Elsevier Editorial System(tm) for Marine Environmental Research

Manuscript Draft

Manuscript Number:

Title: TRANSCRIPTOMIC ANALYSIS OF BOTTLENOSE DOLPHIN (TURSIOPS TRUNCATUS) SKIN BIOPSIES TO ASSESS THE EFFECTS OF EMERGING CONTAMINANTS.

Article Type: Short Communication

Keywords: Bottlenose dolphin; Transcriptome; Emerging contaminants; Biomarkers; Ocean health

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Abstract: Chemicals discovered in water at levels that may be significantly different than expected are referred to as "contaminants of emerging concern" (CECs) because the risk to environmental health associated with their presence and frequency of occurrence is not known. The perfluorooctanoic acid (PFOA), used to make fluoropolymers, and the bisphenol A (BPA), a monomer used in epoxy resins and polycarbonate plastics present in many hard plastic bottles and metal-based food storage are worldwide distributed compounds considered CECs because of their agonist or antagonist effects on the endocrine receptors.
We applied an ex vivo assay using skin biopsy slices from the bottlenose dolphin (Tursiops truncatus) to analyze global gene expression in response to PFOA or BPA exposure. Small slices of skin biopsy were cultured and independently treated with different concentrations of PFOA or BPA prior hybridization to a custom-made microarray. Results illustrate how the skin transcriptome holds information on the contaminant exposure, showing potential long-term effects on the dolphin health status. Most important is the potential of the novel species-specific approach described: the skin transcriptomic signature could be used as classifier for a specific contaminant and the significantly regulated genes used to develop a patented kit of biomarkers of exposure.

Dear Editor,

Please find our attached manuscript entitled "TRANSCRIPTOMIC ANALYSIS OF BOTTLENOSE DOLPHIN (*Tursiopstruncatus*) SKIN BIOPSIES TO ASSESS THE EFFECTS OF EMERGING CONTAMINANTS" by Denise Lunardi, Luigi Abelli, Cristina Panti, Letizia Marsili, Maria Cristina Fossi, and Annalaura Mancia, that we would like to offer as *Short Communication* for publication in Marine Environmental Research. One Table and 3 Figures are included.

We applied an *ex vivo* assay using skin biopsy slices from the bottlenose dolphin (*Tursiops truncatus*) to analyze global gene expression in response to the exposure of two chemicals listed as contaminants of emerging concern. Small slices of skin biopsy were cultured and independently treated with different concentrations of PFOA or BPA prior hybridization to a custom-made microarray. Results illustrate how the skin transcriptome holds information on the contaminant exposure, showing potential long-term effects on the dolphin health status.

Most importantly, this work describes the successful employment of two novel approaches: 1) the use of the dolphin skin biopsy coupled with a transcriptomic analysis, using a species-specific custom made microarray; 2) the assessment of the effects of two non-canonic, but environmentally threatening contaminants, on wild dolphin health.

We hope you will like the manuscript for publication in your journal.

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TRANSCRIPTOMIC ANALYSIS OF BOTTLENOSE DOLPHIN (*TURSIOPS TRUNCATUS)* SKIN BIOPSIES TO ASSESS THE EFFECTS OF EMERGING CONTAMINANTS.

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KEYWORDS

Bottlenose dolphin, Transcriptome, Emerging contaminants, Biomarkers, Ocean health

LIST OF ABBREVATIONS: ADIRF, adipogenesis regulatory factor; BCAP31, B-cell receptor-associated protein 31; BPA, bisphenol A; CDC42, cell division cycle 42; CECs, contaminants of emerging concern; EDs, endocrine disruptors; f, fold; GADPH, glyceraldehyde 3-phosphate dehydrogenase; GAP, GTPase-activating protein; GO, Gene Ontology; GPCR, G protein-coupled receptor; MTSS1, metastasis suppressor 1; PFCs, perfluorinated compounds; PFOA, perfluorooctanoic acid; qPCR, quantitative real-time polymerase chain reaction; RGS2, regulator of G-protein signaling 2; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta.

ABSTRACT

Chemicals discovered in water at levels that may be significantly different than expected are referred to as “contaminants of emerging concern” (CECs) because the risk to environmental health associated with their presence and frequency of occurrence is not known. The perfluorooctanoic acid (PFOA), used to make fluoropolymers, and the bisphenol A (BPA), a monomer used in epoxy resins and polycarbonate plastics present in many hard plastic bottles and metal-based food storage are worldwide distributed compounds considered CECs because of their agonist or antagonist effects on the endocrine receptors.

We applied an *ex vivo* assay using skin biopsy slices from the bottlenose dolphin (*Tursiops truncatus*) to analyze global gene expression in response to PFOA or BPA exposure. Small slices of skin biopsy were cultured and independently treated with different concentrations of PFOA or BPA prior hybridization to a custom-made microarray. Results illustrate how the skin transcriptome holds information on the contaminant exposure, showing potential long-term effects on the dolphin health status. Most important is the potential of the novel species-specific approach described: the skin transcriptomic signature could be used as classifier for a specific contaminant and the significantly regulated genes used to develop a patented kit of biomarkers of exposure.

