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Contamination and sub-lethal toxicological effects of persistent organic pollutants in the European eel (*Anguilla anguilla*) in the Orbetello lagoon (Tuscany, Italy)

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Abstract

This paper reports on contamination levels and their sub-lethal toxicological effects in specimens of the European eel (*Anguilla anguilla*) in the Orbetello Lagoon, (Tuscany, Italy). Organochlorine pesticides (OC) and polychlorinated biphenyls (PCBs) were investigated as priority pollutants in muscle tissue. Phase I P450 enzymes, i.e., EROD, B(a)PMO and the two reductases (NADH ferryred and cyt c.), and cholinesterase (ChE) were assayed in liver and muscle as sensitive biological indicators of fish health. PCBs, lindane and *p,p′DDE* in muscles showed a wide concentration range (0.001–0.025 μg g^{-1} wet weight) and attained the lowest levels in the eastern basin. High homogeneity and relatively low values were observed for phase I P450 enzymes, suggesting that no significant detoxification process of OC pesticides and PCBs occurred. The threat posed by organophosphate insecticides (OP) and CB compounds was also evidenced by ChE activity. The integrated response of phase I P450 enzymes and ChE activity being an indicator of potential effects of toxic contaminant levels on reproductive success and population decline of eels, can be used to assess the overall lagoon quality.

Introduction

Coastal lagoons are ecosystems of great ecological value characterised by moderate biodiversity and trophic migrations that could be at risk without appropriate management strategies (Cognetti & Maltagliati, 2000). The amount and multiple nature of anthropogenic stressors are responsible of adverse impacts on ecological and biological components, which still remain largely unknown.

Traditional methods used to assess ecosystem health mostly rely on classification schemes, which consider abiotic components. Nevertheless, such methods are thought to be insufficient due to the lack of information on the possible effects they may have on the biological integrity of ecosystems as well as on physiological functions of individual organisms (van der Oost et al., 1996b). The presence of a particular chemical does not necessarily imply a measurable decrease in the health of the ecosystem or a toxicological effect on biological components. An ecotoxicological approach, able to integrate toxic contaminant levels with effects on molecular and cellular processes can provide a better understanding of ecosystem health than individual measurements (Schaeffer et al., 1988; Adams et al., 1989; van Gestel & van Brummelen, 1996). Patterns and relationships between contaminant levels and biological responses can also provide early warning signals of susceptibility or damage from single organism up to the whole ecosystem level (Adams et al., 1999).

The Orbetello lagoon is one of the largest brackish water ecosystems in the western Mediterranean: It is located along the Tuscany coast. It is a shallow non-tidal environment with a weak hydrodynamics. Low water volume with limited turnover and partial isolation from the sea drastically reduce the dilution potential of organic matter, nutrients (from urban areas, aquaculture facilities and agriculture waste water) and contaminants of anthropic origin (Barnes, 1995; Innamorati, 1998). The lagoon has been affected by periodic algal blooms and severe dystrophic crises in the past, which caused fish die-offs and a drastic reduction in biodiversity with a low recovery rate of benthic communities (Lenzi, 1992; Cartei & Innamorati, 1997; Lardicci et al., 1997; Cartei et al., 1998; Lardicci et al., 2001).

At present, the main anthropogenic impacts in the lagoon depend upon aquaculture and tourism pressures (Corsi & Focardi, 2002). Eutrophication phenomena have been occurring especially in the eastern basin of the lagoon, which receives sewages from fish farms located in the Ansedonia Canal (Cartei & Innamorati, 1997). The western basin is impacted by pesticides delivered from the Albegna River, surfactants released by the sewage treatment plant of Orbetello, industrial wastewaters and sewages from fish farming (Eljarrat et al., 1999; Corsi & Focardi, 2002; Lenzi et al., 2003; Villa et al., 2003a, b).

The European eel (*Anguilla anguilla*) was selected as the representative fish species of the Orbetello Lagoon. For years, the eel has been prominent in European coastal waters and rivers. The decline in wild stocks and fisheries have been acknowledged by the scientific community since 1980 (Robinet & Feunteun, 2002). Eels breed in the Sargasso Sea and migrate as larval stage to Europe where they metamorphose into more recognisable elvers. Juveniles, or yellow eels, common in rivers, estuaries and coastal lagoons, are relatively stationary and territorial and since they feed on sediments and benthic invertebrates, they are under threat by the sediment-associated contaminants. The extreme high lipidosomatic ratio, up to 31% compared to 3–9% of most freshwater species, coupled with its breeding biology make this species sensitive to bioaccumulation of organic contaminants and to their toxicological effects (Slayter, 1981; Robinet & Feunteun, 2002). Brackish ecosystems including the Orbetello Lagoon were once a suitable habitat for this species, but the increasing anthropic pressure is likely to exert deleterious effects on fish health and populations like the currently documented decline worldwide.

This study aims at describing an integrated ecotoxicological approach to detect the contamination levels and their sub-lethal toxicological effects in specimens of European eel collected from representative sites of the Orbetello Lagoon. Organochlorine (OC) pesticides and polychlorinated biphenyls (PCBs) are investigated as priority pollutants in eels muscle tissue and several enzyme activities are assayed both in liver and muscle as sensitive biological indicators of fish health. Phase I detoxification enzymes are represented by 7-ethoxyresorufin-*O*-deethylase (EROD), benzo(a)pyrene monooxygenase (B(a)PMO), NADH cytochrome c (NADH cyt c red) and NADH ferrycianide (NADH ferry red) reductases. They belong to the mixed function cytochrome P450 oxidase system and are responsible of the oxidative metabolism and detoxification of toxic compounds such as OC pesticides, PCBs and polycyclic aromatic hydrocarbons (Payne et al., 1987; Stegeman & Hahn, 1994). Inhibition of Cholinesterase (ChE) activity is specific bioindicator of exposure to organophosphorus insecticides (OPs) and carbamates (CBs) (Bocquené et al., 1990; Walker & Thompson, 1991; Sturm et al., 1999).

