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Time and dose-dependent impairment of liver metabolism in *Gasterosteus aculeatus* following exposure to diclofenac (DCF) highlighted by LC-HRMS untargeted metabolomics

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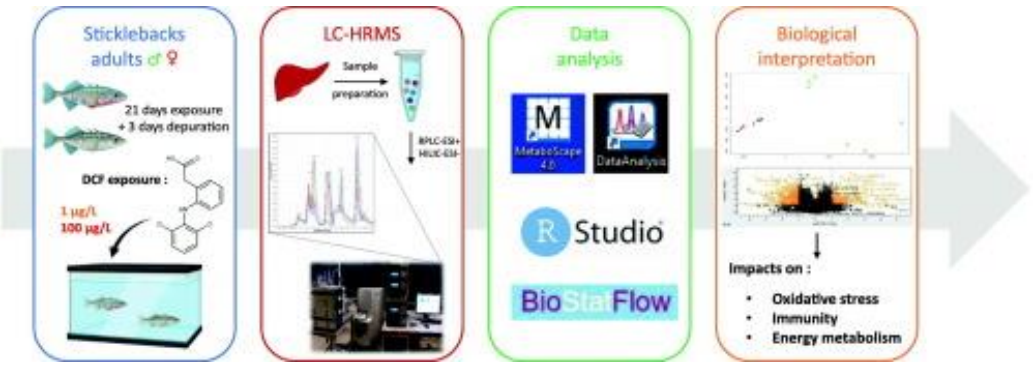
Graphical abstract



Abstract

Anthropogenic chemicals as emerging contaminants, such as pharmaceuticals, increased worldwide in the environment. This study aimed to apply metabolomics-based approaches on the fish model species three-spined stickleback (*Gasterosteus aculeatus*) exposed to diclofenac (DCF) to identify toxicity pathways and potential biomarkers. For this purpose, males and females were exposed to a continuous flow of diclofenac solution in laboratory for 21 days, followed by 3 days of depuration, to nominal concentrations of 1 (low) and 100 µg/L (high) of DCF. A methodology based on liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) was employed. Uni- and multivariate statistical analyses were combined to evaluate the modulations of the liver metabolome of *G. aculeatus* after exposure to DCF. The metabolomics data revealed variations both as a function of time and of the DCF concentration. We observed 2487 altered metabolites, with 1460 and 1027 specific to males and females, respectively. Some of them were significantly impaired by the experimental conditions. However, we showed that several metabolites were impacted by other factors as they were already modulated in the control individuals. The results indicated that the energy metabolism was up-modulated in females and down-modulated in males, with the presence of DCF. The antioxidant system was impacted in males, suggesting oxidative stress in the metabolism, while the immunity system was down-regulated in females following exposure. Moreover, our results revealed 1 and 4 metabolites as potential metabolic biomarkers in male and female sticklebacks, respectively. Among them, the glutaryl-carnitine and the adipoyl-carnitine were putatively identified in females, known to be implicated in the energy metabolism. These 5 metabolites showed to be promising biomarkers since they were early modulated during exposure to the stress and showed a notable trend through time.

Keywords: three-spined stickleback; omics; biomarker; pharmaceuticals; NSAID; gender

Highlights

• Metabololomics was applied to study diclofenac effects on sticklebacks.

• Diclofenac induces oxidative stress and immune system impairment.

• Diclofenac results in modulation of energy metabolism.

• Males and females exhibit sex-dependent metabolism’s responses.

• The glutaryl- and the adipoyl-carnitines present a potential interest as biomarker.

1. Introduction

Numerous studies have demonstrated how anthropogenic pollution, such as those related to agricultural activities, industrial effluents and urbanization can impair aquatic environments (Amoatey and Baawain, 2019; Kümmerer, 2009). Human pharmaceuticals, including their metabolites, mainly enter surface waters, rivers, and lakes by releasing treated effluents from municipal sewage treatment plants (Ebele et al., 2017; Giri and Pal, 2014). These products can lead to serious environmental threats to the resident biota. Despite their ability to be more or less degraded by natural processes in the environment, pharmaceutical compounds are considered as pseudo-persistent since they are continually released at low concentrations into the water bodies (López-Pacheco et al., 2019). Consequently, these contaminants can be found in the aquatic environment with concentrations ranging from ng/L to µg/L (Kümmerer, 2009; Miller et al., 2018). Among the diverse and extensive group of human pharmaceuticals, it is known that the non-steroidal anti-inflammatory drugs (NSAIDs), and especially diclofenac (DCF), are one of the most commonly found within the aquatic environment in receiving waters throughout the western world (Sathishkumar et al., 2020). DCF is prescribed for oral use such as DicloCare (Advacare Pharma) or available as an ointment for local application such as Flector® (Laboratories Genevrier) without a prescription to both humans and livestock to treat various acute or chronic pain and inflammatory conditions, with a global annual consumption of up to 1000 tons (Näslund et al., 2017). It is thus frequently detected in treated wastewaters, streams, rivers, lakes, and even drinking water (Sathishkumar et al., 2020), with maximal concentrations in freshwater reported as high as the µg/L range, such as in Nigeria with a concentration up to 57.16 µg/L (Sathishkumar et al., 2020). DCF has known effects in aquatic non-target fishes at µg/L concentrations such as oxidative stress, cytological and histopathological effects, mortality, or effects on the immune system (Hoeger et al., 2005; Islas-

Flores et al., 2013; Joachim et al., 2021; Näslund et al., 2017; Schwaiger et al., 2004; Schwarz et al., 2017; Triebskorn et al., 2007, 2004).

The three-spined stickleback (*Gasterosteus aculeatus*) is commonly used as a model species in ecotoxicology tests as it is sensitive to a range of different toxicants (Kemble et al., 2013; Pottinger et al., 2011; Van Geest et al., 2014), abundant, and easy to manipulate under laboratory and *in situ* conditions (Catteau et al., 2021; Hani et al., 2018). For example, Joachim et al. (2021) reported effects of DCF on its survival rate and the immune system with traditional endpoints. They highlighted a perturbation of the final population structure, with a decrease of the total abundance of fish and the percentage of juveniles. Näslund et al. (2017) also observed that DCF exposure affects kidney histology of the stickleback also with traditional endpoints. Although the literature has highlighted the development of biomarkers related to various physiological functions such as reproduction, immunity, or oxidative stress (Catteau et al., 2021; Sanchez et al., 2008), discrepancies are observed, reflecting the limitations of the current understanding of the effects of DCF. Indeed, the widely used ecotoxicological tools promoted by research communities for stress evaluation in organisms (genotoxicity tests, histology…), cannot comprehensively picture biological effects upon exposure to stressors, due to their unidimensional nature. Recently, omics approaches, combining biological approaches and modern analytical techniques, have become a promising opportunity to overcome such limitations and provide reliable information of the different biological processes (Brockmeier et al., 2017). Among them, metabolomics is applied in ecotoxicology to measure metabolites (molecular weight < 1000 Da) involved in various biological functions (Pomfret et al., 2019). The production and level of these molecules can be affected by physiological factors or the environment (Pomfret et al., 2019).

The present study uses untargeted metabolomics analyses by LC-HRMS to assess the effects of DCF on *G. aculeatus*. Individuals were exposed for 21 days in laboratory conditions to

relevant environmental concentrations of DCF followed by 3 days of depuration. The DCF exposed fish were compared to unexposed ones. The aim was to investigate whether DCF alters the metabolic profile of the three-spined stickleback's liver at environmental doses and different exposure and depuration times. A secondary goal was also to evaluate the biochemical response between males and females post-exposure.

2. Materials and methods

2.1. Chemicals and reagents

Analytical grade diclofenac sodium salt was supplied by Interchim (Montluçon, France). UPLC-MS grade acetonitrile (ACN), methanol (MeOH) (purity ≥ 99%) and ethyl 3-aminobenzoate methanosulfonate, dimethylsulfoxide (DMSO) (purity ≥ 99.7%) were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Heptane (purity ≥ 90%) was supplied by Merck-Millipore (Saint-Quentin en Yvelines, France). HPLC water was ordered by Fisher (Illkirch, France). Labeled (^{15}N, ^{13}C, D) analytical standards (Table S1) (purity ≥ 97%) were purchased from Sigma-Aldrich or CDN isotopes (Quebec, Canada). Stock solutions of individual standards at concentrations of 0.2 mg/mL were prepared in ACN, ACN/H_2O, DMSO, or MeOH according to their solubility, and stored at -18°C until use. Working standard mixtures were prepared monthly by diluting the stock solutions with ACN.

2.2. Three-spined stickleback maintenance, exposure, sampling and measurements

2.2.1. Fish maintenance

A total of 240 adults (120 males and 120 females) were used. Individuals were collected from the INERIS (French National Institute of Industrial Environment and Risks) husbandry located in Verneuil-en-Halatte (France), in which the population is maintained in an outdoor pool with natural vegetation and macro-invertebrate communities. The fish were collected in Autumn 2019 and were first placed during 7 days into aquaria in a circulating freshwater system for

acclimatization set at 16 ± 1°C with pH at 7.6, at a day/night cycle of 10h/14h to keep them in an inactive reproductive condition. Food (frozen commercial blood worms, 3% of the fish body weight/day, Ocean nutrition, Belgium) was provided once a day (Leloutre et al., 2018). All experiments were conducted in accordance with the European directive 2010/63/UE on the protection of animals used for scientific purposes at INERIS facilities (registration number E60-769-02). Furthermore, the Ethical Committee of INERIS approved all processes.

2.2.2. Fish exposure design and parameters

A flow-through exposure was carried out over 21 days, followed by 3 days of depuration, using the following nominal concentrations: 0 µg/L (control), 1 µg/L (low) or 100 µg/L (high). Fish were exposed into 24 aquaria divided into 2 identical benches containing each 6 aquaria for controls, 3 aquaria for 1 µg/L and for 100 µg/L exposures (Figure S1). Ten organisms (5 males and 5 females) were randomly placed in each 10 L aquarium. All aquaria were visually isolated by placing grey opaque plastic plates and pictures of aquatic decoration on all sides to avoid visual contact between fish from different aquaria (stress named "mirror induced aggression"). A piece of PVC was also placed in each aquarium as a hiding place to limit potential aggressivity.

Aquaria were daily controlled for survival, aeration maintenance, and waste cleanup. A scoring list covering a wide range of clinical aspects was used to provide an overview of the health status of animals over time. Thresholds of severity were used for fish presenting clinical signs to end test procedure earlier in order to avoid pain or prolonged distress. The temperature (16 ± 0.2 °C), the dissolved oxygen (8.3 ± 0.2 mg/L), the pH (7 ± 0.1), and the conductivity (441 ± 34 µS/cm) were stable along the experiment. Exposure concentrations were measured every 3 days in the aquarium water. Mean values were 0.4 µg/L ± 0.1 and 77.8 ± 5.4 µg/L for both low and high doses of DCF. There was no contamination of unexposed aquaria.

2.2.3. Fish sampling and biometric measurements

At each sampling date (0, 3, 7, 21, and 24 days), 40 fish were anesthetized using ethyl 3-aminobenzoate methanesulfonate (100 mg/L, Tricaine Pharma, Overhalla, Norway) to avoid handling stress and then killed by cervical dislocation. Each fish was weighed, measured and sexed to determine Fulton's condition index (K, total body weight/standard length^3, Gabriel et al., 2010). The livers were sampled and weighed to determine the somatic liver index (SLI, 100 × liver weight/total body weight, Slooff et al., 1983). K indicates the reproduction ability of the fish while SLI indicates their physiological state. The collected livers were pooled by sex, by time, and by treatment, freeze-dried and stored at -80°C until analysis. For each condition, the remaining organs (gills, brain, kidney…) were sampled, stored at -80°C and used in another study with other approaches.

2.2.4. Sample preparation for DCF accumulation and metabolomics analysis

Each pool was homogenized by vertical agitation (2 min, 1,000 rpm) with a Genogrinder® SPEX SamplePrep® (Metuchen, USA) in polypropylene centrifuge tubes (50 mL) containing steel beads (3 mm, Retsch, Germany). Depending on the mass available for each pool, 1 to 3 aliquots of 45.0 ± 0.2 mg dry weight (dw) were weighed in a 5 mL tube. Each sample was fortified with a spiking mixture of labeled internal standards (Table S1) prepared in ACN, then a solid-liquid extraction was performed with water, MeOH, and heptane as described in our previous study (Lebeau-Roche et al., 2021). Two aliquots of each final extract were transferred into two 2 mL tubes then dried under a slight nitrogen flow at 35°C. The dry extracts were reconstituted in 100 µL of ACN/H_2O (10:90, v/v) or 100 µL of ACN/H_2O (95:5, v/v) for reversed-phase liquid chromatography (RPLC)-HRMS and hydrophilic interaction liquid chromatography (HILIC)-HRMS analyses, respectively. These extracts were used for both metabolomics analysis and assessment of DCF accumulation. Extraction blanks, corresponding

to extracts without matrix, were also prepared to check for the presence of contaminants throughout the analytical process. Moreover, to ensure the reliability of the results, a quality control sample (QC) was prepared for each analytical condition (RPLC- or HILIC-based) by pooling 15 µL of all the sample extracts.

