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Toxicological assessment of the anti-salmon lice drug diflubenzuron on Atlantic cod *Gadus morhua*

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ABSTRACT: Increasing use of the chitin synthesis inhibitor diflubenzuron against the ectoparasitic salmon louse *Lepeophtheirus salmonis* in marine aquaculture has raised concerns over its environmental impacts. This study evaluated how diflubenzuron affects Atlantic cod *Gadus morhua*, a fish species often found near Atlantic salmon *Salmo salar* farms, focusing on uptake kinetics and hepatic transcriptional responses. Two experiments were conducted, one time-series trial in which the fish were given a daily dose (3 mg kg^{-1} fish) of diflubenzuron for 14 d followed by a 3 wk depuration period, and one dose–response trial with increasing concentrations (3, 10 and 50 mg kg^{-1} fish). The highest diflubenzuron concentrations were found in the liver at Day 15. No detectable levels of diflubenzuron were found in liver or muscle 3 wk after the end of the treatment. At the molecular level, small effects of diflubenzuron treatment on gene transcription were observed. In the time-series experiment, the strongest effects were seen at Day 8, with 2 transcripts being upregulated (*bclx2* and *cpt1a*) and 8 transcripts being downregulated (*gstp1*, *gstm1*, *gstt1*, *ugt1a*, *nat2*, *cat*, *p53* and *slc16a9a*). Five transcripts (*cyp3a*, *cpt1a*, *ptgs2*, *elovl5* and *mapk1*) responded significantly to diflubenzuron exposure in the dose–response experiment. This study shows that diflubenzuron can be taken up by Atlantic cod, that it is rapidly cleared from the body and that when present this pharmaceutical causes only small effects on the expression of genes involved in detoxification pathways. Taken together, our data suggest that accumulated diflubenzuron at the levels studied would have a relatively small effect on wild Atlantic cod.

KEY WORDS: Atlantic cod · Diflubenzuron · Sea lice drug · Transcriptional responses · Uptake kinetics

INTRODUCTION

The salmon louse *Lepeophtheirus salmonis* (Krøyer 1837) is a major problem in the marine Atlantic salmon *Salmo salar* industry in Europe and eastern North America (Pike & Wadsworth 1999, Costello 2006, Burka et al. 2012). Furthermore, several other sea lice genera in the family Caligidae have been shown to parasitize on farmed and wild marine fish (Burka et al. 2012). In addition to reducing the general welfare to the farmed fish, sea lice cause significant economic losses due to reduced growth, increased mortality, downgrading of fish quality and the cost of treatment (MacKinnon 1997). Sea lice derived from aquaculture sources may also have negative impacts on wild populations of sea trout and migrating wild post-smolts of Atlantic salmon (Wagner et al. 2008, Costello 2009).

In Atlantic salmon aquaculture, benzamide insecticides such as diflubenzuron (1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl)urea) and teflubenzuron have been used to control salmon lice infestation. The recommended dose for Atlantic salmon is 3 mg kg^{-1} fish for 14 consecutive days, applying feed-added 0.6 g diflubenzuron kg^{-1}. The use of diflubenzuron spiked in Norway in 2009, 2010 and 2012, after having been

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avoided for almost a decade, due to the risk of potential harmful effects on other aquatic organisms. The consumption of diflubenzuron measured as an active component in Norwegian aquaculture was 1413, 1893, 704 and 1611 kg in 2009, 2010, 2011 and 2012, respectively (FHI 2013). Benzamide insecticides act by inhibition of chitin synthesis, and chitin-synthesizing organisms are sensitive to these chemicals during molting (Branson et al. 2000, Ritchie et al. 2002, Campbell et al. 2006a, 2006b, Merzendorfer et al. 2012). A portion of the diflubenzuron and teflubenzuron that are administered to fish as well as their metabolites ends up in the environment, and release of such chemicals into the environment has raised considerable concern in recent years (Eisler 1992, Fischer & Hall 1992, Haya et al. 2005, Burridge et al. 2010). Oral administration of diflubenzuron and teflubenzuron to farmed salmon may become an environmental problem when uneaten feed and feces accumulate in the sediments beneath and around farming facilities. In the marine environment, these drugs are rather stable and mainly bound to organic compounds in the sediments and are found in a concentration-dependent gradient in sediments away from the fish farms, with the highest environmental levels observed during and immediately after treatment. Bottom-dwelling crustaceans are considered to be among the most vulnerable organisms to diflubenzuron and teflubenzuron contamination, especially those that are at the premolt stage. Fish inhabiting areas next to the farming facilities accustomed to forage on surplus feed pellets may also be exposed to and negatively affected by these compounds. Fish species such as saithe *Pollachius virens* and Atlantic cod *Gadus morhua* are often observed locally around salmon fish farms in Norway, taking advantage of the rich food supplies near the net pens (Ervik et al. 1994). In a recent unpublished study of 134 samples of wild fish harvested in the vicinity of a salmon farm conducting an anti-lice treatment with teflubenzuron, we found residues of this agent in 16 of the 19 fish species examined, with the highest single concentration of 1354 ng g^{-1} found in one sample of saithe. Of the examined samples, 10 were from Atlantic cod. Two of these samples had detectable concentrations, of which the highest was 34 ng g^{-1}.

For diflubenzuron, toxicity assessments exist for several animal species. According to the US EPA (1997), diflubenzuron is practically non-toxic to avian species, small mammals, freshwater fish and marine/estuarine fish on an acute oral dietary basis, while it is slightly toxic to avian species on a subacute dietary basis. Lethal concentration (LC_{50}) values (96 h) for diflubenzuron in various fish species are 660 mg l^{-1} for bluegill sunfish, 240 mg l^{-1} for rainbow trout, 255 mg l^{-1} for saltwater minnows and 180 mg l^{-1} for channel catfish. Fish tissue can show some traces of the metabolites after waterborne exposure of diflubenzuron; however, tissue concentrations decline steadily with time in clean water. For freshwater invertebrates and marine/estuarine crustaceans, diflubenzuron is considered to be very highly toxic. For marine/estuarine mollusks it is considered to be highly toxic. The literature indicates that diflubenzuron affects reproduction, growth and survival in freshwater invertebrates, as well as reproduction in marine/estuarine invertebrates (US EPA 1997). Diflubenzuron is considered to show very low toxicity in humans and has been recommended as an additive to potable water in concentrations of 0.25 mg l^{-1} to suppress the growth of insect vector larvae (WHO 2008). Animal studies (rats, mice, dogs and rabbits) suggest that the liver and spleen are the main target organs for toxicity (EMA 1998). Diflubenzuron has been approved for application in salmonids, and has been given an EU maximum residue limit (MRL) of 1000 µg kg^{-1} in fish products intended for human consumption (EMA 1998, European Commission 2010).

One of the possible metabolites of diflubenzuron is 4-chloroaniline (see Fig. 1). In rats given a very high dose of diflubenzuron (7.8 g kg^{-1} body weight d^{-1}), 4-chloroaniline could be detected in the urine, although in a concentration representing less than 0.01% of the absorbed dose (EMA 1998). This metabolite is considered to be mutagenic, and possibly a human carcinogen (Briggs 2008). According to our knowledge, 4-chloroaniline is not a significant metabolite in fish, even though a minor proportion of a single dose of ^{14}C-labeled diflubenzuron has been found as 4-chloroanilline in Atlantic salmon liver (Schaefer et al. 1979, 1980, EMA 1998). In rats and mice, the route of elimination of unabsorbed diflubenzuron is via feces and from the liver via bile to feces or urine after absorption. From a toxicological viewpoint, the liver is therefore considered to be one of the most important target organs of diflubenzuron toxicity.

The aim of this work was to study whether diflubenzuron exposure may pose a health threat to wild fish inhabiting areas near salmon aquaculture facilities. Uptake kinetics and effect biomarkers were examined in Atlantic cod, one of the wild fish species often found in large numbers next to the fish farms. The experimental fish were fed a standard nominal concentration of diflubenzuron for 2 wk. Tissue concentrations were examined in the liver during 14 d of