Contaminant levels and biological indicators measured in eels from the seven sites are analysed within a multivariate with a canonical discriminant analysis.

Materials and methods

Study area and sampling

The Orbetello lagoon (Fig. 1) is located on the southern coast of Tuscany (Lat. 42° 30′ N; Long. 11° 10′ E). The surface area is 27 km^2. The lagoon is embraced within two sandbars, which link the mainland to Monte Argentario, once an offshore island. The lagoon is composed by two basin, which are separated by an isthmus. Water from the Albegna River flows through the Fibbia channel into the western basin up north and the Nassa Canal connects it to the sea down south. In the

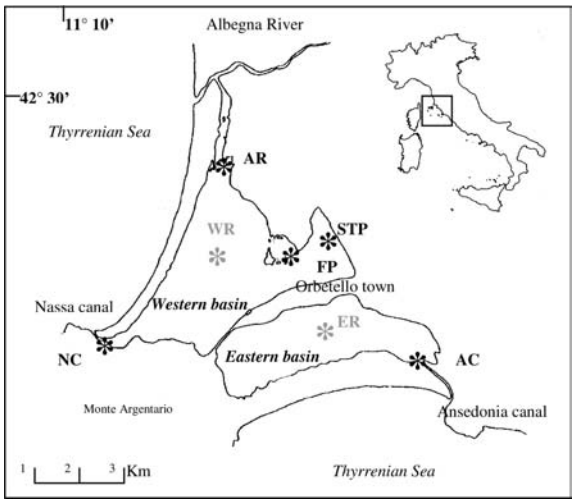

Figure 1. Sampling sites in the Orbetello Lagoon. Western basin: site STP (sewage treatment plant of Orbetello), FP (former fertilizer production plant), site AR (at the mouth of the River Albegna, site NC (Nassa Canal) and reference site WR (middle basin). Eastern basin: site AC (Ansedonia Canal) and reference site ER (middle basin).

eastern basin, exchange with seawater occurs through the Ansedonia Canal. Exchange In the western basin, lagoon and sea exchanges are man-regulated through a scooping plant with a capacity of 16 m^3s^{-1} (Lardicci et al., 2001).

Sampling was conducted in June 2002. Eels were collected using fish traps positioned in seven sites throughout the Orbetello lagoon. Specimens were sampled from seven sites selected as to represent potential sources of contamination: five sites in the western and two in the eastern basins (Fig. 1). Site STP is located near the sewage treatment plant of Orbetello town, which has been described in our previous investigation as moderately contaminated by non-ionic surfactants such as nonylphenols (Corsi & Focardi, 2002). Site FP is located near a former fertilizer production plant where both the presence and the effect of P450 inducers like polychlorodibenzo-*p*-dioxins (PCDDs), -furans (PCDFs) and -naphtalenes (PCNs) have been reported in sediment and biota (Jimenez et al., 1998; Eljarrat et al., 1999; Corsi et al., 2003b). Site AR is at the mouth of the Albegna River which has been reported by Villa et al. (2003a) to have waters polluted by pesticides. Their biological effect were also demonstrated in benthic fish species by Corsi et al., 2003a; Villa, 2003a, b. Site NC is the Nassa Canal which is close to fish farming facilities. Reference site WR, located in the middle of the basin, is less affected by pollution as reported by Nocciolini et al. (2000) based on microbiological parameters of water quality. Two sites were sampled in the eastern basin. Site AC is under the influence of the Ansedonia canal and receives approximately 35 million m^3 $year^{-1}$ of sewages (Lenzi et al., 2003). The reference site ER, located in the middle of the basin, is less affected by pollution.

At each site, 15 sexually immature eels with an average weight of 35 g (silver eel) were collected and shipped to the laboratory in oxygenated coolers with aerated collection-site water. Immediately after being sacrificed, fish were individually weighed and measured. Livers and a portion of dorsal muscle were excised and flash frozen in liquid nitrogen. Samples were analysed individually, half stored at −80 °C for enzyme assays and half at −20 °C for chemical analysis.

Contaminants analysis

OC pesticides including α-, β-, γ-hexachlorocyclohexanes (HCH), hexachlorobenzene (HCB), heptachlor, mirex, α-, γ-chlordane, DDTs and also PCBs were extracted according to Kannan et al. (2001). Eels muscle tissues were homogenised with sodium sulphate and Soxhlet-extracted with methylene chloride/hexane (3:1 v/v) for 16 h. After extraction, a portion of each extract was used for a gravimetric determination of total lipid content.

The extract was rotary evaporated at 34 °C. A multi-layer silica gel column was prepared by packing a glass column (20 mm i.d.) with a series of layers of silica gel. The column was cleaned with 150 ml of hexane and samples were then eluted with 200 ml of hexane and concentrated to 1 ml.