2.2.4.1. Quantification of DCF and some of its metabolites in fish liver

For accumulation assays, the concentrations of DCF and of 4 of its primary metabolites (4'-hydroxy DCF (4'-OH DCF), DCF carboxylic acid (DCF COOH), 2-oxindole and DCF glucuronate) previously detected in fish were evaluated as previously described (Daniele et al., 2016). Briefly, the extracts were vortexed (10 s), centrifuged (10,000 g, 3 min) and 300 µL of supernatant was sampled, evaporated to dryness, and finally reconstituted in 60 µL of H_2O/MeOH (90:10, v/v).

2.2.4.2. LC-HRMS analysis of fish liver

LC-HRMS analyses were performed on an Ultimate 3000 UHPLC system (Thermo Scientific®, MA, USA) coupled to a quadrupole time-of-flight mass spectrometer (QToF) (Maxis Plus, Bruker Daltonics®, Bremen, Germany) equipped with an electrospray ionization interface (ESI). The LC separations were carried out on 2 chromatographic columns. HILIC acquisitions were performed with a Nucleodur HILIC-column (100 mm x 2 mm; 3 µm) from Macherey-Nagel (Hoerdt, France) in negative ionization mode and RPLC acquisitions with an Acquity UPLC® HSS T3 C18-column (100 mm x 2.1 mm; 1.8 µm) in positive ionization mode. The analysis conditions are described in Lebeau-Roche et al. (2021). QCs were run several times at the beginning of the analytical sequence to equilibrate the column and at regular intervals throughout the sequence to check for instrument drift and to control analytical repeatability and sensitivity. Instrument control, data acquisition, and processing were performed using OTOF Control and HystarTM (v4.1, Bruker Daltonics®) softwares. All the

samples were randomly analyzed in full-MS scan over a *m/z* range of 50-1200 Da with a quadrupole energy of 5 eV. QCs were also analyzed in auto MS/MS mode with a collision energy ranging from 7 to 65 eV depending on the mass to obtain fragments information for annotation.

2.3. Statistical analyses and data treatment

2.3.1. Biometric parameters and concentration of DCF and some of its metabolites in fish liver

The variability of morphological parameters (weight, length, K, SLI) and livers' accumulation of DCF and its 4 metabolites were evaluated by analysis of variance (ANOVA) across the different conditions of exposure (time and concentration) using Excel software 2016. If the p-value was significant (< 0.05) and the homogeneity of variance (Fisher's test) and the normal distribution (Shapiro's test) were verified, then a Tukey test was further applied to confirm the discrimination of the values.

2.3.2. LC-HRMS data processing

Statistical analyses of metabolomics data obtained in sticklebacks' liver were performed using Metaboscape (v4.0, from Bruker Daltonics®), R software (v4.1.1) and the BioStatFlow platform (v2.9.2; http://biostatflow.org, a website developed for statistical omics data analyses using R scripts).

The data were preprocessed (peak picking or peak detection, peak grouping and peak alignment) using Metaboscape to generate a buckets table for each sequence containing all the information for 1 bucket i.e. the mass to charge ratio *m/z* of each detected peak, the retention time R_T, the intensity in each sample and the adduct forms.

The generated data were then normalized with the labeled internal standard methionine-d3. This standard was chosen since it was very reproducible during all datasets' analyses (R_T and intensity). After normalization, a matrix cleanup was performed based on the coefficient of variation in the QCs (CVQC) and the dispersion-ratio (d-ratio) calculated for each signal with the following formula (with SD: Standard Deviation):

$$d - ratio\ (\%) = \frac{SD(QC)}{SD(samples)} x\ 100\%$$

It compared the technical variation measured in replicated QCs to the biological variation (Broadhurst et al., 2018). Thus, a feature was discarded from the dataset when it was present in the matrix blanks, or with a CVQC over 30% or a d-ratio over 50%.

Then, uni- and multivariate statistical analyses were performed with the BioStatFlow platform. Missing intensities in a replicate were replaced by the mean intensities in the corresponding group. A descriptive unsupervised Principal Component Analysis (PCA) was first performed with Pareto scaling to observe any differences in the metabolic profiles of the studied conditions. Then, a mean comparison test (Student's test) was implemented to identify the metabolites responsible of the observed discriminations between the experimental groups on the score plots. Features showing abundance differences with a FDR adjusted p-value < 0.05 and a fold change (FC, ratio of the intensity means for a feature) ≤ 0.5 or ≥ 2 were considered significantly different. Visual inspection of the extracted ion chromatograms of raw data for all buckets reported as significantly different was then performed with R to ensure that each integrated signal at a defined couple *m/z* and R_T corresponded to a metabolite presenting a Gaussian chromatographic peak (i.e. not a baseline drift or background noise). If not, features were discarded from the dataset.

2.4. Metabolite annotation and identification strategy

Metabolite annotation and identification were performed using Metaboscape (v4.0, from Bruker Daltonics®) and DataAnalysis (v4.3).

To elucidate the chemical structure of the discriminant features, Metaboscape provides various tools helping the annotation based on the MS/MS data: (i) SmartFormula, providing raw formula of the compounds according to their exact mass and their isotopic signature; (ii) Compound Crawler, searching corresponding molecular structures into available online databases including Kyoto Encyclopedia of Genes and Genomes (KEGG), Human Metabolome Database (HMDB), Chemical Entities of Biological Interest (ChEBI); (iii) MetFrag, corresponding to *in silico* fragmentation and comparing its suggested fragments of a given *m/z* to the list of fragments obtained with DataAnalysis (Wolf et al., 2010), while indicating the 2 main fragments. This comparison of the fragments helps the putative identification by reducing the list of possible molecules. Finally, after obtaining potential identification, we eliminated the biological inconsistencies (e.g. names of antibiotics, chemical products). We putatively identified metabolites according to the level of identification confidence from 1 to 5 (Schymanski et al., 2015).

3. Results and discussion

3.1. Biometric measurements and quantification of DCF and some of its metabolites in fish livers

We did not observed any significant differences in mortality rate or physiological parameters (weight, length, K, SLI) between conditions for both genders (Table S2). Similar results were reported by Näslund et al. (2017) with no impact on three-spined sticklebacks weight and length after 28 days of exposure to DCF at concentrations from 4.6 to 271 µg/L.

Among the studied compounds, DCF and its metabolite 4'-OH DCF were quantified in water and in fish liver along the 24 days of experiment (Table 1), the other metabolites being under

the limit of detection in liver (1.0, 50.0 and 100.0 for DCF COOH, 2-oxindole and DCF glucuronate, respectively). The 4'-OH DCF detected in the aquaria is supposed to come from the photodegradation of DCF, as already observed in previous experiments (Qin et al., 2012; Schmitt-Jansen et al., 2007).

In males, no significant accumulation of DCF was observed for the lowest dose of exposure whereas for the highest dose an accumulation of DCF was observed over time after 7 days of exposure (75.8 ± 2.0 ng/g) with a p-value = 0.0005. The 4'-OH DCF was quantified at significant levels both for low and high doses exposures, after 7 days (0.5 ± 0.4 ng/g and 21.5 ± 1.7 ng/g) with p-values of 0.0093 and 0.0139.

For females, a significant accumulation of DCF was observed in the liver after 7 (0.5 ± 0.1 ng/g) and 3 days (31.9 ± 2.3 ng/g) of exposure at low and high doses with p-values of 0.0292 and 0.00003, respectively. The 4'-OH DCF was also quantified at a significant level after 3 days of exposure (71.8 ± 11.3 ng/g) at the highest dose with a p-value = 0.00008. The internal concentration of 4'-OH DCF was not significant during the exposure at the lowest dose.

Our results are in line with the literature since the uptake and metabolization of DCF in stickleback was already demonstrated during a chronic exposure with the presence of DCF and the phase I metabolite 4'-OH DCF in whole fish exposed 6 months in mesocosms (Daniele et al., 2016). The concentration of DCF was under the limit of quantification while 4'-OH DCF was detected at concentrations from 1.4 to 7.7 ng/g in sticklebacks exposed to a concentration of 4.1 µg/L of DCF. However, in some studies such as Schwaiger et al. (2004), higher internal concentrations of DCF have also been shown after 28 days of exposure to similar doses in rainbow trout liver (*Oncorhynchus mykiss*) with 2,800 and 5,500 ng/g (reported in wet weight and not dry as in our study) for 1 and 100 µg/L, respectively.

On the other hand, our results showed a sex-specific ability to uptake or metabolize the DCF since the internal concentrations of DCF and 4'-OH DCF were different during the experiment between males and females, especially when exposed to the highest dose. Indeed, at the highest dose, DCF was more accumulated in males (60.2-132.4 ng/g) than in females (23.1-38.7 ng/g) whatever the exposure time. On the contrary, after exposure at the highest dose 4'-OH DCF was detected in higher content in females (34.0-71.8 ng/g) than in males (19.9-21.5 ng/g) at all times except 21 days of exposure (males 122.9 ± 33.1 ng/g *vs* females 63.9 ± 3.1 ng/g). This observation agrees with the literature since it was already demonstrated that sex could influence the ability to accumulate and transform compounds in fish (Burger, 2007; Burger et al., 2004). Moreover, the metabolization process and the defence system induce a high energy consumption in an organism (Sokolova, 2021). Therefore, these measured differences could induce different impacts on the energy metabolism between genders. In addition, our results highlighted that the internal concentrations of DCF and 4'-OH DCF in fish liver were readily and efficiently eliminated both in males and females, after 3 days of depuration (Meredith-Williams et al., 2012; Nkoom et al., 2020).

Table 1. Measured concentrations of diclofenac (DCF) and 4'-hydroxy diclofenac (4'-OH DCF) in water (µg/L) for nominal doses of 1 and 100 µg/L DCF and in fish liver (ng/g, dw). Data are represented as mean ± SD; dw: dry weight. LOQ DCF (water) = 0.025 µg/L, LOQ 4'-OH DCF (water) = 0.02 µg/L, LOQ DCF and 4'-OH DCF (liver) = 0.5 ng/g. LOQs were determined as the concentrations that would result in a signal to noise ratio higher than 10.

		Exposure at 1 µg/L				**Exposure at 100 µg/L**			
		Exposure			**Depuration**	**Exposure**			**Depuration**
Diclofenac	**Water (µg/L)**	0.4 ± 0.1			< LOQ	77.8 ± 5.4			< LOQ
	Time (days)	D3	D7	D21	D24	D3	D7	D21	D24
	Males' liver (ng/g)	< LOQ (*n = 3*)	0.7 ± 0.6 (*n = 3*)	1.6 ± 0.2 (*n = 2*)	< LOQ (n = 3)	60.2 (n = 1)	75.8 ± 2.0 (n = 3)	132.4 ± 8.5 (n = 3)	0.6 ± 1.0 (n = 3)
	Females' liver (ng/g)	< LOQ (*n = 3*)	LOQ (*n = 3*)	< LOQ (*n = 3*)	< LOQ (*n = 3*)	31.9 ± 2.3 (*n = 3*)	23.1 ± 2.6 (*n = 3*)	38.7 ± 2.1 (*n = 3*)	< LOQ (*n = 3*)
4'-hydroxy diclofenac	**Water (µg/L)**	0.03 ± 0.01			< LOQ	0.4 ±0.1			< LOQ
	Time (days)	D3	D7	D21	D24	D3	D7	D21	D24
	Males' liver (ng/g)	< LOQ (*n = 3*)	LOQ (*n = 3*)	0.8 ± 0.0 (*n = 2*)	< LOQ (*n = 3*)	19.9 (*n = 1*)	21.5 ± 1.7 (*n = 3*)	122.9 ± 33.1 (*n = 3*)	1.4 ± 0.1 (*n = 3*)
	Females' liver (ng/g)	< LOQ (*n = 3*)	< LOQ (*n = 3*)	< LOQ (*n = 3*)	< LOQ (*n = 3*)	71.8 ± 11.3 (*n = 3*)	34.0 ± 7.2 (*n = 3*)	63.9 ± 3.1 (*n = 3*)	< LOQ (*n = 3*)

3.2. Responses of the sticklebacks' liver metabolome

3.2.1. Distribution of the samples according to the exposure conditions in both sexes

Before any statistical filter, 10059 and 1949 features were initially detected in males, while 10923 and 2062 were detected in females, both with RPLC and HILIC conditions, respectively.