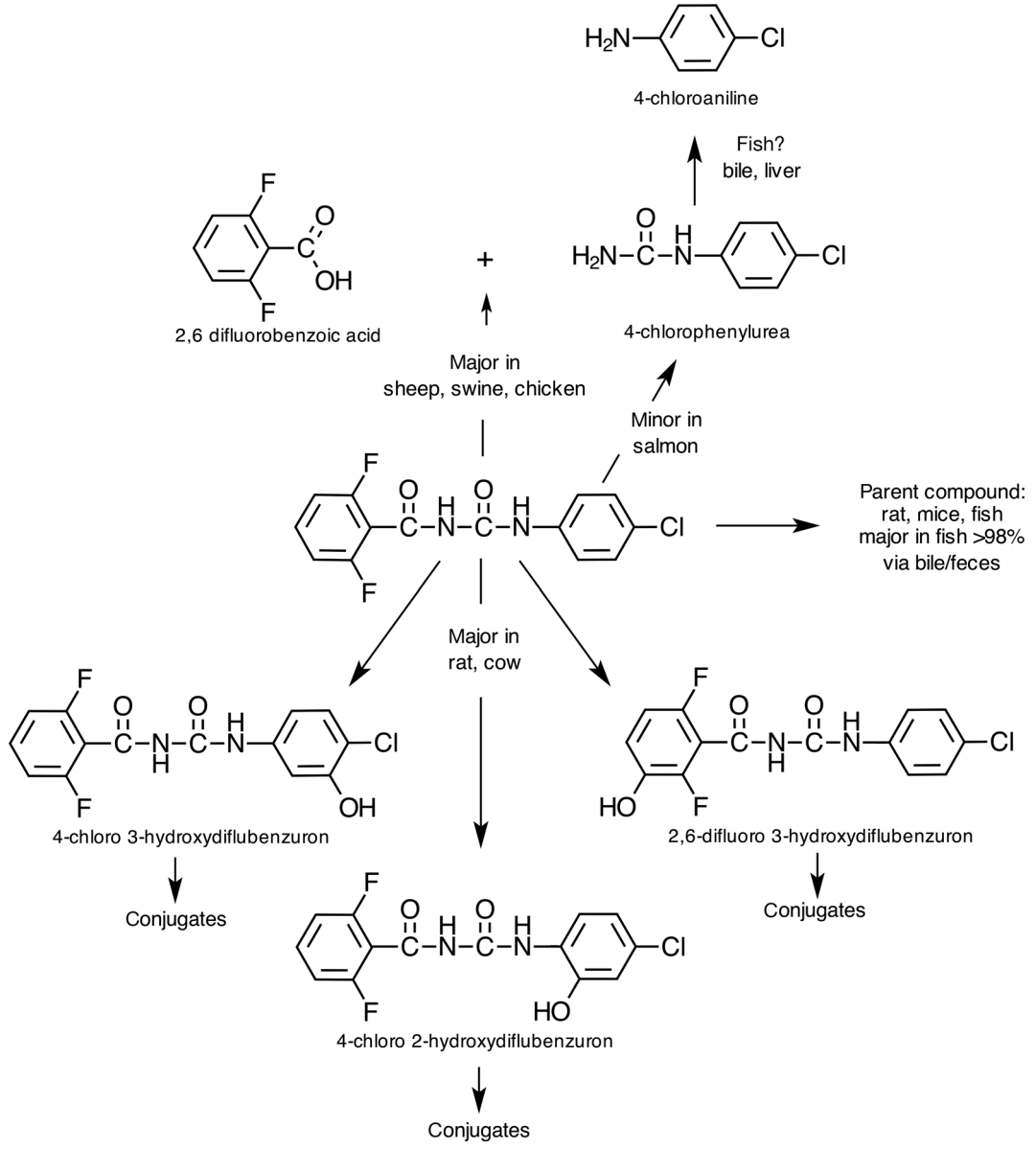

Fig. 1. Diflubenzuron has been assigned a maximum residue limit (MRL) for salmonid species of 1000 µg kg^{-1} in samples of muscle with skin, in natural proportions (FAO 1982, EMA 1998). According to the documentation presented during the establishment of this MRL, the main elimination pathway in Atlantic salmon was the parent drug. When a single dose of radiolabelled diflubenzuron or multiple doses of non-radiolabelled diflubenzuron were followed by one dose (3 mg kg^{-1} body weight) of the labeled compound, diflubenzuron was rapidly excreted as the parent compound (>98%) via bile and feces. Furthermore, the bile contained 2 metabolites in minor concentrations, 4-chlorophenyl urea and a non-identified metabolite, possibly 4-chloroaniline, both in concentrations of 0.23% of the administered dose. In liver, 5 components were isolated, 3 of which were identified as either diflubenzuron, 4-chloroaniline (<0.1%) and 4-chlorophenylurea (<0.3%). The remaining 2 components from the liver were not identified

oral exposure and during a 3 wk depuration period. To assess whether diflubenzuron exposure negatively affects the fish, 28 genes were selected for transcriptional evaluation in liver tissue. The transcriptional assays were selected to include markers for phase I metabolism (*cyp1a*, *cyp2x7*, *cyp4f2*, *cyp3a*), phase II metabolism (*gstp1*, *gstm1*, *gstt1*, *ugt1a*, *sult2*, *nat2*), oxidative stress (*cat*, *gpx1*, *gpx4b*, *hmox1*, *tf*), response to stress (*hsp70*), response to DNA damage (*p53*, *bclx2*), mitogen-activated protein kinases (*mapk1*), apoptosis (*tnfrsf1a*), lipid metabolism (*cpt1a*, *ptgs2*, *elovl5*), membrane transport (*slc16a9a*), estro-

gen response (*esr1*) and potential reference genes (*ubi*, *actb*, *ef1a*). Some of these gene markers were selected particularly to evaluate to what degree diflubenzuron is metabolized in cod liver, and possible mutagenic effects of the metabolite 4-chloroaniline. In addition, a 5 d dose–response exposure experiment was conducted to study hepatic dose-dependent effects on transcriptional markers.

MATERIALS AND METHODS

Animal trials and experimental feeds

For both experiments, fish from the same stock were used. Juvenile Atlantic cod obtained from Parisvatnet Research Station (Institute of Marine Research, Norway) in February 2011 were kept in 500 l flow-through tanks at the Institute of Marine Research (IMR), Bergen, Norway. Temperature was kept constant during the experimental period (7.7 ± 0.2°C), and the fish were kept under a 12 h light:12 h dark cycle. After 3 wk of acclimatization, the fish consumed about 1% of their body weight in feed per day.

Time-series experiment

In the time-series experiment, the fish were orally medicated with feed pellets containing diflubenzuron at a concentration of 0.6 g kg^{-1} feed (Releeze). The fish were fed at 0.5% body weight daily for 14 d, giving a total dose of 42 mg diflubenzuron kg^{-1} fish. Liver samples were collected from 5 fish prior to initiation of treatment followed by 10 medicated and 5 control fish d^{-1} during the treatment (4, 8 and 12 d) and depuration (15, 22 and 36 d) periods. Table 1 shows the size of the fish used in this experiment.

Dose–response trial experiment

Using the same experimental setup as in the time-series experiment, the fish in the dose–response experiment were administered a single dose of diflubenzuron dissolved in propylene glycol–saline (50%:50%) into the caudal vein using a syringe. The doses used were: control (sham injection with propylene glycol), low dose (3 mg kg^{-1}), medium dose (10 mg kg^{-1}) and high dose (50 mg kg^{-1}). Samples of muscle and liver from 4 fish from each dosing were taken 5 d following injection.

Tissue sampling

At samplings, the fish were anesthetized using benzocaine (Benzoak, ACD Pharmaceuticals) and killed with a blow to the head. Liver samples for RNA extraction were immediately dissected out, frozen in liquid nitrogen and stored at –80°C before further processing. Muscle and liver samples for concentration determination were stored at –20°C before analysis.

Table 1. Atlantic cod *Gadus morhua* size and sampling

Time series	Treatment	Date sampled (dd/mm/yy)	Weight (g)	Length (cm)	n
Start		14/03/11	93 ± 32	21.8 ± 1.9	5
Day 4 control		17/03/11	94 ± 24	22.0 ± 1.7	5
Day 4 exposed	Exposure	17/03/11	81 ± 9	21.1 ± 0.6	10
Day 8 control		21/03/11	98 ± 9	21.8 ± 0.3	5
Day 8 exposed	Exposure	21/03/11	97 ± 18	22.0 ± 1.4	10
Day 12 control		25/03/11	100 ± 34	21.8 ± 1.9	5
Day 12 exposed	Exposure	25/03/11	104 ± 14	22.1 ± 0.6	10
Day 15 control		28/03/11	122 ± 15	22.7 ± 0.8	5
Day 15 exposed	Depuration D1	28/03/11	102 ± 30	22.0 ± 1.6	10
Day 22 control		04/04/11	118 ± 27	22.9 ± 1.5	5
Day 22 exposed	Depuration D8	04/04/11	115 ± 18	23.1 ± 0.9	10
Day 36 control		18/04/11	115 ± 30	23.0 ± 1.8	5
Day 36 exposed	Depuration D22	18/04/11	101 ± 13	22.4 ± 1.0	10
Dose–response					
Control		10/02/12	96 ± 21	21.7 ± 1.8	4
Low (3 mg kg^{-1})		10/02/12	88 ± 17	21.2 ± 1.0	4
Medium (10 mg kg^{-1})		10/02/12	99 ± 25	21.7 ± 1.2	4
High (50 mg kg^{-1})		10/02/12	93 ± 12	21.6 ± 1.1	4

Determination of 4-chloroaniline and diflubenzuron

Chemicals

Acetonitrile (HPLC grade), heptane (HPLC grade) and acetone (HPLC grade) were all from Sigma-Aldrich. Diethyl ether (analytical grade), tetrahydrofuran (HPLC grade), 25% (v/v) aqueous ammonia solution (HPLC grade) and formic acid 98–100% purity (HPLC grade) were all from Merck. Dichloromethane HPLC grade was from Riedel-de Haën. Purified water (18.0 MΩ) was used for all analytical purposes. Diflubenzuron (CAS number 35367-38-5) and tefluben-

zuron (CAS number 83121-18-0) for the calibration curve and internal standard were both analytical grade from Aldrich. 4-chloroaniline was purchased from Sigma-Aldrich.

Sample preparation

Samples of muscle and liver (1 g wet weight) were homogenized using a Polytron PT 2100. These homogenates were transferred to 25 ml plastic centrifuge tubes and added to 100 µl of an acetonitrile:water (1:1, v/v) solution containing teflubenzuron (0.5 µg ml^{-1}) as an internal standard. Following addition of 5 ml acetone to each tube, the samples were stirred for approximately 1 min on a whirl mixer, sonicated for 10 min and centrifuged (2500 × *g*) for 3 min. The supernatant was transferred to a 10 ml glass centrifuge tube and in order to remove fat, 1 ml of heptanes was added to the sample and shaken prior to centrifugation (1300 × *g*) for 2 min. The upper heptane layer was discarded, and the extraction was repeated. The acetone layer was evaporated to dryness under a gentle stream of nitrogen gas at 40°C.

