Science of the Total Environment 550 (2016) 934–939

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Zebrafish-based reporter gene assays reveal different estrogenic activities in river waters compared to a conventional human-derived assay

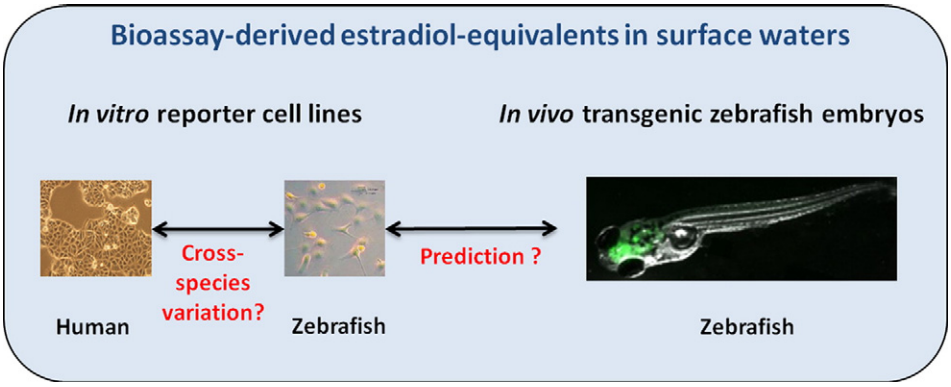
Manoj Sonavane [a], Nicolas Creusot [a], Emmanuelle Maillot-Maréchal [a], Alexandre Péry [b,c], François Brion [a,*], Selim Aït-Aïssa [a,*]

[a] Institut National de l'Environnement Industriel et des risques (INERIS), Unité Ecotoxicologie in vitro et in vivo, Parc Technologique ALATA, BP2, 60550 Verneuil-en-Halatte, France
[b] AgroParisTech, UMR 1402 INRA-AgroParisTech Ecosys, 78850 Thivernal Grignon, France
[c] INRA, UMR 1402 INRA-AgroParisTech Ecosys, 78850 Thivernal Grignon, France

HIGHLIGHTS

- New zebrafish assays as bioanalytical tools for monitoring surface waters
- Different estrogenic patterns are provided by human and zebrafish *in vitro* assays
- *In vivo* assay revealed brain specific responses at hot-spot sites
- Relevance of fish-based integrated assessment for environmental diagnosis

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 6 December 2015
Received in revised form 27 January 2016
Accepted 27 January 2016
Available online 4 February 2016

Editor: D. Barcelo

Keywords:
Zebrafish assays
Estrogen receptor subtypes
Cross-species differences
Complex mixtures
(eco)Toxicological relevance

ABSTRACT

Endocrine disrupting chemicals (EDCs) act on the endocrine system through multiple mechanisms of action, among them interaction with estrogen receptors (ERs) is a well-identified key event in the initiation of adverse outcomes. As the most commonly used estrogen screening assays are either yeast- or human-cell based systems, the question of their (eco)toxicological relevance when assessing risks for aquatic species can be raised. The present study addresses the use of zebrafish (zf) derived reporter gene assays, both *in vitro* (*i.e.* zf liver cell lines stably expressing zfERα, zfERβ1 and zfERβ2 subtypes) and *in vivo* (*i.e.* transgenic *cyp19a1b*-GFP zf embryos), to assess estrogenic contaminants in river waters. By investigating 20 French river sites using passive sampling, high frequencies of *in vitro* zfER-mediated activities in water extracts were measured. Among the different *in vitro* assays, zfERβ2 assay was the most sensitive and responsive one, enabling the detection of active compounds at all investigated sites. In addition, comparison with a conventional human-based *in vitro* assay highlighted sites that were able to active zfERs but not human ER, suggesting the occurrence of zf-specific ER ligands. Furthermore, a significant *in vivo* estrogenic activity was detected at the most active sites *in vitro*, with a good accordance between estradiol equivalent (E2-EQ) concentrations derived from both *in vitro* and *in vivo* assays. Overall, this study shows the relevance and usefulness of such novel zebrafish-based assays as screening tools to monitor estrogenic

* Corresponding authors.
E-mail addresses: francois.brion@ineris.fr (F. Brion), selim.ait-aissa@ineris.fr (S. Aït-Aïssa).

http://dx.doi.org/10.1016/j.scitotenv.2016.01.187

activities in complex mixtures such as water extracts. It also supports their preferred use compared to human-based assays to assess the potential risks caused by endocrine disruptive chemicals for aquatic species such as fish.

1. Introduction

The occurrence of endocrine disrupting chemicals (EDCs) in aquatic ecosystems has raised global concern about their adverse health effects for humans and wildlife (Sumpter, 2005; Hotchkiss et al., 2008). EDCs act on the endocrine system through multiple mechanisms of action, among them interaction with estrogen receptors (ERs) is a well-identified key event in the initiation of adverse outcomes. As a member of the nuclear receptor super family, ER acts as a ligand-dependant transcription factor that modulates the transcription of target genes involved in essential physiological processes (*e.g.* development, reproduction). A number of environmental contaminants of different chemical classes and origins are xeno-estrogens (XEs) (reviewed by Kiyama and Wada-Kiyama, 2015), *i.e.* they have the ability to interfere with ER signaling pathway, modulate its transcriptional activity, and thereby disrupt the normal cellular response to hormones and thus may trigger adverse effects in exposed organisms. While this mechanism is well conserved among species, it can be influenced by several intracellular factors such as the promoter context, the cellular context or the origin species of the receptor. Depending on the cell type or species, one chemical can thus elicit differential estrogenic response in terms of receptor binding affinity, activation and subsequent gene transcription. In this context, the development of species-specific assays has been identified as an important challenge (Hotchkiss et al., 2008). Overall, given the risks posed by these compounds in the environment, increased knowledge about their occurrence, identity and effects in aquatic ecosystems is still needed in order to improve environmental risk assessments.

