Abstracts from Thirteenth International Symposium on Pollutant Responses in Marine Organisms (PRIMO 13) – Endocrine disruption

Less recovery from imposex and organotin pollution in the rock shell, *Thais clavigera* in Korea

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Abstract

The present status on imposex, its recovery rate and the temporal trends of organotin concentrations in tissue of the rock shell, *Thais clavigera* from Korea were investigated between 1995–1997 and 2002. In the first imposex survey conducted in Korea from 1995 to 1997, percentage occurrence of imposex in rock shell populations were 100% in almost all sites surveyed. No imposex populations were only observed in the population from Deukryang Bay situated in the southwestern part of the South Sea. Percentage occurrences of sterile individuals (with blocked vaginal openings by vas deferens formation) were higher (60% or more) in the eastern part than those in the western part of the South Sea. No sterile females were observed in the open-sea-side and other areas. In the second imposex survey in 2002, percentages occurrence of imposex in rock shell populations were still 100% in most of sites surveyed. While, percentages occurrence of imposex in the rock shell populations from Jeju coast were in the range of 0–100%. Geographical distribution of percentages occurrence of sterile females was similar to that of the first survey. TBT as well as butyltin concentrations in tissues of the rock shell collected at several sites in 2002 increased than those in the 1995–1997 survey. These results indicate less recovery from imposex in...
Evaluation of relationships between reproductive metrics, gender and vitellogenin expression in demersal flatfish collected near the municipal wastewater outfall of orange county, California, USA

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Abstract

Estrogenic activity in fish has primarily been evaluated using vitellogenin expression in male and juvenile animals. Although the response has been widespread, the relevance of the response to higher-level adverse effects, particularly in the field is less than clear. Previous evaluations of vitellogenin within flatfish species collected near the Orange County Sanitation District (OCSD) outfall and a reference station 7.7 km downcurrent indicated bioavailable estrogens within demersal flatfish populations. In order to evaluate the persistence of estrogenic activity and relationships to reproduction and development, fish were sampled in the winter and summer of 2003, and the winter and summer of 2004 from the same locations. Vitellogenin, gonadal somatic indices, serum estradiol, and sperm DNA damage were measured. In addition gender ratios were measured in English sole (\textit{Pleuronectes vetulus}) and hornyhead turbot (\textit{Pleuronichthys verticalis}) collected in the summers of 2001–2004, and 1988–2004, respectively. Although vitellogenin levels were elevated at the outfall compared to the reference station, the elevation was not significant. Rather than an expected feminization of populations, there was either no trend or a significant trend toward masculinization, as was observed in hornyhead turbot at the OCSD outfall (64% males at outfall versus 51% males at reference station). Male gonadal somatic indices were significantly higher at the outfall for English sole during the summer 2003 and 2004 sampling periods, and in hornyhead turbot males during the winter of 2004. Serum levels of estradiol were significantly decreased in female English sole from the outfall location during the winter of 2003. These results indicate possibly stronger androgenic activity than estrogenic activity on flatfish residing near the outfall.
Abstract

A recent study showed that exposure to tributyltin (TBT) reduced the formation of testosterone–fatty acid conjugates in female snails [Gooding et al., 2003. EHP 111, 426]. In this work, the freshwater gastropod *Marisa cornuarietis* was used to investigate sex differences in esterified testosterone and estradiol levels, and whether those steroids are modulated by exposure to TBT (30–500 ng/L), methyltestosterone (MT; 30–300 ng/L) and fenarimol (Fen; 300–3000 ng/L). Levels of both, esterified testosterone and estradiol, determined in the digestive gland/gonad complex of *M. cornuarietis* had a clear sexual dimorphism, males having higher levels than females (5- to 9-fold). Accordingly, the activity acyl-CoA: testosterone acyltransferase (ATAT), which catalyzes the esterification of steroids, was higher in males (2-fold). TBT exposure led to a comparatively higher decrease in esterified testosterone (66%) than estradiol (45%) in females, but had no effect in males. Fen and MT did not alter levels of esterified steroids in females, nor in males, although exposed females developed imposex. Changes in esterified steroids were not directly related to ATAT activity, which was marginally induced in TBT-exposed organisms (1.3-fold), and significantly induced in males and females exposed to MT (1.8- and 1.5-fold, respectively). Overall, the study shows that a decrease on steroid esterification is associated to TBT exposure, but not to exposure to other chemicals that had the ability to induce imposex.

In situ assessment of endocrine-mediated effects in fish

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Abstract

The assessment of adverse effects supported by wildlife in consequence to exposure to endocrine disrupting chemicals requires a long-term monitoring. A wide sampling program was held in northwest of France (2002–2004). A total of 120 flounders (Platichthys flesus) from the Seine Bay and 1200 roaches (Rutilus rutilus) from 7 rivers were collected during the study. Fish were analyzed for plasmatic vitellogenin levels (ELISA), bile estrogenic activity (YES assays, HPLC fractionating and GC–MS) and gonadal histology (structure, development). Evidence for endocrine disruption was detected in many fish. Histology revealed occurrence of intersexuality (presence of testicular oocytes) in 0–34% of male fish according to sampling sites. Vitellogenin, the female yolk protein, was detected in numerous males (10% on average) at levels as high as 1 μg/mL. Estrogenic compounds were present in male biles and have been identified as estradiol, estrone and synthetic compounds such as ethinyl-estradiol and nonylphenol. Up to 15 ng of estradiol-equivalent per mL were detected. In females, signs of endocrine disruption were less obvious. A clear relationship between vitellogenin level, bile estrogenicity and developmental stage of the gonads was found. Furthermore, bile estrogenicity and vitellogenin level were significantly correlated to the maturation stage of the gametes. Nevertheless, these links were absent in several individuals and might indicate endocrine dysfunction. In addition to identification of hot-spots of endocrine-mediated effects in males, this study provides a better understanding of regular plasma vitellogenin and biliary estrogen levels in relation to reproductive cycle, allowing a better interpretation of abnormal levels. This work is supported by the European Council through the INTERREG 3 program.