INTRODUCTION

The decline in the health status of the marine ecosystem can be mostly related to anthropogenic impacts such as overfishing, coastal habitat destruction, deep sea mining, oil and gas exploration, and ocean acidification, but it is also strictly correlated to industrial application and the release of chemical contaminants and pollutants. The exposure to contaminants, known as “contaminants of emerging concern” (CECs, http://water.epa.gov/scitech/cec/), is unavoidable: after their release into the environment, they can be transported through air, water and soil, and be a threat to both ecosystem and human health. Several of these chemical products are classified as endocrine disruptors (EDs), since they can interfere with the endocrine system, modifying the synthesis, circulating levels, and peripheral action of hormones [1].

Among the CECs is the well-known bisphenol A [2,2 bis(4-hydroxyphenyl) propane; BPA], an ubiquitous, high-volume-production (>$2.5x10^6$ kg $year^{-1}$) monomer used in the manufacture of polycarbonate plastics [1]. BPA has been shown to leach out of products such as plastic containers, utensils, toys, water bottles and fax paper, and high levels of monomer have been identified in human and animal samples [2]. In humans, higher urinary concentrations of BPA are associated with an increased occurrence of cardiovascular disease, diabetes, and liver enzyme abnormalities [3] and a large body of evidence links BPA to adverse health effects in perinatal, childhood and adult [4]. Due to its ability to bind to estrogen receptors and promote both agonist and antagonist activity, BPA is classified as an ED in the form of a xenoestrogen and has the potential to mimic estrogen activity through the body. BPA binds to aryl hydrocarbon receptors and has diverse endocrine effects on mammalian and non-mammalian health [5].

Another CEC is the perfluorooctanoic acid (PFOA), one of the most important synthetic perfluorinated compounds (PFCs). The chemical structure of PFCs gives them unique properties, such as thermal stability and the ability to repel both water and oil, that make

them useful components in a wide variety of consumer and industrial products including paper, leather and fire-fighting foam. Due to its persistence and bioaccumulation PFOA has been listed as emerging persistent organic pollutant in 2009 Stockholm Convention. High production volumes led to widespread distribution in the environment, and once absorbed PFOA does not undergo biotrasformation, distributing primarily in the liver and plasma, and to a lesser extent in the kidneys and lungs [6]. In several species, PFOA has been found to exert acute and sub-chronic toxic effects with the liver as a primary target organ. Several recent studies detected PFOA in a variety of wildlife animals including fresh water species, marine mammals and shellfish, and suggested that PFOA can be biomagnified at the top levels of the food chain [7]. PFOA can be considered an ED, since the exposure of rodents led to serious impact on phospholipid metabolism, reductions in serum cholesterol [8], hormonal perturbations with decreased testosterone levels and increased estradiol levels [9].

Connections between the health of the environment, animals, and humans are well established. Some CECs can bioaccumulate through the food chain, leading to higher levels of exposure in predator species. The bottlenose dolphin (*Tursiops truncatus*) shares the marine coastal environment with humans, consumes the same food and is exposed to the same contaminants. Due to their long lifespan, bottlenose dolphins could be chronically exposed to CECs and as top-level predators concentrate the contaminants in their body, being also endowed with large blubber stores that can serve as depots for anthropogenic chemicals and toxins. In addition, bottlenose dolphin populations display high site fidelity for coastal locations thus have the potential to be sentinel species for the marine environment reflecting the emerging contamination in that areas [10-14]

Here we report about the effects of BPA and PFOA on bottlenose dolphin skin biopsies studied by transcriptomic analysis. This tool allows the response to treatment of thousands of genes to be examined simultaneously, providing a large amount of information on the

metabolic pathways that are being activated. Understanding the relationship between genes in the cellular context under controlled conditions would allow to identify among the genes differentially expressed those that could be potentially used as biomarkers of exposure. If successful, this work could be informative not only of the impact of CECs on dolphins, and marine mammals in general, but also on the threats posed to the marine ecosystem and have a practical outcome due to the development of real-time tools for rapid detection of contaminant exposure.

METHODS

Dolphin samples

Bottlenose dolphin sampling was conducted on a juvenile female (length 144 cm, weight 31.6 Kg) stranded on Tyrrhenian shores (Italy) in October 2011, after the death (CITES permit: Nat. IT025IS, Int. CITES IT 007 issued to Accademia dei Fisiocritici and University of Siena). Skin samples were obtained 4 hours after death. No relevant pathology, parasites and lesions were detected at *post mortem* examination and the cause of death is unknown.

Ex vivo assay

Slices (about 2 mm-thick) spanning the epidermis and dermis were cut from skin samples of the stranded specimen immediately after collection, to set up the organotypic cultures exposure experiments in 5 ml culture tubes, as previously described [15]. Distinct slices were separately incubated for 24 h at room temperature (ranging from 24 to 28 °C) in cell culture media [16] containing BPA (0.1 µg/ml or 1 µg/ml), or PFOA (0.1 µg/ml or 1 µg/ml), or the vehicle (final concentrations: 0.01% ethanol for BPA and 0.1% methanol for PFOA) in a final volume of 3 ml. Thereafter, they were homogenized using a Tissue Lyser (Qiagen) and RNA was extracted using the Aurum TM Total Fatty and Fibrous Tissue kit (Bio-Rad) following the manufacturer's instructions.