PCBs and OC pesticides were analysed in Certified Reference Material CARP-2 provided by the National Research Council Canada (NRC). Results of two replicates were highly consistent with the certified values with an average error of 5%. A blank sample prepared by the same procedure used for the samples was included every five samples and results were blank corrected. Detection limits were 0.01 ng g^{-1} tissue. OC pesticides and PCB congeners were identified and quantified by using a GC/MS (ion trap detector) from

ThermoFinnigan (Trace^{TM} GC 2000/GCQ plus) equipped with an Rtx-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm) from Restek, using splitless injection mode and helium as carrier gas. Injector temperature was 250 °C. The oven temperature program was 100 °C (held for 2 min), increased at 20 °C/min to 140 (held for 0 min), then increased again at 4 °C/min to 200 °C (held for 13 min) and at 4 °C/min to 300 °C (held for 10 min). The mass spectrometer, tuned on PFTBA, was run with an EI + source, with a source temperature of 250 °C, interface temperature of 280 °C and electron energy of 70 eV. The ions monitored were: 256.1, 258.1 (tri-CB); 290.1, 292.1 (tetra-CB); 326.1, 324.1 (penta-CB); 360.1, 362.1 (hexa-CB); 372.0, 374.0 (^{13}C hexa-CB); 394.0, 396.0 (hepta-CB); 428.0, 430.0 (octa-CB); 181.0, 219.0 (α-, β-, γ-HCH); 284.0, 286.0 (HCB); 373.0, 375.0 (α-, γ- chlordane); 246.0, 248.0 (*p,p′*DDE); 235.0, 237.0 (*o,p′* DDD, *p,p′* DDD, *o,p′* DDT, *p,p′* DDT); 272.0, 274.0 (mirex). The marked ions were used for quantification, the others for confirmation only. A calibration mix of 43 PCB congeners and 14 OC pesticides was used (PCB IUPAC numbers: 18, 22, 28, 31, 41, 44, 49, 52, 54, 56, 60, 64, 70, 74, 87, 90, 95, 99, 101, 104, 110, 114, 118, 123, 132, 141, 149, 151, 153, 155, 156, 157, 158, 167, 170, 174, 180, 187, 188, 189, 194, 199, 203). $^{13}C_{12}$-labelled congener 141 was added as an internal standard. PCB-30 was used as the recovery standard. The concentrations of individually resolved peaks were summated to obtain total PCB concentrations, calculated on wet weight (w.w.) and normalised to lipid weight (l.w.). All reagents were pesticides grade.

Biochemical analysis

The eel livers were homogenised in a 1:4 (w/v) ratio with sucrose buffer (50 mM K_2HPO_4, 0.75 M Sucrose, 1 mM EDTA, 0.5 mM DTT, 400 μM PMSF, pH 7.5) using a Potter-Elvenjem glass/Teflon homogeniser at 2000 rpm. Microsomes were obtained by differential centrifugation in a Sorvall RC28S Ultracentrifuge. Homogenates were first centrifuged at 9000 × *g* for 20 min to remove nuclei, mitochondria, lysosomes and cell debris while the resulting supernatants (S9 fractions) were transferred and centrifuged at 100000 × g for 1 h. The resulting microsomal pellets were subsequently transferred and resuspended in a 1:2.6 (w/v) solution with Tris-(base) buffer (10 mM Tris), 20% p/v glycerol, 0.5 mM DTT, 400 μM PMSF, pH 7.5). All the procedures were carried out at 4 °C as described previously (Corsi et al., 2003a).

Liver microsomal EROD and B(a)PMO activities were measured in triplicate according to the fluorimetric methods of Burke & Mayer (1974) and Kurelec et al., (1977), using a Perkin-Elmer LS50B luminescence spectrofluorimeter. EROD assay conditions in the reaction mixture (final volume 2.25 ml) were as follows: pH 7.5, 30 °C, in a fluorimeter cuvette containing 50 mM Tris-HCl, 25 mM $MgCl_2$ $6H_2O$, 125 μM NADPH and about 50–100 μl of eel liver microsomal fraction. 7-ethoxyresorufin (10 μl 0.1 mgml^{-1} in DMSO) was used as the substrate. The reaction started by adding NADPH and the progressive increase in fluorescence was recorded for 4 min at λ_{EX} = 522 nm /λ_{EM} = 586 nm. The amount of produced resorufin was calculated from a pure resorufin standard calibration curve with a detection limit of 0.05. EROD activity was expressed as picomoles of resorufin produced per minute per milligram of total microsomal protein (pmol min^{-1} mg prot^{-1}).

B(a)PMO assay conditions in the reaction mixture were pH 7.5, 30 °C, 10 mM Tris–HCl, 15 mM $MgCl_2$ $6H_2O$, 1.8 μM NADPH and about 100–150 μl of eel liver microsomal fraction. B[*a*]P (2 mM) was used as the substrate in a 1-h reaction stopped with cool acetone. The amount of produced 3 OH-B[*a*]P was read at λ_{EX} = 396 nm/ λ_{EM} = 522 nm, with 1 M H_2SO_4 and 1 μgml^{-1} quinine sulphate as standards. The fluorimetric assay was carried out according to the method of Nerbert & Gelboin (1968) as modified by Walters et al. (1979). B(a)PMO activity was calculated subtracting blanks obtained by using acetone prior to incubation and expressed as unit of fluorescence per minutes per milligram of total microsomal protein (U.F. min^{-1} mg prot^{-1}).