Transgenerational reproductive and endocrine response following exposure to the androgen 17-β-trenbolone

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Abstract

Trenbolone (TREN), an androgenic feed additive, represents a likely source of environmental androgens for fish exposed to run-off from cattle feed lots. The purpose of this study was to evaluate the potential of TREN to alter endocrine function and reproduction as well as change these responses in the next generation. Reproductively active pairs of adult Japanese medaka were aqueous exposed to TREN for 14 days at concentrations ranging from 2.0 to 2000 μg/L. Egg production was significantly impaired at higher concentrations of TREN, but egg size, fertilization rate and hatching success were not affected. Physiological effects of TREN included inhibition of vitellogenin, altered gonadal size, and gonadal steroid production. Effects on offspring produced by adult pairs during exposure were compared to these responses. Even at the highest exposure concentrations, no significant skew in sex ratio was detected for these in ovo exposed offspring (n = 9) although the resulting sex ratio was male biased. The responses to TREN exposure in these transgenerationally
treated animals were different when compared to the parental exposure. In ovo treated animals did not show a decline in reproductive capacity exhibited by their parents with 200 μg/L TREN. Testis size was not altered in ovo exposed males, even following the second exposure. Thus, TREN exposure may negatively impact the fecundity of adult pairs and concurrently desensitize offspring produced during that exposure.

Androgen metabolism in the sea urchin Paracentrotus lividus: The modulating effect of triphenyltin

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Abstract

Androgen metabolism (androstenedione and testosterone) has been assessed in the digestive tube and gonads of the echinoid Paracentrotus lividus exposed to different concentrations of the biocide triphenyltin (TPT) in a semi-static water regime for 4 weeks. Key enzymatic activities involved in both synthesis and metabolism of androgens, namely 17β-hydroxysteroid dehydrogenases (17β-HSDs), 3β-HSDs, 5α-reductases, P450-aromatase, palmitoyl-CoA: testosterone acyltransferases (ATAT), and testosterone sulfotransferases (SULT), were investigated in digestive tube and/or gonads of control and TPT-exposed specimens in an attempt to see whether androgen metabolism was altered by exposure. In agreement with previous data for vertebrate studies, exposure to TPT led to a dose dependent decrease of P450 aromatase in \textit{P. lividus}. This decrease was statistically significant at the highest TPT dose (225 ng/l). Additionally, increased metabolism of testosterone to form dihydrotestosterone (DHT), and 5α-adrostan-3β,17β-diol was observed, suggesting increased 5α-reductase activity in the gonads of TPT-exposed individuals. Interestingly, exposure to TPT induced testosterone-conjugating activities in organisms exposed to medium (SULT) and high (ATAT and SULT) doses. Despite of these changes on androgen metabolizing enzymes, the levels of testosterone determined in gonads remained stable, and no significant differences between control and exposed organisms were observed. Overall, the data indicates the ability of TPT to modulate androgen metabolism in \textit{P. lividus}, and suggests the existence of regulatory mechanisms to maintain endogenous levels of testosterone stable. This study also contributes to a better knowledge of the endocrinology of this representative marine species.

The stickleback model in endocrine disruption research: An essential tool in the laboratory and the field
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Abstract

The three-spined stickleback, a small teleost species with habitats that stretch from full marine to fresh water bodies across the whole of the North hemisphere, has a number of advantages for endocrine disruption research. It is the only teleost species with an unambiguous biomarker for androgens; the presence of the glue protein spiggin in the male kidney, for which we have developed a sensitive ELISA. We have adopted the androgen assay to detect anti-androgens in two different ways and have also developed a homologous ELISA for stickleback vitellogenin. DNA markers for molecular sex determination are available – thus sex ratios can also be used for in situ bio-monitoring. In addition, the critical period of sexual differentiation has been determined and occurrence of intersex fish has been reported several times. The full genome sequence is expected before the end of 2005. All aspects of stickleback biology (ecology, evolution, behaviour, physiology, endocrinology, etc.) are well documented. Using spiggin and vitellogenin assays in the laboratory and field, we have confirmed androgenic activity in pulp mill effluents and oestrogen activity in sewage treatment works. We have confirmed anti-androgenic activity of a number of pesticides. We are currently in the process of developing a full-life cycle exposure test, incorporating behavioural end-points in contaminant exposures. In European waters, the stickleback is the only fish that can bring the lab and the field studies together and allow the true impact of endocrine disruptors on fish populations to be evaluated.

