A fish scale *in vitro* bioassay to screen for endocrine disrupting compounds

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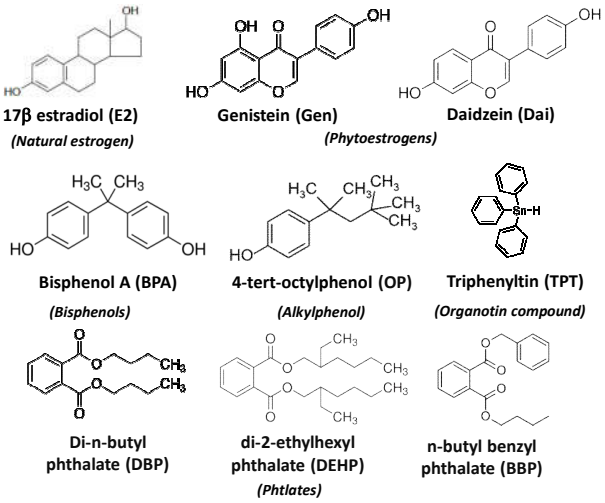
INTRODUCTION

Estrogenic endocrine disruptors (EDCs) constitute a wide range of natural and anthropogenic compounds that are accumulating in the aquatic environment. Many of them can interact with and disrupt the estrogenic system with negative impacts on humans, wildlife and ecosystems and are particularly relevant in aquatic organisms like fish that may experience life-long exposures. The effects of EDCs in fish have mainly been assessed using reproductive endpoints and *in vivo* animal experiments.

We propose the use of other potential endpoints to allow the development of a simple non-invasive assay, to measure the effect of estrogens and disrupting compounds on a mineralized tissues like the fish scale. Fish scales express both membrane and nuclear estrogen receptors, and are targets for natural estrogens and EDCs. Bioassays in which fish scales of different species are incubated with compounds of interest, followed by measurement of enzymatic activities related to calcium turnover (TRAP, tartrate-resistant acid phosphatase and ALP, alkaline phosphatase) have been carried out by several authors but generally using fresh water species. In the present study, a fish scale assay was developed and optimized, using scales from both marine and freshwater species, sea bass (*Dicentrarchus labrax*) and tilapia (*Oreochromis mossambicus*), respectively, and tested with the compounds indicated on the right panel.



Tested compounds - chemical structures and classes

METHODS

***In vitro* scale bioassay conditions:**

Variables such as culture media, different classes and concentrations of compounds and different incubation conditions (e.g. temperature, time) were tested.

Species: Sea bass (150-300g) and Mozambique tilapia (75-125g)

N= 4 scales / fish, 3 fish / treatment (dose and time combinations)

Tested compounds: E2, Gen, Dai, BPA, OP, TPT, DBP, DEHP, BBP (see figure above)

Concentration range: 10^{-6}, 10^{-8} or 10^{-10} M

Incubation time: 30 min and 24h

Measured activities: TRAP- osteoclast (OSC) marker; ALP-osteoblast (OSB) marker

Method: colorimetric method using p-nitrophenyl phosphate (pNPP) as substrate, quantification of the product p-nitrophenol (pNP) at 405nm

Fish scales sampling → *In vitro* incubation → Colorimetric method for enzymatic activity (405 nm)

RESULTS

TRAP and ALP activities of scales from sea bass or Mozambique tilapia with different test compounds *in vitro* (at concentrations 10^{-6}, 10^{-8} or 10^{-10}M) for 30 min or 24h.

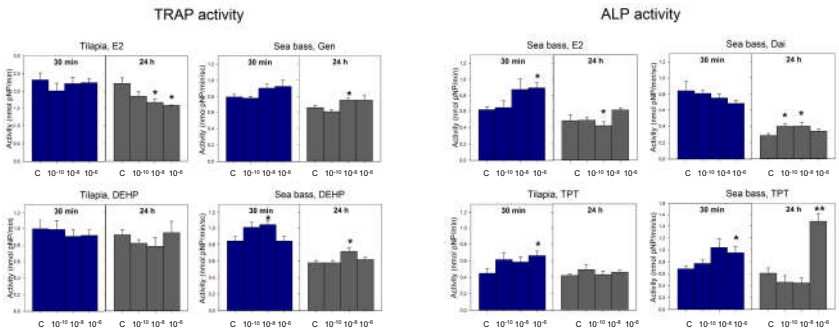
TRAP

	Sea bass		Tilapia	
Compounds	30 min	24 h	30 min	24 h
E2	nc	nc	nc	↓(10^{-6};10^{-8} M)
Gen	nc	↑(10^{-8} M)	nc	nc
Dai	nc	↑(10^{-8} M)	nc	nc
BPA	nc	nc	nc	nc
TPT	nc	nc	nc	nc
OP	↑(10^{-6},10^{-8} M)	↑(10^{-10} M)	nc	nc
DBP	nc	nc	nc	↓(10^{-6} M)
DEHP	↑(10^{-8} M)	↑(10^{-8} M)	nc	nc
BBP	nc	nc	nc	nc