Measurements of NADH-ferrycianide and NADH cyt. c reductases activities were performed according to the method of Livingstone & Farrar (1984) with the following assay conditions: 25 °C, 100 mM Tris–HCl (pH 7.6), 20 mM KCN, 10 mM cytochrome *c* and ferrycianide and 50 μl of liver microsomal suspension. The reaction started

by adding 2 μM NADH and the progressive decrease in absorbance was recorded for 1 min at a wavelength of 420 nm. The resultant NADH-ferryred and NADH cyt. *c* enzymes activities were expressed as nmol min^{-1} mg $prot^{-1}$. Assays were carried out in triplicate using a Shimadzu UV-160A visible recording spectrometer.

ChE activity was assayed in crude dorsal muscle tissue homogenates (at 2000 rpm) at a ratio of 0.06/1 (w/v) in buffer 0.1 M Tris–HCl, 0.1% Triton, pH 8. The pellet containing cellular debris was discarded and the supernatant was immediately assayed for ChE versus ASCh activity. The reaction was carried on at 30 °C with the following reagents: 25 mM Tris–HCl, 1 mM $CaCl_2$, pH 7.6, ASCh (0.277 mM final concentration), DTNB (0.333 mM final concentration) and 50 μl of sample. ChE versus ASCh activity was assayed by the method of Ellman et al. (1961) in which thiocholine derivates are hydrolysed by cholinesterases to yield thiocholine. Subsequent combination with DTNB forms the yellow anion 5-thio-2nitrobenzoic acid that absorbs strongly at 410 nm. The reaction was initiated by adding ASCh and the progressive increase in absorbance (ΔA) was recorded for 5 min at 410 nm. ASCh-cleaving ChE activity was expressed as nmol min^{-1} mg $prot^{-1}$. Biochemical measurements were carried out in triplicate using a Shimadzu UV-160A visible recording spectrometer.

Protein concentration was measured according to Bradford (1976) using a Shimadzu UV-160A visible recording spectrometer and bovine serum albumin as a standard.

Data were reported as mean and standard deviation (SD). Data were analysed using both univariate and multivariate analysis and were log-transformed for achieving normal distribution before running ANOVA. A probability level of 0.05 was assumed. Comparison among sampling sites were also evaluated by the Student *t*-test for bioindicator responses while the Mann–Whitney–Wilcoxon rank sum non-parametric test was used for contaminant levels. Correlations between bioindicator responses and contaminant levels were determined with the Pearson correlation coefficient (*r*). Statistical analyses were performed with Statistica 5.1 (StatSoft, USA). In addition to the analysis of differences among investigated sites of the individual bioindicators and contaminant levels, all variables from the seven sites were evaluated by multivariate technique with principal component analysis (PCA) (Chatfield & Collins, 1980). A significance test for differences among bioindicators and contaminant levels among sampling sites was performed using the ANOSIM randomisation/permutation test based on mean values among replicates within each site (Clarke, 1993). For each site, variables were first grouped in three replicates and then normalised before statistical analysis. The first five components were analysed. The interpretation of biological significance and quality of the graphical representation has been evaluated by using the eigenvalues and on the cumulative percentage of variance.

Results

Contaminants in eels

Eels collected in the reference site (WR) of the western basin were significantly bigger in size (TL) and weight (g) ($p < 0.05$) than those collected from the other sites. This finding was confirmed by the significant differences observed in total lipids in muscle tissue (Table 1). Contaminant levels were thus normalised to lipid weight in order to compare levels among sites.

OC pesticide and PCB concentrations in muscle tissue of eels from the seven sites are reported in Table 1. PCBs, lindane (γ-HCH) and *p,p′*DDE were the main contaminants present in eels at all the sites in the following order: PCBs > lindane > *p,p′*DDE. α- and β-HCH, heptachlor, mirex, α- and γ-chlordane and *ortho* forms of DDTs were not detected in eels from the Orbetello Lagoon. HCB was detected at all sites at low concentrations ($\sim$1 ng g^{-1}) and no differences were observed against reference values of both basins.

*p,p′*DDE, which account for most of total DDTs, and lindane (γ-HCH) were both higher in eels collected at the sewage treatment plant (STP) and at the mouth of the Albegna River (AR) compared to the reference site (WR). On the contrary, no differences in the levels of these contaminants were observed between the two sites (AC and ER) in the eastern basin.

A similar pattern was also observed for PCBs with higher levels in eels from STP and AR sites,

Table 1. OC pesticides and PCBs (expressed as ng g^{-1} lipid weight) in muscle tissue of eels collected in seven sites throughout the Orbetello lagoon

	Western basin				Eastern basin		
	WR	NC	FP	AR	STP	ER	AC
% total lipids	12.67 (4.62)	5.33 (3.21)	3.33 (0.58)	7.33 (6.81)	6.33 (5.13)	6.01 (4.01)	9.01 (5.66)
γ- HCH (lindane)	85.1 (140.8)	10.26 (17.76)	6.33 (10.96)	161.1 (279)	707.3 (1138)	12.46 (11.93)	9.08 (12.84)
HCB	2.95 (3.94)	1.62 (0.05)	1.78 (1.77)	1.02 (0.9)	2.12 (0.9)	1.07 (0.95)	1.74 (0.26)
*p,p′*DDE	60.88 (21.25)	52.56 (16.96)	39.57 (21.31)	82.63 (46.83)	94.61 (76.39)	30.82 (29.19)	17.06 (8.66)
o,p′ DDT + *p,p′* DDD	9.84 (8.14)	7.47 (3.71)	6.15 (1.35)	9.06 (4.18)	3.79 (3.47)	3.76 (3.88)	5.87 (4.32)
Σ DDTs	71.6 (28.65)	60.03 (20.49)	45.72 (22.47)	91.69 (50.32)	98.40 (73.52)	34.57 (33.03)	22.93 (12.98)
Total PCBs	149 (19.68)	158.9 (72.16)	170.7 (72.32)	191.3 (71.86)	186.4 (59.50)	118.1 (70.81)	97.94 (35.28)

