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Determination of estrogenic activity in the river Chienti (Marche Region, Italy) by using *in vivo* and *in vitro* bioassays

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ABSTRACT

Environmental estrogen-like compounds (*i.e.* xenoestrogens) are a variety of pollutants, ranging from synthetic to natural occurring molecules, that are found in surface and waste waters over a wide range of concentrations. In aquatic environment, the overall estrogenic activity is often due to the presence of a mixture of chemicals and their degraded products which can induce synergistic effects. Current strategies for monitoring estrogen-like chemicals are based on the use of a battery of *in vivo* and *in vitro* ecotoxicological tests. In this regard, the aim of the present work was to carry out a bio-monitoring study for testing estrogenicity of the Chienti river (Marche Region, Italy) by using both an E-screen and a vitellogenin (Vtg) induction assay in juvenile goldfish. Three sites were used for analysis, localized at the mouth (sampling point 1), in the middle (sampling point 2) and at the origin (sampling point 3) of Chienti river. For most of the water samples (*i.e.* samples collected at sampling points 2 and 3), clear estrogenic activity was detected in the E-screen assay suggesting different proliferation activities in function of the collecting site. In contrast, the Vtg ELISA demonstrated that water samples collected from each sampling point were estrogenic. Overall, we showed for the first time that the estrogenic activities in water samples from the Chienti river were significant in both *in vivo* and *in vitro*; we also observed a different sensitivity between bioassays.

Introduction

Environmental estrogen-like compounds (*i.e.* xenoestrogens) are a variety of pollutants, ranging from synthetic to natural occurring molecules, that are found in surface and waste waters over a wide range of concentrations. Most of these compounds (*e.g.* pharmaceuticals, synthetic estrogens, pesticides, surfactants, plasticizers, and phytoestrogens) show structure similarity to natural estrogens (Johnson et al., 2008). However, given the wide chemical variety of xenoestrogens, molecular structure is not enough to predict their estrogenic potentials. Overall, estrogenicity is dependent on estrogen receptor (ER) relative binding affinity of the pollutant, transcriptional and post-transcriptional regulation of ER-dependent genes, and the mechanisms related to distribution, metabolism and elimination of xenoestrogens in exposed organism (Beresford et al., 2000; Katzenellenbogen et al., 2000; Tollefsen et al., 2008). Several studies suggest that exposure to xenoestrogens may cause a broad range of adverse effects on reproductive biology of aquatic vertebrates (Arukwe and Goksøyr, 1998; Kloas et al.,

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2009). Xenoestrogens have been reported to impair the Hypothalamic–Pituitary–Gonadal (HPG) axis of fish by resulting in modulation of sex steroid concentrations, gonadal development and vitellogenin (Vtg) production (Soverchia et al., 2005). These effects are mainly related to direct interaction between xenoestrogens and ERs.

In order to assess environmental impact of xenoestrogens found in the aquatic environment extensive chemical monitoring is requested. However, concentration analysis of selected chemicals is often not correlated with the observed biological effects because of possible presence of interactive effects of pollutants. In most cases, the estrogenic effect of a mixture of chemicals and their degraded products may result in different biological potency with respect to the effects related to a single contaminant exposure (Krein et al., 2012). Current strategies for monitoring estrogens are based on the use of a battery of *in vivo* and *in vitro* ecotoxicological tests (Baker et al., 1999a,b, 2000; Fasulo et al., 2010; Isidori et al., 2010; Palermo et al., 2008; Reel et al., 1996; Shelby et al., 1996). In particular, mechanism based-*in vitro* tests (*e.g.* ER binding dependent transcriptional activity) are used as first step screening tools, prior to moving into *in vivo* assays (OECD, 2003). Recently, Isidori et al. (2010) demonstrated that human breast cancer adenocarcinoma cell (MCF-7) proliferation assay (E-screen) and Vtg assay, used for assessing estrogenic potential of alkylphenols and trace elements, showed differences in the bioassays' sensitivity and potency suggesting the following order: E-screen > Vtg. These findings provide further support for the usefulness of *in vitro* assays in detecting estrogenic activity of chemical mixtures.

