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Nonylphenols in a Lagoon Environment: *p*-Nonylphenol and Nonylphenol Ethoxylates in Fish Tissue

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Alcohol ethoxylates (AnE) and alkylphenol polyethoxylates (APnE) are two classes of nonionic surfactants used most by industry over the past 40 years. The most commonly used APnE in consumer products have nine carbon atoms (C9) with branched alkyl chains and are thus known as nonylphenol polyethoxylates (NPnE). These chemicals are discharged into aquatic environments via urban and industrial wastewater where they are biologically degraded into the by-products nonylphenol ethoxylates ($NPEn_{<5}$) and into the final degradation intermediate nonylphenol (NP) (Talmage 1994). Evidence of how such hydrophobic metabolites (log K_{ow} 4-4.6), especially NP and its lower oligomers (NPE_{1-3}), are persistent and toxic to aquatic organisms is increasing (Lewis 1991). Moreover, other studies indicate that these molecules act as oestrogen mimics affecting the endocrine system of sexually mature fish (endocrine disruption) (White et al. 1994) and placing reproduction at risk (Jobling et al. 1998). In terms of oestrogenic potency, several *in vitro* studies on mammal, bird and teleost cells indicate also that NP and NPE_{1-3} are much stronger than most aquatic pollutants because they mimic the female hormone 17β-oestradiol (Mueller et al. 1978). *In vivo* studies on various fish species show effects such as vitellogenin synthesis in male sex organism, testis-ova, inhibition of testicular growth and modified growth and ovosomatic index (Ashfield et al. 1998). The bioaccumulation of NP and NPE_{1-3} has been studied mainly in freshwater fish species since mostly exposed to sewage treatment plant effluents (STPEs). Because little is known about the exposure mechanisms and the entity of biological accumulation involved in environments other than those investigated up to date, the aim of the present study is to measure concentrations of *p*-NP and NPE_{1-3} in fish sampled in a shallow brackish basin off the Italian coast: the Orbetello Lagoon.

The Orbetello Lagoon is located off the southern coast of Tuscany and covers an area of 2600 hectares and is limited by two sand dunes. An isthmus upon which the town of Orbetello stands, divides the lagoon into an eastern and a western basin. Lagoon waters exchange with the sea by three canals.

The increasing development of anthropogenic and economic activities includes intensive and extensive fish farming with four culture installations with an annual production of about 200 tonnes which are directly discharged into the Lagoon (supply 1150l/s for 35 million m^3/year). In recent years, this has increased

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eutrophication, altered shorelines and accelerated filling-up. Increased tourism during spring and summer results in increased urban waste water coupled with insufficient clearance capability of the only STP which is located in the western basin (Innamorati 1998).

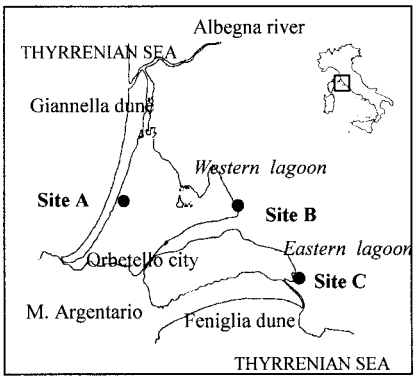

Figure 1. Sampling sites in the Orbetello Lagoon. A Giannella dune; B near the Orbetello sewage treatment plant; C near the settling tank of a phytodepuration plant associated with a fish farm.

MATERIALS AND METHODS

Samples of *Zosterisessor ophiocephalus* were collected in winter 1998 (December) and spring 1999 (March) using fish traps positioned in three different sites of the Lagoon: sites A and B are located in the western basin while site C is located in the eastern basin (Figure 1).
The Grass goby was chosen on the basis of its distribution and abundance in each site. It is a non-migratory bottom dwelling fish species widely distributed in Italian lagoons and successfully used as an indicator organism for monitoring persistent pollutants in aquatic environments. Fish were sacrificed *in situ*. A portion of the midsection from the dorsal fin was withdrawn and wrapped in aluminium foil, stored in liquid nitrogen and shipped to the laboratory where they were stored at –20°C until analysis.