α-, β-HCH, heptachlor, mirex and α-, β-chlordane were below the detection limit of 0.01 ng g^{-1} in all investigated sites. The percentage of lipid varies consistently in eels from seven sites with an average value of 7.14 (2.99). Data are reported as mean value (standard deviation).

although no significant differences were observed. They showed a more homogeneous distribution among sites compared to total DDTs and lindane, with the lowest levels at the Ansedonia Canal (AC) (eastern basin). The mean concentration of PCBs (153 n gg^{-1} l.w.) was similar to that of lindane (142 ng g^{-1} l.w.) and both were three times higher than those of *p,p′*DDE (54 ng g^{-1} l.w.). According to the fingerprint analysis of single PCB congeners, eels collected in the Orbetello Lagoon showed a distribution and relative concentrations characteristic of both Arochlor 1254 and Arochlor 1260 mixtures.

Biological indicators

As an indicator of contaminant exposure, B(a)PMO activities in eels from the western basin showed a similar pattern of spatial distribution regarding PCBs, lindane and *p,p′*DDE with significantly higher activities in site STP (industrial chemicals) compared to reference site (WR). B(a)PMO activity was slightly lower at sites FP (industrial chemicals), NC (western basin sea inlet) and AR (inland run-off) although this activity was higher compared to reference site (WR) (Table 2). The two sites in the eastern basin had higher activities than those of site STP. In particular, eels from the reference site ER had the highest activities in the lagoon ($p < 0.05$).

EROD activities did not significantly differ from reference and impacted sites of both basins and resulted in particularly low numbers for this species according to reports on other *in situ* studies dealing with eels (Bonacci et al., 2003a, 2003b; Regoli et al., 2003)(Table 2).

The two reductases enzymes, NADH cyt *c* and NADH ferrycianide, showed a high spatial correlation ($r = 0.91$) and homogeneity among sites as described for EROD, with the exception of eels from the reference site WR in which activities were the highest and significantly higher than all other sites ($p < 0.05$)(Table 2).

As an indicator of insecticide exposure and effect, ChE activity in muscle of eels, using ASCh as a substrate, showed clear differences among sites (Table 2). Eels collected from the Albegna River mouth (AR)(agricultural run-off) and the two inlets (fish farms) (NC and AC in the western and eastern basins, respectively) showed the lowest activities compared to sites STP and FP (industrial chemicals) and the reference sites (WR and ER).

Discussion

Persistent Organic Pollutants (POPs) including PCBs, lindane and DDTs were all present in muscle tissue of European eels from the Orbetello lagoon. Among OC pesticides, *p,p′*DDE and DDT derivatives dominated in eels from all the sampling sites even though significant concentrations of lindane were detected.

As recently reviewed by (Robinet & Feunteun, 2002), concentrations of OCs in muscle tissue of eels varies consistently among several *in situ*

Table 2. Bioindicator responses of eels at each sampling site throughout Orbetello Lagoon. Phase I P450 enzyme activities in eels liver microcomal fractions and ChE versus ASCh activity in crude muscle tissue homogenates. Data are reported as mean value (standard deviation)

Biological indicators	Western basin				Eastern basin		
	WR	NC	FP	AR	STP	ER	AC
EROD pmol min^{-1}mg $prot^{-1}$	32.54 (4.33)	23.37 (5.26)	28.68 (14.1)	29.02 (12.66)	17.32 (2.07)	21.03 (7.78)	32.25 (4.08)
B(a)PMO U.F. min^{-1} mg $prot^{-1}$	3.86 (0.02)	3.86 (1.05)	5.30 (0.75)	4.94 (0.66)	6.84 (1.23)	7.39 (0.42)	6.59 (0.94)
NADH ferryred μmol min^{-1}mg $prot^{-1}$	2.86 (0.42)	1.19 (0.08)	1.57 (0.17)	1.55 (0.32)	1.67 (0.33)	1.63 (0.19)	1.78 (0.15)
NADH cyt c nmol min^{-1} mg $prot^{-1}$	317.4 (97.25)	99.27 (34.07)	96.53 (15.68)	141.9 (33.09)	127.92 (54.92)	170.15 (48.28)	108.86 (34.42)
ChE vs ASCh nmol min^{-1} mg $prot^{-1}$	78.87 (4.01)	75.49 (13.11)	152.59 (58.44)	100.6 (12.65)	135.59 (83.67)	92.75 (44.62)	104.65 (8.44)

studies both in lagoon and freshwater environments. The authors underline that eels tend to concentrate much more pollutants *in situ* than in test predictions *in vivo* according to contaminant type, location, date and tissue analysed (Hendricks, 1995).

Lindane concentrations detected in eels from Orbetello lagoon (range 0.001–0.04 μg g^{-1} w.w.) were highly comparable to the lowest levels reported in studies conducted in Spain (Hernandez et al., 1987) and England (Hamilton, 1985) (0.02–0.027 μg g^{-1} w.w.) respectively. Similar levels were also reported by van der Oost et al. (1996a) from six Dutch freshwater sites except for the highest levels recorded in the western basin of the Orbetello lagoon at the mouth of the Albegna River (AR) and at the sewage treatment plant (STP).