Endocrine disruption: Vitellogenin induction vs. other biomarkers in rainbow trout

(Oncorhynchus mykiss)

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Abstract

Plasma vitellogenin (Vg) concentration is the most commonly used biomarker for assessing the estrogenic potency of endocrine disrupters (EDs). However, this protein is not always induced in fish subjected to realistic exposure scenarios. Therefore the value of investigating the compromise of other physiological processes to develop alternative biomarkers is readily evident. To address this issue individually tagged rainbow trout were given different doses of 17β-estradiol (E2) or atrazine (A), through a single injection, or exposed to waterborne pulses of 4-nonylphenol (NP) or atrazine. Endocrine responses were monitored by measuring plasma Vg and sex steroid concentrations. Individual specific growth rates (SGR) were recorded and white muscle total protein, RNA and DNA
concentrations were measured as biochemical growth indices. In addition liver catalase (CAT) activity and metallothionein (MT) concentrations were assessed. The effects of these agents on growth were dose dependent. For all three compounds the highest doses resulted in a linear relationship between SGR and white muscle cell size (RNA:DNA ratio), suggesting an increased contribution of growth through hypertrophy in ED-exposed fish. Vg was induced in fish injected with 0.5 mg/kg E2 suggesting these responses are related to endocrine disruption. CAT activity increased in fish 5 days after a 2-h exposure to 0.5 mg/l waterborne atrazine, whereas 10 days after exposure the trout showed reduced CAT activity. A 2-h water exposure to 15 μg/l NP resulted in reduced MT levels. Nevertheless A and NP as known estrogen-inducers failed to increase vitellogenin synthesis, suggesting CAT and MT to be more sensitive responses. Supported by the European Commission’s fellowship 15708-2000-02 P1B20 ISP IT and the European Union’s Marie Curie Grant EVK1-CT-2002-57003.

**Imposex induction in *Hexaplex trunculus* by trybutiltin and other stressors**

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**Abstract**

We investigated experimentally the potential of a class of endocrine disruptors (PCBs – Aroclor 1260) to induce imposex, i.e., the development of additional male sex organs (penis and/or vas deferens), in prosobranch gastropods female. Adults of *Hexaplex trunculus*, with low imposex level (VDSI = 1.3) coming from an Italian MPA, were dosed with different concentrations of TBT and Aroclor 1260 for seven days. The compounds were dissolved in ethanol and organisms were narcotised by immersion in MgCl\(_2\) solution before the injections. Care was paid to unconfound differences in imposex development across toxic compounds and differences due to the experimental artefacts. To achieve this, two artefact controls (narcotic and narcotic plus solvent) were adopted. The results showed that all the treatments, including artefact controls, are able to induce a significant increase of imposex with respect to not treated organisms, thus indicating that, besides the injected toxic compounds, the immersions in a 7% solution of MgCl\(_2\) is able to cause imposex in *Hexaplex trunculus* too. This finding suggests that the level of imposex, at least in this species, could be not considered as a specific biomarker for TBT contamination but, instead, as a non specific response to stress stimuli; these results could be the basis for further researches on mechanisms regulating sexual development in gastropods.

**Thyroid hormone and vitamin responses to endocrine disrupting POPs in turbot**

(*Scophthalmus maximus*)
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Abstract

Over the last decades the commercial use of persistent organic pollutants (POPs) has been of increasing concern, due to their effect as endocrine disrupters. Examples of POPs are phthalate (DAP), bisphenol A (BPA) and tetrabromodiphenyl ether congener 47 (PBDE 47), which are used respectively as plasticizer, antioxidant and flame retardant in plastics. These compounds are often mentioned for their effects on sex hormones. However, in this study we focused on the effects of these POPs on thyroid hormones and the vitamins A, E (tocopherol) and retinyl palmitate, in juvenile turbot (\textit{Scophthalmus maximus}). Vitamin A shares carrier protein with hormones, whereas retinyl palmitate is the stored form of vitamin A. Vitamin E is a known antioxidant. The fish were exposed to either waterborne 50 ppb DAP, 50 ppb BPA or 5 ppb PBDE 47 for three weeks, after which blood plasma and liver were sampled. The plasma samples were analysed for thyroid hormones by radioimmunoassay (RIA) and the vitamin concentrations were measured chromatographically (HPLC). The plasma concentration of total triiodothyronine (TT3) resulted significantly lower in all the exposed groups whereas no effect of any of the compounds was registered on plasma total thyroxine (TT4) levels. In the liver, tocopherol concentrations were significantly higher in fish exposed to BDE-47. Retinyl palmitate showed the same trend when exposed to BPA. Retinol, however, did not respond to any exposure. Our results suggest that although many POPs are studied for their sex steroid disrupting effects, effort should be made to study alternative mechanisms of action of POPs. This study was supported by the BEEP project (EU project number EVK3-CT2000-00025).

Bioaccumulation of the estrogenic compound 4-nonylphenol in the clam \textit{Tapes philippinarum}

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Abstract

Despite the widespread use of nonylphenol ethoxylates (NPEs) in many household and industrial detergents and their accumulation in aquatic environments, mostly as nonylphenol (NP), very few data are available about bioaccumulation of NP in bivalves. Considering that NP can exert estrogenic effects in marine organisms, it is of great concern to improve the knowledge on their capability of bioaccumulating the contaminant. In the present study, bioaccumulation of 4-NP was evaluated.
in the clam *Tapes philippinarum* after exposure for 7 and 14 days to 0, 0.025, 0.05, 0.1, and 0.2 mg NP/L. NP concentrations were also determined before and after water renewal in the experimental tanks. NP concentrations measured after water renewal ranged from 52% to 96% of the nominal concentrations, whereas the values fell to 20–59% after 24 h.