High-purity pesticide HPLC-grade *n*-hexane, isopropanol and acetone were obtained from Sigma-Aldrich (USA). High-purity standards (>99% purity) of *p*-NP was obtained from Fluka while ethoxylates (NPE $_{1-3}$) were obtained from CONDEA (commercial mixture: NONFIX2). Extraction and quantitation method by Marcomini et al. (1990) was modified. Approximately 2 g of fillets were Soxhlet extracted with 160 mL of *n*-hexane for 16h, evaporated to a final volume of 5 mL and then percolated through an aminosilica (NH_2) minicolumn cartridge (Supelco ©). Aliquots (50-200 µL) of the final concentrated extracts were injected into a liquid chromatograph (Waters) connected to a UV spectrophotometer and monitored at 277 nm. Prepacked spherical 3 µm aminopropylsilica columns (Hypersil APS, 100x4 mm i.d., Supelco ©) were used. The *p*-NP and NPE_{1-3} were

eluted under gradient conditions using *n*-hexane and *n*-hexane-isopropanol (100; 85/15) at flow of 2mL min^{-1}. *p*-NP and each ethoxylate (NPE_{1-3}) were quantitated by external calibration curves obtained by plotting measurements of peak areas vs. known weights of each injected compound. Two,4,6-trimethylphenol was added to the extracts as internal standard. Spiked samples showed recoveries greater then 90% for both *p*-NP and NPE_{1-3}. Limits of quantification was 0.01 ngg^{-1} on 1 g of fresh tissue. Residues are reported on a wet weight basis. Data were expressed as mean ± standard deviation (SD). Differences between season and sampling sites were examined by the non-parametric Mann-Whitney *U*-Test. A probability level of less than 0.05 was considered significant. Statistical analyses were performed with Statistica 5.1 (StatSoft, USA).

RESULTS AND DISCUSSION

All the samples collected in winter were males while spring sampling was characterised by a higher occurrence of females (Table 1). In both sampling seasons, no significant differences in length of samples were observed except for fish collected in March in site A which were significantly ($p < 0.01$) bigger than those sampled in December. Although not statistically significant, the highest levels of *p*-NP in fish sampled in winter, were observed in specimens from site B, located closest to the STP of Orbetello city. NPE_{1-3} forms were higher in samples from site C which is closest to the phytodepuration plant. The same trend was observed in samples collected in March with significantly higher levels of *p*-NP in samples from site B ($p < 0.01$) while the highest NPE_{1-3} concentrations were observed in samples from site C ($p < 0.01$). Significant seasonal variations in *p*-NP concentrations were observed in fishes collected in sites B and C. Comparable levels of *p*-NP were in fact observed in samples collected in both seasons in site A while significant increases in *p*-NP concentrations were observed in samples collected in March in site B ($p < 0.01$) and C ($p < 0.05$) (Figure 2). As previously reported for *p*-NP, levels of NPE_1 detected in samples from site A did not show any significant difference between seasons. An opposite trend was observed in the other two sites with significantly ($p < 0.01$) higher levels in samples collected in December respect to those sampled in March (Figure 3). The highest levels of NPE_2 were also detected in site C respect to those reported only for samples from site A (site B below the detection limit; see Table 2) and were also significantly higher in samples collected in March ($p < 0.01$). Detectable levels of NPE_3 were observed only in samples from site C which were significantly higher in samples collected in March ($p < 0.01$).

Concentrations of *p*-NP and NPE_{1-3} in sediment from the selected sampling sites could not be assessed even if they are reported to be more persistent in sediment and on/in macrophytes because of their hydrophobic nature which results in partitioning to solid surfaces (Heinis et al. 1999).

Table 1. Biometric parameters of samples analysed (mean ± SD).

