression. Such dysregulation during the perinatal period, on the other hand, could cause cretinism in the affected person, which is characterized by having a short stature, poor motor skills, moderate to severe mental retardation.

Laboratory Studies

Ishihara et al. (2003) investigated the effect of nonylphenol (NP) on 3,3',5-L-$^{[125I]}$triiodothyronine ($^{[125I]}$T3) binding to purified Japanese quail transthyretin (qTTR), a major thyroid hormone-binding protein in plasma, and to the ligand-binding domain of thyroid hormone receptor beta (qTR LBD). The study revealed two classes of binding sites to qTTR, with binding constant (Kd) values of 6.9 and 185 nM, and a single class of binding sites to qTR LBD, with Kd value of 0.31 nM. NP was effective in completely inhibiting $^{[125I]}$T3 binding to qTTR at higher concentrations. Its potency, however, was two orders of magnitude less than that of T3. NP had an insignificant effect on $^{[125I]}$T3 binding to qTR LBD. These results show that NP targets qTTR rather than qTR LBD.

The pituitary is a target of THs. The endocrine disrupting potential of NP was determined by its effect on the cell proliferation of TH-dependent rat pituitary GH3 cell line (Ghisari and Bonefeld-Jorgensen, 2005). NP elicited an inhibitory effect on cell growth. The authors concluded that NP has the potential to exert TH disruption via the pituitary to increase the risk of a negative impact on fetal brain development. However, in a study on the effect of NP on TH-dependent dendritic development of Purkinje cells in mouse cerebellar cultures using serum-free defined medium, unlike bisphenol A, NP did not induce any inhibition, but significantly promoted the dendritic extension of Purkinje cells in the absence of THs (Kimura-Kuroda et al., 2007).

Okada et al. (2005) previously demonstrated that protein disulfide isomerase (PDI) was a target molecule of bisphenol A. In the current study the investigators extended the testing to NP. PDI plays a key role in protein folding as an isomerase and also possesses a $^{[125I]}$T3-binding activity. NP was shown to possess inhibitory effects on the isomerase activity of PDI. The result suggests that NP not only has inhibitory effects on the isomerase activities of PDI but also infers that NP may compete with T3 for receptor binding.

A 28-day repeated oral dose toxicity study of NP was performed for an international validation of the "Enhanced Organisation for Economic Co-operation and Development Test Guideline 407" paying particular attention to the sensitivity of individual endocrine-related parameters (Woo et al., 2007). Sprague-Dawley rats, each group consisting of ten males and ten females, were administered NP once daily by gavage at doses of 0 (control), 10, 50, or 250 mg/kg body weight. An increase of thyroid weight in males was detected from the 50 mg/kg exposure. However, in a developmental study, an injection of NP into Japanese quail embryos decreased the height of simple cuboidal epithelial cells surrounding the thyroid follicle (Razia et al., 2006), which is inconsistent with the previous finding.

To assess interference with endocrine regulation of the thyroid axis, rats (female, ovariectomised) were treated for 12 weeks with NP and other endocrine disruptors on the background of a soy-free or soy-containing diet, and endpoints relevant for regulation via the thyroid axis were measured (Schmutzler et al., 2004). NP inhibited thyroid peroxidase, but increased levels of T4 in rats on a high soy diet and T3 in rats on a low soy diet. There appears to be no uniform, obvious pattern in the effects, but NP elicited a spectrum of alterations, arguing for multiple targets of interference with the complex network of thyroid hormone action and metabolism.

Summary

The available data demonstrate that NP can potentially interfere with TH functions. NP was shown to interfere with TH biosysnthesis, transport, and receptor binding. While it could be inferred that the disruption of TH functions would adversely impact brain development or skeletal growth, additional scientific evidence will be required to establish such connections.

Immune System

Introduction

The immune system is our main defense mechanism against invading microorganisms or tumor growth. Suppressing the immune system would weaken our defense capabilities. Overstimulation of the immune system during an infection, however, could cause extensive collateral damages—"spill-over" destruction of surrounding but otherwise healthy tissues that may prove fatal in some instances. Dysregulation of the immune system in other situations may lead to autoimmunity—attacking "one's own tissues without cause or provocation."

The immune system is under tight, complex regulation to ensure that it continues to function at the optimal range. Existing data suggest that NP could perturb this regulatory apparatus, leading to weakened defense capabilities or detrimental overstimulation of immune functions as an end result. It appears that NP can also affect the immune system indirectly via the neuroendocrine system. Thyroid and sex hormone systems are immunoregulators (Berczi, 1997), and it should not come as a surprise that NP, which is known to disrupt thyroid and estrogen functions, can potentially impact the immune system.

Laboratory Animal Studies

It is interesting to observe that NP seems to have stimulatory effect on thymus cells *in vivo*. Yamashita et al. (2003) reported that NP at 10^{-5} M (2.2 mg/L) in drinking water given to mice for 6 weeks did not affect the body weight of the mice, spleen and thymus cell numbers, and serum immunogloburin concentration. The proliferative responses of spleen cells cultured *in vitro* were not changed. However, the proliferative responses of thymus cells from NP-treated mice were enhanced.

Razia et al. (2006; 2005) studied the effects of NP on the immune organs of Japanese quail and its embryos. In the 2005 study, birds were injected with NP in doses of 1000, 100 and 10 ng/g body weight from 4 to 7 weeks of age. Injection of NP tended to induce many empty vacuoles and increased connective tissue in the bursa of Fabricius (part of the immune system in birds) but did not affect the structures of the spleen and thymus. In the 2006 study, NP was injected into the yolk of embryonated eggs. Injection of NP increased the disappearance of lymphoid cells from the lymphoid follicles in the bursa.

Lee et al. (2003) investigated the influence of NP on allergic immune responses. In this study, they examined the effects of NP on production of IL-4, a cytokine closely associated with allergic immune responses. Their findings indicate the possible enhancement of allergic response by NP through increasing IL-4 production in CD4+ T cells and antigen-specific IgE levels in the sera via the stimulation of Ca2+/calcineurin-dependent NF-AT activation.

In a two-generation feeding study, Karrow et al. (2004) evaluated the potential for NP to modulate certain immune parameters. Pregnant female Sprague-Dawley rats were exposed to NP (0, 25, 500, and 2000 ppm) in their feed for 65 days, beginning 7 days into gestation. The

F(1) generation male and female offspring were exposed *in utero* at the respective treatment levels, commencing the 7th day of gestation, and continuing through to 64 days of age. Changes in splenic antibody-forming cell response, natural killer cell activity, and leukocyte numbers were used to evaluate NP immunotoxicity. The results from this study indicate that dietary exposure to NP can increase splenic natural killer cell activity and splenocyte subpopulation numbers in the F(1) generation rats, without similar changes to the F(0) generation. This suggests that the *in utero* period is a sensitive window for NP exposure.

In Vitro Studies

To elucidate relevance of estrogen distruption to immune responses, Sakazaki et al. (2002) investigated whether ERα exists in mouse splenic B cells and T cells, and the effect of 17 beta-estradiol and endocrine disrupting chemicals such as NP had on lymphocyte mitogenesis. ERα was identified in both male and female mouse splenic cells. Crude splenic cells were stained with anti-ER antibody, and the distribution of ERα in the splenic B cells and part of the splenic T cells was confirmed by flow cytometry. 17 beta-Estradiol inhibited B cell mitogenesis at the concentration of 10^{-8} M (2.2 µg/L) and T cell mitogenesis at the concentration of 10^{-6} M (220 µg/L). NP suppressed lymphocyte mitogenesis at the concentration of 10^{-6}-10^{-5} M. The authors concluded NP may suppress lymphocyte mitogenesis through ERα in B and T cells.

Yao et al. (2005; 2006) investigated the cytotoxicity of NP. In the 2005 study, the effects *in vitro* of NP on apoptosis (the process of programmed cell death) in rat thymocytes were investigated. Thymocytes were treated with NP at 0.1, 1, and 10 ppm, respectively. The results showed that NP induced apoptotic death in thymocytes. These findings suggest that NP may induce apoptosis so as to affect the immune system function. In the followup 2006 study, Yao et al. showed that the cytotoxic effects of NP involved DNA fragmentation (DNA ladder), characteristic of apoptosis. Staining of NP-treated thymocytes showed the typical apoptotic nuclei condensation and fragmentation of chromatin. The rates of apoptosis of the NP-treated thymocytes increased significantly at 4 and 6 hours.

Several studies were conducted to investigate the effects on NP on compromising macrophage functions. Yoshitake et al. (2008) demonstrated that NP suppressed nitric oxide (NO) production and NF-kappaB activation in lipopolysaccharide (LPS)-stimulated macrophages through an estrogen receptor (ER)-dependent pathway. Yoshitake et al. investigated these effects in a mouse macrophage cell line. The results revealed that NP dose-dependently suppressed LPS-induced NO production, as reflected by decreased NO(x) content. The suppressive effects of NP were blocked by the ER inhibitor, ICI. You et al. (2002) studied the effects of NP on the production of NO and tumor necrosis factor-alpha (TNF-alpha), and on the level of inducible NO synthetase and TNF-alpha gene expression in mouse macrophages. NP alone did not affect NO or TNF-alpha production. In contrast, NP inhibited LPS-induced NO and TNF-alpha production in a dose-dependent manner. Treatment with ICI, an estrogen-receptor antagonist, inhibited the suppressive effects of NP. These results demonstrate that NP may affect the regulation of the immune system function by reducing NO and TNF-alpha production through the ER receptor. In a similar study, Hong et al. (2004) investigated the effect of NP on mouse macrophage production of TNF-alpha and NO in response to bacterial endotoxin *in vitro*. NP was shown to inhibit LPS-induced NO production. Two subsequent experiments suggest that NP effects on TNF-alpha and NO in macrophages are a result of down-regulation of gene transcriptions. The activation of the transcription factor NF-kappaB (Igarashi et al., 2006) and IFN-β promoter (Ohnishi et al., 2008) are essential for the production of TNF-alpha and NO. Igarashi et al. and

Ohnishi et al. demonstrated that NP inhibited LPS-induced activation of NF-kappaB and IFN-β promoter.

Summary

NP appears to possess complex immuno-modulating effects. It could stimulate or suppress the immune system. It could also alter immune response pathways. NP's immunosuppressive effects could potentially compromise our abilities to fight infections. It is more difficult to interpret NP's immune-stimulative effects. Existing data do not provide conclusive evidence that such stimulatory effects could predispose the affected individuals to autoimmunity or allergy.

Nervous System

Introduction

NP has both indirect and direct effects on the nevous system. Since gonadal hormones in conjunction with other neurotrophins regulate cell death, neuronal migration neurogenesis, and neurotransmitter plasticity, NP, in disrupting sex hormone functions, can affect brain development. In disrupting thyroid functions, NP can also affect the development of the nervous system because thyroid hormones play an important role in prenatal and neonatal development of the brain (Porterfield and Hendrich, 1993). Early hypothyroidism, for example, caused stunted dentritic growth in hippocampal Cornu Ammonis zone 3 neurons, resulting in cognitive effects including impaired memory, spatial perception, and attention problems (Schantz and Widholm, 2001). In addition, NP may directly cause neurodegeneration. Experimental data from literature indicate that NP has a significant impact on the dopaminergic system.