For further purification of the samples, an automated solid phase extraction system was used (Gilson ASPEC XL4 system). The dried samples were dissolved in 5 ml of heptanes and applied to a solid phase extraction column packed with silica (Agilent) and pre-conditioned with heptane. The column was washed with 1 ml heptane, diethyl ether:heptane (5:95 and 10:90, v/v) and the analytes were eluted with 1 ml diethyl ether: heptanes (40:60 v/v). Prior to HPLC the eluate was evaporated to dryness under a gentle stream of nitrogen gas at 40°C, dissolved in 250 µl of acetonitrile:water (75:25 v/v) filtered through a 0.45 µm syringe filter and was ready for analysis.

Calibration curve and control samples

Calibration curves were prepared by spiking control samples with 0, 20, 35, 50, 60 and 75 ng g^{-1} of diflubenzuron. In order to assess method validity, 2 control samples were also spiked at the detection limit (LOD) and the quantification limit (LOQ), 10 and 20 ng g^{-1}, respectively, from a separate control solution of 0.5 µg ml^{-1} diflubenzuron in acetonitrile. Samples for calibration curve levels and control samples all received an addition of teflubenzuron as internal standard (100 µl, 0.5 µg ml^{-1}, corresponding to 50 ng g^{-1}).

Equipment for quantitative analysis of diflubenzuron

The samples were separated by reversed-phase HPLC using a Hewlett-Packard HP-1100 autosampler, a quaternary pump (G1311A) and a 4.0 × 125 mm Asahipak ODP-50 4D 4 µm analytical column. The mobile phase was 25% 10 mM aqueous ammonia and 75% acetonitrile (isocratic) at a flow rate of 0.7 ml min^{-1} at ambient temperature, with an expected retention time of approximately 5 min for diflubenzuron. The injection volume was 20 µl.

The mass spectrometry (MS) detector was a Hewlett-Packard Agilent 1100 MSD quadrupole operating with negative electrospray ionization at the following instrumental settings: needle voltage, 3600 V; fragmentor voltage, 70 V; desolvation temperature, 350°C; desolvation gas flow, 4 l min^{-1}; nebulizer pressure, 40 psig (pound force per square inch gauge). To ensure that the chromatogram peaks were assigned correctly to diflubenzuron, qualifier ions were assigned to each peak. Selected ion monitoring was used for the following ions: diflubenzuron 309 u (unified atomic mass unit) (quantifier) and 289 u (qualifier), and teflubenzuron 379 u (quantifier) and 359 u (qualifier). The quantifier ion (309 u) and the qualifier ion (289 u) should appear at the same chromatographic retention time with a quantifier/qualifier ratio of 32–48%. The software used to control the HPLC/MS system and process the acquired data was Agilent ChemStation for liquid chromatography (LC) and LC/MS systems, revision A08.03. For this system, the LOD of diflubenzuron is 10 ng g^{-1} and the LOQ is 20 ng g^{-1}.

Equipment for qualitative analysis of p-chloroaniline

For the detection of 4-chloroaniline, HPLC-MS/MS with electrospray ionization was applied. The samples were separated by reversed-phase HPLC using an Agilent 1200 series system, with an Agilent Eclipse Plus analytical column (2.1 × 100 mm, 1.8 µm particle size). The mobile phase was an isocratic mixture of 75% acetonitrile and 25% formic acid (1% aqueous solution v/v) at a flow rate of 0.20 ml min^{-1} at ambient temperature. The retention time for 4-chloroaniline was expected to be approximately 1.8 min (based on blank samples spiked with 4-chloroaniline and 4-chloroaniline-^{13}C). Final injection volume was 5 µl.

An Agilent 6410 mass spectrometer with triple quadrupole configuration was used operating in the

multiple reaction monitoring (MRM) mode. Positive ionization was applied at the retention times of 4-chloroaniline and diflubenzuron, while negative-ion MRM was used at the retention time of teflubenzuron, as this combination has been found to produce the optimal peaks for each of the analytes. For identification of the compounds, the characteristic transitions were 128 u → 111 u (collision energy = 25 eV, cone voltage = 25 V) for 4-chloroaniline, 315 u → 141 u (collision energy = 30 eV, cone voltage = 25 V) for diflubenzuron and 381 u→ 158 u (collision energy = 25 eV, cone voltage = 25 V) for teflubenzuron. Further MS parameters were as follows: needle voltage, 3500 V; desolvation temperature, 250°C; desolvation gas flow, 3 l min^{-1}; nebulizer pressure, 1 l min^{-1}. The software controlling the HPLC/MS/MS system was Agilent MassHunter Workstation, and the data processing program was Agilent MassHunter Quantitative Analysis. Based on spiking experiments on blank sample tissues, 2 ng g^{-1} is considered to be the LOD of 4-chloroaniline in this study.

RNA isolation

Atlantic cod liver tissue was thoroughly homogenized before RNA extraction using a Precellys 24 homogenizer and ceramic beads CK28 (Bertin Technologies). Total RNA was extracted using the BioRobot EZ1 and RNA Tissue Mini Kit (Qiagen), treated with DNase according to the manufacturer's instructions and eluted in 50 µl RNase-free MilliQ H_2O. The RNA was then stored at –80°C before further processing. RNA quality and integrity were assessed with a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies) and an Agilent 2100 Bioanalyzer (Agilent Technologies). The RNA 6000 Nano LabChip kit (Agilent Technologies) was used to evaluate the RNA integrity of the liver samples. The 260/280 and 260/230 nm ratios of the extracted RNA were 2.1 ± 0.1 and 2.0 ± 0.3 (n = 102), respectively (mean ± SD). The RNA integrity number of a selected set of the liver samples used for RT-qPCR was 9.6 ± 0.3 (n = 18; mean ± SEM).

Quantitative real-time PCR

PCR primer sequences used for quantification of the transcriptional levels of selected genes, as well as the reference genes, are shown in Table 2. In total, 28 genes were quantified with quantitative real-time PCR (RT-qPCR), of which 3 were selected as potential reference genes. BLASTX or BLASTN was used to predict PCR assay specificity. The reaction specificity of each assay was verified by observing a single peak at the expected temperature (T_m) on the melting curve.

Briefly, a 2-step real-time RT-PCR protocol was used to quantify the transcriptional levels of the selected genes. The RT reactions were run in duplicate on a 96-well reaction plate with the GeneAmp PCR 9700 machine (Applied Biosystems) using TaqMan Reverse Transcription Reagent containing Multiscribe Reverse Transcriptase (50 U $µl^{-1}$) (Applied Biosystems). Two-fold serial dilutions of total RNA were made for efficiency calculations. Six serial dilutions (1000–31 ng RNA) in triplicates were analyzed in separate sample wells. Total RNA input was 500 ng in each reaction for all genes. Quality controls 'no template controls' and 'no amplification controls' were run for quality assessment for each PCR assay.

Reverse transcription was performed at 48°C for 60 min using oligo dT primers (2.5 µM) for all genes in 50 µl total volume. The final concentrations of the other chemicals in each RT reaction were $MgCl_2$ 5.5 mM, dNTP 500 mM of each, 10× TaqMan RT buffer (1×), RNase inhibitor 0.4 U $µl^{-1}$ and Multiscribe reverse transcriptase 1.67 U $µl^{-1}$ (Applied Biosystems). Two-fold diluted cDNA (2.0 µl cDNA in each RT reaction) was transferred to 384-well reaction plates and the qPCR was run in 10 µl reactions on the LightCycler 480 Real-Time PCR System (Roche Applied Sciences). RT-qPCR was performed using SYBR Green Master Mix (LightCycler 480 SYBR Green Master Mix Kit, Roche Applied Sciences), which contains FastStart DNA polymerase and gene-specific primers (500 nM of each). PCR was achieved with a 5 min activation and denaturizing step at 95°C, followed by 45 cycles of a 10 s denaturing step at 95°C, a 20 s annealing step at 60°C and a 30 s synthesis step at 72°C. For the time-series experiment, mean normalized expression (MNE) of the target genes was determined using a normalization factor based upon *ubi*, *hsp70* and *tnfrsf1a*, as calculated by the *geNorm* software (Vandesompele et al. 2002). For the dose–response experiment, *ubi*, *hsp70* and *gstm1* were the most stably expressed transcripts. This set of 3 genes were determined as the most stable of all quantified transcripts by GeneX software, all with geNorm M stability values <0.4 and were therefore selected as reference genes for the time-series and dose–response RT-qPCR data.

