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FINAL REPORT

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This final report presents the work progress and the research highlights of the project titled "Environmental risk assessment of four pharmaceutical compounds employing genomic tools and the seabream, *Sparus aurata*." (Acronym ERA4PHARM, Proposal N° 239325, FP7-PEOPLE-ERG-2008)). This report encompasses the work progress during the 36-month of reintegration phase after the completion of a Marie Curie Intra-European Fellowship at the Institute of Aquaculture in Stirling (Scotland). Project title: Evaluation of the genomic effects of four environmentally suspect pharmaceutical compounds on Atlantic salmon (Proposal N° 039691-SALMONPHARM, FP6-2005-Mobility-5).

BACKGROUND AND OBJECTIVES

Human and veterinary pharmaceutical drugs are more and more consumed worldwide. Their detection in the environment and their bioactivity has now raised concern for potential adverse effects on non-target species. Notwithstanding recent attention for their environmental presence, there are significant research gaps for existing pharmaceuticals with regard to their potential ecological consequences. Some acute toxicity data on pharmaceuticals is available in several non-target organisms, however there is only limited data on sublethal and chronic toxicity of pharmaceuticals. As a consequence, the research focus of the Environmental Risk Assessment (ERA) in general, has shifted to the challenge of understanding the chronic effects of contaminant exposure. The generation of more extensive sublethal effects data sets which provide cause-effect information for pharmaceuticals is pivotal to ensure the "level of protection" of the ecosystems from this kind of compounds.

In this study, a selection of the most abundantly used pharmaceuticals: Acetaminophen (APAP), Carbamazepine (CBZ) and Atenolol (AT), was examined for their long term effect on the genomic and proteomic expression of the gilthead seabream, *Sparus aurata*. These genomic endpoints provide useful information on the xenobiotic-induced impairment resulting in the activation and silencing of specific genes by elucidating the underlying molecular mechanisms of higher level damage. Gene expression and protein products were evaluated in liver and brain as target tissues by microarray and 2D differential gel electrophoresis (2D DIGE) techniques, and significantly up and down regulated proteins were identified by liquid chromatography–tandem mass spectrometry (LC–MS–MS). Together with already available toxicity data for this species and the chosen pharmaceutical compounds, we aimed to elucidate toxicity mechanisms at molecular level that are linked with effects at higher organizational level. This will help to improve extrapolation techniques from laboratory tests to derive potential ecosystem effects. Also, the knowledge of molecular pathways and proteins involved in toxic processes forms the base for the development of specific biomarkers which will supply decision makers with information for the further monitoring of the effects of pharmaceuticals in the environment and the regulation of these compounds.

The most obvious application of genomic technologies in toxicology has been in the prediction of toxic mechanism or model of action (MoAs). Gene expression analysis has utility in predicting MoA for chemicals in general. The ability to deduce mechanism from the pattern of genes expressed has applications in 1) prioritising chemicals for more extensive testing regarding the environmental risk they can represent; 2) tailoring test batteries based on presumed MoA; 3) as support for read-across procedures to limit testing in well-studied chemical families, among others. Gene expression analysis may also support comparison of responses and extrapolation across species.

General objectives and expected results. The objectives of the current project were (1) to evaluate the xenobiotic-induced impairment resulting in the activation and silencing of specific genes and proteins in the gilthead sea bream, *Sparus aurata*, due to exposure to selected representative pharmaceuticals, (2) to elucidate the underlying molecular mechanisms of higher level damage and relate effect concentrations for different endpoints (molecular, cellular, organ, organism), (3) to develop biomarkers of pharmaceutical contamination, (4) to use the obtained data to review existing extrapolation techniques in environmental risk assessment.

The results will allow to test the hypotheses (1) that environmentally relevant concentrations of the selected pharmaceutical compounds result in differences in the expression of functional genes, (2) that effects observed at higher organizational levels can be linked to molecular processes and (3) that existing pharmaceutical concentrations may have knock-on ecological effects stressing the need to include this kind of pollutants in the respective priority list of pollutants for their regulation.