In Vitro Studies

NP may directly cause neurodegeneration. The treatment of neural stem cells (NSCs) with NP for 24 hours inhibited cell growth in a concentration-dependent manner (Kudo et al., 2004). In addition, treatment with NP resulted in nuclear condensation and DNA fragmentation (morphological changes due to apoptosis) in NSCs after 12 hours of exposure. Furthermore, an exposure to NP led to the accumulation of cells at a specific point of the cell cycle and a reduction in levels of major regulatory proteins that allow the cell to continue to move through the cycle. Together, these results indicate that NP may exhibit a potent cytotoxicity through apoptosis and suggest that NP may affect neurogenesis in the central nervous system. In another study, Kim et al. (2006) used undifferentiated human embryonic stem (hES) cells and the neural progenitor cells derived from them to investigate the potential toxicity of NP. The results showed that the cytotoxic effects of NP involved DNA fragmentation. The NP-induced apoptosis was concomitant effects seen in other studies. In addition, the investigators observed that hES cell-derived neural progenitor cells had a higher sensitivity to the toxicants than undifferentiated hES cells.

The data provided by Bevan et al. (2006) suggest that NP may elicit very disparate effects along divergent signaling pathways than those that arise from the actions of physiological levels of endogenous estrogens. The data highlight important implications with respect to potentially deleterious effects of NP exposure during early neural development. Treatment of dissociated embryonic *Xenopus* spinal cord neurons with NP did not alter cell survival but inhibited neurotrophin nerve growth factor (NGF)-induced neurite outgrowth. These effects were also

seen with comparable concentrations of 17 beta-estradiol (E2). Effects of NP were not inhibited by the nuclear ER antagonist ICI, but were inhibited by the G-protein antagonist, pertussis toxin. These data suggest that the effects of NP are ER independent but G-protein dependent. The ability of NP to inhibit NGF-induced neurite outgrowth without altering survival was also seen in a rat pheochromocytoma cell line. As with Xenopus neurons, the inhibitory actions of NP in pheochromocytoma cells were not antagonized by ICI. In another study, Khan et al. (2003) investigated the influence of alkylphenol endocrine disrupters and the synthetic estrogen diethylstilbestrol on inositol-1,4,5-trisphosphate (IP(3))-sensitive Ca(2+) channels from porcine cerebellum and rat testicular membranes. All alkylphenols and diethylstilbestrol inhibited the extent of IP(3)-induced Ca(2+) release from both cerebellar and testicular microsomes. NP was the most potent compound tested. These results illustrate another mechanism by which NP can disrupt endocrine function without the involvement of estrogen receptors.

Dopaminergic System

Other experimental data from literature indicate that NP has an adverse impact on the dopaminergic system. The following is a synopsis of relevant background and data.

The phenotypic expression of behaviors is the outcome of interacting cortical neuronal networks that are modulated by subcortical components such as the cholinergic neurons of Myenert's basal nucleus, dopaminergic neurons of the Ventral Tegmental Area (VTA), serotongeric neurons in the Raphe nuclei, norepinephrine neurons in the Locus Coeruleus, and histamine neurons in the posterior hypothalamus (Viggiano et al., 2003). Though behaviors emerge from complex interactions, the dopamine systems are very important for the phenotypic expression of attention and reward. It is recognized that the mesolimbic VTA and the nigrostriatal dopaminergic sytems are essential to reward-based learning, novelty-induced behavior, attention, and activity (Andersen and Teicher, 2000; Berridge and Robinson, 1998; Carlsson, 1993). The dysfunction of dopaminergic systems has been associated with neuropsychiatric disorders such as Parkinson's disease, schizophrenia, attention-deficit hyperactivity disorder (ADHD), and autism. Certain drugs used to treat schizophrenia and ADHD, for example, target the dopamine system. Most of the anti-psychotic medications for schizophrenia are dopamine receptor antagonists, whereas drugs for treating ADHD are usually psycho-stimulants that modify dopamine transmission (Viggiano et al., 2003). Methylphenidate, which blocks dopamine re-uptake and effectively increases the synaptic concentration of dopamine, has been used to treat ADHD (Medscape, 2006). Addictive drugs such as cocaine and amphetamine, on the other hand, create a "reward" reinforced behavior by modifying the dopaminergic transmission of VTA.

Sex differences in striatal dopamine content or density of dopamine receptors (D_1 and D_2) during development suggest that sex steroid hormones may mediate the development of dopamine systems in the brain (Andersen and Teicher, 2000; Ferretti et al., 1992). In adults, estrogen appears to be neuroprotective (Marx and Lieberman, 1998). Prenatal "excess" exposure to estrogen seems to have an opposite effect than in adulthood. That evidence was seen in psychotic patients prenatally exposed to diethylstilbestrol (Katz et al., 1987). On the other hand, Turner syndrome, in which a missing X chromosome that causes an absence of estrogen during perinatal life, is associated with cognitive problems and psychosis (Bamrah and Mackay, 1989).

While the development of the dopamine systems is influenced by sex hormones and disruption of sex hormone functions can impact this system, some data, as discussed, suggest that NP could affect the nervous system including the dopamine pathways via other mechanisms rather than via

estrogen disruption. The following data demonstrates NP's effects on the dopamine systems without defining whether these effects are related to endocrine disruption. NP was shown to increase hydroxyl radical formation in the rat striatum (Obata and Kubota, 2000), and enhance hydroxyl radical formation induced by 1-methyl-4-phenylpyridinium ion (Obata, 2006), known to cause neurodegeneration of the substantia nigra and produce acute Parkinsonian symptoms. Antioxidants, histidine and imidaprilat, on the other hand, were shown to suppress NP and 1-methyl-4-phenylpyridinium ion-induced hydroxyl radical generation in rat striatum (Obata, 2006; Obata et al., 2001).

Several studies investigated the causative relationship of NP and motor hyperactivity because of the observed hyperactivity in patients with pervasive developmental disorders, such as autism and ADHD. Masuo et al. (2004a) studied the effects of intracisternal administration of NP and other endocrine disruptors on spontaneous motor activity in neonatal rats. Treatment with NP caused significant hyperactivity during both dark and light phases in rats aged 4-5 weeks. In another experiment, Masuo et al. (2004b) also showed that intracisternal injection of NP in rats on postnatal day 5 caused an increase in spontaneous motor activities at 4 weeks of age. At the same time Masuo et al. observed that NP caused a deficit in dopamine neurons. Added to the evidence, Ishido et al. (2005) reported a single intracisternal administration of NP into 5-day-old male Wistar rats caused significant hyperactivity at 4-5 weeks of age. It was about 1.3- to 1.6-fold more active in the nocturnal phase than control rats. The gene expression of dopamine receptor D_{1A} was decreased by NP by 0.23- to 0.4-fold, whereas that of dopamine D_2 was increased by NP by 2- to 2.8-fold. The results suggest that neonatal treatment with NP can generate an animal model of ADHD, in which clinical symptoms are pervasive.

In a study to examine the relationship between NP and monoaminergic associated behavioral alterations, Negishi et al.(2004) exposed F344 rats perinatally to NP [0.1 mg/kg/day (low dose) and 10 mg/kg/day (high dose) orally] daily from gestational day 3 to postnatal day 20. NP exposure did not affect behavioral characteristics in an open-field test (8 weeks of age), in a measurement of spontaneous motor activity (12 weeks of age), or in an elevated plus-maze test (14 weeks of age). A passive avoidance test (13 weeks of age) showed that NP-treated offspring tended to delay entry into a dark compartment. An active avoidance test at 15 weeks of age revealed that low-dose NP-treated offspring exhibited slightly fewer avoidance responses. In a monoamine-disruption test using 5 mg/kg (intraperitoneal) tranylcypromine, a monoamine oxidase inhibitor, low-dose NP-treated offspring at 22-24 weeks of age failed to show a significant increment in locomotion in response to tranylcypromine, whereas control and high-dose NP-treated offspring significantly increased locomotion behavior after tranylcypromine injection. The results indicate that perinatal NP exposure irreversibly influenced the reception of fear-provoking stimuli (e.g., electrical shock), as well as monoaminergic neural pathways.

Summary

Studies conducted with cultured cells and tissues suggest that NP could adversely affect brain development and may cause neurodegeneration. Laboratory animal data also suggest that NP can specifically affect the dopamine system. Since the dysfunction of dopaminergic systems has been associated with neuropsychiatric disorders such as attention deficit/hyperactivity and autism, the concern is that NP may be a factor in the pathogenesis of such disorders.

Conclusions

Findings

This toxicological profile on NP describes its effects on freshwater and marine life, humans, and laboratory animals.

- NP is toxic to a wide variety of marine and freshwater vertebrate and invertebrate species in laboratory settings. Toxic effects include reproductive and endocrine effects as well as general and systemic toxicity.
- Most NP effects are associated with water concentrations ranging from 1 to 1000 µg/L, but there are some reports of effects at environmental concentrations less than 1 µg/L. Since most environmental concentrations are less than 1 µg/L, it appears that only the most vulnerable species are likely to be affected and only at the upper range of environmental concentrations. Although extrapolating the results of laboratory studies to environmental settings is common practice, it would be preferable to have data based on free-living marine organisms. Unfortunately, there is a paucity of these more-difficult studies.
- Most environmental concentration data are from fresh water systems. It would be useful to gather data on levels in marine environments, especially near municipal and industrial outfalls, landfills, and other possible point sources of NP.
- The exposure of males and females rodents to NP results in effects consistent with estrogenic activity of NP. Testicular tube diameter and uteri primarily exhibit seemingly minor alterations in size and weight. Alterations in reproductive organ weight do not necessarily indicate toxicity, but indicate estrogenic activity by NP.
- The effects of NP on development in laboratory animals, Japanese quail, and humans are less conclusive compared with reproductive effects. Prenatal exposure to NP appears to have effects consistent with those of other estrogenic compounds. Neurobiological alterations – such as sexually dimorphic behaviors – were also noted as a result of exposure to NP.
- There is very little information on the carcinogenicity of NP in the literature. The information available gives some reason for concern, but significantly more information is needed for a determination on the carcinogenicity of NP.
- The available data demonstrate that NP can potentially interfere with thyroid hormone functions, but more studies are needed before any determination can be made that this is an important effect of NP.
- NP appears to possess complex immuno-modulating effects. It may stimulate or suppress the immune system. Its immunosuppressive effects can potentially compromise an organism's abilities to fight infections. It is more difficult to interpret NP's immune-stimulative effects.
- Studies conducted with cultured cells and tissues suggest that NP could adversely affect brain development and may cause neurodegeneration. Laboratory animal data also suggest that NP can specifically affect the dopamine system. Since the dysfunction of

dopaminergic systems has been associated with neuropsychiatric disorders such as attention deficit/hyperactivity and autism, the concern is that NP may be a factor in the pathogenesis of such disorders.

Data Gaps

- Most of the environmental concentration data on NP are from fresh water systems; NP levels in the marine environment were not identified except around point and area sources.
- Little information on environmental fate and food chain exposure to NP was found.
- It is unknown how much plastics contribute to NP concentrations in the environment.
- Data on toxicity to marine organisms, especially free-living marine organisms, are lacking.
- Environment Exposure levels of human is unknown or at least not investigated in this report.
- Developmental toxicity of NP has not been well elucidated.
- Possible subtle effects in neurological development may occur, but is not well enough studied.

