Food and Chemical Toxicology 73 (2014) 157–174

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Toxicological effect of single contaminants and contaminant mixtures associated with plant ingredients in novel salmon feeds

Liv Søfteland [a,*], Jennifer A. Kirwan [b], Tiago S.F. Hori [c], Trond R. Størseth [d], Ulf Sommer [b], Marc H.G. Berntssen [a], Mark R. Viant [b], Matthew L. Rise [c], Rune Waagbø [a], Bente E. Torstensen [a], Marije Booman [c], Pål A. Olsvik [a]

[a] National Institute of Nutrition and Seafood Research, Norway
[b] School of Biosciences, University of Birmingham, UK
[c] Department of Ocean Sciences, Memorial University of Newfoundland, Canada
[d] SINTEF Fisheries and Aquaculture, Norway

ARTICLE INFO

Article history:
Received 24 February 2014
Accepted 17 August 2014
Available online 2 September 2014

Keywords:
Atlantic salmon
PAH
Pesticides
Metabolomics
Synergy
Toxicogenomics

ABSTRACT

Increasing use of plant feed ingredients may introduce contaminants not previously associated with farming of salmonids, such as pesticides and PAHs from environmental sources or from thermal processing of oil seeds. To screen for interaction effects of contaminants newly introduced in salmon feeds, Atlantic salmon primary hepatocytes were used. The xCELLigence cytotoxicity system was used to select optimal dosages of the PAHs benzo(a)pyrene and phenanthrene, the pesticides chlorpyrifos and endosulfan, and combinations of these. NMR and MS metabolic profiling and microarray transcriptomic profiling was used to identify novel biomarkers. Lipidomic and transcriptomic profiling suggested perturbation of lipid metabolism, as well as endocrine disruption. The pesticides gave the strongest responses, despite having less effect on cell viability than the PAHs. Only weak molecular responses were detected in PAH-exposed hepatocytes. Chlorpyrifos suppressed the synthesis of unsaturated fatty acids. Endosulfan affected steroid hormone synthesis, while benzo(a)pyrene disturbed vitamin D3 metabolism. The primary mixture effect was additive, although at high concentrations the pesticides acted in a synergistic fashion to decrease cell viability and down-regulate CYP3A and FABP4 transcription. This work highlights the usefulness of 'omics techniques and multivariate data analysis to investigate interactions within mixtures of contaminants with different modes of action.

1. Introduction

Marine fish oils was traditionally the main source of the persistent organic environmental pollutants (POPs) in salmon feed and farmed Atlantic salmon (*Salmo salar* L.) (Berntssen et al., 2010). Replacing marine ingredients with plant ingredients has reduced the levels of these traditional POPs in salmon feeds, but as a consequence introduced a new cocktail of plant-oil derived contaminants, such as polycyclic aromatic hydrocarbons (PAHs) and pesticides, that have not previously been associated with farming

Abbreviations: ARP, acidic ribosomal protein; ANOVA, analysis of variance; AhR, aryl hydrocarbon receptor; CI, cell index; CT, crossing point; CYP1A, cytochrome P450 1A; DMSO, dimethyl sulfoxide; EF1AB, elongation factor 1 AB; sEH, epoxide hydrolase; ER, estrogen receptor; EROD, ethoxyresorufin O-deethylase activity; FABP4, fatty acid binding protein 4; FS, fish serum; FC, fold change; GH, Games–Howell; cGRASP, Genomic Research in All Salmonids Project; R^2, goodness of fit; Q^2, goodness of prediction; KEGG, Kyoto Encyclopedia of Genes and Genomes; MNE, mean normalized expression; MAP1LC3B, microtubule-associated proteins 1A/1B light chain 3B precursor; nac, no-amplification control; NCI, normalized cell index; ntc, no-template control; NMR, nuclear magnetic resonance spectroscopy; PLS-DA, Partial Least Squares Discriminant Analysis; PFP, percentage of false positives; PPARγ, peroxisome proliferator activated receptor γ; PPARα, peroxisome proliferator-activated receptor α; POPs, persistent organic environmental pollutants; PMTs, photomultiplier tube settings; PAHs, polycyclic aromatic hydrocarbons; PCA, Principal Component Analysis; QC, quality control; real-time qPCR, quantitative real-time RT-PCR; RTgill-W1, rainbow trout gill cells; RTG-2, rainbow trout gonadal; RTL-W1, rainbow trout liver; RT, reverse transcription; RT-PCR, reverse transcription polymerase chain reaction; SIM, selected ion monitoring; THF-diols, tetrahydrofurandiols; SOX4, transcription factor SOX-4; TAG, triacylglycerols; VTG, vitellogenin.