The aim of the present work is to carry out a biomonitoring study for testing estrogenicity of the Chienti river (Marche Region, Italy) by using both *in vitro* and *in vivo* assays. These ecotoxicological tests consist of an E-screen and a Vtg induction assay in juvenile goldfish (*Carassius auratus*). Previous evaluation of water quality of the Chienti river was carried out by using both biotic and chemical parameters (Scuri et al., 2006). All parameters tested by the authors demonstrated that the water quality gradually deteriorates due to the increased anthropogenic pressure concentrated along the lower section of the river.

1. Materials and methods

1.1. Study area, water sampling and extraction method

The river Chienti has a hydrographic basin of 1298 km^2, flows west east for 91 km, crossing the Marche Region before emptying into the Adriatic Sea, south of Civitanova Marche city. River flow is characterized by a marked seasonality with maximum flow normally in the spring and minimum flow normally occurring toward the end of summer. One of the spring branches of the Chienti river is in the Colfiorito Valley that is an important agricultural area. In addition, at level of lower (or Sub-Appennine) stretch, the river is bordered by industrial establishments, including foodstuff factories, sugar refineries, shoe manufacturing, and tanneries which influence river water quality (Scuri et al., 2006). The sampling activity was conducted in July 2013 at three sampling stations (Fig. 1) using 1-L amber glass bottles. Water samples were kept chilled at 4°C and transferred to the laboratory. Upon arrival at the laboratory, the water samples were filtered to eliminate the suspended matter and extracted according to the procedure previously described by Swart and Pool (2007). The dried water extracts were reconstituted in 10 mL ethanol to give a final volume 1/100 times that of the original sample volume. The samples were stored at −20°C until further use.

1.2. Cell culture

ER-positive human breast MCF-7 adenocarcinoma cells were maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS), 2 mmol/L-glutamine, 1 mmol/L sodium pyruvate, 100 IU/mL penicillin, and 100 μg/mL streptomycin. ER-negative human breast MDA-MB 231 adenocarcinoma cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% HI-FBS, 2 mmol/L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Stock solution of 17β-estradiol (E2) (Sigma) 1 mmol was prepared with ethanol. Cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Constituents and

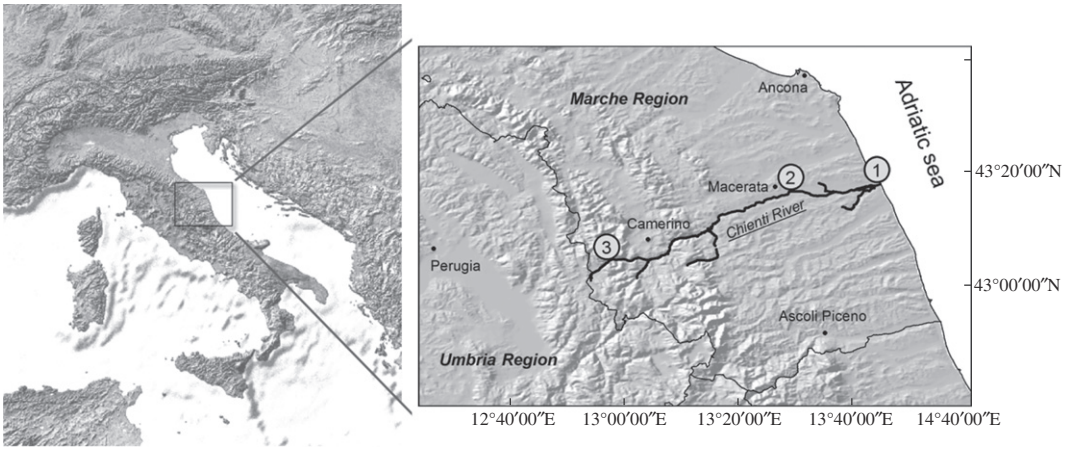

Fig. 1 – Study area. Map of the Chienti river and position of all the sampling points. Sampling point: (1) 43°17′37.86″N, 13°44′34.36″E; (2) 43°16′12.46″N, 13°31′23.59″E; (3) 43°03′2\58.06″N, 12°56′35.82″E.