Dolphin microarray hybridization and gene expression analysis

The microarray used was a species-specific, custom 4X44K Agilent oligo array representing 24,418 unigene sequences [17].

All RNA labeling and microarray hybridizations were performed according to the manufacturer's instructions in the One-Color Microarray-Based Gene Expression Analysis manual (Agilent Technologies, Santa Clara, CA). Five hundred nanograms of RNAs from

each treated slice were hybridized on dolphin 4X44K Agilent oligoarray. One-color gene expression was performed according to the manufacturer's procedure. Briefly, total RNA fraction was obtained from samples by using the RNeasy kit (Qiagen). RNA quality was assessed by the use of Agilent 2100 Bioanalyzer (Agilent Technologies). Low quality RNAs (RNA integrity number below 6) were excluded from microarray analyses. Labeled cRNA was synthesized from 200 ng of total RNA using the Low Imput Quick-Amp Labeling Kit, one color (Agilent Technologies) in the presence of cyanine 3-CTP. Hybridizations were performed at 65 °C for 17 hours in a rotating oven. Images at 5 um resolution were generated by Agilent scanner and the Feature Extraction 10.7.3.1 software (Agilent Technologies) was used to obtain the microarray raw-data. The microarray build and hybridization data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO accession numbers GSM1712791- GSM1712796 (www.ncbi.nlm.nih.gov/geo/).

Microarray results were analyzed using GeneSpring GX v.12 software (Agilent Technologies). Data transformation was applied to set all the negative raw values at 1. Data were normalized using quantile normalization. A filter on low gene expression was used to keep only the probes expressed in at least one sample (flagged as detected). Then, samples were grouped in accordance to their treatment status. BPA- and PFOA-treated samples were analyzed compared with samples incubated in cell media containing the specific vehicles. Differentially expressed genes were selected as having a 1.5-fold expression difference (geometrical mean) between the groups of interest and a statistically significant p-value (<0.05) at moderated t-test statistic, followed by the application of the Benjamini and Hoechberg correction for false positives reduction. Differentially expressed genes were employed for cluster analysis of samples using the Manhattan correlation as a measure of similarity.

Quantitative real time PCR (qPCR): validation of microarray data

Results from the microarray analysis were validated by qPCR by measuring mRNA expression of four selected genes in all the treated samples. *GADPH* (glyceraldehyde 3-phosphate dehydrogenase) and *YWHAZ* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta) genes were used as internal controls.

Relative levels of mRNA were determined using qPCR on a CFX Connect (BioRad, Hercules, CA, USA) with gene-specific primers designed using *Tursiops truncatus* sequences on the arrays and in the NCBI public database. Each primer set was optimized for efficiency and specificity by running standard curves on cDNA resulting in a correlation coefficient R^2>96.9% and efficiencies between 90%-110%. Quantitative qPCR efficiencies were calculated using the equation m=(1/logE), where m is the slope of the line and E is the efficiency [18] (Table S1).

One microgram of total RNA for each treated samples was reverse transcribed using iScript Select cDNA Synthesis Kit (BioRad, Hercules, CA, USA) according to manufacturer's instructions.

Optimized qPCR parameters for each genes were determined using diluted (1:10) cDNA reverse transcribed from 1 µl of total RNA using SsoFast™ EvaGreen® Supermix (BioRad, Hercules, CA, USA) in a total volume of 10 µl of a reaction mix containing 1 µl cDNA, 0.6 µl of each primer (300 µM), 5 µl Evagreen enzyme and 3.4 µl DNase-free sterile water.

qPCR reactions were run as following: 1 cycle of 98 °C for 30 minutes, 49 cycles of 95 °C for 5 minutes, 60 °C for 10 minutes; melting curve 65 °C to 95 °C: increment 0.5 °C every 5 minutes. Each reaction was run in triplicate, together with a triplicate of no-template control. The average Ct values were normalized to the corresponding measured mRNA Ct value of the housekeeping genes *GADPH* and *YWHAZ*. Comparative Ct method of

analysis ($2^{-\Delta\Delta ct}$) was used to determined changes of expression between control and treated samples.

RESULTS AND DISCUSSION

1. Gene expression profiles of BPA-treated skin biopsies

BPA treatment of organotypic cultures differentially regulated (fold ≥1.5 and p value ≤0.05) 1146 genes, of which 449 were up regulated and 697 down regulated (Figure 1, Table S1). Based on Gene Ontology (GO) analyses, the differentially expressed genes annotated are involved in metabolic and cellular process, immune response, developmental process, cell localization, cell signaling (Supplementary Figure 1).