Recommendations

- Need to do more literature research and freshwater and ocean sampling of water columns and sediment to determine if there is a contamination problem.
- While reproductive and developmental effects in aquatic organisms are known to occur, other types of toxicity need further research.
- There is a need for further research on NP tissue levels in aquatic organisms for food chain exposure estimates.
- A review of the Ambient Water Quality Concentration levels developed by US EPA should be done to determine if they are still adequate. (AWQC published December 2005.)
- Research is needed to determine whether plastics are a significant contributor to NP environmental exposure.

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Appendix 1: Effects of nonylphenol on aquatic organisms

Species	Exposure (µg/L)	Effect	Reference
Reproductive Toxicity			
Flounder (*Psetta flesus)*	333	↑ vitellogenin (an egg yolk precursor protein expressed in female fish) levels	(Kirby et al., 2007)
Turbot *(Psetta maxima)*	29	↑ plasma vitellogenin ↑ zona radiata protein	(Larsen et al., 2006)
	29	↓ plasma, testicular, and biliary androstenedione & testosterone	(Labadie and Budzinski, 2006)
	30	↓ testosterone and β estradiol ↓ glucuronidation of testosterone and estradiol ↓ P450 aromatase	(Martin-Skilton et al., 2006)
Cod *(Gadus morhua)*	30	↓ glucuronidation of estradiol	(Martin-Skilton et al., 2006)
	29	↑ plasma vitellogenin	(Larsen et al., 2006)
Medaka *(Oryzias latipes)*	5.4	Hepatic vitellogenin levels (♂)	
	61	↓ Fecundity & fertility,	(Ishibashi et al., 2006)
	100 µmolar	↓ spermatozoa swimming speed ↓ ratio of motile spermatozoa	(Hara et al., 2007)
	30-100	↑ female/male ratio, mixed sex characteristics	(Balch and Metcalfe, 2006)
	25	induction of testis–ova and VTG	
	101	↓ reproduction	(Kang et al., 2003)
Rainbow trout *(Oncorhynchus mykiss).*	0.28 – 0.75	↓ embryo development & survival	(Lahnsteiner et al., 2005)
	0.13	↓ semen volume	
	1.05	↑ liver vitellogenin	(Ackermann et al., 2002)
	10.2	↑ liver zona radiate protein	
	220	Biochemical changes in liver	(Cakmak et al., 2006)
	6	↑ liver vitellogenin	(Thorpe et al., 2001)
	100	↑ liver vitellogenin	(Van den Belt et al., 2003)
	0.1 µM (22 µg/L)	↓ salmon gonadotropin releasing hormone2	(Vetillard and Bailhache, 2006)
Tilapia *(Oreochromis mossambicus)*	10-100 µM	↓ thymidine uptake in cartilage	(Ng et al., 2001)
Fathead minnow *(Pimephales promelas)*	5	EC50 vitellogenin induction	(Brian et al., 2005)
	10	vitellogenin induction	(Pickford et al., 2003)
	10	vitellogenin induction	(Marin and Matozzo, 2004)

Species	Exposure (µg/L)	Effect	Reference
Zebrafish *(Danio rerio)*	10 - 100	↑ Female/male ratio, ↓ swim-up	(Lin and Janz, 2006)
	17.7	↓ population growth	(Lin et al., 2005)
	39-100	Ovatestes @ 60 days, not after100-day recovery	(Hill and Janz, 2003)
	100	↓ ♂/♀ ratio ↓ % viable eggs, hatchability, and swim-up	
	500	vitellogenin induction	(Van den Belt et al., 2003)
	500	↓ ♀ Gonadosomatic index	(Yang et al., 2006)
	100	vitellogenin induction ♂	
	50	Thin F1 eggshells. ↓ Cat D & ↑ malformations in F2	
rare minnow *(Gobiocypris rarus)*	10	♂ liver lesions, ↑ VTG ↑ gonadosomatic index,	(Zha et al., 2007)
	30	Testis-ova	
Atlantic salmon *(Salmo salar)*	5-50	↓ brain p450 aromatase B mRNA ↑ zona radiata protein mRNA ↑liver & brain ER α, ↑VTG	(Meucci and Arukwe, 2006)
	15-50	↑ VTG in plasma & mucus	
	10-60	↑ VTG, ZR, ERα & ERβ mRNA	(Arukwe and Roe, 2008)
	15	↑ plasma VTG	(Meucci and Arukwe, 2005)
	≥5	↑ ZR protein in plasma & mucus	
Platyfish *(Xiphophorus maculates)*	80	Hypertrophied Sertoli cells & efferent duct cells. ↓cysts of spermatogenetic cells	(Kinnberg et al., 2000)
	14	↓ gonad development, ↓ spermiogenesis,	(Magliulo et al., 2002)
Swordtail *(Xiphophorus helleri)*	100	Testicular necrosis	(Kwak et al., 2001)
	0.2	↓ sword length	
Killifish *(Fundulus heteroclitus)*	65	↑ liver vitellogenin mRNA	(Garcia-Reyero et al., 2004)
	10 mg/kg	↑ liver vitellogenin	(Pait and Nelson, 2003)
Sheepshead minnow *(Cyprinodon variegates)*	5.6	Vitellogenin LOEL	(Hemmer et al., 2002)
	5.4	Vitellogenin LOEL	(Hemmer et al., 2001)
Worm *(Tubifex tubifex)*	610 µg/g sediment	LC_{LO}; Surviving adults had empty spermatheca if present, spermatic sacs had few germinal elements and no euspermatozons. Ovaries were present but ovocytes were not developed	(Bettinetti and Provini, 2002)
Pond snail *(Lymnaea stagnalis)*	105-124	↓ egg masses, ↑ embryo mortality, delayed development	(Lalah et al., 2007)

Species	Exposure (µg/L)	Effect	Reference
	100	↓ fecundity	(Czech et al., 2001)
Freshwater mudsnail *(Potamopyrgus antipodarum)*	10 µg/kg sediment	↑ number embryos	(Duft et al., 2003)
Zebra mussel *(Dreissena polymorpha)*	500	↑ vitellogenin	(Quinn et al., 2006)
Sea urchin *(Para-centrotus lividus)*	0.27	↓ fertilization (sperm toxicity EC50)	(Ghirardini et al., 2001)
Frog *(Rana pipiens)*	1-10	↑ intersex, ↓ males, ↑ gonadal development	(Mackenzie et al., 2003)
Daphnia magna	40	↓fecundity	(Brennan et al., 2006)
	25, 50	Altered sex ratio	(Zhang et al., 2003)
Midge *(Chironomus riparius)*	1	Altered sex ratio	(Lee and Choi, 2006)
Mysid shrimp	0.01	↑ VTG (not @ 1 or 100)	(Ghekiere et al., 2006)
Rotifer *(Brachionus calyciflorus)*	≥0.59µM (130 µg/L)	↓ population growth	(Radix et al., 2002)
General Toxicity			
Sea bream	200 µg/kg bw	↓ kidney Na+,K+-ATPase, ↑ plasma osmolality	(Carrera et al., 2007)
Turbot *(Psetta maxima)*	30	↑ micronuclei	(Barsiene et al., 2006)
	30	Borderline ↑ micronuclei	(Bolognesi et al., 2006)
Coho Salmon parr	≤ 2g/kg diet	No effect on smoltification	(Keen et al., 2005)
Rainbow Trout *(Oncorhynchus mykiss)*	1	vacuolation of epidermal mucous cells	(Burkhardt-Holm et al., 2000)
	10 (NOEC = 6.3)	↓ length & weight of fry	(USEPA, 2005)
Fathead minnow *(Pimphales promelas)*	14 (NOEC = 7.4)	↓ fry survival	(USEPA, 2005)
Shrimp *(Americamysis bahia)*	6.7 (NOEC = 3.9)	↓ length of offspring	(USEPA, 2005)
Medaka	61	Increased mortality	(Ishibashi et al., 2006)
Zebrafish	1 µM (220 µg/L)	CYP19A2 transcription	(Kazeto et al., 2004)
Platyfish *(Xiphophorus maculates)*	14	↑ mortality, ↓weight & length	(Magliulo et al., 2002)
Atlantic salmon (*Salmo salar*) smolts	10	↑ plasma cortisol	(Lerner et al., 2007b)
	100	Loss of osmoregulatory control	
	10	↓ gill (Na+,K+-ATPase) ↓ preference for & tolerance of seawater, 20% lower plasma insulin-like growth factor, ↑ mortality, 35% lower plasma T3	(Lerner et al., 2007a)
	20	↓ plasma IGF-I concentrations ↓ Smolt weights	(Arsenault et al., 2004)
Chinook salmon (*Oncorhynchus tshawytscha*)	20	↓Growth, ↓plasma IGF1	(Fernandez et al., 2007)

Species	Exposure (µg/L)	Effect	Reference
Japanese eel (*Anguilla japonica*) cultured testicular cells	≥10 µg/ml	proliferation of type A spermatogonia then developmental arrest enlargement of Sertoli cells	(Miura et al., 2005)
Chironomus riparius	5.65 µmol/L	LC50 water	(Maenpaa and Kukkonen, 2006)
	11.4 µmol/kg	LC50 body burden	
	2.04 µmol/kg	↓ survival, low FOC	
	9.23 µmol/kg	↓ survival high FOC	
	100-200 µg/g	Sediment tox ↓ weight ECLO	(Bettinetti et al., 2002)
	300-600 µg/g	Sediment tox LC50	
	484	24-hr LC10	(Lee et al., 2006)
	1	↑ Heat-shock protein mRNA	
	10 (marginal @ 1)	DNA strand breaks	
	10	↑ glutathione-S-transferase	
Chironomus tentans	1 - 100	↑ heat-shock protein & hemoglobin mRNA	(Lee and Choi, 2006)
Daphnia magna	140	48-hr EC50 mobility	(Brennan et al., 2006)
	130	48-hr EC50 moulting frequency	
	40	↑adult mortality	
	210 (250)	24-hr LC50 (NOEC)	(Milam et al., 2005)
	.46µM	↑ abnormal embryos	(LeBlanc et al., 2000)
Ceriodaphnia cornuta	20	48-hour LC50	(Hong and Li, 2007)
	10	48-hour LOAEL (mortality)	
Ceriodaphnia dubia	220	24-hr EC50	(Isidori et al., 2006)
	8	7-day EC50	
	200 (100)	24-hr LC50 (NOEC)	(Milam et al., 2005)
Copepod *(Eurytemora affinis)*	3	Delayed development	(Forget-Leray et al., 2005)
	15	↑ 10 day mortality	
Copepod *(Tigriopus japonicus)*	200	48-hr EC0 motility	(Marcial et al., 2003)
	0.1-10	Delayed completion of naupliar stages	
Aquatic mesocosm	29	↓ Cladocera, ↓ Copepoda ↑ rotatoria, some phytoplankton ↑, some ↓	(Hense et al., 2003)
Aquatic microcosm	~20	Changes in algal species composition and biomass	
bullfrog	234-936	Delayed tail resorption	(Christensen et al., 2005)
Sea urchin	0.94 - 18	Malformations	(Cakal Arslan and Parlak, 2007)
Clam *(Tapes philippinarum)*	25	↓Respiration rate ↓Absorption efficiency	(Matozzo et al., 2004)
	25	↓Superoxide dismutase	
	100	↓Re-burrowing	
Mussel *(Mytilus sp)*	228	Hemocyte lysomal membrane de-stabilization	(Canesi et al., 2007)
Zebra mussel *(Dreissena polymorpha)*	1000	↓ attachment and siphon extension	(Quinn et al., 2006)