Table 2. PCR primers, accession or contig numbers, amplicon sizes and PCR efficiencies

Gene	Full gene name	Accession no.	Forward primer	Reverse primer	Amplicon size (bp)	PCR efficiency
cyp1a	Cytochrome P450, family 1, subfamily A	EX725014	CCTTGACCTCTCGGAGAAAGAC	CGCCCCGCTAGCTATAGACA	146	1.87
cyp2x7	Cytochrome P450, family 2, subfamily X, polypeptide 7	>CUST_26360_ PI425838995	TCCAAGGGAACGATCATCGT	CACAAACTCTCCCCGGTCAT	117	2.01
cyp3a	Cytochrome P450, family 3, subfamily A	EX727125	GGATCCCGGTGAAGGACATA	CAATGAGTCACAGCGGCTCTT	135	2.03
cyp4f2	Cytochrome P450, family 4, subfamily F, Polypeptide 2	>CUST_22382_ PI425838995	ATTTACGGGACGCACCACAA	AAGGGAATGAATGCGTTGGA	113	2.10
gstp1	Glutathione S-transferase pi 1	EX730032	GTCCCCCTGCTGCCATTC	CCTCCATACACCGCCACCTA	126	1.79
gstt1	Glutathione S-transferase theta 1	>Contig6168	ATCACCCTGCATATACGAAAACG	GCCAAAACCTTCCAGGACAA	123	1.90
gstm1	Glutathione S-transferase mu 1	>Contig6399	CCGGTTGACGGTGTAGATTCA	TCGAGGCTCTGGAGAAGATTTC	119	2.00
ugt1a1	UDP glucuronosyltransferase 1 family, polypeptide A1	>isotig13052 gene= isogroup06602	AGTGCTGGAGGCCATTAACAA	ACGACGAACTCGGTCCAGAA	132	1.94
esr1	Estrogen receptor 1	>CUST_28940_ PI425838995	CCTTGAGCTGTCCCTTCATGA	GTCTTGTGCGAAGATGAGTTTCC	121	2.03
sult2	Sulfotransferase family, cytosolic, 2	EX724532	TGGCCAACTACTCATCATTTGAAA	CTGGGTTACGGTGAAGTGGTTT	101	1.91
cat	Catalase	DQ270487	GCCAAGTTGTTTGAGCACGTT	CTGGGATCACGCACCGTATC	101	1.98
gpx1	Glutathione peroxidase 1	EX725875	CCAAATATGGACGGCATAGGA	CAAACGCTACAGCCGGAACT	101	1.77
gpx4	Glutathione peroxidase 4	EX721840	CCCTGTGGAAGTGGCTGAAG	CATCCAAGGGTCCGTATCTCTT	129	2.07
hmox1	Heme oxygenase 1	EX738947	AGAGAACACAGGGCTGATGTTGA	CGGGTGGCTGCTGTTATTGT	133	1.99
tf	Transferrin	EX722617	ACGACGGGTCAACTCGTTTG	AGTCACTCGGGCACAATGTG	139	2.07
hsp70	Heat shock protein 70	EX741726	CATGACATCGTCCTGGTTGGT	CGTAGGCCACAGCTTCATCA	121	1.99
p53	Tumor protein p53	EX723548	CGCTGCTGCTGAACTTCATG	GGATGGCTCTCCGGTTCAT	63	2.07
bcl-2	B-cell CLL/lymphoma 2	ES471531	GAACGACGGTAACGGCAATG	GCGTGTAGCGCAACTCAAAC	113	1.97
mapk1	Mitogen-activated protein kinase 1	>GmE100127i37392	GTTGCTCAGATGCTGGGTCTT	CGAGAACATCATCGGGATCA	130	1.99
tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1A	>GmE090818c14347	GGCAGCTCCTGCAGTTGTC	TGCCCTCCAGGCTATAAACG	124	1.96
cpt1a	Carnitine palmitoyltransferase 1A (liver)	>GmE100215i04260	CCTGACCAGCTACGCCAAGT	GACCCATGGTGCCGTTCTT	101	2.02
elovl5	ELOVL fatty acid elongase 5	>GmE100215i19804	CCTCACCGTGCTCTCCTTCT	GGTCTCGTCATCGCTGTGTGT	103	1.95
ptgs2	Prostaglandin-endoperoxide synthase 2	EX728598	CGCCGAACACCGTTCATTAT	AGAGCGGGACGTCAGAACAT	113	2.00
slc16a9a	Solute carrier family 16, member 9	>isotig28071 gene= isogroup21621	TGGGTTGCGGTCTGGTCTAC	ACGCTCCGAAGCTGGTACCT	111	2.00
nat2	N-acetyltransferase 2	EX740965	TATCTTTGGCCATGCCAATG	GGGCTCCCAGATTTGAAAGG	124	1.87
ubi	Ubiquitin	EX735613	GGCCGCAAAGATGCAGAT	CTGGGCTCGACCTCAAGAGT	69	1.87
actb	Actin, beta	EX739174	CACAGCCGAGCGTGAGATT	ACGAGCTAGAAGCGGTTTGC	95	1.99
ef1a	Eukaryotic translation elongation factor 1 alpha	EX722124	CGGTATCCTCAAGCCCAACA	GTCAGAGACTCGTGGTGCATCT	93	1.95

Statistics

GraphPad Prism 6.0 software was used for statistical analyses of the gene expression data. For the time-series experiment, comparisons were only made between medicated and control fish at each time point, evaluated with a *t*-test. One-way ANOVA with Dunnett's pairwise multiple comparison *t*-test was used to compare the dose–response treatments against the control mean. In case the Brown Forsythe or Bartlett's tests showed that the variances differed, the MNE data were log-transformed before ANOVA analysis. Spearman rank correlation analysis (Statistica 9.0, StatSoft) was used to search for possible co-regulation among the transcripts. Principal component analysis (PCA) (Sirius 6.5) was used for multivariate analyses, in search for an explanation of the variance in the data. Grubbs test was used to screen for outliers. A significance level of $p < 0.05$ was used for all tests.

RESULTS

Tissue accumulation of diflubenzuron

Fig. 2A shows the accumulated levels of diflubenzuron in liver of Atlantic cod orally treated at a dose of 3 mg kg^{-1} d^{-1} for 14 d, and then kept for 22 additional days during depuration. After 4 d of treatment, the liver contained 76 ± 12 ng (mean ± SEM) diflubenzuron kg^{-1} tissue. One of the medicated fish sampled at Day 4 showed a very high level of diflubenzuron and was thus removed from the data set as an outlier (Grubbs outlier test, $p < 0.01$). The highest levels were observed 1 d after the end of treatment (Day 15), when the level of diflubenzuron reached 181 ± 21 ng kg^{-1} (mean ± SEM). The data showed a dose-dependent accumulation response, except for Day 12, when the level of diflubenzuron was in line with the levels measured at Day 4. After 13 d of depuration (Day 22), detectable levels of diflubenzuron were only observed in liver of 1 of 10 individuals (16.8 ng kg^{-1}). The LOD was 2 ng kg^{-1}. No diflubenzuron was detected in any of the fish at Day 36. As expected, no diflubenzuron was detected in liver of the control fish.

For the dose–response experiment, we were unable to quantify the accumulation level of diflubenzuron in liver tissue due to a technical error that left us with no materials for analysis. However, we were able to quantify the concentration in muscle tissue. Accumulation of diflubenzuron in muscle tissue represents an approximation of the accumulation in liver tissue. For the time-series experiment, the accumulated levels of diflubenzuron in liver and muscle showed a relatively strong correlation (Spearman rank correlation, $r = 0.80$, $p < 0.0001$). In muscle tissue diflubenzuron accumulated in a dose–response manner, but with relatively large individual variation (Fig. 2B). In muscle of one of the fish in the high exposure group, a very high level of diflubenzuron was determined. This value was omitted from the data set since it was a significant outlier (Grubbs outlier test, $p < 0.05$). No fish died during the experiments.

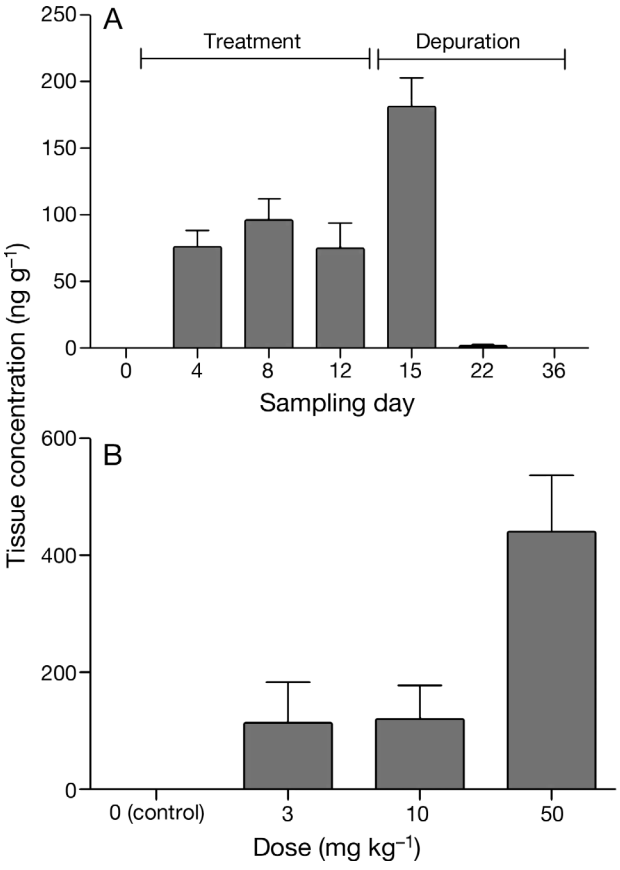

Fig. 2. Accumulated tissue concentrations of diflubenzuron in (A) liver of juvenile Atlantic cod *Gadus morhua* orally administrated with the salmon lice treatment for 14 d, and thereafter during a 3 wk depuration period (control: n = 6; exposed: n = 10), and (B) in muscle of juvenile Atlantic cod injected with 3 different doses of diflubenzuron (n = 4): low, 3 mg kg^{-1} fish; medium, 10 mg kg^{-1} fish; and high, 50 mg kg^{-1} fish