In the 'response to stress' GO category we found genes like the *programmed cell death protein 4* (down, 1.53 fold, f), a tumor suppressor that plays a role in apoptosis and has a role inhibiting neoplastic transformation. Alterations in the concentration of the cellular product of this gene can lead to tumor development and malignant progression, as well as to deregulation of cellular processes such proliferation, differentiation and DNA repair [19-21]. The activation of the genes involved in this GO category after BPA exposure can be linked to a generalized response of the cells to an emergency condition where the cellular pathways involved in the malfunctioning of proteins are interrupted by a deregulation of key genes, and defected cells keep dividing, avoiding physiological apoptosis.

Because of its estrogenic activity, BPA can impact immune signaling pathways in many ways, stimulating T- and B-cell activation, TNF-α production by macrophages of, denditric cells and chronic activation of antigen-presenting cells. We found many down regulated genes in the 'immune system process' category: *complement factor H* (down, 1.6 f), involved in negative regulation of the inflammatory response, *class I histocompatibility alpha chain* (down, 2.1 f), involved in the presentation of foreign antigens, *alpha 2 macroglobulin precursor (*down, 2.4 f*)*, able to inactivate an enormous variety of proteinases, functioning as an inhibitor of fibrinolysis and coagulation, *melanoma inhibitory activity protein 3* (down, 1.7 f), a protein with growth factor activity involved in skin melanoma, *pleckstrin* (down, 5.9 f), a modular platelet protein. Some immune genes were

also up regulated, such as *B-cell receptor-associated protein 31* (up, 2.1 f), involved in antigen processing, and *lymphocyte antigen 96* (up, 1.5 f), cooperating with TLR4 in the innate immune response to bacterial lipopolysaccharide.

BPA exposure, even in low doses, can result in adverse effects both pre- and post-birth. Estrogens have a key role in early development and prenatal exposure of BPA can affect estrogen-responsive tissues. A study showed that maternal behavior can be altered, in part affecting the central nervous system, in particular the neuroendocrine-gonadal axis, which regulates the neuronal development organization [22]. According to GO, we found changes in the transcription of genes involved in embryonic development and growth, like *titin* (down, 5.57 f), a key component in the assembly and functioning of both adult and embryonic striated muscles and muscle tendons, *nuclear distribution protein nude-like 1* (up, 1.64 f), involved in organization of the cellular microtubule array and microtubule anchoring at the centrosome, and in brain development for the migration of newly formed neurons which plays a role in the regulation of neurite outgrowth [23].

Moreover, it is well known that estrogenic effects of BPA can impact adipogenesis and body weight, through complex interactions with the estrogen, thyroid hormone and glucocorticoid receptors, causing metabolic disturbances [1, 24]. We found that BPA may affect pathways involved in fat (blubber) differentiation, down regulating several genes involved in lipid metabolism: *adipogenesis regulatory factor* (down, 1.80 f), which promotes adipogenic differentiation and plays a role in fat cell development, *fatty aldehyde dehydrogenase isoform 2 (*down, 1.62 f), responsible for the catalysis of the oxidation of long-chain aliphatic aldehydes to fatty acids, *apolipoprotein B 100 (*down, 2.39 f), whose function is to recognize the signal for the cellular binding and internalization of LDL particles. Alterations in apolipoprotein B 100 can lead to disorder in lipoprotein metabolism and hypercholesterolemia, and increased proneness to coronary artery disease [25].

2. Gene expression profiles of skin biopsies treated with PFOA

PFOA treatment of organotypic cultures differentially regulated (fold ≥1.5 and p value ≤0.05) transcription of 428 genes in total, of which 191 were up regulated and 237 were down regulated (Figure 2, Table S2). GO analysis of the annotated genes collocated most of them in the following categories: cellular and metabolic processes, single organism process, biological regulation, response to stress, immune response, cellular component organization or biogenesis, localization, multicellular organismal process, developmental process.

The genes up regulated involved in response to stress were: *uv excision repair protein rad23 homolog a* (up, 1.59 f), which plays a central role both in proteasomal degradation of misfolded proteins and DNA repair, and *heat shock protein 90* (up, 1.8 f), a molecular chaperone that promotes the maturation, structural maintenance and proper regulation of specific target proteins involved in cell cycle control and signal transduction. Differently, we found in the same category a down regulated gene: *complement c5 (*down, 5.79 f), a mediator of local inflammatory process. In humans, high levels of PFOA have been associated with significant changes in clinical markers of immune and inflammatory process, and exposure to PFOA can adversely affect immune function [26]. Besides *complement c5*, that can be included also in the inflammatory response GO category, we found variations (down/up) in transcripts of genes engaged in immune system activation, like *acidic mammalian chitinase (*down, 5.23 f), involved in the defense against nematodes, fungi and other pathogens, or *interferon-induced protein* (up, 1.86 f) and *interleukin 8* (up, 6.80 f), which is a chemotactic factor for neutrophils, basophils and T-cells and it is also involved in neutrophil activation.

It is worth noting how PFOA treatment evoked transcription changes of a panel of genes different from that effected by BPA. The different chemical structures and mechanisms of action of the two compounds can likely account for this findings.