Species	Exposure (µg/L)	Effect	Reference
Oyster *(Crassostrea gigas)*	1-100 µg/L	↓ sperm motility	(Nice, 2005)
Leptodea fragilis	570 (130)	24-hr LC50 (NOEC)	(Milam et al., 2005)
Lampsilis cardium	1190 (200)	24-hr LC50 (NOEC)	
Lampsilis siliquoidea	490 (240)	24-hr LC50 (NOEC)	
Megalonaias nervosa	560 (<180)	24-hr LC50 (NOEC)	
Ligumia subrostrata	1040 (240)	24-hr LC50 (NOEC)	
Utterbackia imbecillis	770 (340)	24-hr LC50 (NOEC)	
Pimphales promelas	136	96-hr LC50	(Teneyck and Markee, 2007)
Ceriodaphnia dubia	92.4	48-hr LC50	
Lumbriculus variegatus	6.3 µmol/L	LC50 water	(Maenpaa and Kukkonen, 2006)
	11.5 µmol/kg	LC50 body burden	
Lymnaea stagnalis	100	lesions in foot, mantle	(Czech et al., 2001)
Algae (unspecified)	80 - 530	Algal growth EC50	(Graff et al., 2003)
Algae *(Isochrysis galbana)*	1000	Absence of photosynthesis	(Correa-Reyes et al., 2007)
Diatom *(Skeletonema Costatum)*	27 µg/L.	EC50 growth	
Amphipod *(Eohaustorius estuaries)*	191	LC50	(Hecht and Boese, 2002)
	116	1-hr re-burial EC50	
Nematodes	1 mg/kg sediment	↓ is abundance of some species	(Hoss et al., 2004)

RUNNING HEAD: Ubiquitous distribution of 4-NP in west coast estuaries

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The ubiquitous distribution of 4-nonylphenol in marine organisms in North American Pacific Coast estuaries

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Abstract

Initial surveys of Morro Bay, a small (9 km^2) pristine estuary in central California with low watershed population density and limited pollutant inputs, discovered mud-dwelling arrow gobies (*Clevelandia ios*) with gonadal tumors and liver pathologies. Subsequent chemical analyses of over 120 pollutants in two samples of goby livers found high levels of 4-nonylphenol (4-NP) (716 and 2700 ng/g wet weight), *p, p'*-DDE (82 and 120 ng/g), and total PCBs (25.2 and 25.4 ng/g). A survey of different trophic levels showed that 4-NP accumulates from seawater and sediment to fish, filter-feeders (*Crassostrea* and *Mytilus*), piscivorous birds (*Gavia* and *Aechmophorus*), and marine mammals (*Phocoena*, *Zalophus*, and *Enhydra*). In organisms that are yearlong residents of Morro Bay and are closely linked trophically (e.g. filter-feeders and sea otter), 4-NP accumulates to some of the highest levels recorded. Point sources for 4-NP include a wastewater treatment plant and septic systems, possibly due to 4-NP in toilet paper. Analyses of gobies and filter-feeders from Drakes and San Francisco Bays (California), Netarts Bay (Oregon), and Bamfield Inlet (British Columbia), show similar high levels of 4-NP, suggesting that 4-NP is widespread among Pacific coast estuaries. The liver pathologies in Morro Bay fish are known consequences of exposure to 4-NP, but tumor formation requires tumor-promoting chemicals whose effects could be stimulated by the estrogenic influence of 4-NP.

Key Words: nonylphenol, estrogen mimic, arrow goby, Morro Bay, gonadal tumor

Introduction

Estuaries are important ecosystems: they provide breeding and nursing environments for a number of organisms, some commercially important, and harbor a number of aquaculture operations. Most estuaries are surrounded by human developments and are thus threatened by pollution. It is often difficult to isolate a single pollutant as the cause for biological effects (e.g., tumor occurrence or endocrine disruption) in most bays or larger estuaries. Thus, we focused our study on Morro Bay, a relatively small estuary (9 km^2) located at the base of a 198 km^2 watershed with a low human population density (128 people/ km^2 in 2000) in central California. Morro Bay is considered an estuary in good condition because it lacks contaminants, excepting phosphorus, mercury, DDT, and occasional intrusions of fecal bacteria [1]. A survey of potential indicator species to assess the health of the ecosystem discovered fish with protruding abdomens due to gonadal tumors. Our subsequent histopathological analysis revealed liver pathologies in the mud-dwelling arrow gobies, *Clevelandia ios*, that suggested exposure to organic chemicals. Analysis of 122 pollutants showed that few chemicals are stored in goby tissue and 4-nonylphenol (4-NP) is the only one present at high levels. Further investigations focused on the distribution of 4-NP in Morro Bay, specifically in trophically-related organisms and potential point sources.

Nonylphenol ethoxylates are anthropogenically-produced substances utilized as surfactants in detergents, agricultural sprays, and personal care products, as spermicide in contraceptives, and as stabilizers in plastics. In 2001, approximately 155 million kg of NP were produced in the United States [2]. Although the long-chained, relatively water-soluble ethoxylates degrade during the wastewater treatment process, the breakdown product 4-NP is

hydrophobic, adheres easily to sediment rich in organic material, concentrates in fatty tissue, and persists for decades in anaerobic environments, such as the mudflats of estuaries [3,4].

4-NP is a xenoestrogen [5], resulting in endocrine disruption to a higher degree than expected based on the relative affinity of 4-NP for estrogen receptors [6,7]. Consequences for fish species include induction of the egg-yolk protein vitellogenin, undeveloped gonads, modified testicular structure and decreased sperm count, liver damage, intersex fish, altered sex ratios in populations, and mortality [8,9]. When exposed to 4-NP, marine invertebrates exhibited impaired larval development, decreased growth rates, growth abnormalities, decreased species abundance, and decreased survival [8,9]. 4-NP is known to affect growth of tumor cells [7,10], likely due to its estrogenic properties because 17β-estradiol promotes tumor formation in the livers of trout (*Oncorhynchus mykiss*) and medaka (*Oryzias latipes*) [11,12]. Furthermore, long-term exposures in the laboratory have shown that 4-NP can cause liver pathologies in mosquitofish (*Gambusia holbrooki*) and the rosy barb (*Puntius conchonious*) [13,14].

Because 4-NP adsorbs to marine sediments rich in organic matter [3], it has been shown to specifically accumulate in benthic organisms that live close to the sediment [15]. However, it is unclear if 4-NP biomagnifies along the trophic chain. To date, there are a limited number of studies investigating the biomagnifications of 4-NP, specifically in marine environments [9, 15-18], though its presence has been noted in estuaries of Asia, Europe, and North America [15]. The small Morro Bay estuary provides an ideal test case to assess the distribution of 4-NP, including its sources, since it has a limited number of point sources and a limited trophic chain. We present findings that suggest that 4-NP is indeed biomagnifying along estuarine trophic relationships.

Once we established the presence of 4-NP in Morro Bay, our investigation expanded to determine the spatial extent of contamination in various estuaries on the West coast of North America, focusing on Californian estuaries, but ranging as far north as Vancouver Island, British Columbia, Canada. In addition, we explore the possibility of a link between 4-NP concentration in *C. ios* and tissue pathologies, especially gonadal tumors, in these fish.

Materials and Methods

Sampling of organisms

Organisms were sampled from four California estuaries (Morro Bay, San Francisco Bay, Drakes Bay, and Tomales Bay), one estuary in Oregon (Netarts Bay), and one estuary on Vancouver Island, British Columbia, Canada (Bamfield Inlet).

During low tide arrow gobies (*Clevelandia ios*) from Morro Bay, CA, Tomales Bay, CA, and Bamfield Inlet, Canada, were collected from subsurface burrows with shrimp catchers (aka yabbie pumps), which are hand-pumped, low-pressure suction devices that remove inhabitants of a water-filled burrow to the surface of the mudflats. Gobies were euthanized and stored at -80°C according to our California Polytechnic State University-approved IACUC protocol (#812) and our Bamfield Marine Sciences Centre- approved Animal Utilization Protocol (RS-09-51). *C. ios* from San Francisco Bay were collected by the San Francisco Estuary Institute (SFEI). Thirty-five gobies were collected from four sites in San Francisco Bay in September and October of 2006: Point Isabel Regional Shoreline (near Berkeley, CA), Candlestick Point State Recreation Area (near San Francisco, CA), Martin Luther King Jr. Regional Shoreline (near inner Oakland Harbor), and Bird Island (near Foster City, CA). Ten more *C. ios* were collected from Martin

Luther King Jr. Regional Shoreline by SFEI in October of 2009. The samples were pooled according to collection site for chemical analysis.

Mussels (*Mytilus californianus*) were collected by hand and frozen on dry ice for later storage at -80°C from Morro Bay, CA and Eagle Bay in Bamfield, Canada. Oysters (*Crassostrea gigas*) were purchased and shipped frozen from commercial companies: Giovanni's from Morro Bay, CA, Drakes Bay Oyster Farm from Drakes Bay, CA, and T & S Oyster Farm from Tillamook, OR which harvests oysters in Netarts Bay, OR.

Staghorn sculpins (*Leptocottus armatus*) and Pacific sanddabs (*Citharichthys sordidus*) were collected by net in Morro Bay. Seabird livers were collected by Pacific Wildlife Care Morro Bay, during autopsy from piscivorous birds (grebes of the genus *Aechmorphorus* spp. and Pacific loon *Gavia pacifica*) that were refrigerated upon death, 3 to 5 days prior, then subsequently frozen at -80°C. Marine mammal livers were collected by California Fish and Game (sea otters, *Enhydra lutris nereis*), Santa Barbara Natural History Museum (harbor porpoise, *Phocoena phocoena*), and the Marine Mammal Center in Sausalito, CA (California sea lion, *Zalophus californianus*) during autopsy, then frozen at -80°C. We subsampled interior 2 g sections from female specimens for chemical analysis.

Sampling of water and sediment

Sediment and seawater samples (n = 9, each) were collected from Morro Bay using a strict protocol to avoid contamination of the samples. Metal spoons used to sample the sediment were acetone-soaked for 5 minutes, heated to 300°C for 2 hours, then wrapped in aluminum foil after they cooled for transport to the field. Each sediment sample was scooped from the upper 2

cm with a separate metal spoon held with gloved hands. If the sediment (500 g) adhered to the spoon when it was held upside-down, it was transferred into a glass container and homogenized with the handle of the spoon. Subsurface seawater (1 L) was collected in UV-protected glass bottles from standing pools of water at low tide. Collections of sediment and seawater were made from five sites within Morro Bay in both 2008 and 2009. Samples were stored at -80°C.

Septic tanks were sampled in conjunction with Los Osos Community Services District on November 19, 2008. Los Osos is a community adjacent to Morro Bay whose septage flows into either personal or community septic systems. We sampled two community septic systems: Bayridge, servicing 186 homes, and Vista del Oro, servicing 89 homes. The top biosolid layer, middle liquid portion, and bottom sludge layer (Bayridge only) were each captured using a collecting bucket on a pole, then stored in UV-protected glass bottles at -80°C.