NP was not detected (DL = 0.05 mg/kg FW) in control clams. The highest NP concentration (180.12 mg/kg FW) was measured in the clams exposed for 7 days to 0.2 mg NP/L.

In the clams exposed to the lowest NP concentrations in water (0.025 and 0.05 mg/L), accumulation of NP was not completed in 7 days, whereas NP tissue concentrations observed in 0.1 and 0.2 mg NP/L-exposed clams after 7 days of exposure were maintained after 14 days.

The bioconcentration factor (BCF), calculated using the mean NP water concentration, ranged from 1098, for the clams exposed for 7 days to 0.025 mg NP/L, to 1918, for the clams exposed for 14 days to 0.05 mg NP/L.

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**Estrogenic effects of 17α-ethinyl estradiol in male flounder *Platichthys flesus* after exposure via food and water**

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**Abstract**

Environmental estrogens are natural or synthetic compounds that imitate the effects of endogenous estrogen. Ethinyl estradiol is a potent synthetic estrogen used in contraceptives, and the compound enters natural waters via wastewater. Vitellogenin is a well-known biomarker for testing estrogenic compounds in male fish.

The aim of the present study was to establish the dose-response relationship of vitellogenin in male flounder *Platichthys flesus* exposed to 17α-ethinyl estradiol orally or in the water. Plasma vitellogenin was measured by a direct non-competitive sandwich ELISA. Water samples were extracted by solid phase extraction and concentrations of 17α-ethinyl estradiol were measured by LC–MS.

In the first experiment, oral treatment with 17α-ethinyl estradiol (5, 50, 500 and 5000 ng EE₂ kg⁻¹ bw) every second day for a period of 12 days resulted in a significant increase in plasma vitellogenin concentrations in fish fed 500 and 5000 ng kg⁻¹ on day 6 and 13.

In the water exposure experiment male flounders were exposed to 17α-ethinyl estradiol in a flow-through system at concentrations of 2.5, 5, 10 and 25 ng l⁻¹ for a period of 21 days. Vitellogenin were measured in plasma from day 0, 7, 14 and 21. Responding fish was observed in all groups including the one exposed to 2.5 ng l⁻¹.

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**Vitellogenin induction in the clam *Tapes philippinarum* after exposure to 4-nonylphenol**
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**Abstract**

Nonylphenol (NP) is reported to exert estrogenic effects in marine organisms, being able to mimic the action of endogenous estrogens. As a consequence, NP can induce vitellogenin (Vg) synthesis, the major precursor of the egg-yolk proteins. In our previous study [Matozzo and Marin, 2005. Environ. Res., 97, 43–49], we found that exposure for 7 days of the clams *Tapes philippinarum* to NP caused a dose-related induction of Vg in both haemolymph and digestive gland of males. In the present study, Vg induction was evaluated in sexually undifferentiated clams exposed to NP: for 7 days to 0, 0 + acetone, 0.025, 0.05, 0.1, and 0.2 mg NP/L and for 14 days to 0, 0 + acetone, 0.025, 0.05, and 0.1 mg NP/L. Vg was determined in both haemolymph and digestive gland by the alkali-labile phosphate (ALP) assay. In the haemolymph Ca\(^{2+}\) levels were also measured. Exposure for 7 days to 0.2 mg NP/L resulted in significant increases ($p < 0.05$) in ALP in both haemolymph and digestive gland with respect to controls, whereas no difference was observed in Ca\(^{2+}\) levels. After 14 days exposure, ALP significantly ($p < 0.05$) increased in digestive gland from 0.05 and 0.1 mg NP/L-exposed clams. Increases ($p < 0.05$) in Ca\(^{2+}\) levels were recorded in haemolymph from clams exposed to 0.025 and 0.05 mg NP/L. Results obtained demonstrates that NP induces Vg synthesis in *T. philippinarum* also far from the reproductive period. As endocrine disruption may cause fertility reduction and alteration in sex ratio, a condition of potential risk for clam populations in estuarine areas is highlighted. This study was supported by grants from Co.Ri.La (Second Research Programme, 2004–2006).

**Endocrine disruption in winter flounder**

(*Pseudopleuronectes americanus*) from an urban estuary, Jamaica Bay, NY, USA

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**Abstract**

The aim of this present study was to evaluate estrogenic effects in field-collected winter flounder in an urban estuary, Jamaica Bay, NY. It receives millions of gallons of sewage effluent daily making sewage effluent its primary source of freshwater. Extremely high levels of estrogenic compounds
(e.g., nonylphenol, estradiol, estrone) in sediments led us to postulate that benthic fish residing in this bay were likely targets for endocrine disruption. To assess this population we collected young of the year (YOY) and adult winter flounder from multiple sites within Jamaica Bay and a reference site, Shinnecock Bay, in the spring of 2002, 2003, and 2004. Previously we had found that adult non-spawning female winter flounder had significantly higher levels of vitellogenin VTG while adult males only showed decreased levels of E2 and 11-KT when compared to reference fish. All YOY collected in Jamaica Bay showed increased levels of circulating plasma VTG than reference fish. Fish exposed to nonylphenol-dosed sediment also showed an increase in plasma vitellogenin. EROD activity in adult fish was measured and showed to be significant only in one area of Jamaica Bay. An analysis of various CYP forms (CYP1A, CYP3A and CYP2E) showed differences only in CYP1A where we saw higher protein levels in fish from Jamaica Bay when compared to the reference fish. Our results indicate that both YOY and adult winter flounder are showing signs of endocrine disruption in Jamaica Bay, and that in addition, enzyme inhibition by endocrine disruptors or other contaminants is also influencing these fish.