* Corresponding author.
E-mail address: lso@nifes.no (L. Søfteland).

http://dx.doi.org/10.1016/j.fct.2014.08.008
0278-6915/

of salmonids (Berntssen et al., 2010; Glover et al., 2007). The introduction of these contaminants to salmon feeds has led to concerns about potential effects on fish health, including interactions with nutritional pathways. Plant oils may be contaminated with PAH during the thermal processing of the oil seeds or indirectly from environmental sources, such as exhaust gases from traffic or from atmospheric particles deposited on the crops during growth (Fromberg et al., 2007; SCF, 2002). The combustion process of oil seeds cause a predominant increase in 2–3 ring PAHs (e.g. phenanthrene), while 4–5 ring PAHs (e.g. benzo(a)pyrene) are present to a lesser extent in the plant crude oils (Teixeira et al., 2007; Dennis et al., 1991). In a feeding trial, a 36% increase of phenanthrene was detected in the fillets of Atlantic salmon fed alternative plant feed (3.2 μg kg^{-1} or 0.018 μM) compared to fish fed traditional marine fish feed (2.4 μg kg^{-1} or 0.013 μM). In addition, the levels of benzo(a)pyrene increased from not being detected in the traditional Atlantic salmon fillets to low concentrations being detected (0.3 μg kg^{-1} or 0.0012 μM) in the plant fed fish fillets (Berntssen et al., 2010). The acute toxicity of PAH in exposed rainbow trout (*Oncorhynchus mykiss*) and largemouth bass (*Micropterus salmonides*) is known to increase with increasing number of aromatic rings (Black et al., 1983). Phenanthrene is therefore considered to have a relatively low toxicity. Phenanthrene is a non-cytochrome P450 1A (CYP1A)-inducing PAH, with aryl hydrocarbon receptor (AhR) independent toxicity (Pathiratne and Hemachandra, 2010; Johnson et al., 2008), while the 4–5 ring PAHs have an AhR dependent mode of action. The main toxicological effects of PAHs, however, are in their genotoxicity and potential endocrine disruption in teleosts (Donnelly and Naufal, 2010; van der Oost et al., 2003; Johnson et al., 2008). Suppressed steroid levels and steroid synthesis inhibition (Monteiro et al., 2000; Seruto et al., 2005;Yan et al., 2012) have been detected in PAH-exposed teleosts as well as retinoid signalling disruption (Benisek et al., 2011).

Endosulfan and chlorpyrifos are pesticides used on crops, and residue levels have been reported in products from plants such as soya or maize (Jergentz et al., 2005; Marchis et al., 2012) that are commonly used as ingredients in salmon feeds (Berntssen et al., 2007). In 2011, the concentration range measured in farmed Atlantic salmon were 0.2–5.8 μg/kg (0.0005–0.014 μM) of α-endosulfan and 0.2–1.2 μg/kg (0.0005–0.003 μM) of β-endosulfan (NIFES, 2014) while chlorpyrifos-methyl has recently been detected in salmon feed (Nácher-Mestre et al., 2014). These pesticides act as endocrine disruptors (Krøvel et al., 2010; Grünfeld and Bonefeld-Jorgensen, 2004). Disturbed steroid production and steroid biosynthesis (De Angelis et al., 2009; Viswanath et al., 2010) as well as histopathological changes have been reported in a variety of fish species exposed to chlorpyrifos (Deb and Das, 2012). Adverse effects like liver metabolic perturbations (Ashad et al., 2007; Glover et al., 2007; Krøvel et al., 2010) and disturbed lipid metabolism such as steatosis have been detected in endosulfan exposed Atlantic salmon *in vitro* and *in vivo* (Krøvel et al., 2010; Glover et al., 2007). Elevated ethoxyresorufin O-deethylase activity (EROD) has been observed in endosulfan exposed Atlantic salmon *in vivo* (Glover et al., 2007).*In vitro* models are useful supplements to animal models for the evaluation of underlying mechanisms of drugs and contaminants, and for interaction studies (Bouhifd et al., 2012; Xia et al., 2008; Judson et al., 2010; Walum et al., 2005; Søfteland et al., 2011). To ensure optimal non-cytotoxic exposure concentrations for *in vitro* assessments, cell viability and dose–response curves of well-known transcriptional markers are often evaluated (ISO, 2009; Judson et al., 2010; Søfteland et al., 2011). The xCELLigence system use impedance-based, continuous real-time assessment of cytotoxicity and mode of action, and is especially suitable to determine when, and at which concentration, to collect cells for downstream analyses (Xia et al., 2008; Atienzar et al., 2011; Judson et al., 2010;Walum et al., 2005). The xCELLigence system has an equal, or even higher, cytotoxicity sensitivity than the standardised methods certified by ISO (Atienzar et al., 2011; Ceriotti et al., 2007) and has been used in large-scale screening of toxicants (Judson et al., 2010; Xia et al., 2008). In feed safety evaluations, a contaminant-by-contaminant approach has traditionally been applied in the risk assessment. This approach may however be inappropriate in animals exposed to a cocktail of contaminants (Bandele et al., 2012; Kortenkamp and Altenburger, 2011). A toxicological effect of a mixture can be greater (synergistic interaction) or lesser (antagonistic interaction) than expected, and these outcomes are often difficult to predict. This is especially true when mixtures are composed of contaminants with differing modes of action and knowledge regarding such contaminant mixtures effects is in general lacking (Kortenkamp and Altenburger, 2011).