N	Site	Season	Weight (g)	Length (cm)	Sex
10	A	December	13.71 ± 1.94	10.75 ± 0.29	M
10	B	1998	28.15 ± 8.6	13 ± 2.1	M
10	C		39.94 ± 22.2	15.5 ± 3.5	M
10	A	March	25.55 ± 6.91	12.35 ± 1.11	F/M
10	B	1999	29.96 ± 9.65	13.06 ± 1.15	F
10	C		30.45 ± 17.68	12.84 ± 1.97	F

Table 2. Mean (± SD) concentrations (ng g^{-1} w.w.) of *p*-NP and NPE_{1-3} in dorsal fin of specimens collected in December 1998 and March 1999.

Site	Season	*p*-NP	NPE_1	NPE_2	NPE_3
A	December	0.12 ± 0.21	47.42 ± 77.43	0.3 ± 0.6	-
B	1998	0.55 ± 0.33	187.7 ± 10.75	-	-
C		0.15 ± 0.09	508 ± 434.3	0.7 ± 0.99	1.3 ± 1.84
A	March	0.22 ± 0.28	40.49 ± 34.94	36.72 ± 19.77	-
B	1999	1.64 ± 0.82	28.12 ± 10.27	-	-
C		1.41 ± 1.87	271.4 ± 194.7	48.17 ± 39.65	6.44 ± 25.7

"-" levels below the detection limit.

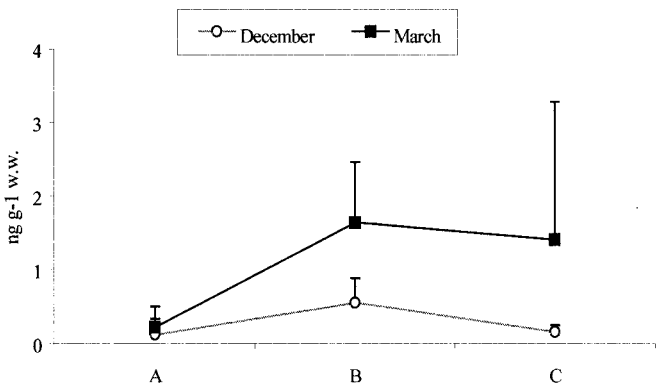

Figure 2. Mean concentrations (± SD) of *p*-NP in samples collected on December 1998 and March 1999 in the three sites: A, B and C.

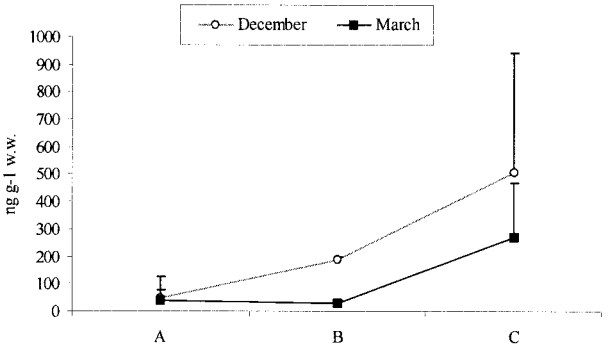

Figure 3. Mean concentrations (± SD) of NPE_1 in samples collected in December 1998 and March 1999 in the three sites: A, B and C.

However benthic feeding and sedentary behaviour of the selected fish species seem to reflect a common source of nonylphenols in an enclosed setting such as the Orbetello Lagoon. These findings were in fact confirmed in both seasons as the highest levels of *p*-NP were found in samples from sites B located near the Orbetello STP, while the highest levels of the ethoxylate forms NPE_{1-3} were found in site C near the settling tank of a fertiliser production plant (phytodepuration) (see Figure 1). No data are currently available on biodegradation efficiency of phytodepuration techniques regarding these compounds shedding no light on the levels detected in site C.