Some studies in adult rats suggest that PFOA interferes with a normal lipid metabolism, affecting serum concentrations of sex hormones (decreasing testosterone and increasing estradiol) and lipids (reducing cholesterol and/or triglycerides) [9, 27]. We have found variations of transcripts of genes regulating lipid metabolic process, like *choline phosphotransferase 1 (*down, 1.50 f*)*, which is involved in phospholipid metabolism, or *trifunctional enzyme subunit beta mitochondrial like (*up, 1.58 f) which is involved in lipid metabolism and fatty acid beta oxidation. Deficiency of this latter gene can lead to hypoglycemia and cardiomyopathy [28].

Furthermore, exposure to PFOA during pregnancy leads to developmental toxicity, reduced birth weight and neonatal mortality in mice [29-31]. This is consistent with our findings, since we report about up regulation of genes involved in embryonic development, such as *vascular endothelial growth factor* (up, 1.54 f), and *Rho GTPase-activating protein 35 (*up, 1.62 f), which may participate in the regulation of retinal development, and *non-lysomal glucosylceramidase* (up, 2.19 f) which plays a role in central nervous system development [32] (Supplementary Figure 2).

3. Quantitative real time PCR

Semi-quantitative gene expression analysis resulting from the microarray study was validated through qPCR, a more specifically quantitative method for the analysis of expressed genes. Four genes among those differentially expressed were chosen for the analysis testing the same RNA used in microarray analysis (Table 1). Two genes were selected from the BPA exposure study: 1) *ADIRF* (adipogenesis regulatory factor), that plays a role in fat cell development and promotes adipogenic differentiation, 2) *BCAP31* (B-cell receptor-associated protein 31), involved in the anterograde transport of membrane proteins from the endoplasmic reticulum to the Golgi and in caspase 8-mediated apoptosis (Figure 3). The two genes selected from the PFOA exposure study were both up

regulated: 1) *RGS2* (regulator of G-protein signaling 2), encoding a protein that binds to heterotrimeric G proteins by way of its RGS domain and acts as GAP (GTPase-activating protein) to turn off G protein-coupled receptor (GPCR) signals, and 2) *CDC42* (cell division cycle 42), involved in regulation of the cell cycle.

qPCR confirmed microarray data for the selected genes at a contaminant concentration (1 ug/ml) already measured in the environment (http://www.epa.gov/oppt/existingchemicals/pubs/actionplans/bpa.html). At this dose, the contaminant is probably toxic and poisoning, hence causing a strong cellular response.

BPA is a selective estrogen receptor modulator that shows a different binding affinity for/and regulation of ERα and ERβ in target cells and can interact differently with transcriptional co-regulators. Furthermore, there is evidence that BPA can elicit rapid cell responses via non-genomic signaling, like estradiol does. Very low concentration of BPA and estradiol can activate cell-signaling pathways via plasma membrane receptors and this very rapid response can produce additive effects to that mediated by nuclear receptors, which takes longer to occur and at higher concentration of BPA [33]. In rat hippocampus, instead, BPA has been shown to antagonize the action of estradiol by locking its stimulatory effect [34]. In another study, BPA seems to disrupt the actions of estradiol acting as potent estradiol mimetic, during cerebellar development [35]. Considering the complex spectrum of mechanisms potentially exerted by BPA, our findings are clearly suggestive of an overall effect of the contaminant, but the underlying mechanisms obviously require more detailed analysis.

PFOA is not metabolized in the body and, unlike BPA, is not lipophylic but binds to serum albumin and is excreted primarily from the kidney. PFOA induces various types of tumors, reduces birth weight in mice and causes neonatal death in rats [36]. In mice it decreases the B-cell and T-cell immune responses and impairs thyroid hormone homeostasis [36, 37]. The observed effects at the concentration tested agreed with previously published data. Unlike

most other well-studied water contaminants, PFOA effects can be observed at low exposure levels in humans. Some effects can be recorded in both humans and animals while others such as on lipid metabolism, can not [38]. In fact, besides the activation of the immune and stress responses we have observed the differential regulation of a suite of genes involved in such pathways. Many studies showed positive correlation between serum level of PFOA and other lipids (i.e., LDL cholesterol and triglycerides) but the underlying mechanisms are not yet clarified. Taken together, our findings suggest that PFOA has an impact on the skin tissue treated, which was not described before and deserves further investigation.

4. Technology potential for cetacean health studies

The implication of the long-term effects of chemical pollution on the marine fauna, and on cetaceans in particular, is mostly unknown. This work describes the successful employment of two novel approaches: 1) the use of the dolphin skin biopsy, an *ex vivo* assay, coupled with a transcriptomic analysis, using a species-specific custom made microarray, and 2) the assessment of the effects of two non-canonic but environmentally threatening contaminants on wild dolphin health. We know from previously published data that exposure to chemical contaminants in the long-term does cause immunosuppression and health problems to marine mammal populations, so the hypothesis to be tested was the activation of contaminant-specific gene signature. The molecular pathways activated by BPA and PFOA had a profile consistent with the cellular response to EDs exposure. Genes involved in the activation of the immune system and response to stress are differentially expressed and mechanisms linked to lipid homeostasis and development seem to be activated. Nevertheless we found a broad spectrum of differentially expressed genes contaminant specific. BPA activates a stress response and many genes affected are also involved in development, which may be inferred to the estrogenic activity of the

chemical compound. PFOA exposure triggers mainly genes related to the immune response, the lipid metabolism and homeostasis, probably depending on the chemical nature of the compound and its mechanism of action.