New investigations on the endocrine disruption effects in the mediterranean population of swordfish 
(*Xiphias gladius*)

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Abstract

Since 1999 the Department of Environmental Science at Siena University has been involved in research into the ecotoxicological effects of Endocrine Disrupting Compounds (EDCs) in a Mediterranean population of swordfish (*Xiphias gladius*). Using sensitive biomarkers such as Vitellogenin (VTG), Zona Radiata Protein (ZRP), sex hormone pathways and CYP1A activities (EROD, BPMO), exposure and effects of anthropogenic chemical have been studied in more than 200 Mediterranean specimens. VTG and ZRP were found to be induced in 35% adult male specimens. These fish showed higher levels of both proteins and 17β-estradiol than mean levels in males from reference sites, suggesting high exposure to xenoestrogens in the Mediterranean Sea. A role of Organochlorines in this induction phenomenon is suggested by statistically significant correlations found between VTG and ZRP levels in males and levels of hexachlorobenzene (HCB) in gonads (*p* < 0.05), plasma 17β-estradiol and polychlorobiphenyl (PCB) in gonads (*p* < 0.05), ZRP and pp′DDE in liver (*p* < 0.06). These results indicate that Mediterranean swordfish could be subject to reproductive alterations. In order to better understand the present results, alterations in swordfish liver and gonads (testis and ovary) will be investigated. Histopathological studies
including the immunohistochemical detection of VTG, ZRP and CYP1A will be performed. Moreover, the mRNA contents of the aromatase and CYP1A genes in swordfish will be measured. All results obtained will be correlated with the quantitative analysis of TCDD, PCBs and organochlorine pesticides in tissues.

Acute toxicity of several ionic liquids to zebrafish (Danio rerio)

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Abstract

Although the information about physical, thermodynamic, kinetic or engineering data had been extended continuously, only little data with regard to the toxicity and ecotoxicity of ionic liquids have been available until now. The “green character” of ionic liquids has usually been justified with their negligible vapour pressure, but even if ionic liquids do not evaporate and do not contribute to air pollution most of them are water soluble and might enter the environment by this path (e.g., accidental spills, effluents). Due to the immense range of possible ionic liquids, there is also a real need for quick toxicological screening process for new and existing ionic liquids. The aim of this work was to test the acute lethal toxicity and the histological changes on fish (zebrafish, Danio rerio) of fifteen ionic liquids of different source. According to ECB guidance, each ionic liquid was evaluated in a limit test performed at the concentration of 100 mg/L. No mortality was observed in limit tests performed with ionic liquids except for AMMOENG\textsuperscript{C228}110 and AMMOENG\textsuperscript{C228}130 (Solvent Innovation) which induced acute mortality over 2 h of exposure. Histological evaluation of dead fish showed skin alteration represented by epithelial hyperplasia with single keratinocyte vesciculation and wide erosions, and disepithelialization of gill lamellae.

Steroid levels and steroid metabolism in the mussel Mytilus edulis: The modulating effect of dispersed crude oil and alkylphenols

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Abstract

Significant amounts of oil and alkylphenols are released into the sea by petroleum installations as a result of discharges of produced water. Some of these pollutants elicit estrogenic responses in fish, but their effects on the endocrine system of molluscs are largely unknown. In this study, mussels *Mytilus edulis* were exposed to North Sea oil (O) and the mixture of North Sea oil + alkylphenols (OAP), and the effects on tissue steroid levels and steroid metabolism (P450-aromatase and estradiol-sulfotransferase) were monitored. Levels of free testosterone and estradiol determined in gonads were not affected by treatment, whereas the amount of esterified steroids (released after saponification), significantly increased in OAP exposed mussels (up to 2.4-fold). The results support the hypothesis that fatty acid steroid conjugates may play a key role regulating physiological levels of free steroids in tissues. The sulfation of estradiol was investigated as an alternative conjugation pathway, and increased activities were observed in digestive gland cytosol of both O and OAP exposure groups (up to 2.8-fold). Additionally, increased P450-aromatase activity was determined in OAP exposed mussels (up to 3-fold, both in gonad and digestive gland), but not in the O group. Altogether, the results indicate that North Sea oil leads to increased sulfation of estradiol (a potential endocrine disruptive effect), and that in combination with alkylphenols, additional alterations are observed: increased P450-aromatase, and increased levels of esterified-steroids. Nonetheless, mussels are able to maintain gonad concentrations of free steroids unaltered, possibly via homeostatic mechanisms such as the conjugation with fatty acid or the formation of sulphate conjugates.