To provide an overview for both genders, an unsupervised PCA was initially performed considering all exposure conditions, including the 3 concentrations of DCF (control, low and high doses) and all times of exposure (3, 7, and 21 days) and depuration (24 days). The resulting score plots PC1 *vs* PC2 obtained in both RPLC and HILIC are respectively presented in Figure 1A for males on C18-column, (Figure S2 on HILIC-column), and Figure 1B for females on C18-column, (Figure S3 on HILIC-column). First of all, the tightly clustering of the QCs confirmed the quality of the data in terms of analytical repeatability. Secondly, for both genders the replicates are relatively clustered but no clear separation was observed according to the treatments.

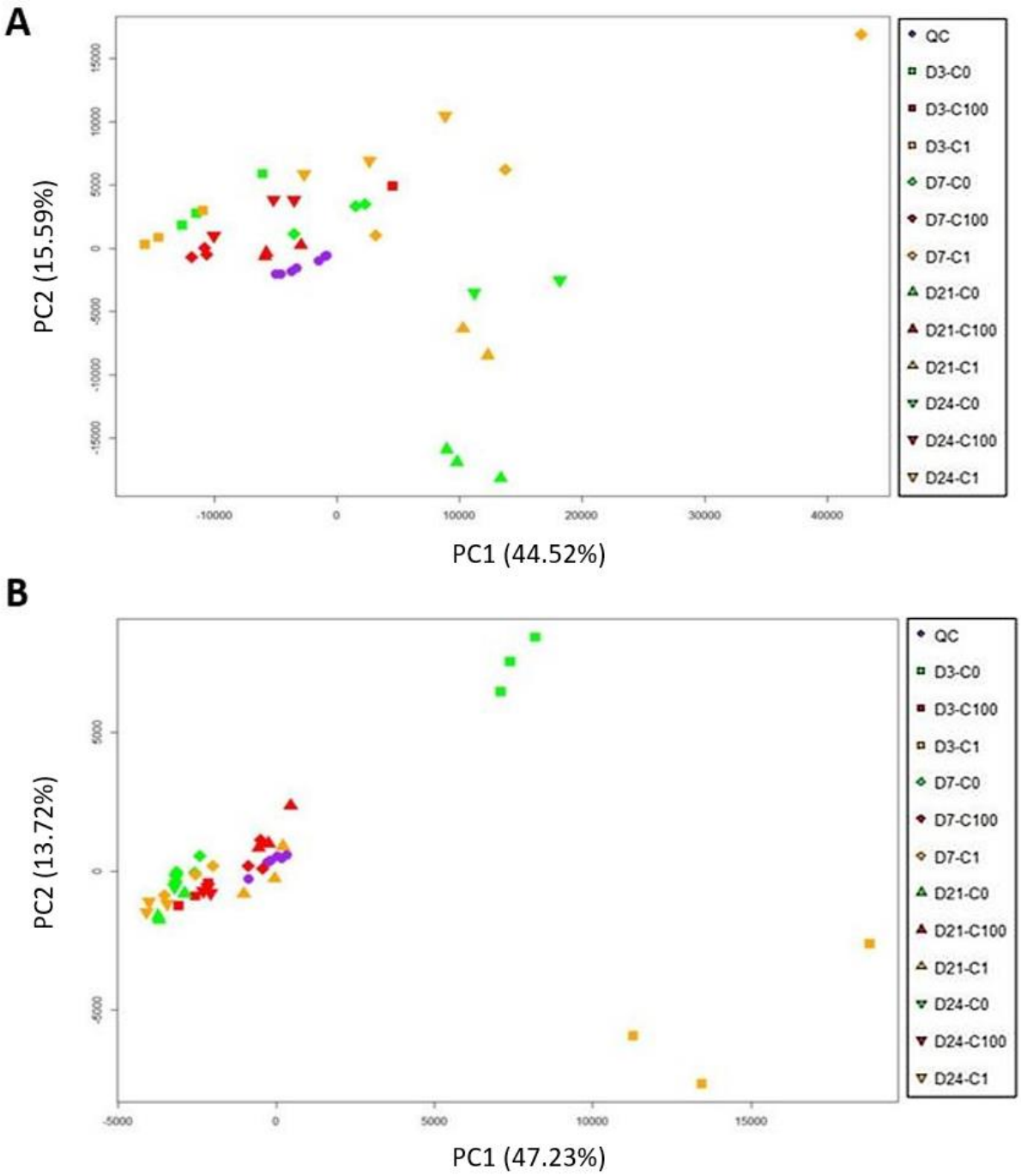


Figure 1. Principal Component Analysis score plots of (A) male and (B) female fish (LC-HRMS performed on the C18-column with electrospray ionisation in positive mode) according to the dose and time of exposure. C0 : controls, C1 : low concentration, C100 : high concentration; Exposure : D3 : 3 days, D7 : 7 days, D21 : 21 days ; depuration : D24 : 24 days (corresponding to 3 days of depuration); QC : control quality.

However, a clear discrimination is observed according to the exposure concentration when we consider each sampling date separately (Figure 2). Consequently, the exposure concentrations were considered at each time of exposure and depuration for both genders and the exposed individuals were compared to the controls as reference (C0 at each time). In this case, the metabolomics profiles differed among concentrations at each date, indicating that the DCF exposure influenced the sticklebacks' metabolic state, for both males and females (Figures 2). The same PCA score plots were obtained for the other dates on the C18-column and also on the HILIC-column. These plots are presented in supplementary information (Figures S4-S17).

The first 2 dimensions expressed between 69 and 82% of the total variance for males, mainly explained by PC1 (from 47 to 69%). The first 2 dimensions expressed between 61 and 79% of the total variance for females, mainly explained by PC1 (from 38 to 56%).

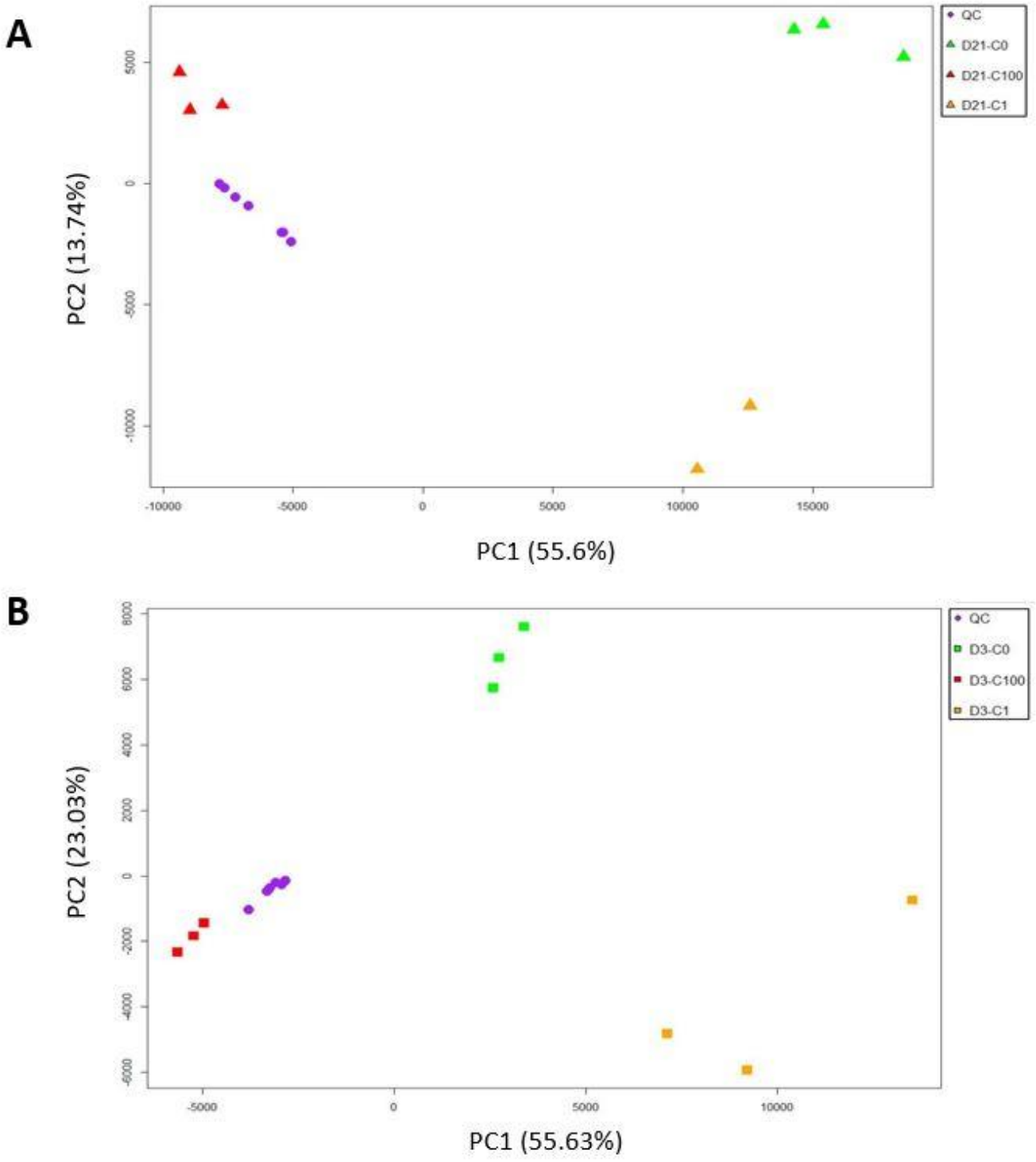


Figure 2. Principal Component Analysis score plots of (A) male fish after 21 days of exposure and (B) female fish after 3 days of exposure (LC-HRMS performed on the C18-colmun with electrospray ionisation in positive mode). C0 : controls, C1 : low concentration, C100 : high concentration; QC : control quality.

However, it is important to note that the temporal natural evolution of the sticklebacks metabolome over time could influence the results. This is particularly apparent for control organisms (C0) that were not exposed to DCF (Figure 3). These results suggest that the potential impact of DCF exposure may be partly masked by a natural evolution of the metabolome over time. It is therefore necessary to consider this natural variation over time to interpret the effects of DCF exposure on sticklebacks (Marie, 2020; Wei et al., 2018).

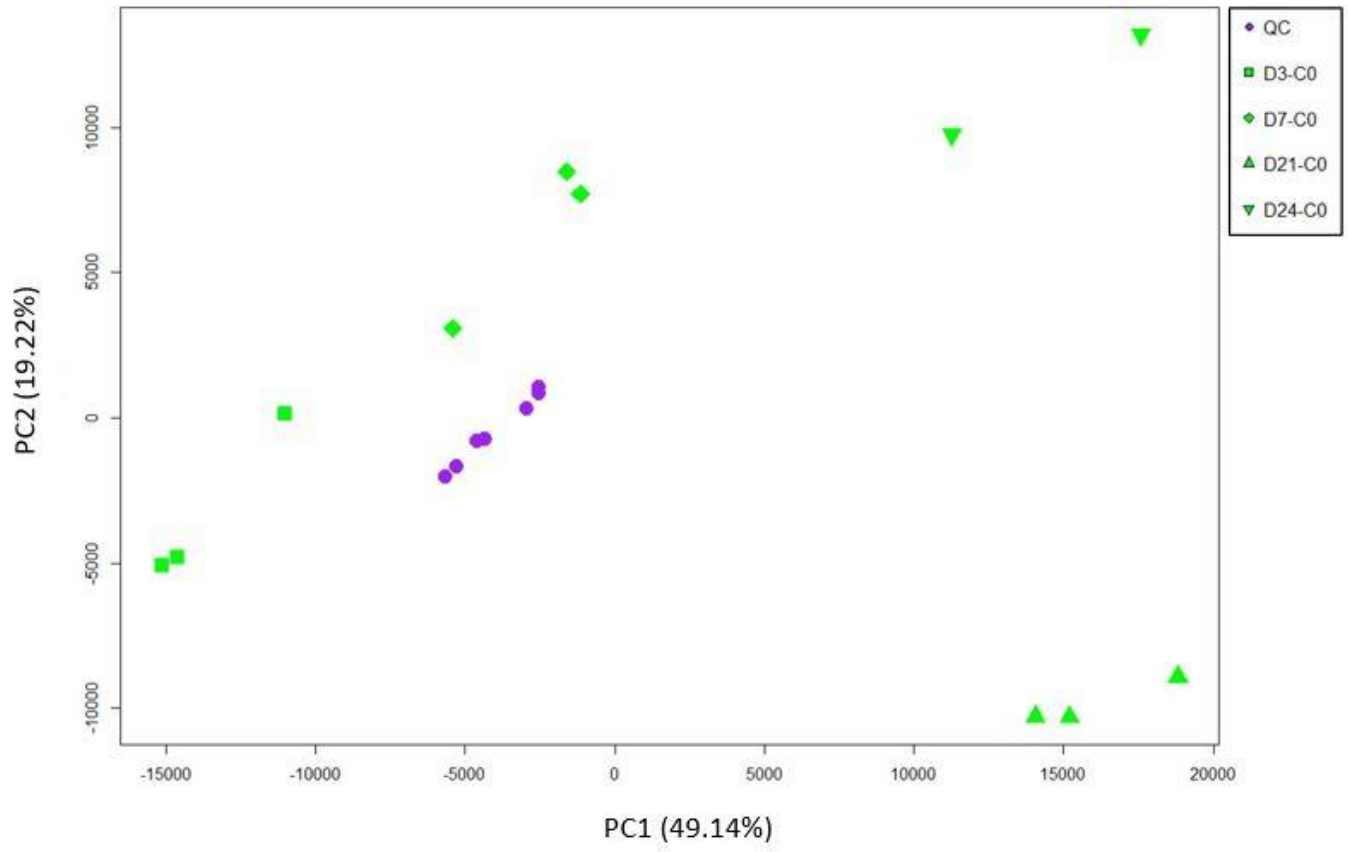


Figure 3. Principal Component Analysis score plots of unexposed male fish (controls) (LC-HRMS performed on the C18-column with electrospray ionisation in positive mode). Exposure : D3 : 3 days, D7 : 7 days, D21 : 21 days ; depuration : D24 : 24 days (corresponding to 3 days of depuration) ; QC : control quality.