We identified some potential biomarkers that will have to be tested on wild animals in order to establish a reliable diagnostic tool. Skin samples from marine mammals inhabiting areas with different level of contamination are currently being collected to test which, between the genes differentially expressed upon treatment, would be fully representative of BPA and PFOA exposure.

The skin biopsy is a sample with many potential applications. It can be used as a reference for diagnosis of stress in cetaceans, or for the detection of the nature of contamination by the quantification of old and emerging contaminants. Target genes selected as biomarkers can be a useful tool to quickly assess exposure and health of free-ranging mammals as well as causes of stranding, and ultimately result in development of a Patented kit. The skin is a excellent tissue to analyze because it forms an effective barrier between the organism and the environment and at the same time offers many advantages: easy to collect from stranded animals but also the easier tissue to collect (dart biopsies) during wild marine mammals sampling, in a rather non-invasive way. Moreover, the skin is a tissue with potential biomedical applications. Regenerative power of dolphin skin is well documented and the possibility to have a skin biopsy system associated with a transcriptomic tool may allow specific gene networks and pathways to be identified, with potential for therapeutic aids of wound healing in humans as well. A better understanding of dolphin's wound healing mechanisms may lead to the identification of new antimicrobial, analgesic and tissue-regenerating mechanisms, that may apply to patent new drugs, and establish new therapies for debilitating and life-threatening diseases.

Acknowledgements

The Authors wish to thank Manuela Ferracin (University of Ferrara) for her helpful advises about microarray data analysis, Matteo Baini, Daniele Coppola and Silvia Maltese (University of Siena) who greatly contributed to the tissue sample collection, and “Museo Zoologico dell’Accademia dei Fisiocritici, Onlus” for providing the facilities and staff for necroscopy.

FIGURE LEGENDS

Figure 1. **Hierarchical clustering of microarray data showing the differentially expressed genes of dolphin skin organotypic cultures treated with different concentrations of BPA (0.1 and 1 µg/ml).**

The dendrogram on the left depicts the 1146 genes differentially expressed (fold change ≥1.5 and p-value ≤0.05). Red: up-regulated genes, green: down-regulated genes.

Figure 2. **Hierarchical clustering of microarray data showing the differentially expressed genes in dolphin skin organotypic cultures treated with different concentrations of PFOA (0.1 and 1 µg/ml).**

The dendrogram on the left depicts the 428 genes differentially expressed (fold change ≥1.5 and p-value ≤0.05). Red: up-regulated genes, green: down-regulated genes.

Figure 3. **Real time PCR data for 4 selected genes on treated organotypic cultures.**

RNA from 2 treated samples (1 µg/ml) was individually reverse transcribed, and each gene was amplified in triplicate from the cDNA. Data were normalized to *GAPDH* and *YWHAZ* genes by ΔΔct method. From left to right: *ADIRF*, *BCAP31*, *RGS2*, *CDC42*. Bottom: green and red brackets, genes up- and down-regulated in microarray analysis.

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Highlights

- Effects of contaminants of emerging concern (CECs) in the marine environment
- Analysis of the impact of BPA and PFOA on marine organisms
- Ex vivo assay using skin biopsies combined to global gene expression analysis
- Transcriptomic signature of the bottlenose dolphin skin to reveal important information on CECs exposure

Table 1. List of genes used in real time PCR, differentially expressed in microarray analysis.

Gene name	Conting number	Treatment	Regulation	Fold change (Microarray)
adipogenesis regulatory factor	Contig-c2315-3	BPA	down	1,7
B-cell receptor-associated protein 31	Contig-c9258-1	BPA	up	2,13
regulator of G-protein signaling 2	Contig-c23388-1	PFOA	up	3,2
cell division cycle 42	SRR027946.159665	PFOA	up	4,5