Regarding *p,p′*DDE and total PCBs, Orbetello eels showed drastically lower levels, of respectively two and one order of magnitude (range 0.001–0.010 μg g^{-1} w.w. and 0.005–0.025 μg g^{-1} w.w., respectively), than those reported in England (0.049–0.3 μg g^{-1} w.w.) (Hamilton, 1985), Spain (0.19 μg g^{-1} w.w.) (Hernandez et al., 1987) and Ireland (<0.1 μg g^{-1} w.w.) (Weatherley et al., 1997). In addition, *p,p′*DDE levels in Orbetello eels were similar to the lowest levels reported for eels from six Dutch freshwater sites (range 0.156–2.17 μg g^{-1} lipid weight) in which *p,p′*DDE appeared to be exposure-related since it increased with increasing levels of pollution in sediments (van der Oost et al., 1996a).

Few data are available on PCB levels in European eels from Italian waters. The only data are those reported by Bressa et al. (1995; 1997) in eels collected from the highly polluted Po River (Italy) (mean value: 0.019 ± 0.006 μg g^{-1} dry weight) and from the Po Delta (mean values: 0.265 ± 0.009; 0.21 ± 0.007 μg g^{-1} w.w.) which are significantly higher than those reported in the present study. Similar levels of total PCBs were also reported in wild eels from an eutrophic lake in southern Scandinavia (range: 0.21–6.58 μg g^{-1} l.w.) (Larsson et al., 1991) and from six Dutch freshwater sites (range: 5.61–14.62 μg g^{-1} l.w.) (van der Oost et al., 1996a). The authors also report higher levels of HCB, mainly accumulated through bioconcentration and bioaccumulation (van der Oost et al., 1996a), than those detected in the present study (van der Oost et al., 1996a; Bressa et al., 1997) confirming the absence of local sources in the Orbetello Lagoon.

The occurrence, accumulation and sublethal toxicity of POPs in long-lived fish species with high lipid storage such as European eel, has been recently discussed as a potential cause for the acknowledged decline of eels (Robinet & Feunteun, 2002).

It has been recently pointed out by several authors that in order to get the energy necessary for migration, gametes production and spawning, the total stored lipids in eels must exceed 20% of their body weight (Boetius & Boetius, 1980). Lindane (Yadav & Singh, 1987) as well as some PCBs (Safe, 1990) are able not only to disturb lipid storage

mechanism and their metabolism by affecting thyroid functioning in fish but also reduce the spawning success by interfering with ovarian development. In particular, lindane exposure leads to an abnormal configuration of the vitellus especially in lipid vesicle (Wester et al., 1985) while PCBs significantly decreases the mean weight of eggs (Johnson et al., 1988). Our previous investigation on the Orbetello Lagoon using two benthic fish species showed abnormal oocyte development resulting in oocytes deformities and smaller diameters probably due to pesticides exposure during the early phase of gonad recrudescence (Kime, 1997; Corsi et al., 2003a). Therefore it is reasonable to assume that if lindane levels detected in muscle tissue of Orbetello eels remain very close to these levels they may potentially affect lipidogenesis and subsequently reduce migration efficiency and breeding success.

Despite lindane has been reported to be metabolised and eliminated to an higher extent than other OC pesticides such as DDTs in fish (Murty, 1986), its long biological half-life in European eels of 480 days (de Boer & Hagel, 1994) seems to suggest that the high levels observed in eels from sites AR and STP in the Orbetello Lagoon could have toxicological implications on their breeding biology including migration and reproduction (Robinet & Feunteun, 2002).

Organic pollutants are known to persist in fish according to their K_{ow} (from 4.4 to 7.4). However, other mechanisms might reduce their retention time in organism tissues such as liver phase I P450 biotransformation enzymes, which alter the chemical properties of the parent molecule (oxidation, reduction or hydrolysis) facilitating its excretion (Goksøyr & Förlin, 1992).

As a prerequisite of our proposed integrated ecotoxicological approach, measurements of bioindicator responses including phase I P450 enzyme activities, will provide information on detoxification mechanisms in the same population of eels collected at the same sites and at the same period. In fact, the induction of P450 enzymes such as EROD, represent a valid bioindicator of OC pesticides and PCBs exposure and it has been commonly accepted as suitable tool for revealing detoxification process in fish exposed to this class of contaminants (Goksøyr & Förlin, 1992).

However, slight differences were observed in EROD activities among the investigated sites of the Orbetello lagoon, which were generally low (average value 26.31 pmol min^{-1} mg $prot^{-1}$) and resembled those reported in our previous studies in unexposed eels (Bonacci et al., 2003a, b) (Table 2). Eels collected in 2001 from the Orbetello Lagoon showed similar low EROD activities in the order of 32.1 ± 2.3 pmol min^{-1} mg $prot^{-1}$ while those *in vivo* exposed to several toxic pollutants including the PCB mixture Aroclor 1254, B(a)P and 2,3,7,8-TCDD (in the range of 0.1–50 mg kg^{-1} body weight) showed a significant dose-dependent induction of this enzyme (Bonacci et al., 2003a, b) In addition, highly positive correlation ($r = 0.82$; $p < 0.01$) was also found between PCB levels in muscle tissue and EROD activity in liver microsomal fraction in those injected with increasing doses of Aroclor 1254 mixture (0.1–50 mg kg^{-1} body weight) (Mariottini et al., 2003). As a result, rather than considering the EROD activities recorded in the present study as indicative of a detoxification process, they can be considered a physiological response of eels to changes in biological and environmental conditions. In fact, several factors including hormone metabolisation during gonad recrudescence as well as changes in water temperature and salinity are known to affect P450 metabolising enzymes including EROD activity in fishes (Anderson & Förlin, 1992; Sleiderink et al., 1995).