3.2.2. Modulated metabolites during the exposure

The Student's t-test confirmed the differences in metabolomic profiles in the controls and between the treatments. It was used to identify the features that were significantly modulated in the controls and in the exposed individuals at each time of exposure and depuration. The evolution through time of each metabolite of interest was then studied. After the application of the following filters: CVQC < 30%, d-ratio < 50%, absence in the matrix blanks, gaussian chromatographic peak, 6865 and 1185 signals were highlighted in males, and 1760 and 395 in females, both with C18 and HILIC conditions, respectively. Among the features detected with both C18 and HILIC columns, 2487 were differentially expressed in exposed compared to controls males and females individuals with 1217 and 243 features for males and 1023 and 4 features for females, for each column respectively. Thus, a more significant number of discriminant features was reported in total (RPLC and HILIC conditions) in males than in females, with 1460 and 1027, respectively. The significance was established when a FC ≤ 0.5 or ≥ 2 and a p-value corrected by False Discovery Rate (FDR) < 0.05 were recorded.

For all these discriminant signals, when MS/MS data were available, DataAnalysis was used to obtain a list of fragments. Then the databases were queried to identify the corresponding compound of interest. Because databases are incremented with spectra originating from different separation and detection systems (chromatographic columns, mass spectrometers, collision energies…), we only assigned 30 putative compounds in males and females including 19 features with one name proposition (18 metabolites at level 2 and 1 metabolite at level 3 of the identification confidence) and 11 features assigned to several possible names (level 3 of the identification confidence) (Table 2).

The signaling pathway of 10 putative molecules (level of the identification confidence 2) were identified, including metabolisms of arginine/proline, methionine/cysteine, tyrosine, purines, pyrimidine, and pyridoxine. In these biological pathways, 6 other metabolites were also identified (level of the identification confidence 2 to 3) among the features (homoserine, hypoxanthine, adenine, glutamate/methyl aspartate, valine/amino pentanoate, uracil), but they were not significantly impaired during the experiment (Table S3).

Table 2. Annotated metabolites modulated in males and females three-spined sticklebacks exposed at 1 and 100 µg/L of DCF for 21 days followed by 3 days of depuration.

[1]FC : Fold Change was considered as a significant change if ≤ 0.5 or ≥ 2

[2]p-value was corrected by False Discovery Rate (FDR) and considered as significantly different if < 0.05

Metabolism pathway	Level of the identification confidence	Annotation/Identification	Sex	*m/z*	Error mass (ppm)	R_T (min)	Time	Comparison	Trend ↗↘	Origin of discrimination	FC[1]	p-value[2]	Molecular ion species detected
Cysteine and methionine metabolism	Level 2	Methionine	♂	150.0584	3.33	1.40	J21	C100 C0	↘	C0 > C100	0.40	0.01	$[M+H]^+$
								C100 C1		C1 > C100	0.44	0.025	
	Level 2	Methionine-sulfoxide		166.0534	2.41	0.86	J21	C100 C0	↘	C0 > C100	0.35	0.010	$[M+H]^+$ $[M+Na]^+$
	Level 2	Ophtalmate		290.1350	< 1	2.04	J7	C100 C0	↘	C0 > C100	0.41	0.014	$[M+H]^+$ $[M+Na]^+$
							J21		↗	C100 > C0	4.00	0.016	
Arginine and proline metabolism	Level 2	Arginine	♀	175.1191	2.28	0.77	J24	C100 C0	↘	C0 > C100	0.44	0.047	$[M+H]^+$
Pyrimidine metabolism	Level 2	Cytidine	♂	244.0930	1.23	1.36	J21	C100 C0	↘	C0 > C100	0.34	0.012	$[M+H]^+$
Pyridoxin metabolism	Level 2	Pyridoxine	♂	168.0666	2.98	8.12	J21	C100 C0	↘	C0 > C100	0.25	0.032	$[M-H]^-$ $[M-H-CO_2]^-$
Purin metabolism	Level 2	Xanthine	♂	153.0408	3.27	5.21	J21	C100 C0	↘	C0 > C100	0.22	0.034	$[M+H]^+$ $[M+Na]^+$
	Level 2	Deoxyinosine	♂	251.0780	< 1	2.90	J24	C100 C0	↘	C0 > C100	0.40	0.027	$[M-H]^-$
			♀	251.0780		2.89	J21	C100 C0	↗	C0 < C100	2.86	0.006	
Tyrosine metabolism	Level 2	Norepinephrine	♂	170.0814	1.76	5.01	J21	C100 C0	↘	C0 > C100	0.32	0.033	$[M+H]^+$ $[M+Na]^+$
Several propositions	Level 3	8 hydoxy deoxyguanosine ; guanosine ; croonoside ; adenosine 1-oxide ; isoguanosine	♂	284.0994	-	4.25	J7	C100 C0	↘	C0 > C100	0.49	0.015	$[M+H]^+$
	Level 3	Anthranilate ; amino-benzoate	♂	120.0446	-	7.64	J7	C100 C0	↘	C0 > C100	0.38	0.027	$[M+H]^+$ $[M-H_2O+H]^+$
	Level 3	Arachidonoyl-sn-glycero-phosphocholine ; lysoPC(20:4(5z,8z,11z,14z))	♂	544.3389	-	15.69	J7	C100 C0	↘	C0 > C100	0.48	0.032	$[M+H]^+$
	Level 3	lysoPC(0:0/18:0) ; lysoPC(18:0) ; O-acetyl-O-hexadecyl-sn-glycero-phosphocholine	♂	524.3708	-	16.47	J7	C100 C0	↘	C0 > C100	0.46	0.020	$[M+H]^+$ $[M+Na]^+$
	Level 3	Docosapentaenoyl-sn-glycero-phosphocholine ;	♂	570.3551	-	15.86	J7	C100 C0	↘	C0 > C100	0.41	0.035	$[M+H]^+$ $[M+K]^+$

		lysoPC(22:5(7z,10z,13z,16z,19z)) ; lysoPC(22:5(4z,7z,10z,13z,16z))											$[M+Na]^+$
	Level 3	Urocanic acid ; aminonicotinic acid	♂	139.0503	-	1.39	J21	C100 C0	↘	C0 > C100	0.28	0.013	$[M+H]^+$
	Level 3	AMP and derivatives	♂	348.0710	-	4.55	J21	C100 C0	↘	C0 > C100	0.18	0.016	$[M+H]^+$
	Level 3	> 10 propositions	♂	235.1692	-	13.72	J21	C100 C0	↘	C0 > C100	0.39	0.032	$[M+H]^+$
	Level 3	> 10 propositions	♂	277.2163	-	14.15	J24	C100 C0	↘	C0 > C100	0.38	0.045	$[M+H]^+$
	Level 3	Sphingosine ; dehydrosphinganine ; palmitoyl-ethanolamide	♂	300.2899	-	16.16	J24	C100 C0	↘	C0 > C100	0.32	0.032	$[M+H]^+$
	Level 3	γ-glutamyl-isoleucine ; γ-glutamyl-leucine ; γ-glutamine-leucine ; glutamine-leucine	♂	261.1449	-	6.46	J21	C100 C0	↘	C0 > C100	0.30	0.015	$[M+H]^+$ $[M+Na]^+$ $[M-H_2O+H]^+$
								C100 C1		C1 > C100	0.36	0.023	
	Level 3	Acetyl-ornithine. Hydroxy-methyl-amino methyl-pyrrolidine carboxylic acid	♀	175.1079	-	1.04	J3	C100 C1	↘	C1 > C100	0.12	0.022	$[M+H]^+$ $[M+Na]^+$ $[M-H_2O+H]^+$
								C1 C0	↗	C1 > C0	7.69	0.022	
Undefined	Level 2	N-decanoyl glycine	♂	230.1754	< 1	8.89	J7	C100 C0	↘	C0 > C100	0.43	0.024	$[M+H]^+$ $[M+Na]^+$
	Level 2	O-propionyl carnitine	♂	218.1390	< 1	3.01	J21	C100 C0	↗	C100 > C0	2.33	0.009	$[M+H]^+$ $[M+Na]^+$
	Level 2	Succinyl-adenosine	♂	384.1152	< 1	5.72	J21	C100 C0	↘	C0 > C100	0.25	0.001	$[M+H]^+$ $[M+Na]^+$
	Level 2	Onchidal	♂	277.1797	2.53	13.27	J7	C100 C0	↘	C0 > C100	0.49	0.027	$[M+H]^+$
							J21	C100 C0		C0 > C100	0.49	0.030	
	Level 2	N-oleoyl phenylalanine	♂	430.3315	1.39	17.26	J21	C100 C1	↘	C1 > C100 > C0	0.45	0.025	$[M+H]^+$ $[M+NH_4]^+$
	Level 2	Oleoyl-glycerol	♂	357.2999	1.68	16.41	J21	C100 C0	↘	C0 > C100	0.32	0.039	$[M+H]^+$
	Level 2	O-methyl-glutarylcarnitine	♂	290.1603	< 1	4.51	J21	C100 C0	↗	C100 > C0	2.63	0.009	$[M+H]^+$
								C100 C1		C100 > C1	4.76	0.047	
	Level 2	Glutaryl-carnitine	♂	276.1445	< 1	4.13	J7	C100 C0	↘	C0 > C100	0.43	0.029	$[M+H]^+$
			♀	276.1446	< 1	3.99	J7	C100 C1	↗	C100 > C1~C0	2.33	0.040	
							J24			C100 > C0 > C1	4.17	0.046	
	Level 2	Adipoyl-carnitine	♂	290.1603	< 1	5.39	J7	C100 C0	↘	C0 > C100	0.34	0.024	$[M+H]^+$
			♀			5.37	J21	C100 C0		C100 > C0	5.56	0.023	