Raw influent and post-polymerization sludge were sampled on August 28, 2008 from the California Men's Colony wastewater treatment plant whose discharge enters into Chorro Creek then flows into Morro Bay. In addition, water and sediment samples were collected 100 m upstream and downstream of the discharge point according to the protocol for water and sediment above, one sample per location.

Products commonly found in septic and wastewater systems, such as toilet paper and tampons, were purchased from local stores for 4-NP analysis.

Sample preparation for chemical analyses

To prepare samples for chemical analysis, dissection tools and glassware were soaked in 99.5% acetone for 5 min, then heated at 300°C for 2 hours. Organisms were dissected on

prepared glassware over ice. Livers were dissected from fish for liver tissue analysis; whole fish tissue analysis required removal of all digestive tissue to avoid contamination from ingested sediment. Mussel and oyster tissues were separated from each organism's valves, and all tissue types were included in the sample.

We analyzed whole fish samples of *C. ios* (minus digestive tracts) collected in April and July 2009 for 122 analytes, including PCBs, PBDEs, PAHs, hormones, legacy and current-use pesticides, butyltins, heavy metals, and industrial and personal use chemicals. Whole fish samples for butyltin species analysis required 52 fish (23 male and 29 female, 1 with a gross tumor). A combined analysis for heavy metals, BPA, BHA, BHT, and bis (2-ethylhexyl) phthalate required 80 fish (30 male and 50 female, 2 with visible tumors). Analysis for PAHs in whole fish tissue required 35 fish (5 male and 30 female, 2 with gross tumors).

We also analyzed *C.ios* liver tissue for 74 of the same analytes in samples of fish collected in January 2008 and April 2009. In order to meet the minimum tissue requirements for analyses of analytes in goby livers, 230 livers were pooled for the January 2008 sample and 209 livers were pooled in the April 2009 sample. The livers in the January 2008 sample were obtained from 65 male and 165 female gobies, 15 of which had gross gonadal tumors; the April 2009 sample was comprised of livers from 52 male and 157 female gobies, 18 of which had gross gonadal tumors.

Whole fish samples contained between 2 and 10 gobies for 4-NP analyses. Regression analysis showed no relationship between number of fish in a sample and the concentration of 4-NP detected ($y = 145 + 2.4x$; $r^2 = 0.001$, $f_{1, 25} = 0.03$, $p = 0.875$), data not shown; similarly, early samples segregating males and female fish for 4-NP analysis failed to show any gender bias (t-

test: t_{13} = -0.01, p = 0.99), so subsequent samples combined genders indiscriminately, data not shown.

Tissues were stored in EPA certified glass vials at -80°C and subsequently shipped overnight on dry ice for chemical analysis to Mississippi State Chemical Laboratory (MSCL), Creek Environmental Laboratories (CEL), or Control Laboratories (CL). MSCL completed the analyses detecting levels of 4-NP in whole fish as well as the contaminants listed in Table 1 in fish livers and whole fish. CL analyzed whole fish tissue for butyltins (Table 2); CEL analyzed whole fish tissue for heavy metals (Table 3) as well as bis (2-ethylhexyl phthalate, bisphenol A (BPA), butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) (Table 2).

Chemical analyses

At MSCL, samples were extracted, purified, and quantified by GC-MS according to the methods of Das and Xia [19] for 4-NP. All isomers of 4-NP detected in the samples are included in reported concentration values. Procedural blanks, duplicate analyses, and spike analyses were conducted every 20 samples for quality control and assurance. Detailed analytical methods for 4-NP, PBDEs, and hormones and personal use chemicals can be found in Das and Xia [19]. Methods for PCBs, PAHs, and legacy and current-use pesticides were developed by MSCL (SOP No. 1.668, SOP No. 1.681, SOP No. 1.667, and SOP No. 1.672).

Histopathology

To prepare *C.ios* for histopathological analyses, the fish were euthanized with an overdose of tricaine methanesulfonate according to our California Polytechnic State University-

approved IACUC protocol (#812), their abdomens were slit open, and they were preserved in a 10% neutral buffered formalin solution. Forty-eight *C. ios* collected in Morro Bay in October 2008 (15 male and 9 female) and April 2009 (5 male and 19 female) and 15 *C. ios* (12 male and 3 female) collected in Bamfield, Canada in November 2009 were analyzed.

Histopathological analysis was conducted on liver and gonads. Tissues were dehydrated in a graded ethanol series and embedded in paraffin. For each tissue block, serial sections (3-4 μm thick) were cut and stained with hematoxylin and eosin. Tissues were screened for a variety of histopathological features and lesions. Livers were analyzed for lesions of glycogen depletion (GD), macrophage aggregate (MA), lipidosis (LIP), and single cell necrosis (SCN). Glycogen depletion is characterized by decreased size of hepatocytes, loss of the 'lacy', irregular, and poorly demarcated cytoplasmic vacuolation typical of glycogen, and increased cytoplasmic basophilia (i.e., blue coloration). Macrophage aggregate is characterized as a cluster of macrophages packed with coarsely granular yellow-brown pigment. Single cell necrosis is characterized by cells having eosinophilic (i.e., pink coloration) cytoplasm with nuclear pyknosis and karyorrhexis. Fatty vacuolar degeneration or lipidosis is characterized by excess lipid appears as clear, round, and well demarcated cytoplasmic vacuoles; eosinophilic protein droplets characterized by the presence of proteins appears as refractile, eosinophilic (pink coloration), round, and well demarcated cytoplasmic vacuoles. Testis and ovary were screened for testicular and ovarian germ cell necrosis. All lesions were scored on an ordinal ranking system of 0 = none/minimal, 1 = mild, 2 = moderate, and 3 = severe using a BH-2 Olympus microscope. Because of the importance of primordial germ cell tumors in the ovary, these lesions are enumerated rather than scored by severity.

Statistical analyses

All statistical tests were conducted with MiniTab (version 15). As stated above, the 4-NP concentrations in *C. ios* were compared with a Student's t-test. The relationship between the number of gobies in a sample and the concentration of 4-NP in the sample was assessed with simple linear regression. In addition, we used simple linear regression to determine whether there were relationships between the percent lipid present in *M. californianus* or *C. ios* tissue and 4-NP concentration. Comparisons of mean concentration of 4-NP in *C. ios* tissue or *C. gigas* tissue among locations were tested using the Kruskal Wallace h statistic, and a similar comparison for *M. californianus* was tested with a Mann-Whitney w statistic. One standard error of the mean is presented with all data.

Results

Chemical contamination of C. ios *in Morro Bay*

C. ios livers contained only four analytes: 4-NP, DDT metabolites, some PCBs, and trans-nonachlor. 4-NP levels in the goby livers were the highest of the contaminants tested: 716 ng/g and 2700 ng/g wet weight (ww), from the January 2008 and April 2009 samples, respectively (Table 1). Whole fish tissue also contained 4-NP and DDT metabolites, though at lower concentrations than in lipidic liver tissue; however, PCBs and trans-nonachlor were not detected (Table 1).

Butyltin species, metals, and PAHs were only analyzed in whole fish samples, and we found that goby tissue contains the four butyltin species, many metals (Al, Fe, Hg, Mn, As, Cr,

Cu, Ni, Se, Zn), diazepam, triclosan, and only one PAH (phenanthrene), present at the detection limit of 10 ng/g (Tables 1-3). Tributyltin and its degradation compounds, dibutyltin and monobutyltin, are introduced into marine environments from anti-fouling paints and are a concern for marine life due to their inhibition of growth and reproduction in marine invertebrates [20]. Wet-weight concentrations of monobutyltin and dibutyltin in whole tissue of *C. ios* were comparable to or lower than levels reported in muscle of marine fishes of previous studies, but levels of tributyltin in *C. ios* were considerably lower than levels in marine fishes of Japan and the Baltic Sea [21,22]. The relatively low levels of the parent compound, tributyltin, to its degradation products in Morro Bay indicate that inputs of tributyltin to the bay have decreased or ceased in recent years (Table 2.).

Many of the heavy metals present in *C. ios* tissue were at concentrations greater than the other analytes tested (Table 3). Toxicity levels of heavy metals can be assessed with the Effects Range Low (ERL) values for marine sediments where each ERL represents the concentration below which less than 10% of studies found an adverse biological effect of the metal [23,24]. Since ERL levels are dry weight values, we used the average percent moisture from all whole goby samples (81.4%) to calculate the dry weight values of heavy metals in goby tissue. Only zinc and mercury exceeded ERL values.

Interestingly, the only chemical to exceed its ERL guideline, 2.2 ng/g (dw), was a breakdown product of the well-known pesticide DDT; *p,p'*-DDE was the second most concentrated chemical in whole fish and liver tissue despite the cessation of agricultural application of DDT to the environment in 1972 (Table 1). DDT and its degradation product *p,p'*-DDE are synthetic chemicals similar to 4-NP in that they are hydrophobic, adhere to organic

matter and soil, persist for decades, and act as endocrine disruptors [25]. 4-NP is not currently a chemical of concern, so ERL guidelines are not available.

NP in the trophic system of Morro Bay

In order to compare concentrations of 4-NP in seawater, sediment, and various organismal tissues within Morro Bay, the concentrations of 4-NP were standardized for this section of the paper according to the percent recovery of the quality control spike and reported as if there had been 100% recovery of 4-NP from each sample. Elsewhere in the paper, following conventional reporting methods, concentrations of 4-NP are given with percent recovery information as reported by the chemical analysis laboratories.

Although the concentration of 4-NP in seawater of Morro Bay did not exceed 0.9 µg/L at any collection time, it was most often present at the detection limit of 0.1 µg/L (Fig. 1). However, the 4-NP concentration in nine samples of anaerobic sediments from five sites in Morro Bay ranged from undetected to 157 µg/kg, averaging 53.1 µg/kg 4-NP (dw), and a single sample of eelgrass contained 33 ng/g 4-NP (ww) (Fig. 1).

Whole organism tissues contained 4-NP levels an order of magnitude higher than the sediment: oysters averaged 203.6 ng/g (ww), mussels averaged 290 ng/g (ww), and arrow gobies had a mean of 185 ng/g (ww) (Fig. 1). Two staghorn sculpin *Leptocottus armatus*, small fish that prey upon *C. ios* but otherwise have a similar diet to that of *C. ios*, had liver concentrations of 1708 and 1809 ng/g (ww) 4-NP, similar to the average of the arrow goby liver samples. However, a piscivorous fish higher in the trophic chain, the Pacific sanddab *Citharichthys sordidus*, contained 4-NP at a concentration of 2914 ng/g (ww) in the single liver sampled,

higher than that of the other fish livers analyzed (Fig. 1).

Piscivorous seabirds (grebes *Aechmophorus spp*. and loon *Gavia pacifica*), contained lower levels of 4-NP in their livers than *C.ios* and the piscivorous fish species (Fig. 1); it is unlikely that these animals are directly linked trophically, but rather simply occupy different trophic levels. The livers of three female harbor porpoise *Phocoena phocoena* and three female California sea lions *Zalophus californianus* had higher concentrations of 4-NP than was found in seabird livers, but lower than that found in *C. ios* and the piscivorous fish group (Fig. 1). On the other hand, three female sea otters *Enhydra lutris nereis* contained the highest levels of 4-NP in their livers that we encountered (Fig. 1).