The proposed objectives were developed through the realization of the following **specific tasks**:

1. Long term exposure (30 days) of individuals of *Sparus aurata* to environmentally relevant concentrations of the selected pharmaceuticals.

2. Evaluate the effects of this exposure on the transcriptome level in liver and brain of the exposed organisms by means of microarray approach.

3. Evaluate the effects of this exposure on the proteome level in liver and brain of the exposed organisms by means of 2D differential gel electrophoresis (2D DIGE) approach.

4. Identify differently expressed proteins by liquid chromatography–tandem mass spectrometry (LC–MS–MS).

5. Ideally develop contaminant specific biomarkers of exposure to pharmaceutical compounds.

SCIENTIFIC ACTIVITIES

- *WORK PROGRESS*

Task 1– Design and performance of pharmaceutical exposure experiments , exposure concentration analysis

1.1 *Development and construction of a continuous flow through exposure system* – A system that allowed the simultaneous exposure of the chosen test species to the selected pharmaceuticals including a control (pure SW) and a solvent control (SW with DMSO) tank was developed and built. This system had a continuous SW input and the contaminant was added by means of a peristaltic pump from a stock solution to achieve environmentally relevant concentrations of the test compound.

1.2 *Exposure of test organisms and sampling* – 15 individuals (approximately one-year-old) were exposed for 30 days to the following nominal concentrations of pharmaceuticals: APAP: 31.90 ± 11.07 $\mu g \cdot L^{-1}$; AT: 0.95 ± 0.38 $\mu g \cdot L^{-1}$ and CBZ: 6.95 ± 0.13 $\mu g \cdot L^{-1}$ (measured concentrations). Stock solutions were renewed every 24h to avoid (photo-) degradation of the substances. Fish were exposed under controlled laboratory conditions (T: 20±1^{o}C, salinity: 35, photoperiod 16h light:8h darkness). At the end of the exposure period, the organisms were killed on ice and rapidly dissected. Brain, liver, spleen and heart tissues were taken from groups of five fish and processed accordingly to the analyses to be carried out on them: for transcriptomic analysis the tissues were rapidly homogenised on ice in Tri reagent (Sigma), for proteomic analysis, tissues were frozen immediately in liquid nitrogen. For both treatments, the tissues were then stored at -80ºC until their use in the different analyses. A third part of tissued was stored in formaline buffer for posterior histochemical analysis.

1.3 *Sample treatment* - Water samples (200mL) from the exposure tanks were taken at days 0, 3, 7 15, 22 and 30 of the exposure period. Prior to extraction, 200 mL water samples were filtered through a 0.45 µm glass fiber membrane filter (Whatman, Mainstone, UK) to remove suspended matter. For solid phase extraction (SPE) cartridges (Oasis HLB, 60 mg, 3 mL) were conditioned with 5mL of methanol followed by 5mL of deionized water (HPLC grade) at a flow rate of 1 mL/min. After the conditioning step, water samples (200mL) were passed through the cartridges at a flow rate of 10 mL/min. Finally, the cartridge was rinsed with 5mL of HPLC-grade water. The cartridge was then dried under vacuum for 15–20 min, to remove any excess of water. Elution was performed with 2×4mL of methanol at 1 mL/min. The extract was evaporated under a gentle nitrogen stream and reconstituted. The residue was then dissolved in 0.5 ml of methanol and injected into the HPLC system.

1.4. High Performance Liquid Chromatography (HPLC) - Exposure concentrations were measured as described by Santos et al., 2005. Briefly, after solid phase extraction (OASIS HLB cartridges; 60 mg, 3mL; Waters, Milford, MA, USA), the analytes were separated under isocratic conditions with acetonitrile and a 50mM potassium dihydrogen phosphate solution at a flow rate of 1$mL \cdot min^{-1}$ in a Waters HPLC module. Peak areas were used for quantitative analyses. APAP and CBZ were measured using the UV signal at 250nm. AT was quantified using the fluorescence signal at 271nm. Compounds were identified by comparing retention times and peaks in the sample and in standard solution chromatograms.