Time-series experiment

The effects of oral administration of diflubenzuron at a rate of 3 mg kg^{-1} d^{-1} for 14 d on Atlantic cod liver

gene expression were determined (Fig. 3). Oral administration of diflubenzuron did not mediate a distinct change in gene transcription pattern in Atlantic cod liver. Most of the 22 evaluated genes did not show a time-dependent change in expression over the 14 d of treatment or over the 22 d of depuration. The largest number of significant differences between control and medicated fish were observed

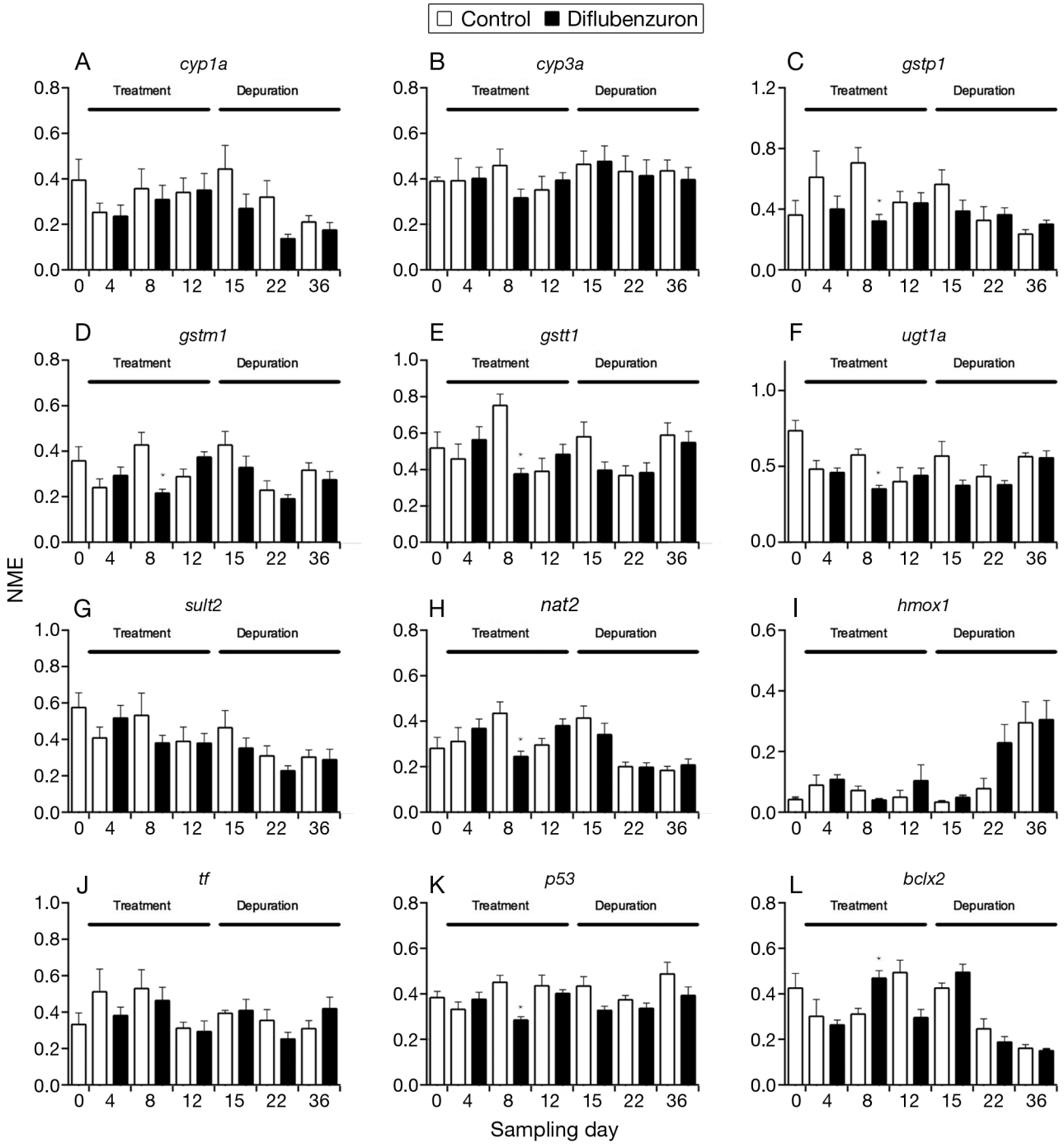

Fig. 3. (Above and next page.) Mean normalized expression (MNE) of a selected set of transcripts in liver of juvenile Atlantic cod *Gadus morhua* orally administrated 3 mg diflubenzuron per kg fish for 14 d, and during the 22 d depuration period (control: n = 6; exposed: n = 10). (A) *cyp1a*, (B) *cyp3a*, (C) *gstp1*, (D) *gstm1*, (E) *gstt1*, (F) *ugt1a*, (G) *sult2*, (H) *nat2*, (I) *hmox1*, (J) *tf*, (K) *p53*, (L) *bclx2*, (M) *gpx1*, (N) *gpx4b*, (O) *cat*, (P) *mapk1*, (Q) *slc16a9a*, (R) *ptgs2*, (S) *cpt1a*, (T) *actb* and (U) *ef1a*. Open bars: controls; dark bars: treated. *Significant difference between control and medicated fish ($p < 0.05$)

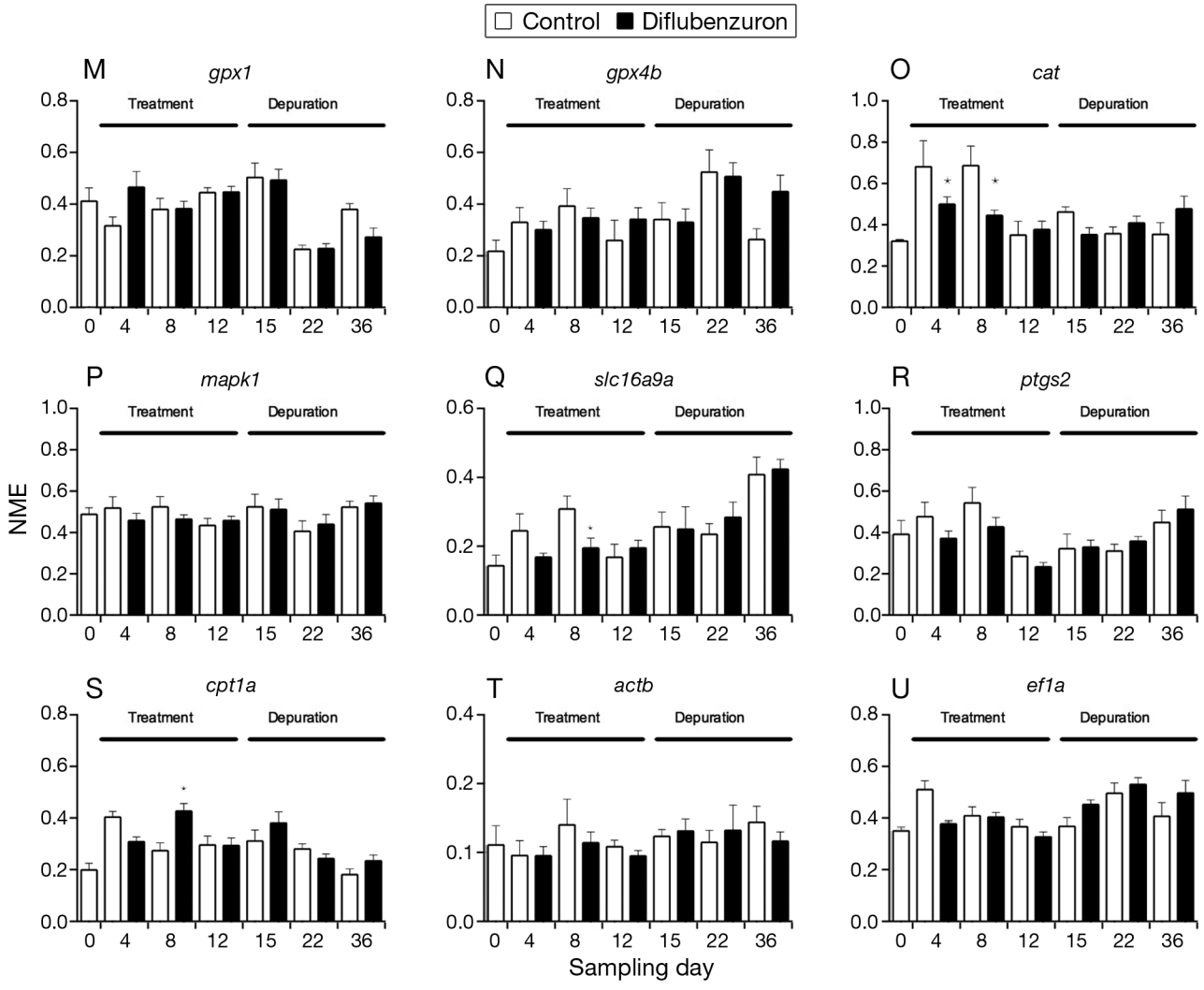

Fig. 3. (continued)

at Day 8, with 9 transcripts of the 21 examined showing lower expression and 2 transcripts showing higher expression compared with their corresponding controls. Expression of the transcripts *gstp1* (Fig. 3C), *gstm1* (Fig. 3D), *gstt1* (Fig. 3E), *ugt1a* (Fig. 3F), *nat2* (Fig. 3H), *cat* (Fig. 3O), *p53* (Fig. 3K) and *slc16a9a* (Fig. 3Q) were significantly lower, whereas *bclx2* (Fig. 3L) and *cpt1a* (Fig. 3S) were significantly higher expressed (*t*-test, $p < 0.05$) at this time point.