If the other P450 enzymes, like B(a)PMO, are considered, eels showed less homogeneous activities among sites since they were significantly higher in sites STP and AR ($p < 0.05$) and were consistent with the highest levels of OC pesticides and PCBs reported in muscle tissue at the same sites (Table 1 and 2). However, this enzyme is considered to be more sensitive to aromatic hydrocarbons exposure, in particular to (B(a)P) (Kurelec et al., 1977), a class of chemicals, which has not been reported yet neither in sediment nor in organisms in the Orbetello lagoon. Moreover, despite our recent investigations showed a dose-dependent B(a)PMO induction in eels treated with 10–50 mg kg^{-1} (body weight) of B(a)P and β-naphthoflavone (β-NP), its induction has not yet been validated as suitable bioindicator of exposure in *in situ* studies (Bonacci et al., 2003a).

Despite the fact that in our study, B(a)PMO activities in eels seem to better reflect the pattern of OC contaminants distribution among sites (higher activities in the more impacted STP and AR sites), further studies both *in vivo* and *in situ* are needed to validate its suitability as a bioindicator of exposure in fishes.

Regarding the two P450 NADH reductases activities, which were highly correlated among sites ($r = 0.91$), they showed a homogenous pattern among sites except for the highest activities recorded in reference site WR (western basin) ($p < 0.05$). A dose-dependent increase in reductase activities in pollution-exposed eels was found by van der Oost et al. (1996b) who concluded that they cannot be considered a suitable bioindicator of OC exposure, even though they are involved in the metabolisation process. This was also confirmed by our previous investigation in which OC-treated eels did not show any dose-dependent induction of both reductases (ferryred and cyt. *c*) activities.

Nevertheless, the high homogeneity and relatively low values observed among sites of both B(a)PMO and two reductases (NADH ferryred and cyt. *c*) seem to confirm the hypothesis on EROD that no significant detoxification/metabolisation process of OC pesticides and PCBs occurred in eels from Orbetello lagoon at the time of sampling.

The absence of an evident metabolisation process coupled with the long residence time of these contaminants in eels tissue (from 1 to 4 years) (de Boer & Hagel, 1994) strongly suggest that contaminants levels reported in muscle tissue of eels from Orbetello lagoon are likely to remain close to these values even during migration and spawning. Moreover, eels are particularly exposed to contaminant accumulation through their diet and specimens over 30–40 cm like those collected in the present study become top piscivorous predators. Eels are benthic feeders and are thus exposed to contaminants accumulated and adsorbed on to sediment particles (van der Oost et al., 1996a) and because they spawn once in their lifetime they do not benefit from this indirect but effective defence mechanism against the accumulation of pollutants (Robinet & Feunteun, 2002).

Our integrated approach included the measurement of ChE activity. The inhibition of this enzyme is not only a valid bioindicator of OP pesticides and CBs exposure in fishes but also affects lipidogenesis by inducing a fast lipid mobilisation by involuntarily and continuous muscular activity (Hughes et al., 1997). The significantly low ChE activity measured in eels collected from the Albegna River mouth (AR – agricultural runoff) and the two inlets (NC and AC – fish farms) of less then 30% respect to that detected in the reference sites (WR and ER) clearly suggest OPs and CBs exposure in the Orbetello Lagoon with possible effects on lipidogenesis in eels. Indeed, eels exposed to sublethal concentrations of the OP pesticide fenitrothion and two CB herbicides, thiobencarb and molinate, showed a significantly lower fat content compared to unexposed eels and also a dose-dependent inhibition (<50% activity) and a significantly prolonged depression of ChE in muscle and plasma (Fernandez-Vega et al., 1999; Sancho et al., 1997, 2000). Even though OP residues in water and fish usually disappear within several days from exposure, ChE activity remains inhibited in fish for several weeks (Sancho et al., 1997). Several studies on the Orbetello lagoon have pointed out both the presence and the effects of OPs and CBs exposure by producing predictive models on insecticide loads and by showing ChE inhibitions over than 30% (in both brain and muscle of selected fish species (Villa et al., 2003a, b). From the results of our previous investigation on the western basin, abnormal ovarian and oocyte development coupled with inhibition of AChE less than 50% (in fish brain were hypothesised as clear sign of OPs and CBs exposure in fish from the Lagoon (Shukla & Pandey, 1985; Rastogi & Kulshrestha, 1990; Corsi et al., 2003a).

The PCA-analysis run with three replicates for each investigated site showed how the first three components explain 73.3% of cumulative variance (Table 3). The first component showed a significant separation between replicates of reference site WR, even though with a high dispersion and all other sites including the eastern basin (Fig. 2). The second component grouped the WR site with the two sites of the eastern basin, AC and ER. The ANOSIM among the seven sampling sites showed significant differences (ANOSIM Statistic $R = 0.185$, $p < 0.01$). According to the eigenvalues of the first component (Table 4), positive values on the first axis are those related to eels length and

Table 3. Eigenvalues and their percentage (%) variation for the first 5 principal components (PC) extracted for bioindicator responses and contaminant concentrations from three replicates for each sampling site

PC	Eigenvalues	% variation	Cum. % variation
1	7.58	37.9	37.9
2	5.02	25.1	63.0
3	2.06	10.3	73.3
4	1.56	7.8	81.1
5	1.23	6.2	97.3

The cumulate (Cum.) percentage of variation is also reported.