NP is known to accumulate in the gallbladder, liver, and intestines after waterborne exposure in rainbow trout, *Oncorhynchus mykiss* [26] and Atlantic salmon, *Salmo salar* [27], likely due to its lipophilic nature. Using simple linear regression analysis, we found no relationship between the percent lipid in whole goby samples and the amount of 4-NP in the tissue ($y = 300- 83x$, $r^2= 0.016$, $f_{1,26} = 0.43$, $p = 0.52$), data not shown; however, there was a significant positive relationship between percent lipid and 4-NP concentration in California mussels from the outer coast near Morro Bay ($y = 23.7 + 43.7x$, $r^2= 0.54$, $f_{1,19} = 22.15$, $p = 0.0003$), data not shown.

NP in WWTP/septic near Morro Bay

Downstream of the wastewater treatment plants (WWTP) on Chorro Creek, which flows into Morro Bay, single samples of stream water and sediment contained 4-NP on the day of sampling; upstream of the WWTP the sample of creek water contained 4-NP, but none was

353 detected in the sediment (Table 4). Less 4-NP was measured in raw influent to the WWTP
354 than exited in dewatered sludge (Table 4) because 4-NP levels increase as nonylphenol
355 ethoxylates degrade during wastewater treatment processes, which are not effective at
356 completely removing 4-NP [28].

357 Local community septic tanks near Morro Bay, CA had high levels of 4-NP in the liquid
358 portions of the tanks, especially considering the hydrophobic nature of 4-NP (Table 5). The
359 sludge that accumulated on the bottom of the septic tanks had a 4-NP concentration over
360 5,000,000 µg/kg (dw) (Table 5). The sampled sludge had been accumulating for eight years,
361 highlighting the affinity of 4-NP for and persistence in anaerobic sediment.

362 We analyzed five types of toilet paper for 4-NP and found that two brands made from
363 100% virgin wood pulp (Scott 1000 and Quilted Northern) contained less 4-NP than two
364 environmentally-friendly brands (Green Forest and 7th Generation) made from 100% recycled
365 paper (Fig. 2). Although Charmin Ultra Strong is made from 100% virgin wood pulp, it has high
366 levels of 4-NP. An organic brand of tampons, Natracare, had no detectable 4-NP, but two
367 mainstream brands (Tampax and o.b.) contained 4-NP (Fig. 2).

368

369 *NP in west coast estuaries*

370 By sampling organisms in three California estuaries and one each in Oregon and Canada,
371 we determined that 4-NP contamination was high regardless of local population density or
372 nearby industries or agriculture. Average concentrations of 4-NP in arrow gobies were consistent
373 among 3 bays in California: Morro Bay, Tomales Bay, and San Francisco Bay, as well as with
374 Bamfield, Canada (Table 5; Kruskal-Wallis $h_3 = 1.3$, $p = 0.73$). Similarly, 4-NP concentrations in

the Pacific oyster *C. gigas* did not differ among Morro Bay CA, Drakes Bay CA, and Netarts Bay, OR (Table 5; Kruskal-Wallis, $h_2 = 1.91$, $p = 0.385$) nor were there differences in 4-NP levels between California mussels in Morro Bay, CA and Bamfield, Canada (Table 5; Mann-Whitney, $w = 9.0$, $p = 0.15$).

Histopathology of C. ios *in Morro Bay and Canada*

We have collected 1115 arrow gobies in Morro Bay between 2006 and 2010, and gross gonadal tumors appeared only in female *C. ios* at a rate of 5.7%. (Fig. 3). We analyzed 28 female Morro Bay gobies histopathologically, and although 7% of these fish in this sample had observable gonadal tumors, histopathological analysis detected gonadal tumors in 29% (Table 6).

Histopathological analyses of 48 *C. ios* (28 female and 20 male) from Morro Bay, CA, revealed liver pathologies in both genders though male goby livers tend to have a higher proportion of pathologies than females (Table 6; Fig. 4). In addition, lipidosis is only present in male goby liver tissue. Morro Bay gobies exhibited mild, moderate, and severe levels of all liver pathologies observed.

C. ios from Bamfield displayed one type of liver pathology at only mild levels (42% of male gobies had mild macrophage aggregates in liver tissue) and no gonadal tumors (Table 6). Although only 3 female gobies were examined histopathologically, a further 8 female gobies from Bamfield were dissected for chemical analysis, none of which contained a gross gonadal tumor.

Discussion

NP pathways in Morro Bay

Chemical analysis of whole fish tissue of *C. ios* did not detect most analytes (Tables 1-3), which is expected for relatively pristine Morro Bay. Two of the contaminants, DDT and mercury, were previously known to be at levels of concern, but this study is the first to discover that 4-NP is contaminating Morro Bay and that it occurs at several trophic levels.

The levels of 4-NP increased from seawater to sediment to organisms and accumulated in some, e.g., sea otter, but not all organisms towards the highest trophic level in Morro Bay, CA (Fig. 1).

Measurements of 4-NP levels from adjacent septic systems and river sediment downstream of a wastewater treatment plant suggest that there are continuous sources by which 4-NP enters the bay. These concentrations are extremely high, especially in the anaerobic sludge layer of the septic tanks (Table 4). The liquid portion of the septage is pumped to a leach field atop a hill adjacent to the bay where it can contaminate the bay through underground drainage as well as wind and rain erosion. We did not test the 4-NP levels in the many individually owned septic tanks adjacent to Morro Bay, but they are potential sources of 4-NP to Morro Bay, especially if they are not pumped frequently, have leach fields that enter the estuary, or contain cracks to allow sludge to leak into the soil. We also found evidence that effluent of a WWTP discharging into a tributary of Morro Bay is a source of 4-NP, found to be accumulating in sediment downstream of the discharge point (Table 4).

While Morro Bay sediment has accumulated 4-NP to levels that are high, the values we measured are not the highest recorded, a distinction that belongs to estuarine sediment in Tokyo Bay, Japan and Jamaica Bay, USA [15]. Estuarine sediment is particularly likely to retain 4-NP due to its hydrophobic nature and affinity for adsorption to organic matter as well as its

documented slow rate of degradation, with a half-life of years to decades, under anaerobic conditions [3].

In the case of Morro Bay, the mud-dwelling arrow goby (*C. ios*) further accumulates 4-NP at the level of the whole organism, and even more so in liver tissue (Fig. 1), suggesting that the arrow gobies of Morro Bay are fulfilling the role of mud-dwelling organisms in other estuaries as a common entry route of 4-NP into the trophic chain [15]. The fact that 4-NP is present at higher levels in liver tissue than other tissues of *C. ios*, confirms previous findings where intravenous administration of 4-NP to rainbow trout (*Oncorhynchus mykiss*) led to higher concentrations in bile, feces, and liver compared to kidney, brain, skin, and muscle tissue [26] and where waterborne exposure of 4-NP to Atlantic salmon (*Salmo salar*) led to higher concentrations of 4-NP in the gall bladder, disgestive tract, and liver compared to gill, skin, fat, or brain tissue [27]. In addition, filter-feeders and benthic-feeding fish that are primary consumers (e.g., the Pacific staghorn sculpin feeds on arrow gobies) and live in contact with sediment contain higher levels of 4-NP than the sediment in Morro Bay. Levels of 4-NP recorded in fish and filter-feeders in Morro Bay are in general at least an order of magnitude higher than previously reported for Asian, European, and other North American estuaries [15].

In general, levels of 4-NP in liver tissue were lower in marine mammals and birds than in fish, suggesting that concentrations do not consistently increase with trophic level [16-18] (Fig. 1). Some of these higher-order consumers use Morro Bay transiently, such as seabirds, porpoise, and sea lions. The marine mammal that tends to remain more local, the sea otter, had the highest concentrations of 4-NP in liver tissue of any organism tested (Fig.1). Although we have not established a direct trophic link between our sea otter samples and filter-feeder samples, the otter

liver samples came from animals that were likely residents of Morro Bay. In addition, the bulk of the sea otters' diet consists of a variety of invertebrates, such as sea urchins, bivalves, and gastropods, placing them in a different trophic pathway than the other marine mammals, which consume mainly fish and cephalopods. An additional factor that contributes to the reduced levels of 4-NP in some marine mammals in comparison to fish is the higher clearance rate for 4-NP that distinguishes mammalian from fish hepatocytes [29].

In summary, estuarine sediments seem to act as sinks of 4-NP from sources of the adjacent watershed. 4-NP then enters the trophic chain when mud-dwelling or bottom-feeding organisms absorb the chemical during contact with or ingestion of sediment, then further accumulating 4-NP in specific tissues (e.g., liver). We present evidence for an increase in 4-NP levels up one branch of the local food chain to the level of the resident top predator, e.g. sea otters. However, other marine mammals are not biomagnifying 4-NP, possibly due a combination of factors, such as a higher clearance rate and a weak link to the estuarine trophic chain.

Occurrence of 4-NP in Pacific coast estuaries

We extended our survey of 4-NP levels of marine organisms beyond Morro Bay in part to find an arrow goby population free of nonylphenol. Average levels of 4-NP (whole fish) in arrow gobies from three estuaries in California and one in British Columbia ranged from 105 to 219 ng/g ww; Table 5). Although average 4-NP levels in arrow gobies were lowest at the most remote site, the Bamfield Inlet in British Columbia, the NP levels were not statistically different from those of *C. ios* in California: Morro Bay, San Francisco Bay, and Tomales Bay. However,

463 the concentrations of 4-NP in whole arrow goby tissue were similar to levels of 4-NP in fish of
464 other estuaries in Europe [30,31] and as much as 37 times greater than fish from US rivers or
465 Asian and European seas [15,32]. Mussels showed a similar (but not significant) difference
466 between Morro Bay and the Bamfield Inlet. Compared to previously reported concentrations of
467 4-NP in marine mollusks, the levels of 4-NP we measured in mussels and oysters are in general
468 one or two orders of magnitude larger than elsewhere worldwide [15].

469 Our data strongly suggest that 4-NP is widespread in marine organisms inhabiting Pacific
470 coast estuaries, regardless of proximity to major metropolitan areas. The levels of 4-NP we
471 found are among the highest recorded for marine organisms, suggesting that Pacific coast
472 estuaries are specifically exposed to 4-NP or particularly prone to accumulating 4-NP or both.
473 The consequences of these high 4-NP levels for marine organisms inhabiting these estuaries are
474 unknown.

475

476 *Sources of NP to all estuaries*

477 Bamfield, Canada, a remote town with a human population of about 250 in 2001, is near
478 the northern range limit of *C. ios*. Even this isolated area contains average 4-NP contamination
479 levels in whole fish tissue and mussel tissue similar to average levels found in heavily populated
480 estuaries (Table 5). One common factor influencing all of these areas is the presence of
481 household waste effluent, either through WWTP discharge, septic leach fields, or both. A
482 ubiquitous element in these systems is toilet paper, which has been considered a major input of
483 NP to German wastewater [33]. All five types of toilet paper analyzed contained measurable
484 levels of 4-NP, with higher concentrations associated with higher content of recycled paper (Fig.