PCA showed no distinct spatial distribution for any of the fish groups (Fig. 4A). Several genes encoding phase II metabolism enzymes grouped together, both when looking exclusively at the medicated fish, and when looking at all 102 examined fish together. Thus, the PCA result suggests that the phase II metabolism transcripts *gstp1*, *gstm1*, *gstt1*, *ugt1a*, *sult2* and *nat2* are co-regulated in the liver of Atlantic cod exposed to diflubenzuron. Many of the phase II metabolism transcripts were significantly co-regulated, as determined by Spearman rank correlation analysis. Interestingly, *nat2* showed significant ($p < 0.001$) co-regulation with *gstp1* ($r = 0.62$), *gstm1* ($r = 0.70$), *gstt1* ($r = 0.63$) and *sult2* ($r = 0.58$), as well as *cyp1a* ($r = 0.46$), *gpx1* ($r = 0.55$) and *tf* ($r = 0.48$). *gstp1* showed a significant ($p < 0.001$) correlation with *gstt1* ($r = 0.65$), *cyp1a* ($r = 0.62$) and *tf* ($r = 0.71$), whereas *gstm1* showed a significant correlation with *gstt1* ($r = 0.65$), *p53* ($r = 0.68$) and *gpx1* ($r = 0.56$) and also weaker but still significant correlation against *ugt1a* ($r = 0.37$), *sult2* ($r = 0.51$) and *cyp1a* ($r = 0.38$).

The PCA plot from the time-series data suggested that *slc16a9a*, *mapk1*, *cat* and *gpx4b* displayed a size-dependent transcriptional expression in the diflubenzuron-treated fish (Fig. 4A). Of these transcripts, only *gpx4b*, *slc16a9a* and *mapk1* showed a significant correlation with the weight of the fish (Spearman rank correlation, *gpx4b*: $r = 0.36$, *mapk1*:

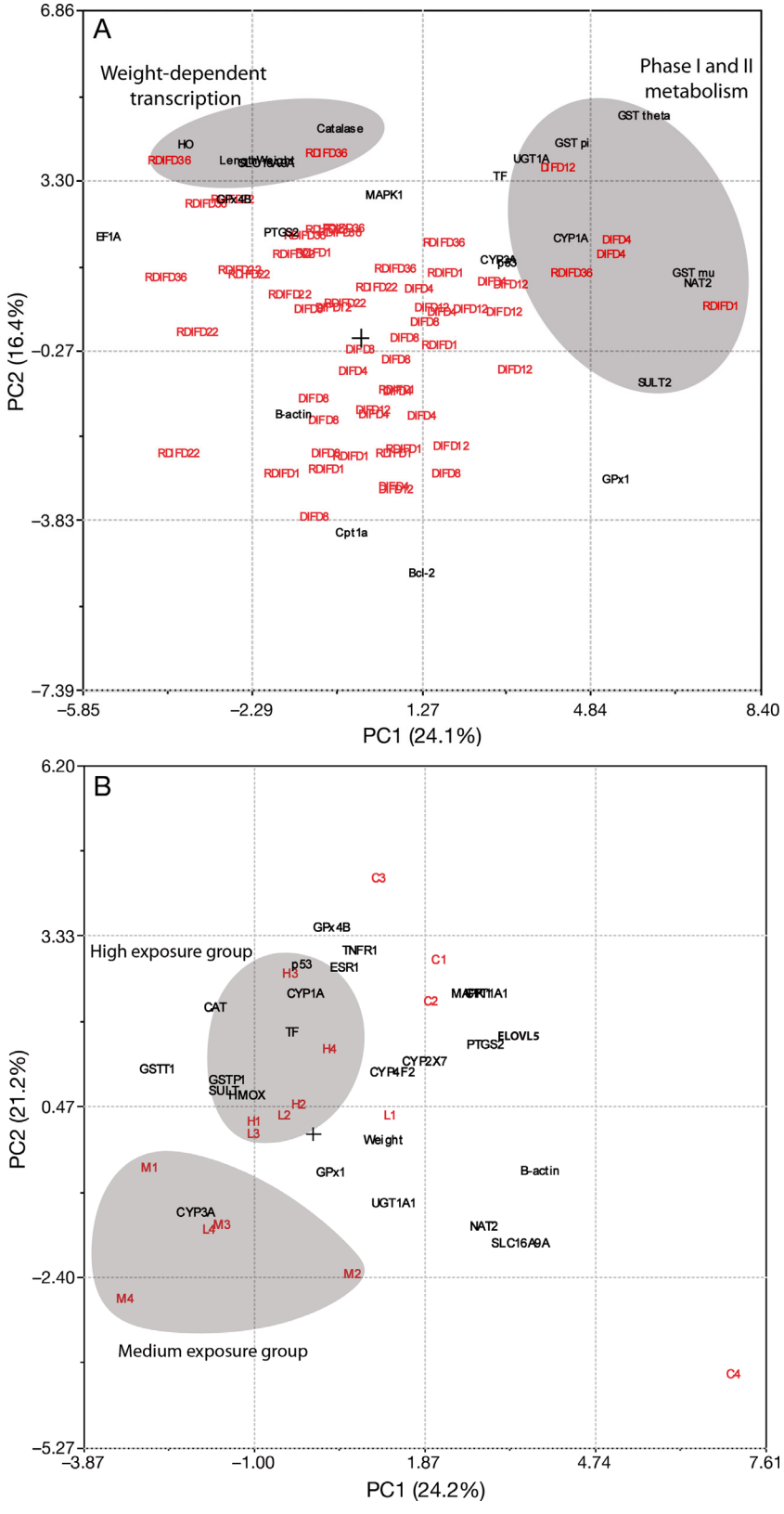

Fig. 4. (A) Principal component analysis (PCA) plot of all samples from the time-series experiment; n = 102. (B) PCA plot of only fish from the dose–response experiment; n = 16. Control: C1–4; Low: L1–4; Medium: M1–4; High: H1–4

r = 0.034, *slc16a9a*: r = 0.33, p < 0.001). In addition, *actb* showed a size-dependent transcription in the studied fish (r=0.36, p < 0.001).

Dose–response experiment

Since no distinct patterns in gene transcription were discovered in the time-series experiment, we decided to supplement the data with a limited set of fish exposed to increasing levels of diflubenzuron (Fig. 5). This was done to evaluate whether any of the selected gene transcripts showed a dose-dependent response to the anti-salmon lice treatment, and whether the transcription of any of the studied genes were affected at higher exposure concentrations. Instead of oral administration, the drug was dissolved in propylene glycol and injected directly into the vein of the fish, and tissue specimens were collected 4 d later. The low dose used in the dose–response exposure experiment was similar to the regular salmon lice treatment of Atlantic salmon (3 mg kg^{-1}) used in the time-series experiment, whereas the medium concentration (10 mg kg^{-1} fish) was about 3 times above the regular dose and the high concentration (50 mg kg^{-1} fish) was 17 times above the regular dose.

Of the 22 genes examined, only a few gene transcripts showed a significant effect of diflubenzuron exposure in the dose–response trial. One single injection with diflubenzuron into the blood significantly increased the transcription of *cyp3a* in liver of cod exposed to the medium concentration when compared with control fish that received only an injection of propylene glycol (1-way ANOVA, p < 0.01; Fig. 5C). None of the evaluated oxidative stress markers were differentially expressed. Three transcripts encoding proteins involved in lipid metabolism were significantly downregulated by the treatment compared with controls. Prostaglandin-endoperoxide synthase