1.5. *Calibration curve and limits of detection and quantification* - The calibration curves were constructed in the expected concentration range of each pharmaceutical. Calibration standards were prepared by dilution of the stock standard solutions in methanol. Calibration curves were generated by linear regression of peak areas of standard solutions against their respective concentrations. Limits of detection (LOD) and limits of quantification (LOQ) were calculated by using a signal-to-noise ratio of 3 and 10, respectively (the ratio between peak intensity and intensity of the noise was used).

Task 2 – Transcriptomic analysis

2.1. RNA isolation and microarray hybridisation - Gene expression profiling has been carried out using an updated version of the *Sparus aurata* oligo DNA microarray, SAPD_V3.0-031723 (Ferraresso et al., 2008). A total of 13,605 probes, targeting 12,881 transcripts, were successfully designed. Probe sequences and further details on the microarray platform can be found in the GEO repository (http://www.ncbi.nlm.nih.gov/geo/) under accession number GPL15601.

RNA was isolated from individual brain samples according to the manufacturer's instructions (Sigma). For each treatment (SW control, DMSO vehicle, APAP, AT and CBZ), five microarrays were used resulting in a total of 25 microarrays hybridized for expression analysis. Additionally, a further five microarrays were used for technical replicates (carried out with five individual control samples) to check on the repeatability of the technique. Sample labeling and hybridization were performed according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol. Slides were incubated for 17 h at 65°C in an Agilent hybridization oven.

2.2. Image acquisition and microarray data analysis - Hybridized slides were scanned using an Agilent G2565BA DNA microarray scanner. Data were extracted and background subtracted using the standard procedures contained in the Agilent Feature Extraction (FE) Software version 9.5.1. A Lowess normalization procedure was performed using R statistical software (http://www.r-project.org) and normalized data were used for all subsequent analyses. Normalized fluorescence data for these comparisons have been deposited in the GEO database under accession numbers GSE38195. Significance Analysis of Microarray (SAM) (Tusher et al., 2001) was used to identify differentially expressed genes between pharmaceutical exposed and SW-and DMSO control tissues. False discovery rate (FDR) and minimum fold change (FC) were set at 0.05 and 1.5, respectively. Obtained candidate gene lists were submitted to Venn analysis (Oliveiros, 2007) in order to detect treatment specific features and/or those common to two or all three treatments. In order to extract biological significance behind the obtained gene lists, DAVID (Database for Annotation, Visualization and Integrated Discovery; http://david.abcc.ncifcrf.gov/) was used to perform functional annotation analysis. Gene ontologies biological process (BP), cellular component (CC) molecular function (MF) and KEGG (Kyoto Enzyclopedia of Genes and Genome) pathway analysis were carried out. This provided a systematic interpretation of the set of differentially expressed genes based on an integrated biological knowledgebase.

Task 3 – Proteomic analysis

3.1. Proteomic analysis - Frozen liver tissues were homogenised in lysis buffer Following homogenization the tissue lysates were centrifuged and protein concentration was determined by the method based on Bradford (Bio-Rad Protein Assay, Hercules, USA). Five biological replicate protein samples per treatment were labeled randomly with fluorescent dyes Cy3 or Cy5, whereas the pooled internal standard was labeled with Cy2 (GE Healthcare, Munich, Germany). Isoelectric focussing (1^{st} dimension) was carried out on an IPGphor system (GE Healthcare) in four stages with a ramped voltage change between each step. The second dimensional separations were carried out on 12.5% SDS-polyacrylamide gels on the Ettan DaltSix system (GE Healthcare) at 1W/gel for one hour and subsequently at 15W/gel.

