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

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

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

NP in west coast estuaries

By sampling organisms in three California estuaries and one each in Oregon and Canada, we determined that 4-NP contamination was high regardless of local population density or nearby industries or agriculture. Average concentrations of 4-NP in arrow gobies were consistent among 3 bays in California: Morro Bay, Tomales Bay, and San Francisco Bay, as well as with 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, the concentrations of 4-NP in whole arrow goby tissue were similar to levels of 4-NP in fish of other estuaries in Europe [30,31] and as much as 37 times greater than fish from US rivers or Asian and European seas [15,32]. Mussels showed a similar (but not significant) difference between Morro Bay and the Bamfield Inlet. Compared to previously reported concentrations of 4-NP in marine mollusks, the levels of 4-NP we measured in mussels and oysters are in general one or two orders of magnitude larger than elsewhere worldwide [15].

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

Sources of NP to all estuaries

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

Acknowledgement

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Figure Legends

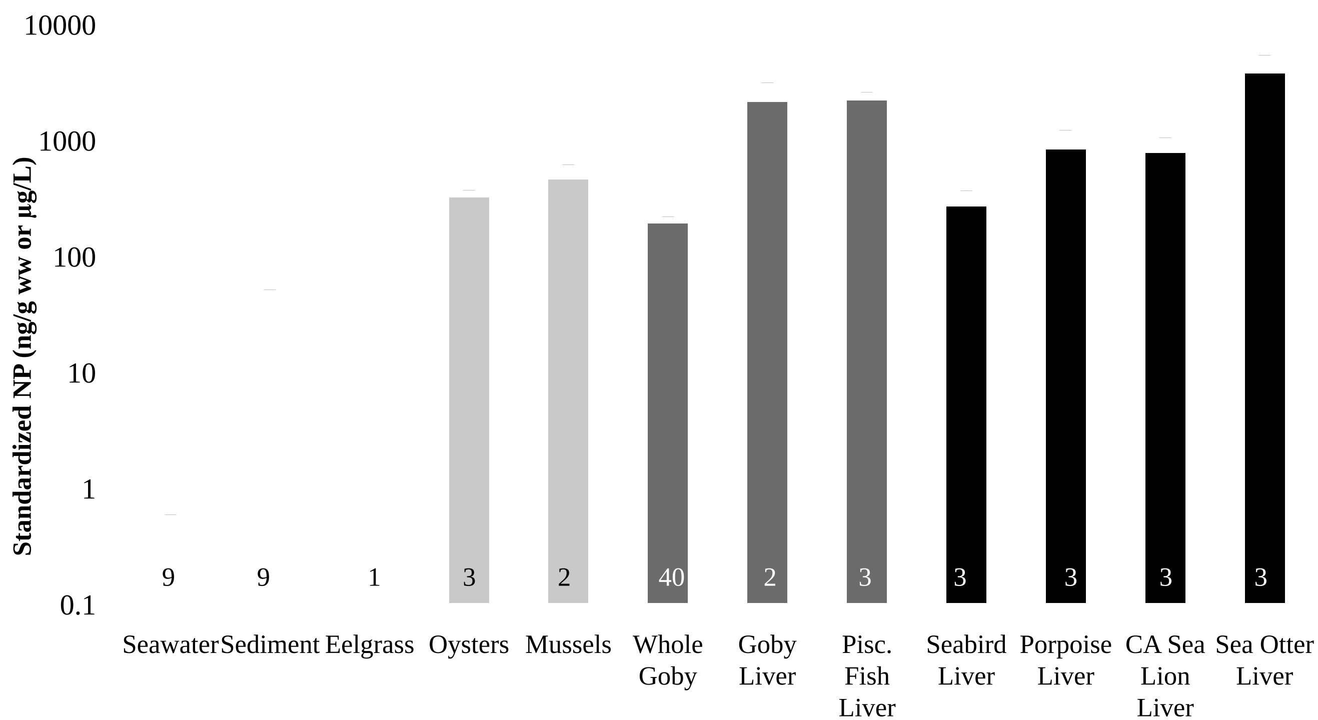
Fig. 1. 4-NP concentrations in Morro Bay, CA. Mean standardized (to 100% recovery values) wet-weight concentrations of 4-NP in Morro Bay seawater (μg/L), sediment (μg/kg), and organisms (ng/g). Error bars are standard error. Sample sizes are on each bar.

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



Standardized NP (ng/g ww or µg/L)
10000
1000
100
10
1
0.1
9
9
1
3
2
40
2
3
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

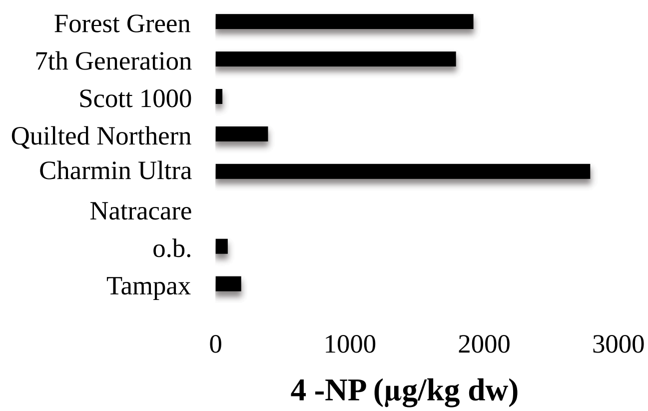
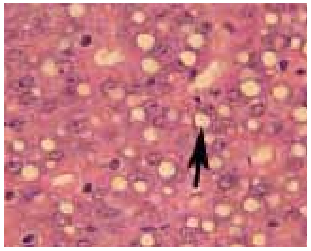
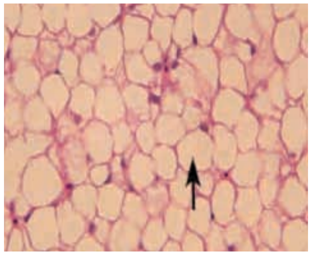

Forest Green
7th Generation
Scott 1000
Quilted Northern
Charmin Ultra
Natracare
o.b.
Tampax
0
1000
2000
3000
4 -NP (µg/kg dw)

Figure 3

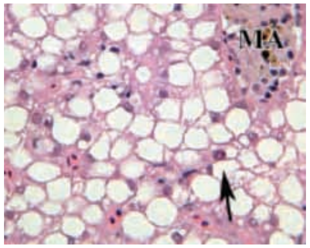
??????????



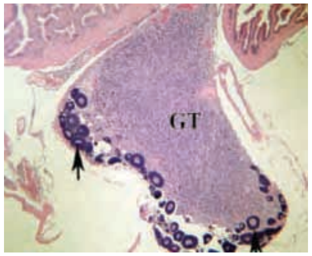
Hyperplysia, severe glycogen depletion in liver tissue



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%