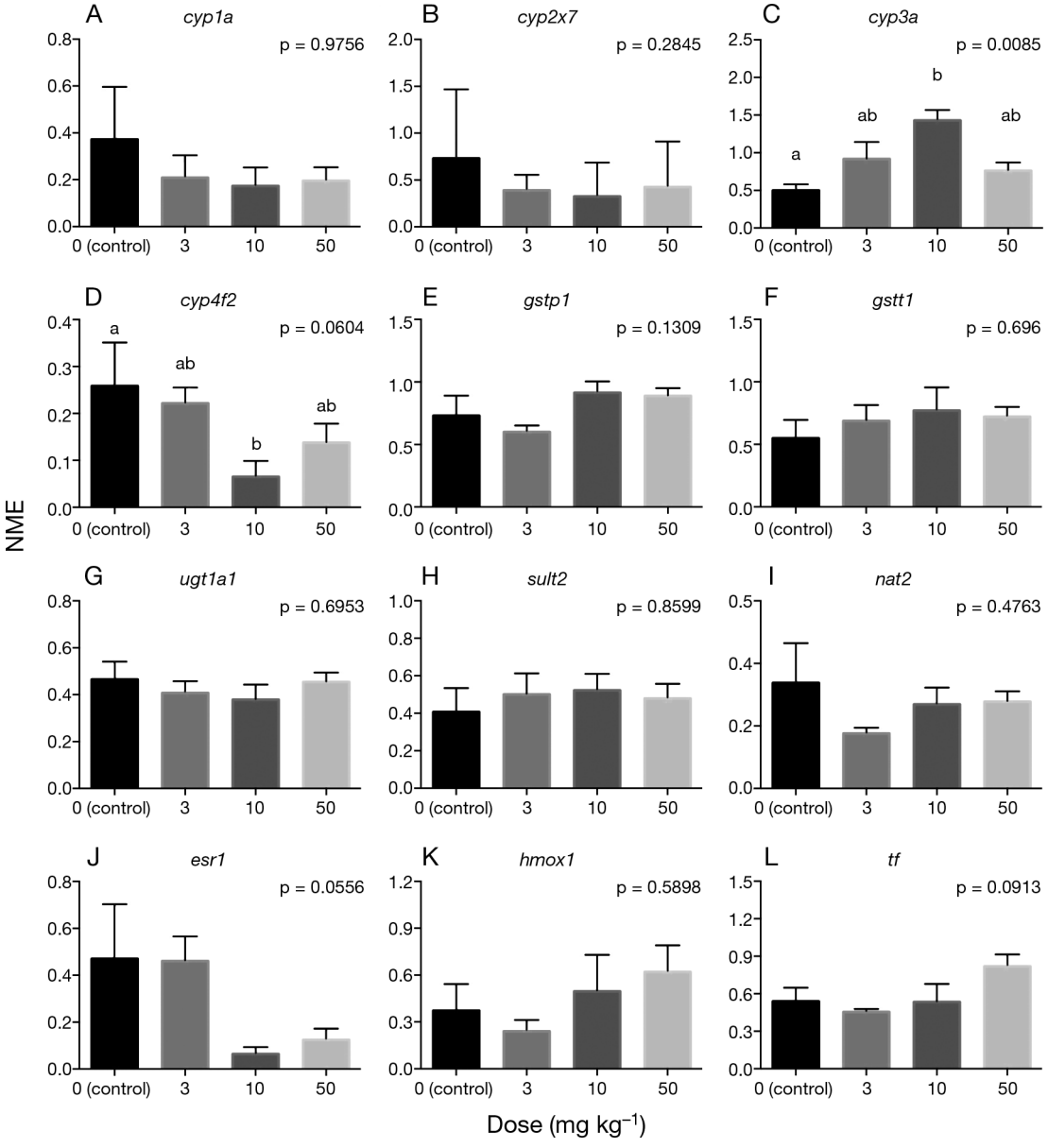

Fig. 5. (Above and next page.) Mean normalized expression (MNE) of a selected set of transcripts in juvenile Atlantic cod *Gadus morhua* treated with 3 doses of diflubenzuron for 4 d. The drug was injected into the fish through the caudal vein at Day 1, and tissues were collected for examination at Day 4. Control fish were injected with carrier only. (A) *cyp1a*, (B) *cyp3a*, (C) *cyp2x7*, (D) *cyp4f2*, (E) *gstp1*, (F) *gstt1*, (G) *ugt1a*, (H) *sult2*, (I) *nat2*, (J) *esr1*, (K) *hmox1*, (L) *tf*, (M) *gpx1*, (N) *gpx4b*, (O) *cat*, (P) *mapk1*, (Q) *tnfrsf1a*, (R) *p53*, (S) *ptgs2*, (T) *cpt1a*, (U) *elovl5* and (V) *slc16a9a.* p-values (ANOVA) are given in the plots. Letters above the columns denote significant differences between the doses. Data are means ± SEM

2 (*ptgs2*), or *cox2*, was significantly downregulated in fish that received the low and medium doses (1-way ANOVA, $p < 0.01$; Fig. 5S), but not in fish from the high exposure group. *cpt1a* was significantly downregulated in fish from the medium exposure group (1-way ANOVA, $p < 0.01$; Fig. 5T), whereas *elovl5* was significantly downregulated in fish from all 3 exposure groups (low and medium groups, 1-way ANOVA, $p < 0.001$, high group, $p < 0.01$; Fig. 5U). In addition, *mapk1* was significantly downregulated in fish from the medium exposure group (1-way ANOVA, $p < 0.05$; Fig. 5P). A few additional gene transcripts, *cyp2x7* (an ortholog to human *cyp2j2*), *cyp4f2*, *elovl5* and *esr1*, were analyzed for the dose–response exposure experiment. These markers were selected because we observed significant transcrip-

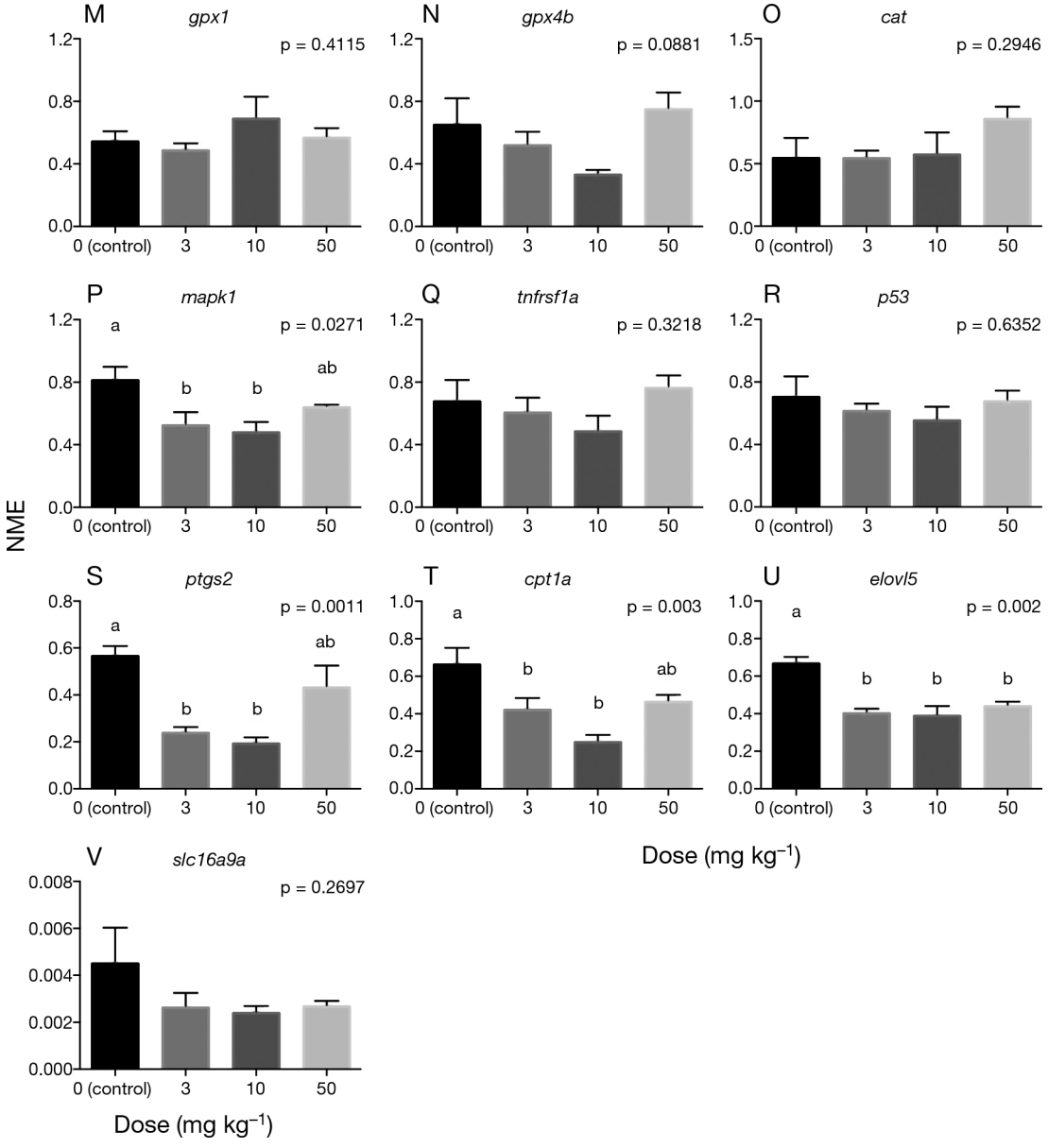

Fig. 5. (continued)

tional responses for *cpt1a* and *ptgs2*, indicating an effect on polyunsaturated omega-6 fatty acids and particularly on arachidonic acid metabolism. Cytochrome P450 4F2 (*cyp4f2*) was downregulated in the medium concentration group (1-way ANOVA, $p < 0.05$; Fig. 5D). No significant effects were observed for *cyp2x7* (Fig. 5B) or *esr1* (Fig. 5J), while *elovl5* (Fig. 5U) was significantly downregulated at all 3 concentrations.

Multivariate analysis of the MNE data from the dose–response exposure experiment showed that *cyp3a* transcription grouped nicely with fish from the medium exposure group (Fig. 4B). No separation was observed between fish from the low and high exposure groups. Thus, based on the gene transcription data, the medium exposure concentration (10 mg kg^{-1} fish) appears to have triggered a stronger molecular response than the high exposure concentration (50 mg kg^{-1} fish).