					-		J24	C100 C1	↗	C100 > C0 > C1	2.22	0.032	
-	Level 5	Uncharacterized metabolite	♂	425.2036	-	5.18	J7	C100 C0	↘	C0 > C100	0.30	0.021	$[M+H]^+$
							J21	C100 C0		C0 > C100	0.36	0.012	
								C100 C1		C1 > C100	0.36	0.023	
			♀			5.12	J21	C100 C0	↗	C100 > C0	14.29	0.025	
								C1 C0		C1 > C0	10.00	0.050	
	Level 5	Uncharacterized metabolite	♂	220.0974	-	8.57	J21	C100 C0	↘	C0 > C100	0.22	0.014	$[M+H]^+$ $[M+Na]^+$
			♀	220.0971	-	8.51	J3	C100 C0	↘	C0 > C100	0.32	0.027	
								C100 C1		C1 > C100	0.11	0.030	
								C1 C0	↗	C1 > C0	3.03	0.045	
							J21	C100 C0	↗	C100 > C0	2.33	0.018	
								C1 C0		C1 > C0	2.50	0.025	
	Level 5	Uncharacterized metabolite	♂	172.0970	-	6.71	J21	C100 C0	↘	C0 > C100	0.28	0.019	$[M+H]^+$ $[M+Na]^+$
			♀	172.0972	-	6.65	J3	C100 C1	↘	C1 > C100	0.13	0.031	
								C1 C0	↗	C1 > C0	3.03	0.032	
							J21	C100 C0		C100 > C0	2.38	0.012	
								C1 C0		C1 > C0	2.94	0.014	
	Level 5	Uncharacterized metabolite	♂	175.0715	-	0.9	J7	C100 C0	↘	C0 > C100	0.49	0.031	$[M+H]^+$ $[M-H_2O+H]^+$
	Level 5	Uncharacterized metabolite	♂	171.0765	-	1.05	J7	C100 C0	↘	C0 > C100	0.24	0.030	$[M+H]^+$
							J21	C1 C0		C0 > C1	0.09	0.041	
	Level 5	Uncharacterized metabolite	♂	188.1284	-	3.43	J7	C100 C0	↘	C0 > C100	0.41	0.024	$[M+H]^+$
	Level 5	Uncharacterized metabolite	♂	186.1128	-	8.30	J7	C100 C0	↘	C0 > C100	0.48	0.024	$[M+H]^+$ $[M+Na]^+$
							J21			C0 > C100	0.25	0.017	
			♀			8.25	J3	C100 C0	↘	C0 > C100	0.32	0.028	
								C100 C1		C1 > C100	0.11	0.027	
								C1 C0	↗	C1 > C0	2.94	0.038	
							J21	C100 C0	↗	C100 > C0	2.38	0.015	
								C1 C0		C1 > C0	2.70	0.018	
	Level 5	Uncharacterized metabolite	♂	146.0602	-	5.56	J21	C100 C0	↘	C0 > C100	0.31	0.012	$[M+H]^+$
								C100 C1		C1 > C100	0.38	0.027	
			♀			5.52	J3	C100 C0	↘	C0 > C100	0.29	0.020	
							J21		↗	C100 < C0	2.08	0.021	

	Level 5	Uncharacterized metabolite	♂	328.1396	-	4.92	J7	C100 C0	↘	C0 > C100	0.45	0.023	$[M+H]^+$ $[M+Na]^+$ $[M-H_2O+H]^+$
	Level 5	Uncharacterized metabolite	♂	120.1021	-	0.77	J21	C100 C0	↗	C100 > C0	12.5	0.029	$[M+H]^+$
	Level 5	Uncharacterized metabolite	♂	278.1755	-	10.86	J7	C100 C0	↘	C0 > C100	0.44	0.015	$[M+H]^+$
	Level 5	Uncharacterized metabolite	♂	243.0768	-	9.14	J21	C100 C0	↗	C100 > C0	3.33	0.002	$[M+H]^+$
	Level 5	Uncharacterized metabolite	♂	133.0321	-	1.40	J21	C100 C0	↘	C0 > C100	0.38	0.010	$[M+H]^+$
								C100 C1		C1 > C100	0.41	0.025	
	Level 5	Uncharacterized metabolite	♂	205.0974	-	5.56	J21	C100 C0	↘	C0 > C100	0.32	0.010	$[M+H]^+$ $[M+Na]^+$
								C100 C1		C1 > C100	0.39	0.023	
	Level 5	Uncharacterized metabolite	♂	144.0658	-	3.79	J21	C100 C0	↘	C0 > C100	0.30	0.012	$[M+H]^+$ $[M+Na]^+$
			♀	144.0659	-	3.78	J3	C100 C1	↘	C1 > C100	0.24	0.021	
								C1 C0	↗	C1 > C0	4.00	0.016	
	Level 5	Uncharacterized metabolite	♂	237.2214	-	15.81	J21	C100 C0	↘	C0 > C100	0.34	0.004	$[M+H]^+$ $[M-H_2O+H]^+$

3.2.2.1. Modulated metabolites in the control sticklebacks

In unexposed males we putatively identified 15 molecules modulated over time (level 2 to 3 of the identification confidence) implicated in the neurotransmitters production and the nervous transmission (pyridoxin, norepinephrine, onchidal), the energy metabolism (acyl-carnitines, n-decenoyl glycine, oleoyl glycerol), the aging process (methionine sulfoxide), the anti-oxidant system (methionine), the DNA/RNA damages and the transmission of cellular messages (xanthine, cytidine, AMP and derivatives, succinyl-adenosine). On the other hand, in unexposed females we putatively identified 1 metabolite (level 2 of the identification confidence) modulated over time: the adipoyl-carnitine, which is implicated in the energy metabolism. The deoxyinosine which is an indicator of DNA damages was discriminated in both males and females (level 2 of the identification confidence).

A temporal evolution of metabolites involved in various physiological functions (energy metabolism, anti-oxidant system, hormones release…) due to other factors (age, sexual maturity…) than a chemical stress has already been observed in controlled laboratory conditions (Ivanisevic et al., 2016; Marie, 2020; Wei et al., 2018). These variations could be induced by fish internal biological differences. This substantial natural evolution of metabolomic signatures over time could be due to an evolution of the sexual maturity of individuals. Indeed, although our study was conducted when sticklebacks were at sexual rest, the fish could start to evolve during the experiment. Moreover, the physiological and behavioral responses could be affected through time by changes in the sticklebacks' environment (space, density…) as demonstrated in the literature (Bell et al., 2010; Kopecka and Pempkowiak, 2008; Sanchez et al., 2008). Indeed, when males are confined in fewer individuals in a smaller container, they can be more aggressive and territorial. Moreover, it is generally observed that in sticklebacks' behavior 1 male become the most dominant compared to the others (Mehlis et al., 2009; Norton and Gutiérrez, 2019). These changes may be more critical than the induction

or inhibition factors due to DCF. These differences between control male and female could also be imply in the difference of DCF accumulation and metabolization (see above).

3.2.2.2. Modulated metabolites by the DCF exposure in the sticklebacks' liver

a. Male sticklebacks

Two metabolites involved in the energy metabolism were impaired in males due to the exposure to DCF (Table 2), namely adipoyl-carnitine and n-oleoyl-phenylalanine. At the highest dose, the adipoyl-carnitine level is reduced after 7 days compared to control group (FC = 0.34, J7), suggesting a disturbance of the energy metabolism. Indeed, the adipoyl-carnitine is involved in fatty and organic acids transport through the mitochondrial membrane and plays a key role in fatty acids β-oxydation, leading to ATP production providing energy (lipids metabolism) (Houten et al., 2020; Li et al., 2017; Ramsay et al., 2001). DCF at the highest dose induced a higher energy consumption in fish after only 7 days.

N-oleoyl-phenylalanine, that allows glucose homeostasis and glucose storage, was significantly higher in fish exposed 21 days to the lowest dose of DCF compared to control, with p-value = 0.118 and FC = 2.86 at 21 days. This effect is less pronounced for the highest concentration of DCF (FC = 0.45, J21). In addition, this modulation was also observed after 7 days, although it was not yet statistically significant (p-value = 0.464, FC = 4.72). When comparing the lowest dose to the controls, a similar trend for this metabolite was observed with p-value = 0.118 and FC = 2.86 at 21 days, likely indicating impairment of this metabolite by DCF. We could suggest that n-oleoyl-phenylalanine was impaired by longer exposure to DCF at low concentration, triggering an increase in the energy demand resulting in a glucose stores reduction. Triebskorn et al. (2007) demonstrated as well that the exposure to 1 µg/L of DCF in rainbow trout and common carp induced an increase in energy consumption resulting in glycogen reduction. Gravel et al. (2009) also reported similar results with other NSAIDs (ibuprofen and salicylate)

in rainbow trout at 1 mg/L. Both drugs significantly depressed liver glycogen and glucose content. Other studies have previously shown an enhanced capacity for glycogen depletion in stress in fishes (Mommsen et al., 1999; Singer et al., 2007). Consequently, the disturbance of glucose homeostasis seen here with DCF may limits the adaptive glucose response critical to coping with the increased energy demand for liver functions.

A third metabolite, the opthtalmate, involved in the antioxidant defence system as a biomarker of glutathione (GSH) depletion in liver (Contaifer et al., 2019; Soga et al., 2006) was impaired by the exposure to DCF. GSH acts as a reducing agent and reacts with oxidant species before interacting with macromolecules like proteins or DNA (Pompella et al., 2003). The decrease of this compound after 7 days followed by an increase after 21 days of exposure to the highest dose of DCF may reflect changes in the antioxidant system (FC = 0.41, J7, FC = 4, J21). The redox imbalance may be triggered by antioxidant defence responses activated to counteract enhanced Reactive Oxygen Species (ROS) levels. Indeed, DCF seems to cause oxidative stress, leading to the organism's consumption of this metabolite followed by its higher production to continue to face this stress. The disturbance of the antioxidant system by DCF exposure was previously observed in several non-target species such as fish (Guiloski et al., 2017) or invertebrates (Ajima et al., 2021; Gonzalez-Rey and Bebianno, 2014; Trombini et al., 2019). This stress seems to be a common response to NSAIDs contamination. Interestingly, oxidative stress was only observed at a high concentration of DCF, while Guiloski et al. (2017) observed these effects at lower concentrations of DCF (0.2, 2 and 20 µg/L) in silver catfish. Sticklebacks might be slightly less sensitive to DCF than other investigated fish species, as already suggested by Näslund et al. (2017). Moreover, as observed in the invertebrates *Dreissena polymorpha* by Schmidt et al. (2011), we could suppose that the increase of the ophtalmate may have avoided DNA damage in the sticklebacks' liver due to an exposure that lasts over time.

The fold change of ophtalmate (FC = 4) showed a higher degree of modulation than the adipoyl-carnitine and n-oleoyl-phenylalanine, suggesting a more important effect of DCF on this compound and thus on the antioxidant defence system. It is important to note that none of these metabolites were still significantly modulated by the DCF exposure after the depuration period.

b. Female sticklebacks

Two metabolites involved in the energy metabolism were impaired in females due to the exposure to DCF (Table 2), namely adipoyl-carnitine and glutaryl-carnitine. At the highest dose, the latter was significantly higher in fish compared to the lowest dose after 7 days of exposure. These results could suggest changes in fatty acids β-oxydation (lipids metabolism) implicated in the energy metabolism of sticklebacks. The DCF increased the level of this metabolite suggesting a metabolic slowdown or an overactivation of energy metabolism to meet the energy demand, leading to increased energy production by β-oxydation (lipids metabolism) in females at the highest dose of DCF. The organism has to maintain an energy balance to cover all the biological functions (i.e. basic functions, defences to a chemical stress) requirements. However, to a certain degree of stress exposure (long time or high concentration), this increase could have adverse effects on physiological parameters such as growth or reproduction, since the energy balance is now considering the maintenance and the repair of the essential functions of the organism as a priority (Ficke et al., 2007).

After the depuration, glutaryl-carnitine and adipoyl-carnitine were still significantly higher in fish previously exposed at high dose of DCF compared to low dose. Comparison of fish previously exposed to the highest dose to controls indicates that there is probably still modulation of these metabolites in the body after 3 days of depuration. Furthermore, the fold change of glutaryl-carnitine (FC = 4.17) showed a high modulation of this compound after 3 days of depuration, underlying a more significant change on this metabolite and, consequently, the enhancement of the energy production.

A third metabolite, namely the amino acid arginine involved in immunes functions (Azeredo et al., 2019; Salze and Davis, 2015) and antioxidant defences in fish (Hoseini et al., 2020) was impaired by the exposure to DCF. It was decreased at the highest dose of DCF after 3 days of depuration. The reduction of this amino acid may be associated with a higher mobilization of protective defences in the organism to face the backlash of the DCF exposure. These results are consistent with other studies on different fish species (Islas-Flores et al., 2013; Saravanan et al., 2011).

c. Comparison of the DCF effects on the biological functions in both genders

Stress does not seem to similarly affect males and females. As we previously reported, males and females showed different internal concentrations of DCF and 4'-OH DCF according to the concentration and time of exposure (Table 1), demonstrating a different ability to metabolize the DCF and thus a different cost in energy between genders. These observations seemed to be attested by the opposite impairments measured in males and females on the energy metabolism (decreasing in males, increasing in females). This could be due to a different metabolism as it was already suggested in the literature (Marie, 2020; Wei et al., 2018).

The sex-dependent responses observed in this study highlight the importance of distinguishing sexes before interpreting untargeted omics data on ecotoxicological issues such as pollutant contamination, and to specifically explored males and females. Our results were in line with a recent review, which confirmed that sex-specific molecular responses induced by environmental stress or toxicity appear to be almost universally observed (Liang et al., 2018). In addition, our study is based on data of the metabolome of the liver, a tissue that is known to present distinct structure and functions among species and fish genders (Le Manach et al., 2016; Qiao et al., 2016), which could explain these strong sex-dependent responses.