To gain toxicological knowledge about contaminants found in elevated levels in novel plant-based salmon feeds, the aim of this *in vitro* study was to screen for interaction effects using metabolomic, lipidomic and transcriptomic profiling. To ensure we used non-cytotoxic exposure concentrations, and to find the most potent mixture concentrations, the xCELLigence system was applied for cytotoxicity assessment. RT-qPCR gene expression analysis of well-known and new biomarkers were used for contaminant dose–response determination and interaction evaluation. Atlantic salmon primary hepatocytes were selected as an experimental model.

2. Materials and methods

2.1. Chemicals

Endosulfan (6,7,8,9,10-hexachloro-1,5,5a,6,9,9,a-hexahydro-6,9-metano-2,4,3-benza-dioxathiepin-3-oxide, $\alpha + \beta \sim 2 + 1$; PESTANAL®, analytical standard), chlorpyrifos (O,O-diethyl-O-3,5,6-trichlor-2-pyridyl phosphorothioate, PESTANAL®, analytical standard), phenanthrene (⩾98% pure) and benzo(*a*)pyrene (⩾96% pure) were all purchased from Sigma–Aldrich (Oslo, Norway). Dimethyl sulfoxide (DMSO) stock solution was purchased from Scientific and Chemical Supplies Ltd. (Bilston, UK), chloroform (HPLC grade) was purchased from Fisher Scientific (Loughborough, UK) and ammonium acetate was purchased from Sigma–Aldrich Co. Ltd. (Dorset, UK).

2.2. Isolation of primary cultures of hepatocytes

Juvenile Atlantic salmon (*Salmo salar* L.) were obtained and kept at the animal holding facility at the Institute of Marine Research, Bergen, Norway at Havbruksstasjonen, Matre. The fish were fed once daily with a special feed produced without addition of synthetic antioxidants and with low levels of contaminants, supplied by EWOS, Norway (Harmony Nature Transfer 75). Feed concentrations of chlorpyrifos, endosulfan, benzo(a)pyrene and phenanthrene were all under the level of quantification. All glassware, instruments and solutions were autoclaved prior to liver perfusion. Hepatocytes were isolated from 8 Atlantic salmon (325–515 g) with a two-step perfusion method previously described in Søfteland et al. (2009). The final cell pellet was resuspended in L-15 medium containing 10% fish serum (FS) from salmon (Nordic BioSite, Oslo, Norway), 1% glutamax (Invitrogen, Norway) and 1% penicillin–streptomycin–amphotericin (10,000 units/ml potassium penicillin, 10,000 μg/ml steptomycin sulfate and 25 μg/ml amphotericin B.) (Lonzo, Medprobe, Oslo, Norway). The Trypan Blue exclusion method, performed in accordance with the manufacturer's protocol (Lonzo), was used to determine cell viability. The different cell suspensions used in this study had cell viability between 83% and 94%. The cell suspensions were plated on 2 μg/cm^2 laminin (Sigma–Aldrich, Oslo, Norway) coated culture plates (TPP, Trasadingen, Switzerland), and the hepatocytes were kept at 10 °C in a sterile incubator without additional O_2/CO_2 (Sanyo, CFC FREE, Etten Leur, Netherland). The following cell concentrations were used; 7.2×10^6 cells per well in 6-well plates (in 3 ml complete L-15 medium), 2.6×10^6 cells per well in 12-well plates (in 2 ml complete L-15 medium), 0.2×10^6 cells per well in xCELLigence 96-well plates (in 0.2 ml complete L-15 medium).

2.3. Chemical exposure

The primary cells were cultured for 36–40 h prior to chemical exposure with a change of medium (containing 10% FS) after 18–20 h. The cells were exposed for 24 h to single contaminants, i.e. endosulfan, phenanthrene and benzo(a)pyrene (0.01, 0.1, 1, 10, 100 μM), chlorpyrifos (0.1, 1, 10, 100, 1000 μM) or to simple