3.2. Image analysis - Labeled proteins were visualized using the Typhoon 9000 series imager (GE Healthcare) at three different wavelengths corresponding to the fluorescent dyes. The Cy2, Cy3, and Cy5 components of each gel were individually imaged using excitation/emission wavelengths specific for Cy2 (488/520 nm), Cy3 (532/580 nm) and Cy5 (633/670 nm). PMT was varied in order to equalise fluorescence intensities between channels and to prevent over saturation of the signal. Gel analysis and protein fingerprint analysis was performed using DeCyder 2-D Differential Analysis Software v6.5 (GE Healthcare). Within the Biological Variation Analysis (BVA) module, each comparison was filtered to find the spots having a p-value <0.05 using the paired T-test.

Task 4 – Protein identification

4.3 In gel digestion and mass spectrometry - A preparative gel was used for spot picking and visualized with silver staining to pick spots of interest. The gel pieces were digested by trypsin (Promega, Madison, WI). MS/MS analyses were performed on a 4800 MALDI-TOF/ TOF instrument (Applied Biosystems, Foster City, CA). Measurements were taken in the positive ion mode between 900 and 3000 m/z. Sequences were automatically acquired by scanning first in peptide mass fingerprint (MS) mode. A database search (NCBI nr) was performed, combining the results of peptide mass fingerprint (MS) with subsequent fragmentation (MS/MS) of up to twelve peptides from each spot according to the quality of the MS spectrum, using MASCOT (Version 2.0.00, release date: 19.02.2007) as a search engine. Scores greater than the given cutoff value for MS/MS fragmentation data were taken as significant ($p<0.05$).

4.4 Data evaluation by multivariate statistical analyses - Multivariate analyses were performed on datasets constituted by the relative levels of all the spots that were consistently matched between the 20 gels, in order to avoid the replacement of null values by inference. PCA was based on the obtained spots of interest where hierarchical clustering was performed on all differently expressed spots. PCA and cluster analysis were carried out using the DeCyder software package (DeCyder 2-D Differential Analysis Software v6.5; GE Healthcare).

Task 5 – Development of contaminant specific biomarkers

5.1. *Discrimination of treatment common and specific features* – After transcriptomic analysis, Significance Analysis of Microarray (SAM) was used to identify differentially expressed genes between pharmaceutical exposed and SW-and DMSO control tissues. SAM identifies genes with statistically significant changes in expression by assimilating a set of gene-specific t-tests assigning a score to each gene on the basis of change in gene expression relative to the standard deviation of repeated measurements. For genes with scores greater than an adjustable threshold, SAM uses permutations of the repeated measurements to estimate the percentage of genes identified by chance, the false discovery rate (FDR). In this case, FDR and minimum fold change (FC) were set at 0.05 and 1.5, respectively. These candidate gene lists were submitted to Venn analysis (Oliveiros, 2007) to separate treatment common from treatment specific features. Those features that are exclusive for a special treatment, as well as the treatment specific identified proteins after 2D gel electrophoresis separation may be useful candidates for the development of contaminant specific biomarkers.

References

ECETOC (2007) Workshop on the Application of 'Omic Technologies in Toxicology and Ecotoxicology: Case Studies and Risk Assessment 6-7 December 2007, Malaga

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- *ACCOMPLISHMENT OF RESEARCH OBJECTIVES AS PRESENTED IN THE ORIGINAL PROPOSAL*

Tasks 1 – 4 *have been largely achieved and* ***task 5*** *is currently being carried out.* ***Tasks 1 and 2*** *had been almost completely accomplished during the first reporting period. The transcriptomic analysis of the brain samples was initiated during 2009 and completed in 2010.* ***Tasks 3 and 4*** *have been carried out during the second reporting period, and* ***task 5*** *is currently being finished. Therefore, the project was very largely on schedule with the overall project almost entirely completed. However, slight modifications were required which will be detailed below, but with effective management, flexibility and planning, the impact of these modifications was minimised and the overall schedule of the ERA4PHARM project is, with some slight delays, on track.*