DISCUSSION

With increasing use of the benzamide drugs diflubenzuron and teflubenzuron against salmon lice in Atlantic salmon aquaculture, their ecological effects

on nontarget animals inhabiting the areas near the fish farms have been questioned. These chemicals are used against salmon lice infestation because they are highly toxic to crustaceans, especially during molting, because of chitin synthesis inhibition, at the same time showing very low toxicity to the medicated fish. This study suggests that diflubenzuron can be taken up in fish eating leftover medicated feed pellets near Atlantic salmon fish farms but with relatively large individual variation in uptake rate. Orally administered diflubenzuron at a standard dose of 3 mg kg^{-1} for 14 d regularly given to Atlantic salmon will accumulate in liver and muscle tissues in Atlantic cod. The highest levels of accumulated diflubenzuron were observed just after the end of the administration. Thereafter, the pharmaceutical drug was cleared from the body relatively fast. In liver, accumulated diflubenzuron after oral administration has only moderate effects on the detoxifying system at the molecular level, suggesting that the drug poses only a minor risk to wild fish living in the vicinity of fish farms. In the dose–response experiment, the highest accumulated concentration in muscle tissue was observed in the high exposure group, but only with a 3.7-fold higher level than in the low and medium exposure groups. Mammalian studies have shown decreasing intestinal uptake with increasing dose level (Dost et al. 1985). Studies have also shown that diflubenzurons are only moderately absorbed in the intestine of Atlantic salmon and metabolized to a minor degree by the fish (Horsberg & Høy 1991, SEPA 1998). Therefore, most of the drug is excreted as unaltered parent compound. Horsberg & Høy (1991) showed that unchanged diflubenzuron accounted for 94.8% of the total levels of diflubenzuron and its metabolites in muscle and 72.3% in the liver. Our study thus suggests a similar decreased uptake rate with increasing dose level from the blood. In Atlantic cod with diflubenzuron injected directly into the blood, the drug induced the hepatic detoxifying system in a dose-dependent manner but with a high-dose inhibition. Our study also suggests that although wild fish may accumulate diflubenzuron when Atlantic salmon are being orally treated with the anti-salmon lice treatment, the potential effect of the drug on wild fish in terms of hepatic transcription of detoxifying enzyme genes appears to be low.

According to the Comparative Toxicogenomic Database (http://ctdbase.org/), benzamides show interactions with a number of enzymes. Bezafibrate (propanoic acid, 2-(4-(2-((4-chlorobenzoyl)amino)-ethyl)phenoxy)-2-methyl), one of the best studied benzamides, shows interactions with several peroxisome proliferator-activated receptors (PPARs), palmitoylacyl-CoA oxidase 1 (ACOX1), nitric oxide synthase 3 (NOS3) and many other proteins, while DEET (benzamide, *N,N*-diethyl-3-dimethyl-), a commonly used insecticide against mosquitoes, shows interactions with acetylcholinesterase (ACHE) and many phase I cytochrome P450 enzymes (CYPs). Several studies have reported altered expression of CYP3A enzymes after exposure to benzamides. For example, Nishimura et al. (2004) evaluated the effect of the benzamide diethyl 4-[(4-bromo-2-cyanophenyl)carbamoyl] benzylphosphonate on primary cultures of cryopreserved human hepatocytes and observed increased mRNA expression of CYP3A4 after 48 h exposure. Lemaire et al. (2006) studied how the pregnane X receptor (PXR) is activated by various chemicals using HeLa cells, and showed that diflubenzuron activate the PXR in humans. PXR activation stimulates increased expression of cytochrome P450 3A (CYP3A) in the liver of most animals, suggesting that CYP3A may be a potential biomarker for diflubenzuron exposure in fish. Dubois et al. (1996) showed that diflubenzuron exposure induced the expression of CYP3A1 protein in liver of rats, whereas Sapone et al. (2005) showed that diflubenzuron has gender- and tissue-specific effects in mice. The authors concluded that diflubenzuron may be mutagenic, a phenomenon linked to the altered expression of CYP genes, requiring more research on the toxicity of the pesticide. Maduenho & Martinez (2008) examined the effects of diflubenzuron on the freshwater fish *Prochilodus lineatus* and observed a reduction in the number of red blood cells and hemoglobin. These authors also observed an increase in blood glucose (hyperglycemic response), suggesting altered energy metabolism, and upregulation of liver glutathione S-transferase (GST) and catalase gene expression, suggesting increased detoxification and biotransformation as well as increased oxidative stress (Maduenho & Martinez 2008). They also observed altered liver function and concluded that the drug affects fish health and suggested that more studies are needed on the effects of diflubenzuron in fish before continued use of the drug in aquaculture. In the present dose–response study, *cyp3a* was induced 2.8-fold in the liver of Atlantic cod in the medium exposure group (10 mg kg^{-1}) but showed no alteration in the high exposure group (30 mg kg^{-1}). No significant effects were observed for the other 3 evaluated phase I enzyme transcripts (*cyp1a*, *cyp2x7* and *cyp4f2*) in any of the experiments. These results clearly suggest that diflubenzuron is metabolized by phase I enzymes and particularly CYP3A after PXR activation in cod.

Interestingly, Sapone et al. (2005) observed a similar suppressed CYP3A response in mice at the highest examined dose (1075 mg kg^{-1}) and suggested that this suppression may rely on toxic metabolites and reactive oxygen species generated by the induction itself. Inverted U-shape response curves have often been observed for CYP induction (Sapone et al. 2005, Chu et al. 2009), as seen for *cyp3a* in the present study. Even though chemical solubility and/or cytotoxicity often can explain an inverted U-shape dose–response curve, hormesis is now generally accepted as a real and reproducible biological phenomenon (Calabrese 2010). Whether the observed inhibited response for *cyp3a* in liver of fish in the high exposure group relies on a hormetic phenomenon or lower chemical solubility is unknown, and needs further study.

Maduenho & Martinez (2008) observed an increased hepatic activity of GST and CAT in the freshwater fish *Prochilodus lineatus* after 96 h of exposure to 25 mg l^{-1} diflubenzuron, suggesting an activation of detoxification and antioxidative defenses. In the present study, no significant effects were observed on phase II enzyme transcripts (*gstp1*, *gstt1*, *ugt1a*, *sult2* and *nat2*) or in any of the examined oxidative stress markers (*gpx1*, *gpx4b* and *cat*). The *nat2* gene encodes a phase II enzyme involved in the detoxification of a plethora of hydrazine and arylamine drugs and was therefore examined in this study. The reason for this discrepancy is not known, but increased transcription of phase I genes are often associated with a weaker but still significant increased transcription of phase II enzyme genes. However, PCA clearly suggests that the phase I and II enzyme transcripts are co-regulated also in the examined Atlantic cod liver, both in fish from the time-series experiment and in fish from the dose–response experiment.

In the time-series experiment, the oxidative stress marker *cat* was one of the transcripts that showed significantly decreased expression at Day 4 and 8, compared with the control fish sampled the same day. However, compared with the fish sampled at the start of the experiment, expression of *cat* was significantly higher at Day 4 and 8 in the medicated group (*t*-test, $p < 0.05$). An increase in the expression of several transcripts in the control fish at Day 4 and Day 8 partly explains this finding, i.e. the decreased *cat* transcription. This unexpected expression pattern was observed for all 3 *gst* transcripts, as well as for *slc16a9a* and particularly for *cat*. One possible explanation for this surprising finding may be that fish transfer and initial feeding have imposed handling stress in both the control and medicated fish. A size-dependent transcription may partly explain the higher expression of a few genes observed in the liver of the control fish sampled at Day 4, with the control fish weighing on average 94 g versus 82 g in the medicated fish. This observed difference in weight is probably not a result of drug exposure, as also supported by Zaidi & Soltani (2011). These authors examined the possible effects of diflubenzuron on growth and condition factors in the western mosquito fish *Gambusia affinis* and concluded that after 28 d exposure no significant effects of diflubenzuron exposure on growth, hepato-somatic and gonado-somatic indices could be seen.

Unfortunately, we were not able to quantify the diflubenzuron level in the bile in the present study, probably due to the high fat content in cod liver and bile. As diflubenzuron is excreted mainly via the bile, increased concentration of the drug and its metabolites in the bile would reflect active metabolism. In follow-up studies, more emphasis should therefore be put on the sensitivity of the method for detection of diflubenzuron and its metabolites in bile. Since some mammalian studies have suggested that the 4-chloroaniline metabolite may have a mutagenic effect, we included a couple of markers for cell death and DNA damage. However, no effects were observed on these markers (*tnfrsf1a*, *bclx2* and *p53*), either in the time-series experiment or in the dose–response experiment. Therefore, based on the evaluated transcripts, we are not able to conclude on a possible mutagenic effect of diflubenzuron in fish. On the contrary, *mapk1* transcription showed a significant decreased transcription in the medium concentration group of fish. MAPK1 plays an important role in the MAPK/ERK cascade, which mediates diverse biological functions such as cell growth, adhesion, survival and differentiation through the regulation of transcription, translation, cytoskeletal rearrangements mitosis and apoptosis (Genecards database, www.genecards.org). In humans, benzamide exposure has been shown to result in a decreased activity and expression of MAPK1 protein (Mattingly et al. 2006, Thomas et al. 2010), in line with our finding for the transcript. Also *mapk1* transcription showed a U-shaped type of transcription, with no significant response in the high exposure group.

Lastly, 3 transcripts encoding proteins involved in fatty acid metabolism showed a significantly reduced transcription after diflubenzuron treatment in the dose–response exposure experiment. Many xenobiotics are known to affect lipid metabolism in fish (Olsvik et al. 2011, http://ctdbase.org), and we therefore decided to examine the transcription of *ptgs2*,

cpt1a and *elovl5* in this study. The proteins encoded by these genes are involved in a number of cellular mechanisms. Analyzing the data through the use of IPA (Ingenuity® Systems, www.ingenuity.com), with the 5 regulated transcripts as input, it is only possible to conclude that diflubenzuron mediated a biased fatty acid metabolism ($p = 1.38 \times 10^5$, activation score –0.893). According to the PCA (Fig. 5B), these 3 transcripts showed a co-varied transcription, but appear to be linked to the control fish. A possible explanation is that the carrier, also sham injected in the control fish, might have triggered an oxidative stress response in the dose–response fish (Božić et al. 2003) that was decreased by diflubenzuron, as discussed above.

In conclusion, our data suggest that diflubenzuron has low toxicity on Atlantic cod at the molecular level, even at concentrations well above environmental levels observed around Atlantic salmon fish farms during salmon lice treatment.

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