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Abstract

Imposex, the superimposition of male sexual characters onto females of some marine gastropods, is an excellent example of endocrine disruption. Its causative agent is tributyltin (TBT), a biocide used in antifouling paints. An exhaustive assessment of organotin (OT) pollution in Galicia was performed in 1996 when 36 populations of the dogwhelk *Nucella lapillus* were examined for imposex and bioaccumulation [monobutyltin (MBT), dibutyltin (DBT), tributyltin (TBT) and triphenyltin (TPhT); levels determined in 19 of those populations]. Imposex and OTs were present in every site included in that survey. Values of imposex ranged from 6% to 59% for Relative Penis Size Index (RPSI); from 3.2 to 4.6 for Vas Deferens Sequence Index (VDSI) and from 3% to 54% (33 sites) for sterile females. Regarding to OTs bioaccumulation; while TBT (from 36 to 974 ng Sn/g dry weight, ppb) and DBT (169–909 ppb) were detected in every sample examined, MBT (65–387 ppb) and TPhT (39–250 ppb) were quantifiable in only 15 and 11 of them.
In October 2001, the United Nations promoted an international convention stating that no ship should apply or re-apply OT-based coatings starting on January 1st, 2003. Anticipating that OT levels in the marine environment might decrease from that year on, we re-surveyed the same populations in the summer of 2003. Although imposex and OTs were again detected in every re-surveyed population, our results evidence that OT pollution has already experienced some reduction along this eight year period. This decreasing trend was detected for all imposex indicators (2–50% for RPSI, 2.2–4.3 for VDSI and 3–27% (20 sites) for sterile females) as well as for the bioaccumulation of the several organotin species measured in female tissues (15–677 ppb for TBT, 15–643 ppb for DBT, 4–296 ppb for MBT and TPhT below LOD everywhere). MBT was also non quantifiable in 4 sites.

Other than providing an updated reassessment of OT pollution, our results are intended to serve as a baseline for the future monitoring of the effectiveness of the aforementioned international agreement in the NW Iberian Peninsula.

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A new case of endocrine disruption: Imposex in the invasive gastropod *Cyclope neritea* L.

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**Abstract**

*Cyclope neritea* is a nassariid gastropod whose native range extends over the Mediterranean, the Black Sea, and the Atlantic coasts of the southern Iberian Peninsula. Since the early 1970s, it has been reported as an invasive alien species along the Atlantic coasts of France and Galicia (NW Iberian Peninsula) where its introduction has been linked to shellfish aquaculture. In Galicia, this snail seems to be currently circumscribed to some enclaves in the southernmost rias.

Imposex, the superimposition of male sexual characters onto females, is a malformation shown by marine gastropods when exposed to tributyltin (TBT), a pollutant leaked from antifouling paints. Worldwide, more than one hundred species of meso- and neogastropods have been reported to exhibit this response. In this study, we checked whether *C. neritea* is also afflicted by the same phenomenon. Samples of this non-native nassariid were collected from 3 sites in NW Iberian Peninsula in February-March 2005. The magnitude of TBT pollution at those sites was assessed by quantifying the intensity of imposex in samples of *Nassarius reticulatus* whose relationship imposex-TBT pollution has been previously well established. The sequence of imposex development in *C. neritea* is similar to that described for *N. reticulatus*. One female suffering from advanced imposex was found to carry a mass of aborted egg capsules, somehow resembling what has been previously reported for *N. reticulatus*. Although care must be exercised given our reduced data set, both species
Mechanisms of endocrine toxicity: Carbonic anhydrase, calcium metabolism and imposex

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Abstract

In polluted habitats, endocrine disruption is unlikely to occur in isolation, but to be just one of a suite of toxicological effects, each of which contributes to adverse health effects and population declines. Here, we address the hypothesis that alterations in calcium metabolism may contribute to the detrimental effects of tributyltin (TBT) exposure in marine molluscs. Two species, the dogwhelk *Nucella lapillus* and the mussel *Mytilus edulis*, were collected from sites in South West England impacted to varying degrees by TBT. The activity of carbonic anhydrase, a key regulatory enzyme in the mobilisation of calcium stores, was determined in the accessory boring organ (ABO) of *N. lapillus* and the gill tissue of *M. edulis*. Carbonic anhydrase activity for *N. lapillus* collected from Whitsand Bay (TBT impacted) was 8.5 ± 1.38 units/mg protein, whilst mean activity for Port Quin (pristine) was significantly higher (18.3 ± 2.87 units/mg, *p* < 0.0001). Corresponding values in *M. edulis* were 5.64 ± 0.98 and 9.32 ± 1.22 units/mg (*p* < 0.001). Imposex stage was used to group female *N. lapillus*, and a negative relationship with carbonic anhydrase activity was confirmed (*r*² = 0.8463). In bivalves, carbonic anhydrase is an absolute requirement for incorporation of calcium carbonate into the shell, whilst in predatory gastropods it is secreted to dissolve the shell of prey. Alterations in activity will therefore perturb the balance of the predator: prey interaction. This study illustrates that in addition to endocrine disruption, other aspects of organism health may contribute to population declines in organisms exposed to TBT.

Keywords: Biomarkers, tributyltin exposure, carbonic anhydrase, dog whelks, mussels

Oral exposure to the xenoestrogen 4-tert-octylphenol in flounder *Platichthys flesus*; induction of vitellogenin and 4-tert-octylphenol disposition
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Abstract

In a previous experiment oral exposure to 4-tert-octylphenol resulted in an induction of vitellogenin and a significant accumulation of 4-tert-octylphenol in liver, muscle and testis tissue of male flounder Platichthys flesus. The aim of the present investigation was to study the effects and disposition over time of a single oral dose of 4-tert-octylphenol (50 mg OP kg\(^{-1}\)) in male flounder. Tissue (liver, muscle and testis) and plasma concentrations of 4-tert-octylphenol (Extraction followed by LC–MS detection) as well as plasma vitellogenin (ELISA) were measured 3, 6, 12, 18, 24, 48, 72, 144 and 216 h post exposure.