Objective 1: To evaluate the pharmaceutical-induced impairment resulting in the activation and silencing of specific genes and proteins in the gilthead sea bream, *Sparus aurata*, due to exposure to selected representative pharmaceuticals.
Progress: The exposure and exposure concentration analysis (task 1), as well as the transcriptomic part of this objective (task 2) has been largely performed during the first reporting period and several aspects have been already listed in the first annual report. The proteomic analysis (task 3 and 4) has been carried out during the second reporting period.
Changes to the original project: Risk assessment was originally planned to be performed based on dose-response curves for each pharmaceutical compound. However, this would have resulted in at least 100 microarrays to be hybridized (5 exposure concentrations x 5 replicates x 4 compounds) instead of 25 (20 for the treatments and 5 for the technical replicates) with a significant increase (4x) of the costs to be employed in the microarray analysis. The costs for 25 microarrays including required reagents was 7530,46€. Thus, the performance of a whole cassette of dose response experiments would have consumed a mayor part of the budget of the grant. Instead, we decided to base our risk assessment on the recommendations of the ECETOC report on "omic" techniques (ECETOC, 2007) which is based on the determination of the mode of action of a specific contaminant and its subsequent categorization and risk evaluation based on structure activity relationships (SAR).
Results and degree to which the objectives were met: The exposure experiments and transcriptome analyses carried out were successful in that the employed microarrays (SAPD_V3.0-031723) provided an effective research tool for the detection of induced or impaired gene expression even under low, environmentally relevant exposure concentrations of pharmaceuticals. After AT treatment, only seven features were significantly altered in comparison to the control treatment, presumably due to the lower exposure concentration. However, a clear effect was observed on the gene expression after APAP and CBZ exposure, with several hundreds of significantly changed genes. A list of the most significantly changed genes is supplied in the manuscript submitted to PlosONE. Also, the protein extraction protocol and subsequent proteome analyses have been optimized and carried out successfully. Different protein expression profiles were obtained after the applied treatments. Therefore, this objective was successfully met, and a manuscript is currently submitted to PlosONE and another under preparation for the publication of these results.

Objective 2: to elucidate the underlying molecular mechanisms and mode of actions of higher level damage.
Progress: The bioinformatic analyses of both transcriptomic (task 2.2.) and proteomic data outcome (tasks 3.2 and 4.4), have been performed during the first (transcriptomic) and second (proteomic) reporting period.
Changes to the original project: None.
Results and degree to which the objectives were met: The obtained results show important relationships and similarities between effects of APAP and CBZ consumption/exposure in humans and rodents and our test species, indicating a high degree of conservation of the genetic targets of these drugs at different organisational levels. It is yet to be determined if low level exposure through several generations will alter physiological processes in non target organisms. Thus, this objective has been completely performed, with the results being under process of publication.

Objective 3: to develop biomarkers of pharmaceutical contamination.
Progress: The Venn analysis of the transcriptomic data (task 2.2.) has been performed during the first reporting period. The proteomic data (task 4.3) revealed several candidate proteins for the further confirmation and development of biomarkers of contamination of the selected pharmaceuticals.
Changes to the original project: This task is still ongoing and further research is necessary to confirm, together with the results obtained from the proteome analysis, and validate these biomarkers in the field.
Results and degree to which the objectives were met: After retrieving the candidate gene lists from the transcriptomic data sets, Venn analysis was carried out (task 2.2) revealing a number of treatment specific alterations that can be useful candidates for the development of compound specific biomarkers of contamination. The proteomic data revealed several candidate proteins that may be useful as biomarkers but more studies have to be carried out in order to confirm this findings.