Figure 1

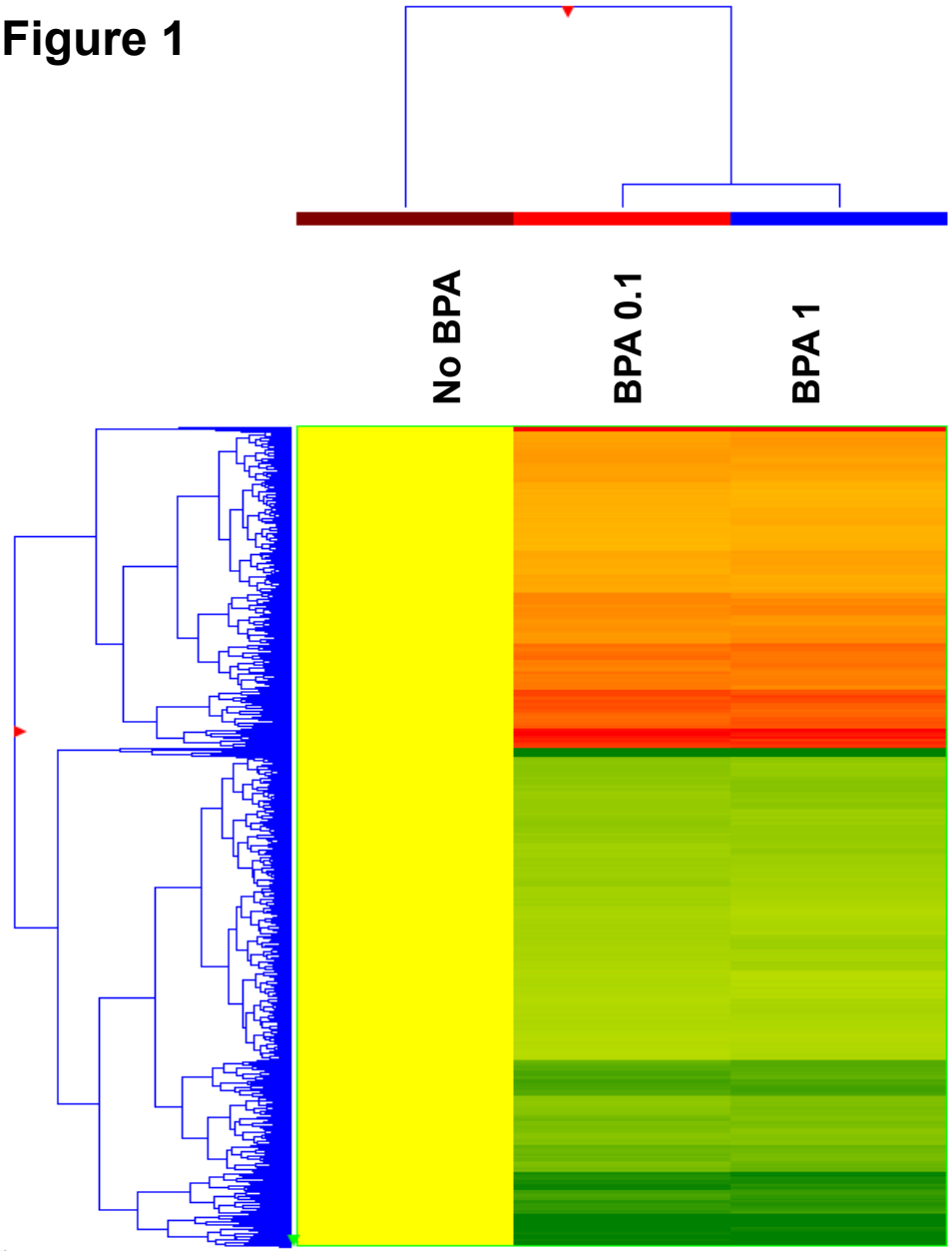


Figure 2

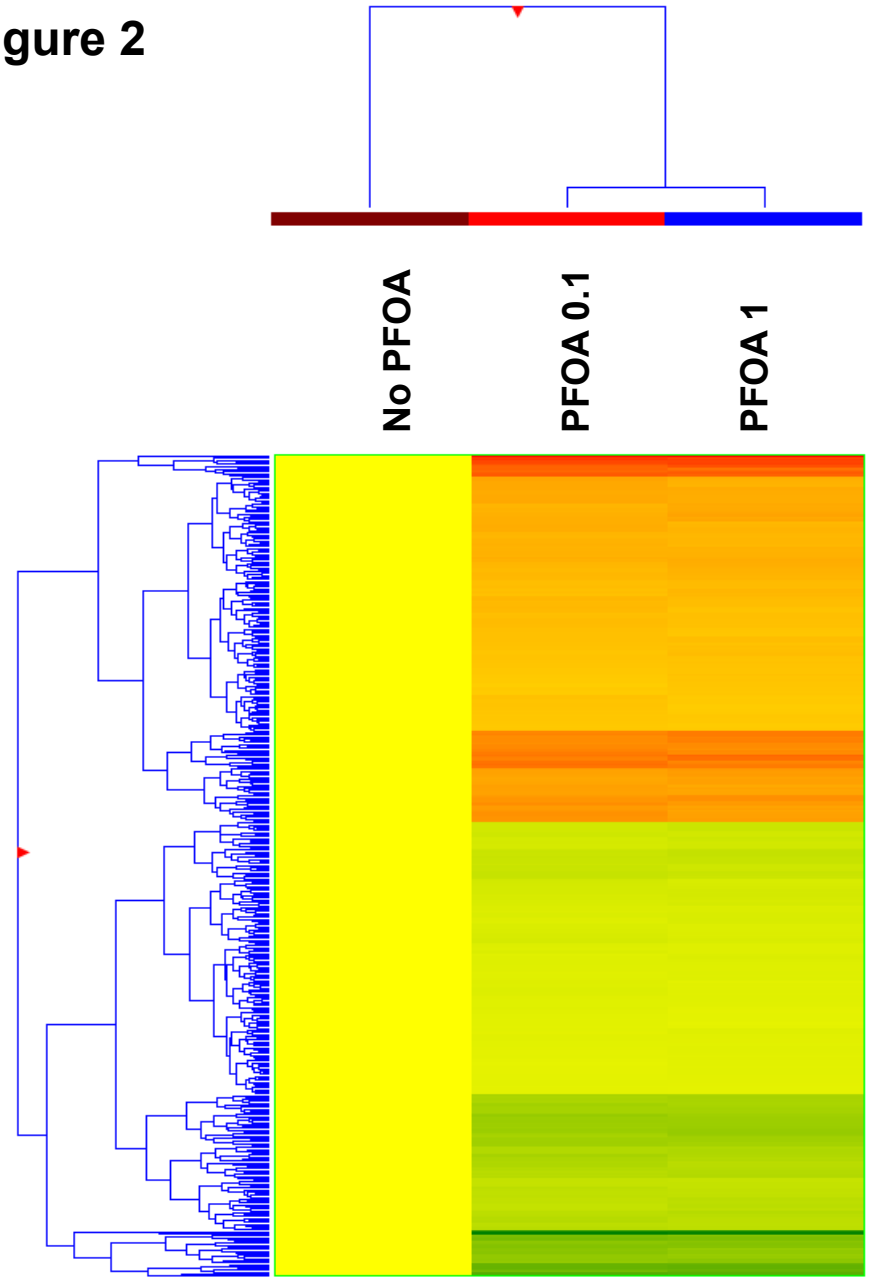
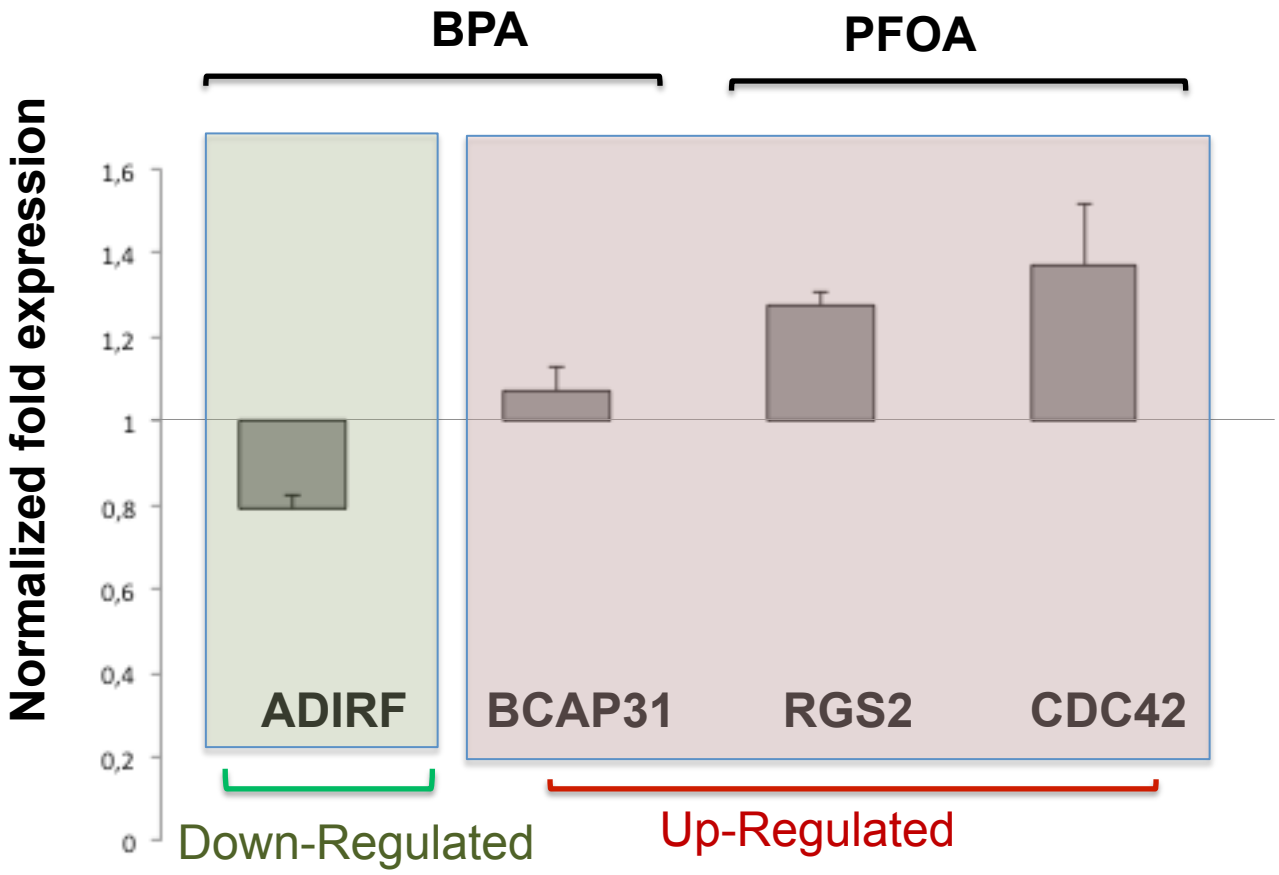


Figure 3


BPA
PFOA
Normalized fold expression
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
ADIRF
BCAP31
RGS2
CDC42
Down-Regulated
Up-Regulated

Supplementary files

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