4-tert-Octylphenol was detectable in liver, testis, muscle and plasma 3 h post exposure and an accumulation was observed in liver, muscle and plasma up to 12 h post exposure and 18 h post exposure in testis. The maximum concentrations of 4-tert-octylphenol in liver, muscle and testis were 67.0, 3.2 and 6.8 µg g\(^{-1}\), respectively. Half lives of 4-tert-octylphenol in plasma, muscle and testis were 20–23 h. In liver, 4-tert-octylphenol was eliminated from two compartments, with half lives 4.4 and 61 h, respectively. An increase in plasma vitellogenin levels were seen 48 h after exposure and the vitellogenin level continued to increase until the end of the experiment 9 days post exposure.

In vivo modulation of vitellogenin synthesis by 3,3′,4,4′,5-pentachlorobiphenyl (PCB126) in the sea bass (Dicentrarchus labrax)

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Abstract

Many chemicals released into the aquatic environment are capable of affecting reproduction and developmental processes of both wildlife and humans. These xenobiotics include some organochlorine pesticides, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzodioxins (PCDDs), plasticizers, and some natural chemicals (phytoestrogens and mycoestrogens). PCBs, depending on the congener studied, have exhibited both antiestrogenic and estrogenic activity in vitro and in vivo. The aim of the present study was
to evaluate the effects of PCB126 on vitellogenin (VTG) synthesis in mature male sea bass. Fish were injected ip with a single dose of 17β-estradiol (E2) (0.1, 0.5, 2.5 and 5 mg/kg) and PCB126 (10 and 100 µg/kg) alone or in combination with E2 (0.5 mg/kg). Blood and mucus samples were collected after 3, 7, 14 and 28 days post-injection. Induction of VTG levels was analysed using immunoblotting and ELISA methods with homologous antibodies. Immunoblotting of fish treated with E2 showed an induced detectable VTG protein (~180 kDa) both in plasma and in mucus samples. E2 induced an increase in plasma and mucus VTG levels showing a dose- and time-dependent effect. By contrast treatment with PCB126 alone was without effect on VTG synthesis. Furthermore, PCB126 treatment in combination with E2 resulted in a dose- and time-dependent reduction of both plasma and mucus VTG levels, compared with E2 treatment alone. These results indicate that after E2 treatment levels of VTG in plasma and surface mucus samples showed similar and parallel expression patterns in the sea bass. The present results also suggest that PCB126 is antiestrogenic in vivo; this characteristic combined with its persistent and bioaccumulative behaviour, make it a potential active environmental antiestrogen in wildlife and humans.

Abstract

Several substances, both man-made and of natural origin, are known to potentially affect the endocrine system of fish. They generally enter the aquatic environment through the discharges of domestic and industrial origin, and particularly estrogens, perhaps the most investigated of the endocrine disrupters, are suspected to cause altered gonadal development in wild fish populations. After the observation of intersexual cyprinids in the River Po, many studies have been undertaken, particularly along its middle section where a polluted tributary, the River Lambro, is suspected to be an effective source of EDs to the major Italian watercourse. An objective of these studies was to investigate whether fish diet may be a significant route of uptake of estrogens. To this aim, several multiplate samplers were placed upstream and downstream from the confluence of the River Lambro, and after one month of exposure, macroinvertebrates were collected and identified to order or family. Gammaridae, Ephemeroptera and Trichoptera were analysed for target estrogenic compounds (E1, E2, E3, EE2, OP, NP, BPA) in high performance liquid chromatography/multiple reaction monitor (HPLC/MRM). The concentrations of EDs determined in the three groups of macroinvertebrates were consistently higher downstream from the confluence of the River Lambro. In the most polluted downstream stretch, t-OP was found at 100 ng/g, whereas both NP and BPA reached levels of 600 ng/g. Surprisingly, also estriol was bioaccumulated up to 800 ng/g. Marked differences in the levels of contaminations were evident among the three groups of invertebrates also suggesting that diet may differently contribute to estrogens uptake. This study is part of the EC.

Potential for dietary uptake of estrogens via macroinvertebrates in River Po fish

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Abstract

Several substances, both man-made and of natural origin, are known to potentially affect the endocrine system of fish. They generally enter the aquatic environment through the discharges of domestic and industrial origin, and particularly estrogens, perhaps the most investigated of the endocrine disrupters, are suspected to cause altered gonadal development in wild fish populations. After the observation of intersexual cyprinids in the River Po, many studies have been undertaken, particularly along its middle section where a polluted tributary, the River Lambro, is suspected to be an effective source of EDs to the major Italian watercourse. An objective of these studies was to investigate whether fish diet may be a significant route of uptake of estrogens. To this aim, several multiplate samplers were placed upstream and downstream from the confluence of the River Lambro, and after one month of exposure, macroinvertebrates were collected and identified to order or family. Gammaridae, Ephemeroptera and Trichoptera were analysed for target estrogenic compounds (E1, E2, E3, EE2, OP, NP, BPA) in high performance liquid chromatography/multiple reaction monitor (HPLC/MRM). The concentrations of EDs determined in the three groups of macroinvertebrates were consistently higher downstream from the confluence of the River Lambro. In the most polluted downstream stretch, t-OP was found at 100 ng/g, whereas both NP and BPA reached levels of 600 ng/g. Surprisingly, also estriol was bioaccumulated up to 800 ng/g. Marked differences in the levels of contaminations were evident among the three groups of invertebrates also suggesting that diet may differently contribute to estrogens uptake. This study is part of the EC.
Agonistic and antagonistic activities of UV filters towards the human estrogen and androgen receptor and activities of UV filter combinations in vitro