2). Nonylphenol ethoxylates are introduced to paper during the manufacturing process, so toilet paper that utilizes post-consumer waste contains a greater proportion of 4-NP due to the degradation of nonylphenol ethoxylates and their probable adherence to pulp during the recycling process. Though not made from recycled paper, Charmin Ultra Strong has high levels of 4-NP, perhaps due to its patented manufacturing methods (patent no. 6540880). Tampons, in much smaller volume and frequency, are another common household input to WWTP and septic systems. Although tampons are contributing less 4-NP to the environment than toilet paper, their use by women places this endocrine disruptor in direct contact with mucosal membranes.

Water quality criteria

Currently, the US EPA recommends that the one-hour average NP concentration and the 4-day average NP concentrations in seawater do not exceed 7.0 μg/L and 1.7 μg/L, respectively [34]. The water samples from intertidal pools remaining on the mudflats at low tide ranged in 4-NP concentration from undetected (detection limit: 0.1 μg/L) to 1.2 μg/L. These samples represent 4-NP concentrations localized in space and time and are within the limits established by the EPA for one hour and 4-day average concentrations of NP in seawater. Liber et. al [35] provide an approach to estimating the long term average exposure levels of fish to 4-NP via a significant ($p < 0.001$) relationship between the average 4-NP concentration in water (X, μg/L) and the measured 4-NP tissue concentration in fish (Y, μg/g ww): $\log(Y) = -1.12 + 0.79 \log(X)$. Their field experiment administered 4-NP to littoral enclosures in a Minnesotan pond and assessed the effects on juvenile bluegill sunfish (*Lepomis macrochirus*). Using the average 4-NP concentration in arrow gobies of Morro Bay, 185 ng/g ww, the equation of Liber et. al [35]

estimates the average 4-NP concentration in Morro Bay waters to be 3.09 μg/L, which exceeds the EPA 4-day average recommendation. It also exceeds Canadian marine water quality guideline that stipulates the concentration of NP and associated ethoxylates should not exceed 0.7 μg/L [36].

Theoretical effects of NP on C. ios

A higher proportion of male, relative to female, gobies in Morro Bay exhibit liver pathologies, but only female gobies have gonadal irregularities, including tumors (Table 6; Fig. 4).

Our histopathological analysis showed both glycogen depletion and lipidosis in liver tissue (15% and 20%, respectively) of male arrow gobies but only glycogen depletion in 14% of female gobies examined from Morro Bay (Table 6; Fig. 4). Glycogen depletion and lipidosis of liver tissue have been observed in response to high levels of estrogen or xenoestrogenic compounds; this response is likely due to the activation of increased synthesis of egg-yolk proteins that deplete the liver of its energy (or glycogen) stores while simultaneously requiring the recruitment of lipids for lipovitellin, a building block of vitellogenin, the main egg-yolk protein [37]. The formation of macrophage aggregates, observed in liver tissue from 30% of male and 4% of female Morro Bay arrow gobies (Table 6; Fig. 4), is a general response of liver tissue to stress, especially with regard to cell detoxification. Single cell necrosis, loss of hepatocellular membranes, and vacuolization of the cytoplasm, have been observed in response to two to four weeklong exposures to 4-NP in liver tissue of rainbow trout and rosy barb [14,38]. General signs of liver pathology in response to long-term exposure to 4-NP (75 days) were also

observed in mosquitofish [13].

Although our histopathological results for liver tissue in Morro Bay are consistent with the known mode of action of NP, none of the Bamfield samples showed the same complete set of pathologies. Liver tissues from *C. ios* collected at Bamfield, Canada, showed only macrophage aggregates in males and no signs of glycogen depletion, lipidosis or necrosis (Table 6; Fig. 4). We propose that this is related to the concentrations of 4-NP in the tissues of fish at these two locations, which while not statistically different, do show differing ranges of contamination that may be biologically meaningful. Four samples of whole fish tissue from Bamfield, Canada show almost no variability, ranging from concentrations of 100 to 110 ng/g 4-NP ww, while forty Morro Bay samples display a large range of variability, between 0 and 550 ng/g 4-NP ww, 53% of which contained more than 121 ng/g 4-NP ww. Uguz et al. [38] have shown that liver pathologies, especially vacuolization and degeneration of cell borders, occur in juvenile rainbow trout in response to 2 weeks of waterborne exposure to 220 µg/L 4-NP once tissue concentrations of 4-NP exceeded 121 ng/g. Furthermore, they determined that activity of the enzyme glutathione-S-transferase, indicative of cellular detoxification, almost doubled in a 66 µg/L 4-NP exposed group of rainbow trout as their tissue concentrations exceeded 166 ng/g. Their study argues for a threshold level, not exceeded by Bamfield fish, above which 4-NP affects the structure of liver tissue as well as initiates a cellular detoxification response.

Thus, so far our findings are consistent with nonylphenol playing a role in the formation of liver pathologies in arrow gobies in Morro Bay. It is less clear that 4-NP plays an exclusive role in stimulating tumor growth in the gonads of female arrow gobies. Instead, 4-NP increases the permeability of cellular membranes, potentially allowing carcinogenic substances access to

bodily organs. 4-NP and its ethoxylates can affect surface tension and surface area of phospholipid membranes, thereby increasing the permeability of cells to toxins and making them more susceptible to their effects [39]. The mucosal cells of rainbow trout epidermis increased vacuolization, had severely deformed nuclei, became larger and irregularly shaped, and were associated with an increased number of infiltrating leukocytes; irregularities were linked more closely with duration of exposure rather than concentration of 4-NP to which they were exposed [40].

The estrogenic mimicry of 4-NP may provide a stimulant to tumor growth in concert with other pollutants. For example, *p, p'*-DDE, the second most prevalent contaminant in Morro Bay (Table 1), is an anti-androgenic compound that up-regulates the expression of the genes for estrogen receptor α in livers of Japanese medaka (*Oryzias latipes*) according to a dose-response relationship for concentrations ranging from 1 to 100 μg/L [41]. An increase in estrogen receptors could increase the effect of any naturally occurring estrogen or xenoestrogens for the fish, thereby allowing a stronger estrogenic influence on the fish [5]. Estrogen, particularly in the form of 17β-estradiol, has been shown to increase the rates of tumor formation in livers of both medaka and juvenile rainbow trout in the presence of tumor promoters [11,12]. In addition, breast tumor cell lines exhibit cell proliferation in the presence of 4-NP and other alkylphenols, likely related to their ability to bind to estrogen receptors [7,10],

There are no studies directly addressing the relationship between 4-NP and tumor formation in gonadal tissue of fish. Our hypothesis is that 4-NP acts synergistically with unidentified tumor-promoting chemicals in the environment by weakening membrane structure and creating an entry point for tumor-initiating substances, then acting as a xenoestrogen to

facilitate tumor progression. In Morro Bay, the presence of *p, p'*-DDE and other anti-androgens may amplify the estrogenic effects of 4-NP by increasing the estrogen receptors present in *C. ios*.

Conclusion

Although we have not established a causal relationship between 4-NP and the tumors in *C. ios*, there is evidence that 4-NP affects the livers of *C. ios* in Morro Bay, CA. More significantly, our study has revealed a major contamination problem in all western North American estuaries that we sampled. A common source to all these estuaries is the infiltration of wastewater or septage containing 4-NP introduced through household products, especially toilet paper. Even dilute inputs of 4-NP to anaerobic environments yield accumulation at extreme concentrations in the sediment and residents of estuaries. Measured levels in seawater are below the EPA regulations, but accumulation in organisms suggests that the inputs to Morro Bay exceed regulations and can lead to biological effects. Restricting levels of 4-NP in water may not be the most effective means of regulating 4-NP entry to the environment given its hydrophobic chemistry and ability to accumulate.

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Figure Legends

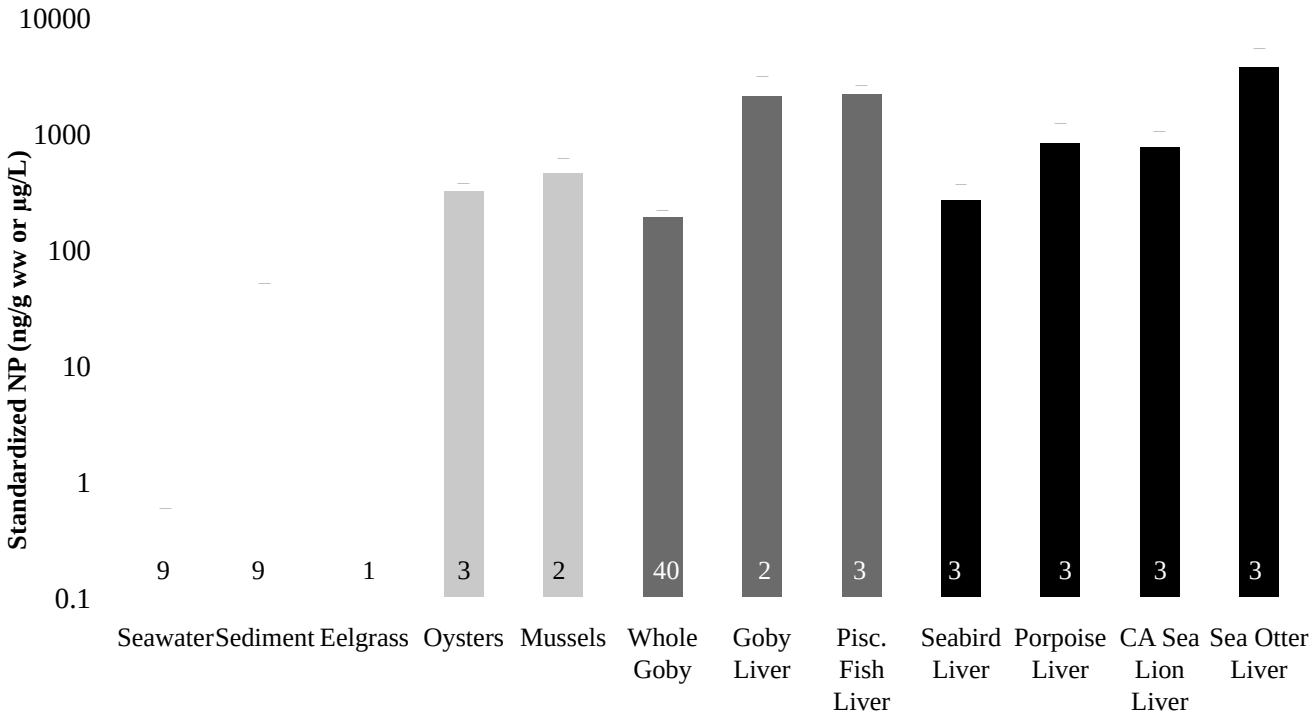
Fig. 1. 4-NP concentrations in Morro Bay, CA. Mean standardized (to 100% recovery values) wet-weight concentrations of 4-NP in Morro Bay seawater (µg/L), sediment (µg/kg), and organisms (ng/g). Error bars are standard error. Sample sizes are on each bar.

Fig. 2. Dry-weight concentrations (µg/kg) of 4-NP in toilet paper and tampons. Quality control reported 100% recovery of spiked value. Sample sizes for all values are n = 1.

Fig. 3. Female *C. ios* dissected to reveal gross gonadal tumor.