With regard to the behaviour of these molecules in the environment, there are many factors influencing their persistence such as physical and chemical properties and ecosystem-specific properties like the nature and concentration of microbial populations, dissolved and suspended material, temperature and degree of isolation (Ahel et al. 1998).

Even if the levels of both *p*-NP and ethoxylates (NPE_{1-3}) we measured in lagoon fish species seem very low compared to those reported by Ahel et al. (1993), Lye et al. (1999) and Keith et al. (2001) in freshwater fish species from rivers heavily discharged as STPEs, our levels could increase seasonally depending on environmental conditions of the Lagoon. In fact very little water exchange with sea water occurs in Orbetello Lagoon which may be delaying the dilution process leading to an enrichment of sediment accumulation and /or retention of toxic compounds. In shallow waters, the fate of waterborne contaminants is regulated by resuspension and deposition, two processes that strongly depend on currents, wind waves and tides (Marcomini et al. 1990).

Fish mortality, dramatic decrease of biodiversity and environmental stress have been recently reported in Orbetello Lagoon during spring and summer (Fossi et al.1995; Lardicci et al.1997) which is likely to be related to poor water exchange and to low water volume, lack of consistent tides and high temperature variations reaching up to 28°C. During warm seasons nutrient seasonal fluctuations and dissolved oxygen oversaturation cause extreme conditions of hypoxia and

periodically of anoxia. The latter could seriously influence NPnE biodegradation increasing the occurrence of the more persistent oligomers: in fact aerobic conditions seem to produce ethoxylate oligomers (NPE_{2-3}) while an anaerobic pathway can lead to the formation of more persistent oligomers, the monoethoxylate (NPE_1) and *p*-NP (Maguire 1999).

Considerable microalgae blooms also are frequent in spring and summer as they could play an important role in influencing the dynamics of these pollutants taken up by growing algae (BCF up to 10000 in freshwater algae reported by Ahel and co-workers, 1993) to be then released, in particulate form, to the sediment increasing exposure concentrations and bioavailability (Ahel et al. 1993).

The high concentrations of *p*-NP and ethoxylates (NPE_{1-3}) in samples collected in March in which many females were present may be alarming because of the *in vitro* and *in vivo* estrogenic properties reported for these compounds (Ashfield et al. 1998). If greater exposure occurs in a "critical window" of oestrogen sensitivity such as during vitellogenesis, hydrophobic compounds like most aquatic pollutants including NPnE could be passed on to the eggs affecting ontogenesis and resulting in malformed or unviable larvae and eggs (Kime 1997). *Z. ophiocephalus* in March, during the spawning season, lays its eggs in a sort of bottom hole where they are strictly in contact with sediment and sludge.

Moreover, Lewis and Lech (1996) report that the concentrations of *p*-NP needed to produce an estrogenic effect in fish are significantly lower than those being lethal (114 ppb and 193 ppb respectively) and that *p*-NP half-life in muscle were three-fold higher than that in liver (20h to 6h) (Lewis and Lech 1996). Thus a great accumulation of the biologically active form of these molecules, such as *p*-NP and NPE_{1-3}, in the skeletal mass of the fish could have serious implications for the organisms itself in terms of oestrogenic action and for humans and wildlife through dietary exposure (Coldham et al. 1998; Keith et al. 2001).

The results of this study confirm the capacity of *p*-NP and NPE_{1-3} to reach detectable levels even in fish from a brackish environment with higher concentrations of the ethoxylates forms (NPE_{1-3}) than those of the final metabolite *p*-NP. Spatial variability is confirmed by the fact that fishes collected near the Orbetello STP and those from the phytodepuration plant associated to the fish farm contain higher levels of *p*-NP as well as NPE_{1-3}. Seasonal variation has also been observed with higher concentrations of the final *p*-NP and the NPE_2 form in samples collected on March while highest levels of NPE_1 were recorded in December. Further investigation on concentrations in sediment and water are needed to piece together accumulation and environmental exposure of *p*-NP and NPE_{1-3} in fish from brackish ecosystems.

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