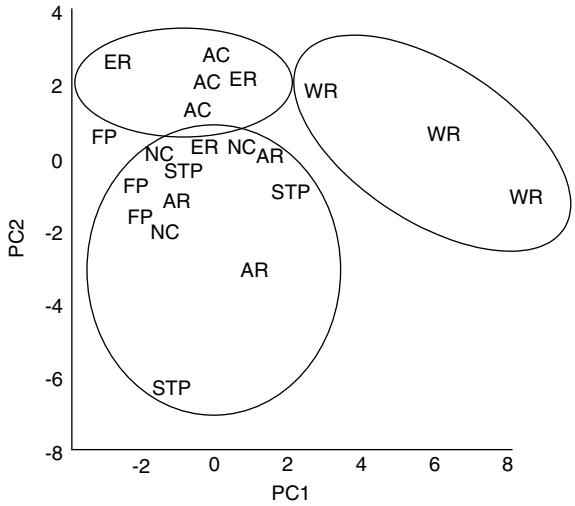

Figure 2. A plot of PC1 versus PC2 based on all bioindicator responses and contaminant levels in eels at each site.

weight, to the highest NADH ferryred and cyt. *c* activities and to contaminants levels on a fresh weight basis as well as to the lowest ChE. It might be hypothesised that the three bioindicator responses as well as contaminant levels expressed on a wet weight basis are affected by both size and weight of eels, which were in fact significantly higher in the reference site WR ($p < 0.05$). In addition, lipid normalisation, which better reflects size and weight of the fish, might be necessary for a better comparison among sampling sites in *in situ* studies with this species.

The eigenvalues of the second component showed that positive scores are observed for the two NADH reductases, the ChE and the contaminant levels expressed on lipid weight basis.

Table 4. Eigenvector coefficients in the linear combinations of variables making up the first 5 PC

Variables	PC1	PC2	PC3	PC4	PC5
Length	0.255	0.142	−0.149	−0.123	0.258
Weight	0.248	0.210	−0.241	0.038	0.182
EROD	0.062	0.052	0.258	−0.072	0.683
B(a)PMO	−0.092	0.049	−0.220	0.395	−0.318
NADH cyt c	0.311	0.078	0.044	0.112	−0.04
NADH Ferryred	0.290	0.111	0.121	0.266	0.174
ChE	−0.139	−0.231	−0.031	0.264	0.379
Total PCBs w.w.	0.304	0.029	−0.293	−0.136	−0.093
Total PCBs l.w.	0.033	−0.335	−0.047	−0.424	−0.136
Lindane w.w.	0.061	−0.390	−0.027	0.274	0.128
Lindane l.w.	−0.026	−0.377	−0.193	0.295	0.142
HCB w.w.	0.290	−0.035	0.278	0.277	−0.166
HCB l.w.	0.184	−0.189	0.365	0.291	−0.212
*p,p′*DDD + *o,p′*DDT w.w.	0.336	−0.017	0.227	0.020	−0.089
*p,p′*DDD + *o,p′*DDT l.w.	0.175	−0.107	0.483	−0.316	−0.055
p,p′ DDE w.w.	0.323	−0.107	−0.214	−0.086	−0.048
p,p′ DDE l.w.	0.076	−0.420	−0.139	−0.100	0.045
Σ DDTs w.w.	0.334	−0.096	−0.168	−0.078	−0.052
Σ DDTs l.w.	0.94	−0.418	−0.88	−0.131	0.039
% lipids	0.296	0.179	−0.255	0.08	0.05

Conclusion

PCBs, lindane and DDTs were detected in muscle tissue of European eels from the Orbetello lagoon (Italy). Although their levels vary consistently throughout the lagoon, the lowest were found in the eastern basin. Lindane levels were comparable to those reported across Europe while *p,p′*DDE and total PCBs were particularly low. Nevertheless, the high homogeneity and relatively low values observed of B(a)PMO and the two reductases (NADH ferryred and cyt *c*.) among sites, seems to confirm the hypothesis on EROD, that no significant detoxification/metabolisation process of OC pesticides and PCBs occurred in eels from Orbetello lagoon at the time of sampling. The absence of an evident metabolisation process coupled with the long residence time of these contaminants in eel muscle tissues strongly suggest that they will remain very close to their levels, potentially affect lipidogenesis and subsequently reduce migration efficiency and the breeding success. Moreover, the presence of other contaminants which affect lipidogenesis, such as OPs and CBs revealed by ChE activity, increases the threat posed by the environmental conditions to this species. In conclusion, by the application of an integrated ecotoxicological approach, is possible to link toxic contaminant levels in eels and their potential capability to affect directly (lipidogenesis) or indirectly through sublethal toxicity mechanism (phase I biotransformation P450 enzymes and ChE inhibition), the reproductive success and subsequently their population decline. Further investigation on gonad morphology and indicators of reproductive competence are needed in order to evaluate if the levels of toxic contaminant detected in muscle tissue of European eels from Orbetello lagoon will affect their gametogenesis and spawning success.

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