

## **EASYRING**

### **Environmental Agents Susceptibility assessment utilizing existing and novel biomarker as Rapid non-invasive testing methods**

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Over the past few decades, evidence has emerged that has indicated environmental pollutants can interfere with the endocrine systems of wildlife and humans. These substances are called Endocrine Disrupting Chemicals (EDCs) and can cause adverse effects on the reproductive biology in vertebrates, including humans, via ingestion and enrichment of the food chain. These compounds may mimic or antagonise the effects of naturally occurring sexual steroids or other natural hormones by binding to their corresponding receptors or affecting their circulating levels. In general, there are various principle actions of EDCs which may have a marked impact on reproductive biology via (anti)estrogenic and (anti)androgenic effects. Estrogenic compounds lead to feminisation, anti-estrogenic substances neutralise sexual differentiation, androgenic agents cause masculinisation, while anti-androgenic compounds have feminising effects. Starting from this knowledge, EASYRING aimed to improve the information relating to the environmental levels of some known EDCs and their biological effects on reproduction as measured with traditional and newly developed innovative tools to aquatic species and for mammalian risk assessment.

In Northern Italy, the River Lambro, the most polluted tributary of the River Po, is contaminated with domestic and industrial waste water as well as with agricultural run-off. Recent evidence of intersexual barbels in the downstream stretch of the River Po has raised concern for this area. Thus, part of our studies were devoted to the comparison of the upstream, relatively unpolluted, reach of the middle River Po with the downstream, polluted, reach of the same intermediate section by considering the structure and reproductive health of the fish community. The fish assemblage is a crucial component of water quality monitoring programs. They are relatively long-lived and mobile, so they serve as good indicators of long-term environmental effects. Conversely fish mobility can be a source of undesired variability which is difficult to control unless other studies (e.g. caged fish) are undertaken in the same areas of study. From the start of the in field activities of EASYRING, more than 9000 specimens were captured, identified at the species level and recorded for biometric parameters. Many individuals belonging to the species at risk were also characterised in much more detail and the analyses of these data (collected over a three year study) provided evidence of several important differences and dramatic changes largely depending on the River Lambro. Both the number of allochthonous species and their abundance are increasing, and this is dramatically evident comparing present results with those of studies undertaken 30 years ago. Upstream and downstream of the Lambro River confluence, the fish community has similar composition. However, the species are not uniformly distributed in the two areas. This difference, supported by comparable fishing efforts, seems to be related both to the load of particulate organic material transported by the River Lambro (more food available), and to a higher tolerance of the present fish community and in general of the allochthonous fish species.

In addition to the animal studies, sediment and water samples were collected from the River Lambro and analysed chemically to determine the levels of EDCs. The moderately low level of contamination of the River Po and particularly of the middle section was documented clearly by chemical analyses of water samples collected upstream and downstream of the River Lambro through the development of new extraction techniques. With the exception of bisphenol A determined at hundreds of nanograms per litre, the other chemicals were found at low concentration levels in river waters. The results of sediments and macro invertebrates on the contrary, suggested a clear distinction between the upstream and downstream stretches of the middle River Po showing the consequences of EDC loads from its polluted tributary. The discrepancy between sediment and overlying water is not surprising, and can be explained by the moderate lipophilicity of EDCs and the higher recording capacity of bed sediments compared to grab water samples. Furthermore, fish sampled downstream

showed a trend towards increasing plasma levels of vitellogenin (VTG), the confirmed biomarker for estrogenicity, altered steroid plasma levels and morphological alteration of gonads and liver.

The water and sediment of the River Lambro were analysed for their estrogenic and androgenic potential using a toxicity identification evaluation (TIE) approach that combined samples' fractioning with a battery of *in vitro* tests. Different *in vitro* assays were employed as a preliminary screening of the water and sediment fractions in order to gain more knowledge about the presence of EDCs and their potential for (anti)androgenic and (anti)estrogenic properties. For this purpose two recombinant stable human cell lines, MVLN and MDA-kb2, in parallel with the yeast oestrogen assay (YES) and the yeast androgen assay (YAS), which are able to identify compounds that bind to oestrogen or androgen receptors (ER and AR agonist and/or antagonist), respectively were chosen. The screening revealed the presence of a mix of compounds with different mode of actions, namely estrogenic, androgenic and anti-androgenic compounds. The main mode of actions seemed to be related to chemicals with estrogenic and anti-androgenic modes of action since the total extracts of the water and sediment were either estrogenic or anti-androgenic. This is the first study reporting high levels of anti-androgenic activity in the environment, in both water and sediments of a polluted river in Italy. Evaluation that anti-androgenic activity might be one of the most important endocrine modes of action in the River Lambro suggests that future research efforts should be focussed on the parallel study of estrogenicity and anti-androgenicity.