ALP

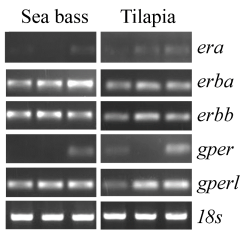
	Sea bass		Tilapia	
Compounds	30 min	24 h	30 min	24 h
E2	↑(10^{-6}M)	↓(10^{-8} M)	nc	nc
Gen	nc	nc	nc	nc
Dai	nc	↑(10^{-8},10^{-10} M)	nc	nc
BPA	nc	nc	nc	nc
TPT	↑(10^{-6}M)	↑(10^{-6}M)	↑(10^{-6}M)	nc
OP	nc	nc	nc	nc
DBP	nc	nc	nc	nc
DEHP	nc	nc	nc	nc
BBP	nc	nc	nc	↓(10^{-6} M)

Arrows indicate significant increases (↑) or decreases (↓) in activity, at p<0.05 (t test), relative to control scales.



Examples of TRAP and ALP activities responses to *in vitro* incubations of scales from sea bass or Mozambique tilapia with selected compounds at concentrations 10^{-6}, 10^{-8} or 10^{-10}M, for 30 min or 24h; * significant differences to control at p<0.05 (t test)

- Some natural and synthetic estrogenic compounds may increase calcium mobilization and deposition (indicated by TRAP and ALP activity, respectively) in sea bass and tilapia.
- In sea bass, OP and DEHP induced both rapid (30 min) and slow (24h) dose dependent increases in TRAP activity. Both phytoestrogens (Gen and Dai, 10^{-8} M) induced an increase in TRAP activity after 24 h exposure. E2 (10^{-6} M) increased ALP activity after 30 min but decreased it at 24 h (10^{-8} M). Dai (10^{-8} and 10^{-10} M) and TPT (10^{-6} M) increased ALP activity after 24 h exposure, and TPT (10^{-6} M) also after 30min.
- In tilapia, E2 (10^{-6} and 10^{-8} M) and DBP (10^{-6} M) decreased TRAP activity and BBP (10^{-6} M) decreased ALP activities, suggesting a tendency to slow down calcium turnover. TPT at a higher dose (10^{-6} M), however, caused a rapid increase in ALP.
- Estrogen receptors (*erba, erbb* and *gperl*) were detected by RT-PCR in the scales of both species (see below).
- *Erba/b* and *gperl* in the scales may mediate both rapid and long term actions of EDCs on fish scales, via membrane and nuclear receptors, respectively.



mRNA expression of nuclear (*era*, *erba* and *erbb*) and membrane G protein-coupled (*gper* and *gperl*) estrogen receptors in scales of sea bass and tilapia, determined by RT-PCR and using as internal control 18S ribosomal RNA

CONCLUSIONS

- Exposure to natural estrogens and EDCs disrupt TRAP and ALP activity in mineralized scales in both marine and freshwater teleost species. Despite extensive assay optimization, changes were of low magnitude, limiting the sensitivity of TRAP/ALP assays and the application of these endpoints in an *in vitro* scale EDC screening assay.
- The tested compounds act through both rapid and slow responses, potentially via different estrogen receptors, and these responses varied with both the compound and the species evaluated.
- Mineral turnover of marine sea bass appears to be more affected by EDCs than that of fresh water tilapia; extending these tests to additional teleost fish species may allow to investigate if this reflects a general higher sensitivity of marine species mineral turnover to EDCs or a species-specific effect.
- The mechanisms of action of tested EDCs remain unknown but may involve disruption of the estrogenic system via *erba/b* and *gperl* in fish scales.

References: Agas, D. *et al.* Arch. Toxicol. 2013, 87, 735–751; Pinto, P.I. *et al.* Gen. Comp. Endocrinol. 2009, 160, 19–29; Pinto, P.I. *et al.* Mar. Drugs 2014, 12, 4474-4494.

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