Our results suggest a general state of stress of both stickleback genders induced by the 2 concentrations of exposure to DCF. However, the effects were more pronounced at the highest concentration for both sexes. We did not show evidence that 1 signaling pathway was mainly modulated but different biological functions related to each other reflecting global stress of the organism.

Moreover, during the depuration period, some metabolites were still significantly impaired by DCF exposure in females whereas none were altered in males.

3.3. Metabolites as potential metabolic biomarkers

Numerous biochemical and physiological biomarkers of defense (e.g. metallothionein (MT) induction) and damage (e.g. DNA strand breaks) have been proposed in ecotoxicology (Amiard-Triquet et al., 2012; de Lafontaine et al., 2000). However, these "core" biomarkers related to various physiological functions such as reproduction, immunity, or oxidative stress (Catteau et al., 2021; Sanchez et al., 2008), discrepancies are observed, reflecting the limitations of the current understanding of the effects of emerging contaminants including pharmaceuticals. Omics approaches, combining biological approaches and modern analytical techniques, have become a promising opportunity to overcome such limitations and provide reliable information of the different biological processes and their impairment due to chemical exposure (Trapp et al., 2014). Generally, a promising biomarker is an early warning marker modulated (significantly or not) traducing an exposure or biological effect (toxicity) of a chemical stress on organisms. Its variations need to be measurable through time, showing a notable trend during exposure to the stress. Based, on the metabolomic approach apply in these study, we proposed several metabolites (identified or not) as potential metabolic biomarkers, traducing an impairment of physiological function, which could be more relevant than actual core biomarkers. Among the 2487 significantly modulated features ($FC \leq 0.5$ or ≥ 2, p-value < 0.05) detected in males and females, the most discriminant signals according to the FC values ($FC \leq$

0.2 or ≥ 5) were sorted out. In that respect, 65 and 148 highly modulated metabolites were highlighted in males and females, respectively. Then, we eliminated the features only detected at one time and those with no notable, constant and explainable trend. This sorting finally led to 1 and 4 compounds in males and in females, respectively. Among these metabolites, the glutaryl-carnitine and the adipoyl-carnitine were identified (level 2 of the identification confidence) in females (section 3.2.2.2., Table 2). The 3 other were unidentified (level 5 of the identification confidence). The metabolite discriminated by DCF exposure in males (detected at R_T = 1.04 min with *m/z* = 287.1969) showed a severe decrease between the control and the exposure condition. An evolution over time is observed at the highest dose of DCF, between 7 (FC = $1.85x10^{-4}$) and 21 days (FC = 0.02) of exposure (Figure 4). This constant diminution over time highlights the interest in this compound as metabolic biomarkers to traduce metabolism impairment. Three days of depuration were sufficient to return to a basal level.

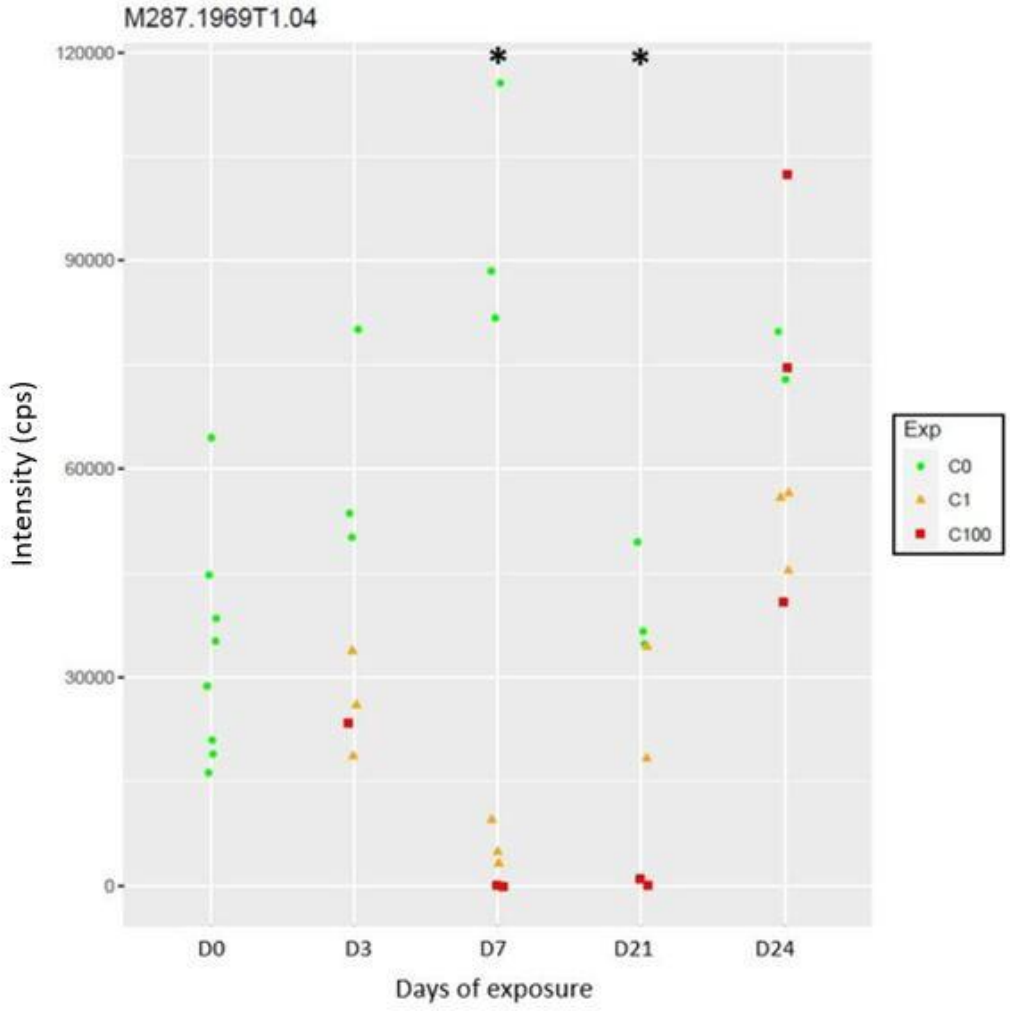


Figure 4. Metabolite (R_T = 1.04 min, *m/z* = 287.1969) impaired by diclofenac (DCF) exposure after 21 days of exposure and 3 days of depuration in stickleback males exposed to 1 and 100 µg/L. For the same time, different colors indicate different conditions. Asterisks indicate significant differences between treatments and control (*p-value < 0.05 and FC ≤ 0.5 or ≥ 2).

Among the metabolites discriminated by DCF exposure detected in females, glutaryl-carnitine and adipoyl-carnitine (level 2 of the identification confidence) (Table 2) and 2 unidentified (level 5 of the identification confidence) may be potential metabolic biomarkers. L-carnitine is proposed as metabolic biomarker and could be utilized as an effective tool for the early detection of gastric cancer in human (Han et al., 2022). Glutaryl-carnitine and adipoyl-carnitine were increased throughout exposure at the highest dose of DCF after 7 and 21 days, respectively. This increase was maintained for both compounds during the depuration time. The two unidentified metabolites showed to be considerably impaired ($FC \leq 0.2$ or $FC \geq 5$) by the exposure to the highest dose of DCF over time. One showed a decrease after 3 days of exposure followed by a progressive increase, maintained during the depuration period (R_T = 5.54 min, *m/z* = 425.2239). The second one (R_T = 4.77 min, *m/z* = 453.1232) was first not impacted after 3 days of exposure, then constantly increased during the rest of the exposure and the depuration times. The maintained modulations over time measured for these 2 compounds suggest them as potential metabolic markers of interest to traduce a metabolism impairment in organisms, but, as for metabolite proposed for male, further researches are necessary to identify these metabolites and their metabolic rule. The evolution of the intensity of these 4 metabolites according to the experimental conditions over time is shown in Figures 5 and 6.

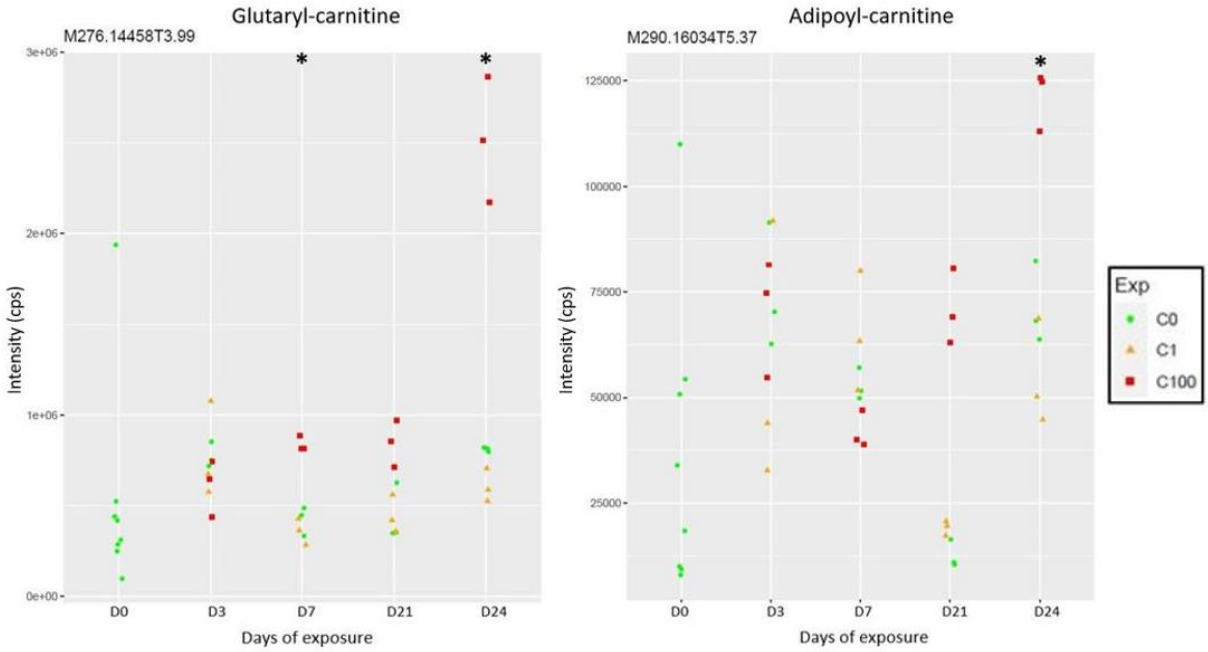


Figure 5. Metabolites (level 2 of the identification confidence) impaired by diclofenac (DCF) exposure after 21 days of exposure and 3 days of depuration in stickleback females exposed to 1 and 100 µg/L. For the same time, different colors indicate different conditions. Asterisks indicate significant differences between treatments and control (*p-value < 0.05 and FC ≤ 0.5 or ≥ 2).

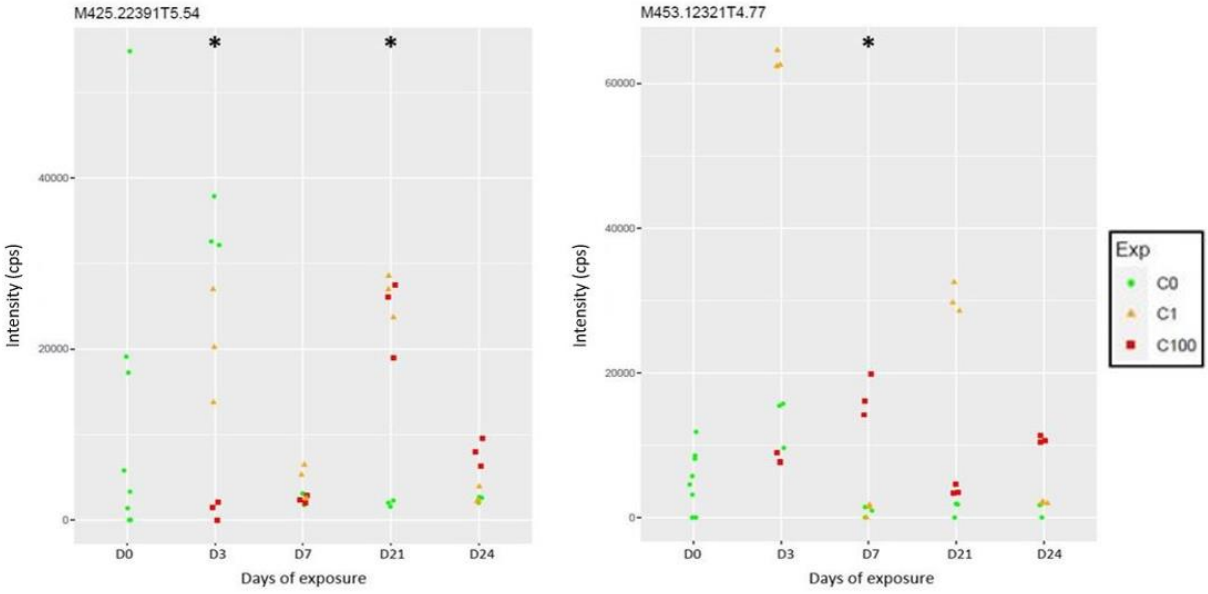


Figure 6. Metabolites (unidentified, level 5 of the identification confidence) impaired by diclofenac (DCF) exposure after 21 days of exposure and 3 days of depuration in stickleback females exposed to 1 and 100 µg/L. For the same time, different colors indicate different conditions. Asterisks indicate significant differences between treatments and control (*p-value < 0.05 and FC ≤ 0.5 or ≥ 2).