*In vitro* tests consistently identified a small number of fractions as being mainly responsible for estrogenicity. Chemical analyses undertaken on these fractions identifying a small number of known estrogenic chemicals i.e. estrone (E1), estradiol-17 $\beta$  (E2), estriol (E3), nonylphenols mix (NPs), bisphenol A (BPA), and t-octylphenol (t OP). The three natural estrogens and the three chemicals identified in the highly estrogenic fractions were chosen to be used in the "environmental mixture" (1x Lambro) for *in vivo* exposures of common carp chosen as the sentinel species. With this premise, and with the aim of interpreting the alterations found in feral fish of the River Po, it was deemed necessary to investigate in the laboratory the effects of graded concentrations of the most interesting of the above-noted estrogenic chemicals as well as of a mixture of them, in such a way to mimic environmentally relevant levels and compositions. One more aspect was taken into account to increase the relevance of laboratory investigations, i.e. the exposure length. This aspect is fundamental whenever it is necessary to investigate the effects on the early development of gonads and on sexual differentiation. Both these processes are very sensitive to hormonal imbalance and therefore to the action of those chemicals which can affect the complex functions of the reproductive system. To investigate these processes, and specifically how the River Lambro may affect them, carp fingerlings having undifferentiated gonads and being still under development, were exposed to the 1x Lambro mixture for five months. The study was completed by two other treatments in which carp fingerlings were exposed to River Po bed sediments, collected upstream and downstream of the confluence of the River Lambro. In this way, carp were also exposed to the "natural" mixtures of EDCs present in the main Italian river particularly after the net enrichment transported by the River Lambro. Such a long study was therefore designed to investigate the long term effects of relevant mixtures of EDCs (synthetic and "natural") on a common cyprinid species, starting when primordial germ cells begins to multiply, until the sexual differentiation of the gonads to male or female structure is complete. Both plasma VTG increase and alteration of gonad differentiation, gonad morphology, sex ratio and steroid ratio were indeed observed in carp juveniles exposed to environmental mixtures and downstream sediment.

One of the main targets of EAYRING was the improvement of analytical methods. Both solid phase extraction technique (SPE) and innovative LC-MS procedures have been developed for the extraction, recoveries and identification-quantification of target compounds from samples of several matrices coming from all treated animals. The methods established, allowing for the simultaneous determination of EDCs with a wide range of polarity, were successfully applied to bile and liver samples from those both exposed in lab and feral fish.

An organism's response to toxicant exposure may ultimately manifest itself at the protein level and induce a change in the proteome by affecting gene expression, protein synthesis and the various

processes of post-translational modifications. mRNA levels do not always correspond with the levels of translated proteins, and post-translational modifications may play a pivotal role in protein function. Another aim of EASYRING was to identify novel biomarkers for EDCs by proteomic analysis of plasma from the common carp, *Cyprinus carpio*, and the African clawed frog, *Xenopus laevis*, treated with four model compounds. Tamoxifen (TAM), ethinylestradiol (EE2), methyl-dihydro-testosterone (MDHT) and flutamide (FLU) were chosen as prototype chemicals with anti-estrogenic, estrogenic, anti-androgenic and androgenic actions, respectively. Two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) were used in a proteomic-based strategy to detect and identify differentially regulated proteins in both plasma, liver and mucus. MALDI-TOF mass spectrometry and MS/MS techniques like MALDI-TOF/TOF and nESI-MS/MS were used to generate “peptide-mass fingerprints” (PMF) and sequence information for identification of these proteins through MASCOT searches in protein and DNA databases (MSDB, Swiss-Prot, NCBIInr, Gen-Bank). These studies allowed the detection of a series of proteins whose expression changed in response to EDCs treatment. The most prominent response of the four different model EDCs tested was observed with the synthetic estrogen EE2 which induced a marked change in the protein pattern of plasma from treated individuals when compared to control. Mass spectrometric analyses identified the changes to be due to an up-regulation of VTG and Ep45. EP45 is a member of the serpin protein family with a close sequence similarity to human alpha-1-antitrypsin. Moreover, the total absence of Ep45 protein in plasma from control males and its subsequent appearance following estrogen treatment suggests this protein as a good non-destructive candidate biomarker for xenoestrogens in *Xenopus laevis*. New potential candidate biomarkers have been individuated for both carp and *Xenopus*, synthetic peptides were obtained and antibodies (Abs) against these biomarker candidates were raised. Furthermore, using these Abs, ELISA assays were developed in order to detect and/or quantify routinely the respective biomarker proteins. Three types of ELISA formats were tested, the semi-quantitative (qualitative) Ab capture ELISA and the potentially quantitative competitive and sandwich ELISA formats. The HRP-labelled Abs were used in the two latter assay formats, whereas unlabelled Abs were used in the first format. The different ELISA formats were tested to assess their suitability for detecting and / or quantifying the biomarkers. Lacking purified protein standards for the different biomarkers, plasma and liver samples from control and treated fish, as well as synthetic peptides, were used in order to validate the performance of the assays we have developed. Finally, the feasibility of the new biomarker proteins have been assessed to determine how well they are suited to act as biomarkers of EDCs in fish.