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Abstract

An increasing number of products contain UV filters, and their residues have been detected in human breast milk, surface water, wastewater, sewage sludge and fish. Being environmentally persistent and lipophilic, UV filters are of potential environmental concern. Moreover, some UV filters have estrogenic activity, others were found having antiestrogenic and antiandrogenic activities. Little is known, however, how frequent different hormonal activities in UV filters are. In the present work, we assessed 18 commonly used UV filters systematically for estrogenic, antiestrogenic, androgenic and antiandrogenic activities utilizing recombinant yeast systems carrying either the human estrogen receptor alpha or androgen receptor.

Our results reveal a novel picture of the hormonal activities UV filters. While 9 compounds possessed estrogenic activity, as many as 14 were found to be antiestrogenic. Androgenic activity was observed in 6 compounds, and as many as 16 UV filters were antiandrogenic. Surprisingly, most UV filters possessed multiple activities; 13 compounds displayed as much as three distinct hormonal activities each. The antiestrogenicity and antiandrogenicity was even more important than estrogenicity or androgenicity. Further studies on combinations of two and multiple estrogenic UV filters demonstrated that UV filter mixtures act according to the concentration addition model. Combinatory actions and multiple hormonal activities of UV filters lead to a new level of complexity in hormonal reaction. Ongoing in vivo studies will show whether this new findings are also of concern for the hormonal activity in vivo. Supported by the Swiss National Science Foundation (NRP50, Grant 4050-066554 to K. Fent).

Vitellogenin in black turtle (Chelonia mydas agassizii): Purification, partial characterization and validation of an ELISA for its detection
Environmental pollutants can mimic the action of the hormone 17β-estradiol (E2), resulting in negative impacts on the reproductive success of wildlife. This is of particular concern in endangered species such as black turtles (Chelonia mydas agassizii). This research was conducted to develop an assay to measure Vitellogenin (Vtg) in black turtle plasma as a biomarker of estrogenic compounds. Black turtle plasmatic Vtg was purified from E2-induced males using ion exchange chromatography. The isolated protein was identified as Vtg by its glyco-lipo-protein nature and amino acid sequence homology with other vertebrate Vtg. It was characterized as a 500 kDa dimer composed of two 200–240 kDa monomers. Polyclonal antibodies raised against black turtle Vtg showed high titer and specificity as demonstrated by ELISA and Western blotting, respectively. The sensitivity of the assay was estimated between 15 ng/ml and 2 μg/ml, intra and inter-assay coefficients of variation were less than 8% and 10%, respectively. Plasma dilution curves from E2-induced turtles showed parallelism with the standard curve. The matrix effect was reduced at 1:5000 dilution. Additionally, black turtle antibody cross-reacted with Vtg of two other sea turtle species, Caretta caretta (loggerhead) and Eretmochelys imbricata (hawksbill), extending the applicability of the assay as part of a sea turtle health assessment program. This work was funded by the Centro de Investigaciones Biológicas del Noroeste (La Paz, B.C.S., México) and by a grant to SCG from the Consejo Nacional de Ciencia y Tecnología (CONACYT, G35437-B).

Abstract

The effect of 17-α-estradiol (E) and 4-α-nonylphenol (NP) was evaluated in sexually differentiated male and female mussels (Mytilus galloprovincialis). Target compounds were injected into the posterior adductor muscle and after 48 h mussel tissues – hemolymph, digestive gland and gonad were sampled and utilised for the analysis.
In oviparous vertebrates and invertebrates, the production of vitellogenin, a yolk-precursor lipophosphoprotein, is induced by estradiol. In fish, several anthropogenic compounds, including nonylphenol, are known to mimic the effects of estradiol, leading to vitellogenin (Vg) synthesis, which may then be used as a biomarker of estrogenic exposure.

In this study, the presence of Vg-like proteins was determined by an indirect test, alkali-labile phosphate (ALP) assay, based on the determination of labile phosphates released by Vg after hydrolysis with alkalis. The release of inorganic phosphates may therefore be considered a biomarker response to estrogens, even though it cannot provide a quantitative measure of yolk protein concentration.

In parallel the development state of gonads was determined by histological analysis and a biochemical method.

Vg-like proteins was detected in all tissues in both male and female mussels. However, only gonads were found to respond to endocrine disrupters. Indeed, a significant increase in Vg-like proteins, dose related, was measured in gonads of mussels exposed to either E or NP.

Negative effects, due to the exposures, were also determined in mussels: an increase in lipid peroxidation and a decrease in lysosomal membrane stability were recorded in mussels exposed to E and NP at all tested concentrations.