In environmental monitoring, analytical strategies based on only target chemical analyses are insufficient to depict environmental contamination by XEs. It is now well admitted that effect-based tools are needed to take into account the complexity of environmental contamination (Altenburger et al., 2015). Mechanism-based bioassays were shown to be powerful bioanalytical tools to assess contamination of environmental matrices and are being increasingly used to monitor estrogenic activity in complex mixtures (Wernersson et al., 2015). In particular, *in vitro* assays based on stable reporter gene expression driven by ER provide efficient screening tools as they allow specific, sensitive and quantitative detection of active compounds (Leusch and Snyder, 2015). To date, most of these bioassays are based on the use of human ER alpha (hERα) expressed in either human (Legler et al., 1999) or yeast cells (Leskinen et al., 2005). However, since cross-species species variations may affect ER transactivation by environmental ligands (Matthews et al., 2000; Molina-Molina et al., 2008, Cosnefroy et al., 2009, Miyagawa et al., 2014), the relevance of human based reporter cell lines to address environmental hazard of XE for aquatic species can be questioned. In this context, efforts have been made to develop screening tools based on model fish species. These include both *in vitro* assays based on fish ER expressed in either human (Tohyama et al., 2015) or fish cells (Ackermann et al., 2002; Cosnefroy et al., 2009, Cosnefroy et al., 2012) and *in vivo* assays based on transgenic fish models (Brion et al., 2012). Although *in vitro* assays are useful and relevant for the integrative quantification of estrogenic chemicals in environmental matrices, they remain simplified biological models that provide only limited information on estrogenic hazard of environmental contaminants. For this purpose, assessing a response at the whole organism level is crucial as it allows addressing these mechanisms while taking into account pharmacokinetics (*e.g.* bioavailability, metabolism, transport, excretion) process in a model species.

In the present study, recently developed zebrafish-based *in vitro* and *in vivo* reporter gene assays were used for the detection of (xeno)-estrogens in surface waters. The *in vitro* assays consist of zebrafish liver cells (ZFL) that were stably transfected by an ERE-driven luciferase gene and the three different zfER subtypes (*i.e.* zfERα, zfERβ1 and zfERβ2) (Cosnefroy et al., 2012). *In vivo* estrogenic activity was monitored by using the transgenic *cyp19a1b*-GFP zebrafish line (Tong et al., 2009, Brion et al., 2012). This fluorescent reporter system allows sensitive detection of environmental estrogens at early developmental stages (0 to 4 days post fertilization) by activation of *cyp19a1b*, an ER-regulated gene coding for brain aromatase (Menuet et al., 2005). This *in vivo* assay has been shown to sensitively respond to a diversity of ER-active compounds that belong to different chemical classes (Brion et al., 2012). In this study, the application of this panel of *in vitro* and *in vivo* bioassays to assess estrogenic activity in river water extracts showed their functionality, sensitivity and complementarity as biomonitoring tools to quantify E2-EQs in complex matrices. Furthermore, the comparison of zebrafish bioassay responses to a human-based *in vitro* assay was performed to assess the possible occurrence of species-specific estrogenic responses in the samples.

2. Materials and methods

2.1. Chemicals, cell culture and bioassay reagents

17β-estradiol (E2), 17α-ethinylestradiol (EE2), 4-tert-octylphenol (4tOP), bisphenol A (BPA), and zearalenone were purchased from Sigma-Aldrich (France). Dichloromethane (DCM), methanol (MeOH), heptane and acetone (HPLC reagent grade) were purchased from VWR (France). Dimethylsulfoxide (DMSO), Leibovitz 15 culture medium (L-15), fetal calf serum (FCS), 4-(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid (HEPES), epidermal growth factor (EGF), G418, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) and D-luciferin were purchased from Sigma Aldrich (St-Quentin Fallavier, France); Dulbecco's Modified Eagle Medium High Glucose (DMEM HG) powder, F-12 nutrient mixture (Ham's F12) powder, penicillin and streptomycin were from Gibco (France); insulin, hygromycin B and sodium bicarbonate were from Dominique Dutscher (France).

2.2. Study sites, sampling and extraction procedures

Twenty French river sites were investigated to assess the environmental occurrence of estrogen-like compounds in the water phase, using passive sampling based on polar organic chemical integrative samplers (POCIS). POCIS are designed to monitor polar to mid-polar organic bioavailable compounds in the water soluble phase (Alvarez et al., 2004) and have been shown to be suitable for bioassay-based monitoring of bioactive compounds (Tapie et al., 2011, Creusot et al., 2014). These investigated sites were characterized by different anthropogenic pressures (urban, industrial and agricultural) (Table S1) and are currently included in surveillance programs in the frame of the Water Framework Directive (WFD).

Sampling was carried out from September to November 2012. At each site, POCIS (obtained from CDTA, EPOC-LPTC, Bordeaux, France) were deployed for a period of three weeks. POCIS preparation and extraction procedures were performed as previously described with