Improvement of *in vitro* and *in vivo* techniques led to the establishment of several genetic biomarkers. In particular, it has been demonstrated that mRNA for vitellogenin (VTG), oestrogen receptors (ER), retinol binding protein (RBP), androgen receptor (AR) and transthyretin (TTR) of *Xenopus* can be used as genetic biomarkers for EDCs. Moreover, the mRNA expression of transferrin (TF) can be used as (anti)estrogenic biomarker, since it is down-regulated in the liver by estrogens in males and females but also up-regulated by antiestrogens in females. Moreover the mRNA expression of gonadotropins in the brain was examined in *Xenopus* to assess whether EDC can interfere with the hypothalamus-pituitary-gonad axis indicating disturbances of reproductive processes by EDC. In general reproduction is mediated via the hypothalamus-pituitary-gonad axis. Several inputs to the central nervous system lead to secretion of gonadotropin releasing hormone (GnRH), which is produced by the cells of the hypothalamus. In turn, the peptide hormone GnRH is transported to the pituitary stimulating the secretion of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) into blood circulation. The gonadotropins increase synthesis and release of sex steroids in the testis and ovary. On the one hand attenuated blood levels of sex steroids can act via negative feed-backs on the hypothalamus and pituitary, inhibiting secretion of GnRH and gonadotropins. On the other hand, passing a target organ such as the liver, sex steroids can activate their receptors in order to induce estrogen or androgen specific gene expression. It is reported for the first time in amphibians that gonadotropin mRNA expression is differentially regulated by (anti)estrogenic and (anti)androgenic EDCs indicating disturbance of reproductive processes at higher regulatory centres. The mRNA expression of the potential biomarker genes from the liver of *Xenopus* were changed in response to (anti)estrogenic but not to (anti)androgenic treatments. Consequently, RBP and TTR can serve as estrogenic biomarker while TF can be used as (anti)estrogenic biomarker. Biomarkers for (anti)androgenic modes of action are still under development.

Cytotoxic and teratogenic effects of low doses of the same four representative EDCs (TAM, EE2, MDHT and FLU), environmental mixture and E3 and BPA used for aquatic species were verified on G2 to M phase transition taking place at the onset of oocyte maturation (part I), on completion of meiosis from metaphase II stage to the extrusion of the second polar body (part II), zygote cleavage, cell proliferation and blastocyst formation and finally on *in vitro* proliferation and differentiation of embryonic teratocarcinoma cells. Dose-effect on Germinal Vesicle Breakdown (GVBD) and first Polar Body (PB) extrusion was evaluated and statistically analysed. Tamoxifen up to 400  $\mu\text{M}$ , FLU from 9 to 126  $\mu\text{M}$  and EE2 up to 400  $\mu\text{M}$  activities on GVBD and PB extrusion were evaluated. These experiments were applied to standardise experimental conditions and avoid any variability in the results. Specific factors (e.g. molecular stability and a mouse's age) which might influence the results were also considered. Pooled results indicated that TAM has no effect at concentrations up to 100  $\mu\text{M}$ , while being totally lethal at 400  $\mu\text{M}$ . The  $\text{LD}_{50}$  was estimated to be approximately 250  $\mu\text{M}$ . These results also show that TAM inhibits the extrusion of the first PB in a concentration-dependent manner. For TAM, it was concluded that FLU exerts an inhibitory effect on both resumption of meiosis and Metaphase I to Metaphase II transition. This effect seems to be dependent on the age of the mice. EE2 is totally lethal at concentrations higher than 400  $\mu\text{M}$ , the  $\text{LD}_{50}$  being between 300 and 350  $\mu\text{M}$  and it has no effect at concentration lower than 25  $\mu\text{M}$ . It appears that EE2 strongly inhibits oocyte maturation in a concentration dependent manner. Most of oocytes being treated with EE2 at concentrations ranging from 50 to 100  $\mu\text{M}$ , were indeed arrested at the GVBD stage. Moreover, at 200  $\mu\text{M}$ , EE2 is totally inhibitory even on resumption of meiosis (GVBD). EE2 exerts a clearcut effect on both resumption of meiosis and Metaphase I to Metaphase II transition (between GVBD-PB stages). In contrast to TAM and FLU, it is strictly dependent on the concentration.