Objective 4: to use the obtained data to review existing extrapolation techniques in environmental risk assessment.
Progress: Functional enrichment and KEGG pathway analysis have been carried out with the obtained transcriptomic data (The Venn analysis of the transcriptomic data (task 2.2.) has been performed during the first reporting period. The proteomic data (task 4.3) revealed several candidate proteins for the further confirmation and development of biomarkers of contamination of the selected pharmaceuticals.
Changes to the original project: The risk evaluation of the selected pharmaceutical compounds based on their specific mode of action instead of dose-response behavior, implies the application of *in silico* and chemoinformatic based techniques which are not considered in this project. However, the existing contacts collaborations the researcher has established during her career will allow further exploiting the obtained results and enhancing recent developments for environmental risk assessment based on "omic" techniques.
Results and degree to which the objectives were met: *A different mode of action has been revealed for APAP and CBZ treatment, respectively. These results allow these compounds to be grouped by in silico and chemoinformatic based techniques with compounds with similar MoA and elucidate their potential environmental and human health risk at existing environmental concentrations.*

OTHER SCIENTIFIC ACTIVITIES

- *STAGES*

The researcher has established a fruitful collaboration with the research group of the Professor Luca Bargelloni from the Department of Comparative Biomedicine and Food Science of the University of Padova. In April 2011, the researcher performed a research stage of one month at Prof Bargelloni's lab to perform the microarray experiments. This included the use of a 15k *Sparus aurata* oligo DNA microarray, (Agilent, SAPD_V3.0-031723) developed by this group, as well as the use of associated bioinformatic approaches. This stage was supported by a mobility aid from the Spanish National Council for Scientific Research (CSIC).

- *PARTICIPATION IN MEETINGS AND WORKSHOPS*

1) 15th International Symposium on Pollutant Responses in Marine Organisms, 17-20 May 2009, Bordeaux (France)
2) Genomics in Aquaculture, 5-7 July 2009, Bodo (Norway)
3) 20th SETAC Europe Congress, 20-24 May 2010 Seville, (Spain)
4) 16th International Symposium on Pollutant Responses in Marine Organisms, 15-28 May 2011, Long Beach (USA)
5) 16th Iberian Seminary for Marine Chemistry, 24-26 January 2012, Cádiz (Spain)
6) 6th SETAC World Congress 2012, 20-24 May 2012 Berlin (Germany)

DEVIATIONS FROM THE PLAN

The project was very largely on schedule. However, the project was slightly delayed due to the reason detailed below, but with effective management, flexibility and planning, the impact of this delay was minimised and the overall schedule of the ERA4PHARM project fulfilled.

The researcher took off for maternity leave for four month from 20th of September 2009 to 23rd of January 2010. This produced a delay in the performance of the scheduled plans of which the fellow duly informed the corresponding project officer Ms Chantal Huts. However, and regarding these four month, the overall tasks scheduled in the project have followed the work program (added below) and there have been no significant deviations from the original plan. Nevertheless, this delay didn't affect the achievement of the objectives outlined and basically, the planned activities were continued according to the work program.

Table 1. Original work plan

Activity	Milestone	Trimester											
		1	2	3	4	5	6	7	8	9	10	11	12
1. Exposure trials													
Exposure system setup and acclimatization of test organisms. Preliminary survey	Preparation and validation of adequate test conditions and functionning, ensurance of reliability of posterior experiment outcome. Review of the state of the art in the field.	x	x										
Exposure experiment: AC; TR; CA; AT; Control.	Availability of target tissues.	x	x	x									
2. Exposure concentration analysis													
Analysis of AC; TR; CA and AT concentration in samples.	Knowledge of exposure and environmental concentrations to establish dose response relationships.			x	x								
3. Molecular effect assessment													
Transcriptomics, RT-PCR	Data about effects in gene expression.				x	x	x	x	x				
Proteomics	Data about effects in protein expression.					x	x	x	x	x			
4. Data analysis and integration													
Analysis of obtained expression data, statistical treatment	Evaluation and interpretation of obtained data; ERA									x	x	x	
5. Reports/Publications/Conferences													
Conferences	Dissemination of results		x				x				x		
Preparation of publications / final reports										x	x	x	x