4. Conclusions

Our results highlighted an accumulation of DCF and one of its main metabolites, namely 4'-OH DCF, in fish liver. Moreover, we observed that the internal concentrations of both compounds were readily and efficiently eliminated after 3 days of depuration.

Furthermore, our study without *a priori* showed a clear separation of sticklebacks' profiles exposed to DCF according to the concentration and time of exposure. We revealed 2487 metabolites that were significantly impaired during the experiment, including 30 annotated compounds. The 5 molecules modulated by the exposure to DCF putatively identified suggest that DCF alters the antioxidant defence system, the immunity system, and the energy metabolism triggering a loss of protective defences of three-spined sticklebacks. These results suggest a general state of stress of the sticklebacks induced by the 2 concentrations of exposure to DCF, although the effects were more pronounced at the higher dose.

Finally, our metabolomics approach revealed 1 and 4 metabolites as potential metabolic biomarkers in male and female sticklebacks, respectively. Among these compounds, 2 acyl-carnitines, namely glutaryl-carnitine and adipoyl-carnitine, were putatively identified in females and showed to be promising metabolic biomarkers of energy metabolism impairment. Moreover, both metabolites were also detected as discriminant signals in males. Although they did not show evolution through time in males, it should be interesting to validate the potential of these 2 metabolites as metabolic biomarkers in both genders. In addition, our results highlight that male and female sticklebacks exhibit sex-dependent molecular responses to the same experimental conditions. The examination of the gonads may confirm or not an evolution of the sexual maturity of the individuals inducing the important natural changes reported in the liver of the unexposed individuals. This substantial evolution should also be considered for the analysis of other organs since the other compartments could be differently prone to fluctuations.

It is thus important to better understand and assess this natural evolution to determine how it could impact the metabolomic profiles under stress exposure.

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SUPPLEMENTARY INFORMATION

Time and dose-dependent impairment of liver metabolism in *Gasterosteus aculeatus* following exposure to diclofenac (DCF) highlighted by LC-HRMS untargeted metabolomics

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Figure S1. Distribution of fish in 24 aquaria divided into 2 identical benches for the DCF exposure for 21 days followed by 3 days of depuration. M : male, F : female.

Figure S2. Principal Component Analysis score plots of male fish (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode) according to the concentration and time of exposure. C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; Exposure : D3 : 3 days, D7 : 7 days, D21 : 21 days; depuration : D24 : 24 days (corresponding to 3 days of depuration); QC : control quality.

Figure S3. Principal Component Analysis score plots of female fish (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode) according to the dose and time of exposure. C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; Exposure : D3 : 3 days, D7 : 7 days, D21 : 21 days; depuration : D24 : 24 days (corresponding to 3 days of depuration); QC : control quality.

Figure S4. Principal Component Analysis score plots of male fish after 3 days of exposure (LC-HRMS performed on the C18-colmun with electrospray ionisation in positive mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.

Figure S5. Principal Component Analysis score plots of male fish after 7 days of exposure (LC-HRMS performed on the C18-column with electrospray in positive mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.

Figure S6. Principal Component Analysis score plots of male fish after 3 days of depuration (D24) (LC-HRMS performed on the C18-colmun with electrospray ionisation in positive mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.

Figure S7. Principal Component Analysis score plots of male fish after 3 days of exposure (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.

Figure S8. Principal Component Analysis score plots of male fish after 7 days of exposure (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.

Figure S9. Principal Component Analysis score plots of male fish after 21 days of exposure (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.

Figure S10. Principal Component Analysis score plots of male fish after 3 days of depuration (D24) (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.

Figure S11. Principal Component Analysis score plots of female fish after 7 days of exposure (LC-HRMS performed on the C18-column with electrospray ionisation in positive mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.

Figure S12. Principal Component Analysis score plots of female fish after 21 days of exposure (LC-HRMS performed on the C18-column with electrospray ionisation in positive mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.

Figure S13. Principal Component Analysis score plots of female fish after 3 days of depuration (D24) (LC-HRMS performed on the C18-column with electrospray ionisation in positive mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.

Figure S14. Principal Component Analysis score plots of female fish after 3 days of exposure (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.

Figure S15. Principal Component Analysis score plots of female fish after 7 days of exposure (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.

Figure S16. Principal Component Analysis score plots of female fish after 21 days of exposure (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.

Figure S17. Principal Component Analysis score plots of female fish after 3 days of depuration (D24) (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.

Table S1. Labeled internal standards employed and various adducts used during the untargeted LC-HRMS method.

Table S2. Biometric parameters (mean ± SD) measured in males and females three-spined stickleback exposed to diclofenac (DCF) during 21 days followed by 3 days of depuration (D24). Controls (ctrl): n = 20, DCF 1 and DCF 100 : n = 10; HSI = 0.1

Table S3. Annotated metabolites not significantly impaired, detected in males and females three-spined sticklebacks exposed at 1 and 100 µg/L of diclofenac (DCF) for 21 days followed by 3 days of depuration.

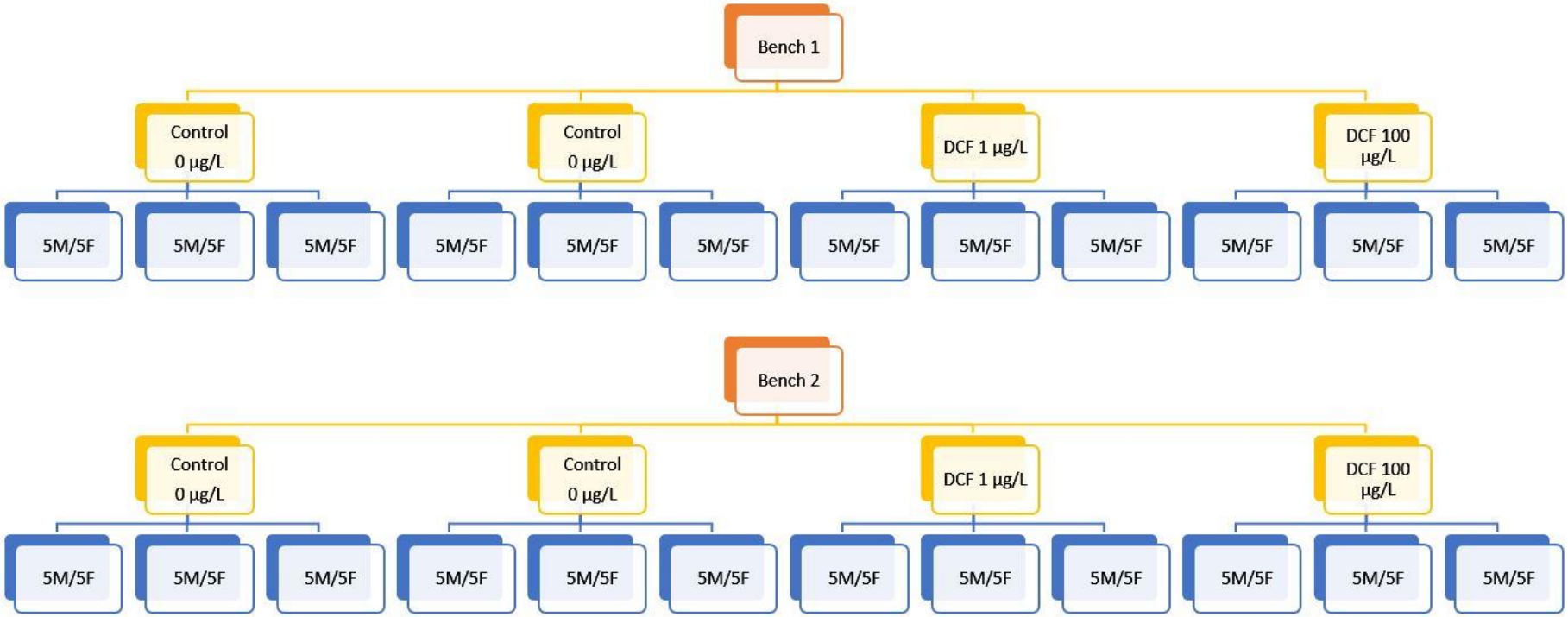

Figure S1. *Distribution of fish in 24 aquaria divided into 2 identical benches for the DCF exposure for 21 days followed by 3 days of depuration. M : male, F : female.*

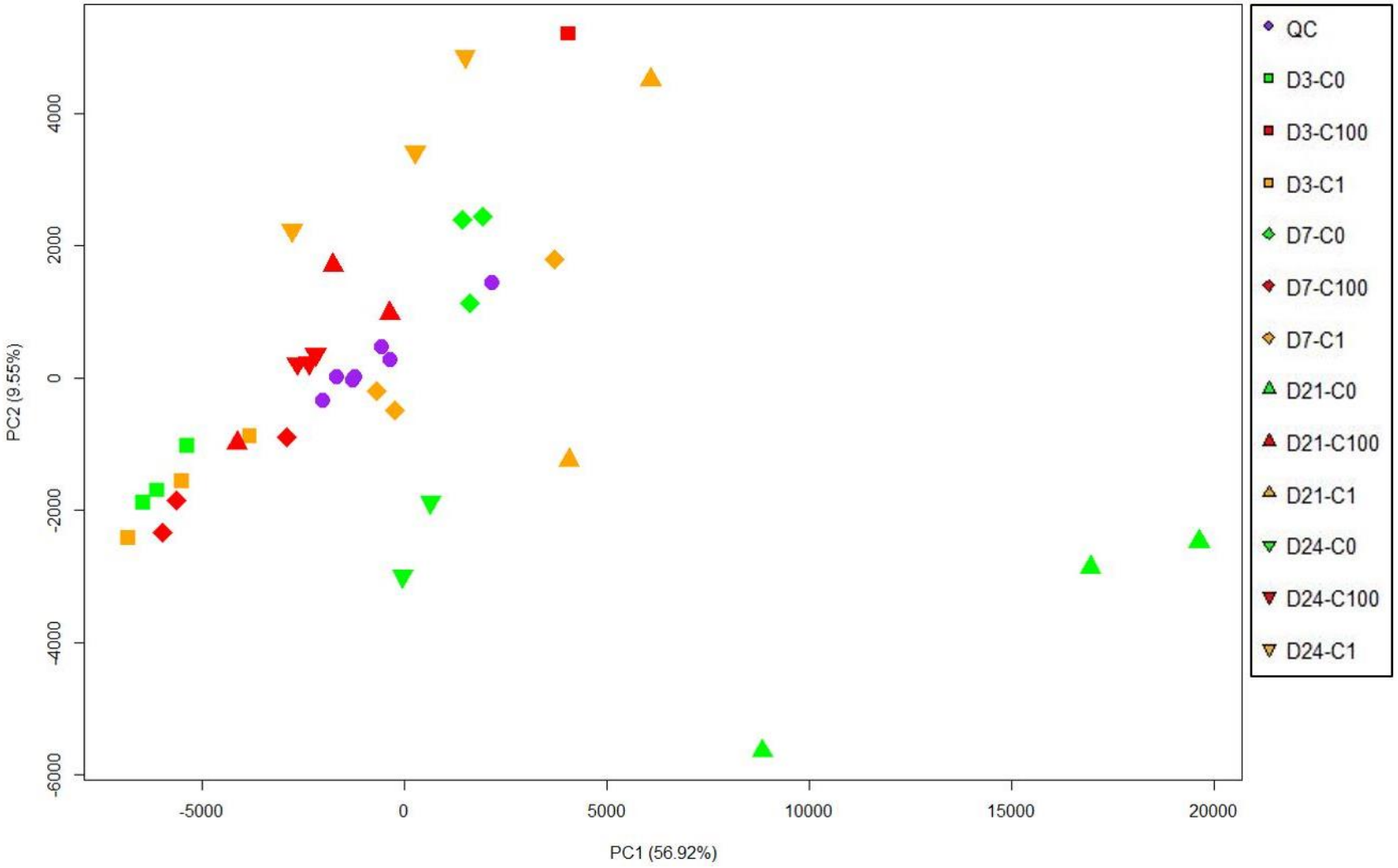

Figure S2. *Principal Component Analysis score plots of male fish (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode) according to the concentration and time of exposure. C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; Exposure : D3 : 3 days, D7 : 7 days, D21 : 21 days; depuration : D24 : 24 days (corresponding to 3 days of depuration); QC : control quality.*