Fig. 4. Pathologies in *C. ios* liver and gonadal tissue. Arrows in liver tissue sections indicate fatty vacuolated hepatocytes.

Figure 1


10000
1000
100
10
1
0.1
Standardized NP (ng/g ww or µg/L)
9
9
1
3
2
40
2
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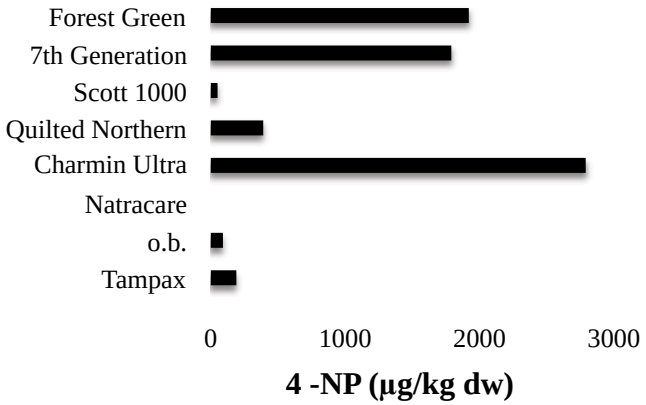
3
3
3
3
Seawater
Sediment
Eelgrass
Oysters
Mussels
Whole Goby
Goby Liver
Pisc. Fish Liver
Seabird Liver
Porpoise Liver
CA Sea Lion Liver
Sea Otter Liver


Forest Green
7th Generation
Scott 1000
Quilted Northern
Charmin Ultra
Natracare
o.b.
Tampax
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4 -NP (µg/kg dw)

Figure 3

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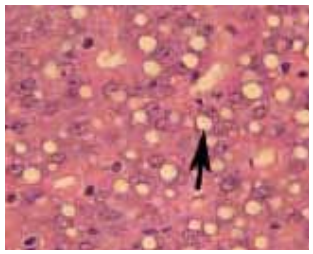
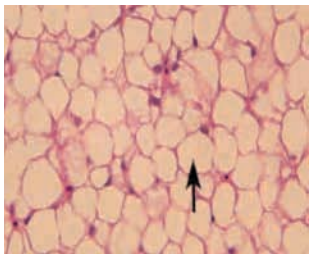
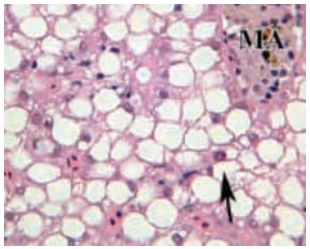


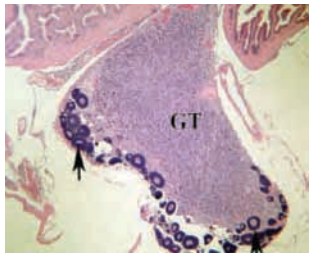
Hyperplysia, severe glycogen depletion in liver tissue



Lipoma in liver tissue



Macrophage aggregates (MA) in liver tissue



Female gonad with primordial germ cell tumor (GT)

Table 1. Wet-weight concentrations (ng/g) of analytes in *C. ios* livers and whole fish tissue, minus digestive tract.

	Livers January 2008[b]	**Livers April 2009[c]**	**Whole fish April 2009[d]**	**LLD[e]**	**% recovery January**	**% recovery April**
4-nonylphenol	716	2700	220	50	66.5	88
4-octylphenol		ND	ND	50		73
4-tert-octylphenol		ND	ND	50		53
Total DDT	94	125	8	2	76-87	91-100
p,p'-DDE	82	120	8	2	78.5	89
trans-nonachlor	ND	5	ND	2	69.5	95
heptachlor	ND			2	NS	NS
cis-nonachlor	ND	ND	ND	2	71.5	99
heptachlor epoxide	ND	ND	ND	2	65.5	96
oxychlordane	ND	ND	ND	2	69.5	95
gamma chlordane	ND	ND	ND	2	72	95
alpha chlordane	ND	ND	ND	2	68	94
dieldrin	ND	ND	ND	2	29.5	95
aldrin	ND			2	NS	NS
endrin	ND	ND	ND	2	59.5	104
hexachlorobenzene		ND	ND	2		78
alpha benzene hexachloride		ND	ND	2		93
gamma BHC	ND	ND	ND	2	62	100
beta BHC		ND	ND	2		86
delta BHC		ND	ND	2		100
mirex		ND	ND	2		100

DDMU	ND	ND	ND	2	NS	NS
diazinon	ND	ND	ND	20	27	NS
chlorpyrifos	ND	ND	ND	10/2	66	NS
methoxychlor	ND	ND	ND	10/2	NS	NS
toxaphene	ND	ND	ND	50	NS	NS
linuron			ND	50		53
Total PBDE	ND	ND	ND	1	58-76	115-125
Total PCBs	25.4	25.2	ND	1	50-114	43-119
17 α-ethynylestradiol	ND	ND	ND	25/4.4	88	96
17 β-estradiol		ND	ND	3.6		98
triclosan	ND		7.37	10/4.92	NS	112
diazepam	ND		2.06	10/1.6	80	91
oxybenzone	ND		ND	10/6.24	80	97
simvastatin	ND		ND	10/29.6	68	98
carbamazepine	ND		ND	40/2	NS	104
phenanthrene			10	10		85
biphenyl			ND	10		86
1,6,7- trimethylnaphthalene			ND	10		81
1- methylnaphthalene			ND	10		91
1- methylphenanthrene			ND	10		80
2, 6 –dimethylnaphthalene			ND	10		71
2- methylnaphthalene			ND	10		86
acenaphthene			ND	10		87
acenaphthylene			ND	10		83

anthracene	ND	10	82
benzo(a)anthracene	ND	10	74
benzo(a)pyrene	ND	10	74
benzo(b)fluoranthene	ND	10	74
benzo(e)pyrene	ND	10	75
benzo(g, h, i)perylene	ND	10	86
benzo(k)fluoranthene	ND	10	76
chrysene	ND	10	82
dibenz(a,h)anthracene	ND	10	82
fluoranthene	ND	10	91
fluorene	ND	10	85
indeno(1,2,3-c,d)pyrene	ND	10	82
napththalene	ND	10	68
perylene	ND	10	73
pyrene	ND	10	90

[a]Concentrations are corrected values based on sample blank.

[b]74.6% moisture, 7.35% lipid, 6.9 g

[c]77.1% moisture, 3.42% lipid, 2.82 g

[d]83% moisture, 0.63% lipid, 41.4 g

[e]If LLD differed for samples, they are presented as Jan./April.

ND = not detected, NS = not spiked

Table 2.Wet-weight concentrations (ng/g) of butyltins and organic compounds tested in *C. ios*.

	Whole fish July 2009	**LLD**	**% recovery spike/duplicate spike**
Monobutyltin	16[a]	2	8.5/9.2
Dibutyltin	14	5	59.5/63.4
Tributyltin	2.2	2	59.4/67.3
Tetrabutyltin	1.4	2	53.3/58.7
Bis (2-ethyylhexyl) phthalate	ND	200	82
Bisphenol A	ND	200	85
Butylated Hydroxyanisole (BHA)	ND	200	61
Butylated Hydroxytoluene (BHT)	ND	200	68

[a]79.8% moisture, 11.85 g

[b]31.6g

ND = not detected

Table 3. Wet-weight concentrations (mg/kg) of metals tested in *C. ios* and approximate dry-weight concentrations. Accepted Effects Range Low (ERL) dry-weight values for metals in sediments.

	Whole fish July 2009[a]	**LLD**	**% recovery spike/duplicate spike**	**Approx. dry-weight equivalent**[b]	**ERL [23]**
Iron (Fe)	43	1	103/99		
Zinc (Zn)	28	4	76/64	150.5	150
Aluminum (Al)	27	2	94/92		
Manganese (Mn)	2.7	0.1	88/89		
Arsenic (As)	1.1	0.4	66/73	5.9	8.2
Chromium (Cr)	0.8	0.4	257/87	4.3	81
Selenium (Se)	0.6	0.5	86/91		
Copper (Cu)	0.5	0.4	68/66	2.7	34
Nickel (Ni)	0.5	0.4	126/92	2.7	20.9
Mercury (Hg)	0.06	0.04	98/98	0.3	0.15
Tin (Sn)	ND	10	16/8		
Antimony (Sb)	ND	0.4	15/20		
Lead (Pb)	ND	0.4	96/87		46.7
Cadmium (Cd)	ND	0.4	90/95		1.2
Silver (Ag)	ND	0.4	83/84		1
Thallium (Tl)	ND	0.4	101/97		

[a]11.1 g

[b]Based on average 81.4% moisture

ND = not detected

Table 4. 4-NP wet-weight concentrations of water (µg/L) and sediment (µg/kg) associated with a WWTP and septic systems near Morro Bay, CA. Reported solid/sediment/sludge concentrations are dry weight with percent moisture in parentheses while liquid concentrations are wet weight. Sample size for each value is n = 1.

Septic Systems

Los Osos Community	Solids[a]	Liquid[b]	Sludge[a]
Bayridge	16,500 (94.7)	7.2	5,073,000 (95.7)
		27.7	
Vista Del Oro	923,000 (99.2)	75.7	
	11,067,000 (94.1)	57.7	

WWTP and Chorro Creek

	Liquid[c]	Sediment/Solids[d]
Raw Influent	16.8	
100 m upstream	3.4	ND (64.7)
100 m downstream	1.3	610 (79.1)
Dewatered Sludge		260 (90.3)

[a] 64% recovery, LLD 100 µg/kg

[b] 124% recovery, LLD 0.1 µg/L

[c] 80% recovery, LLD 0.1 µg/L

[d]115% recovery, LLD 10 µg/kg

Table 5. Mean 4-NP wet-weight concentration (ng/g) and standard error in three species collected in estuaries from California, Oregon, and Canada. Number in parentheses is sample size.

	C. ios	*M. californianus*	*C. gigas*
Morro Bay, CA, USA	167.7± 22.5 (40)[a]	290±100 (2)[e]	203.6±32.1 (3)[e]
SF Bay, CA, USA	161.7±55.4 (6)[b]		
Drakes Bay, CA, USA			211.7 ±44.8 (3)[f]
Tomales Bay, CA, USA	219.7±59.7 (6)[c]		
Netarts Bay, OR, USA			368.0±92.9 (5)[f]
Bamfield Inlet, BC, Canada	105±2.9 (4)[d]	62.5±10.3 (4)[d]	

[a]81-116% recovery

[b]92% recovery

[c]99.5% recovery

[d]88% recovery

[e]65% recovery

[f]89% recovery

Table 6. Percentage of *C. ios* with mild to severe liver pathologies and gonadal germ cell irregularities.

	Morro Bay, CA		**Bamfield, Canada**	
	Male (n=20)	Female (n=28)	Male (n=12)	Female (n=3)
Liver				
Glycogen Depletion	15%	14%	0%	0%
Macrophage Aggregate	30%	4%	0%	0%
Lipidosis	20%	0%	42%	0%
Single Cell Necrosis	10%	21%	0%	0%
Gonad				
Follicular Atresia	0%	14%	0%	0%
Gonadal Tumor	0%	29%	0%	0%
Gross Gonadal Tumor	0%	7%	0%	0%