One of the major objectives of the project was to develop a new non-invasive system for the detection of the exposure to EDCs. The development and performance of a simple, rapid, non-invasive method for assessing estrogenic responses in the carp, a common fish found in European waterways and regularly used in environmental monitoring programmes across Europe was determined in the EASYRING project. The data show that the well-established biomarker for estrogens, vitellogenin, can be detected in fish mucus using a lateral-flow immunoassay (dipstick assay) performed within a few minutes of sampling. "Proof-of-principle" has been established that the assay can be used in non-invasive assessment of estrogenic exposure of fish using mucus samples at a level of sensitivity that is sufficient to detect estrogen exposure in the environment. To check the applicability of the Dipstick tool and its related protocol, the participants also operated in presence of personnel from ARPA, the Regional Environmental Protection Agency. Comparison of VTG levels in plasma and mucus of the same fish using a quantitative sandwich ELISA previously established indicates a positive correlation in almost all groups of carp investigated. Interestingly, in some of the fish groups, no correlation or a negative correlation was observed. This was most pronounced in samples from the field and from the TAM exposure groups. The lack of correlation between plasma and mucus VTG indicates different turnover of VTG in these sample types. In the field sampling, there was a general lack of VTG in the mucus, although varying levels of VTG were found in plasma. In the TAM group, higher VTG levels in mucus were found in fish with relatively low plasma VTG levels. One possible explanation is that exposure to TAM, being an anti-estrogen, results in a decreased VTG synthesis that will first be detected in plasma while mucus levels will need a longer time to be reduced. It is also possible that fish of various age groups show different VTG kinetics, and that fish displaying correlations that were more consistent and significant, like the EE2, FLU, MDHT, and mixed exposure, were from more homogeneous populations.

The VTG LFIA was developed on the basis of the same Ab pair as is used in the sandwich ELISA with the monoclonal Ab as the capture Ab in the test line, and the polyclonal Ab as the detecting Ab (conjugated to colloidal gold). The LFIA was scored at two response levels (1 and 2), based on intensity of the test line, compared to 0, where no test line was visible. Apparently, 0 and 1 response level did not distinguish sufficiently between VTG levels (quantified by ELISA), whereas the 2 response level reflected a clear difference from the 0 level. In plasma, all samples containing  $>5\mu\text{g/ml}$  VTG were positive (level 1 or 2) in LFIA. In mucus, this value was 0.5  $\mu\text{g/ml}$ . This corresponds well with the theoretical detection limits observed with purified VTG. Overall, this shows that the carp

VTG LFIA is able to report significant VTG induction (level 2) at VTG concentrations low enough to detect estrogenic effects in the environment.

EASYRING also aimed to develop predictive models and for the preparation of a database of publicly available information regarding the ability of chemicals to elicit endocrine disruption, to take account of the potential of the different experimental models, and to integrate information from different species and different sources in order to extend mathematical models to human toxicity. The present study dealt with several important aspects for the successful (quantitative) structure-activity relationship ((Q)SAR) modelling. It has been demonstrated that the CoMFA alignment of the compounds can be reliable when considered with regard to the information of their binding mode to the receptor site. Until the current time no automated approach for alignment is known that can recognise the differences in the binding mode of compounds with a very similar structural skeleton. Thus, such an alignment relies on experimental results derived from crystal structures. Another aspect of this study shows that, unlike classical QSAR, the concept of variability of the biological response is incorporated in the development and testing of QSAR models. The statistical results derived from internal and external validation, indicate successful modelling of the relative binding affinity to both ER $\alpha$  and ER $\beta$  for *in vitro* measurements. It is demonstrated that the “quality” of an appropriate for QSAR data set should not be judged on well-performed measurements solely. All steps starting from molecular modelling, going through careful consideration of the methods applied to the particular QSAR modelling and ending up with well-argued data analysis are important for the development of mechanistically interpretable model. In addition, the identification of outliers is shown to be crucial for the explanation of hidden information with respect to the entire data set. Finally, in order to introduce a meaningful mechanistic interpretation of the QSAR models, SAR investigation is also a very helpful tool.