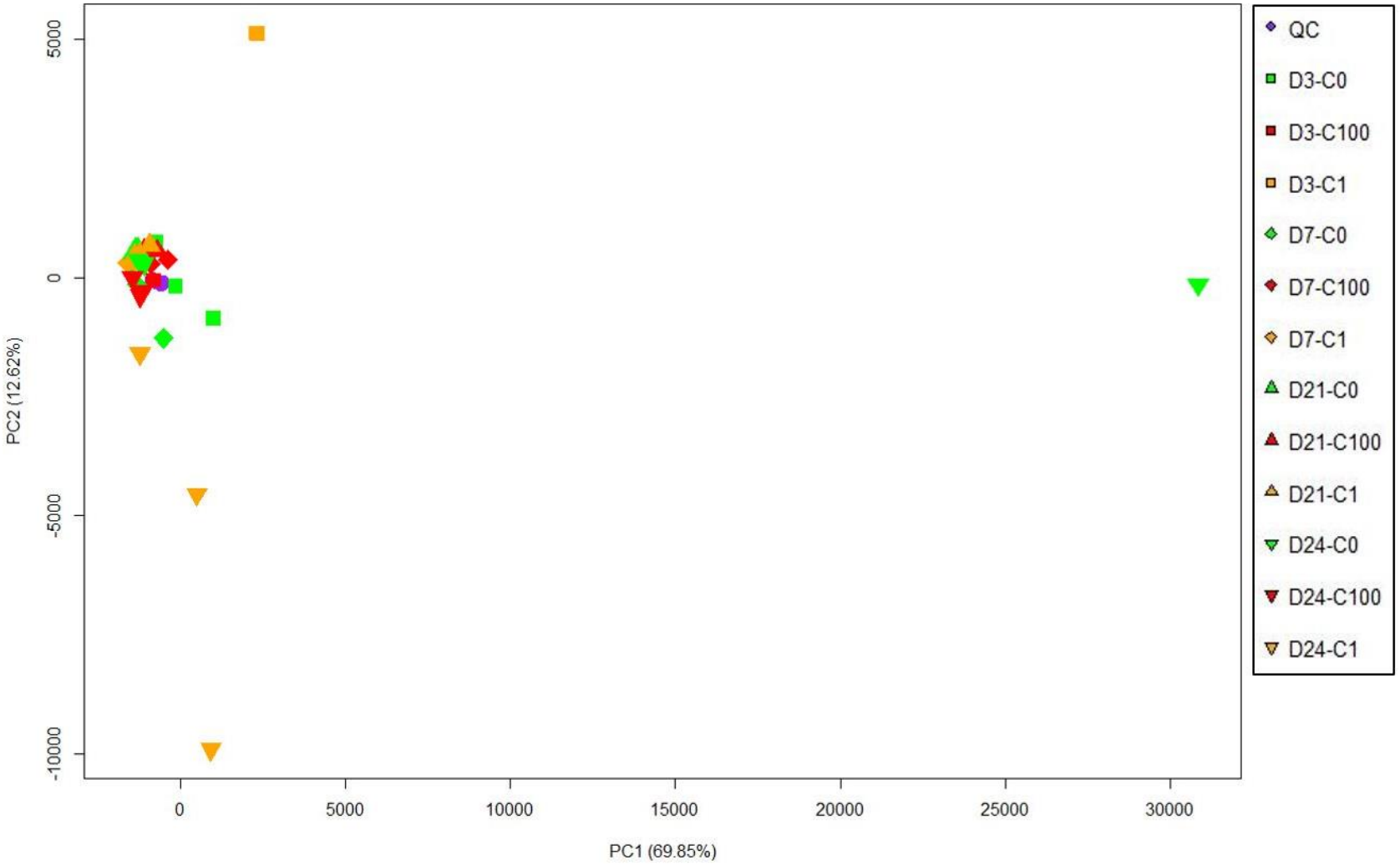

Figure S3. *Principal Component Analysis score plots of female fish (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode) according to the dose and time of exposure. C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; Exposure : D3 : 3 days, D7 : 7 days, D21 : 21 days; depuration : D24 : 24 days (corresponding to 3 days of depuration) ; QC : control quality.*

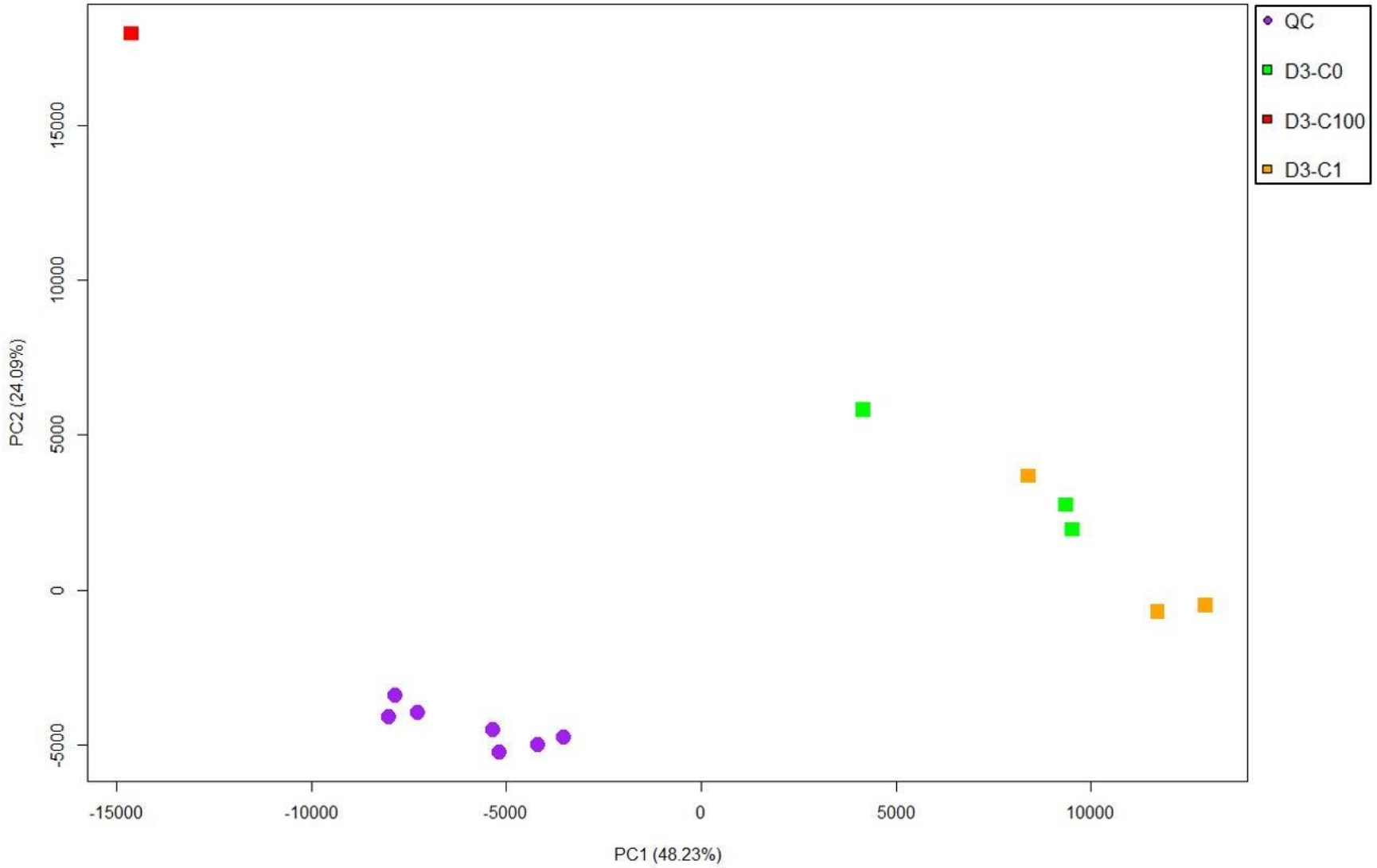

Figure S4. *Principal Component Analysis score plots of male fish after 3 days of exposure (LC-HRMS performed on the C18-colmun with electrospray ionisation in positive mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.*

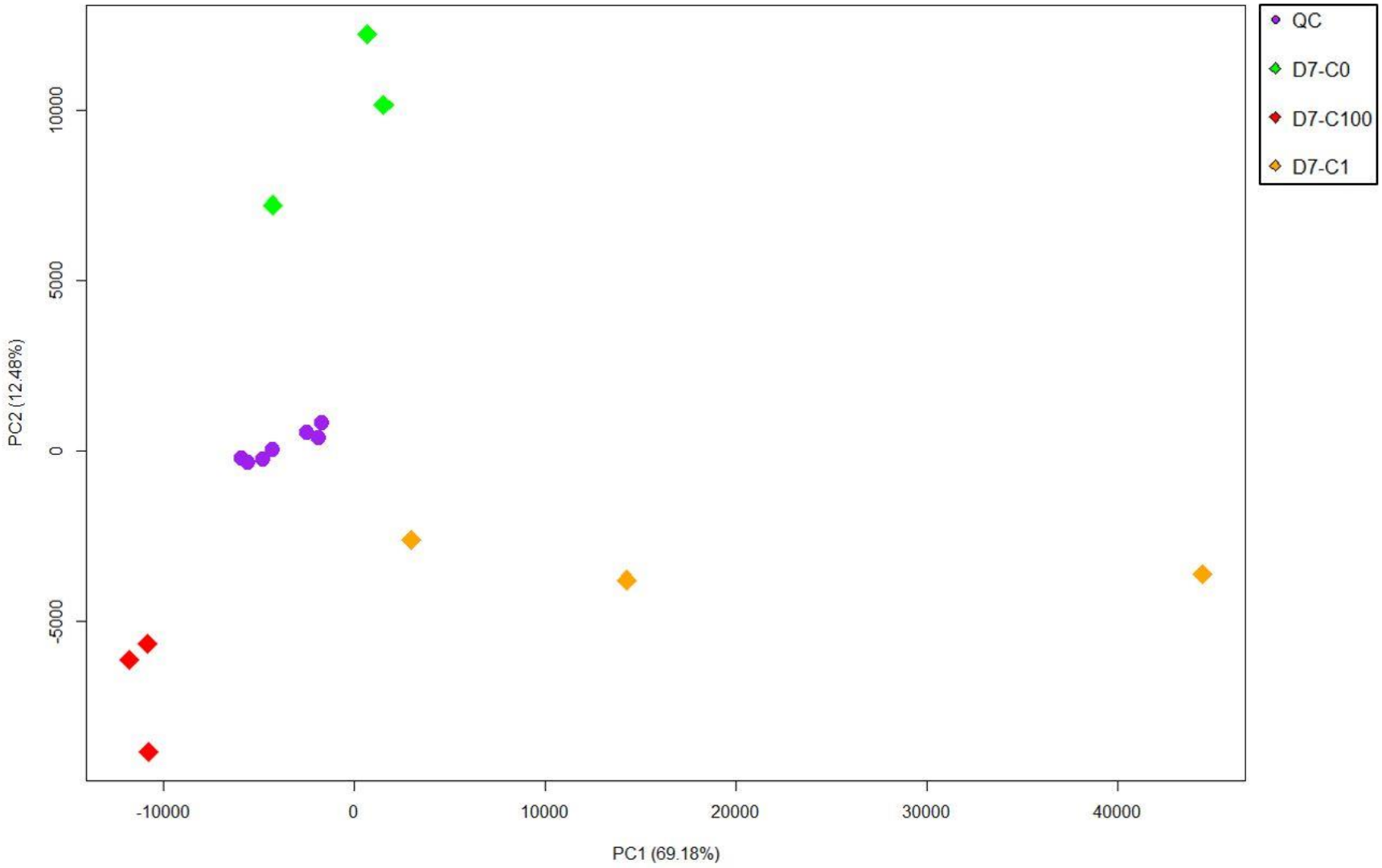

Figure S5. *Principal Component Analysis score plots of male fish after 7 days of exposure (LC-HRMS performed on the C18-column with electrospray in positive mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.*

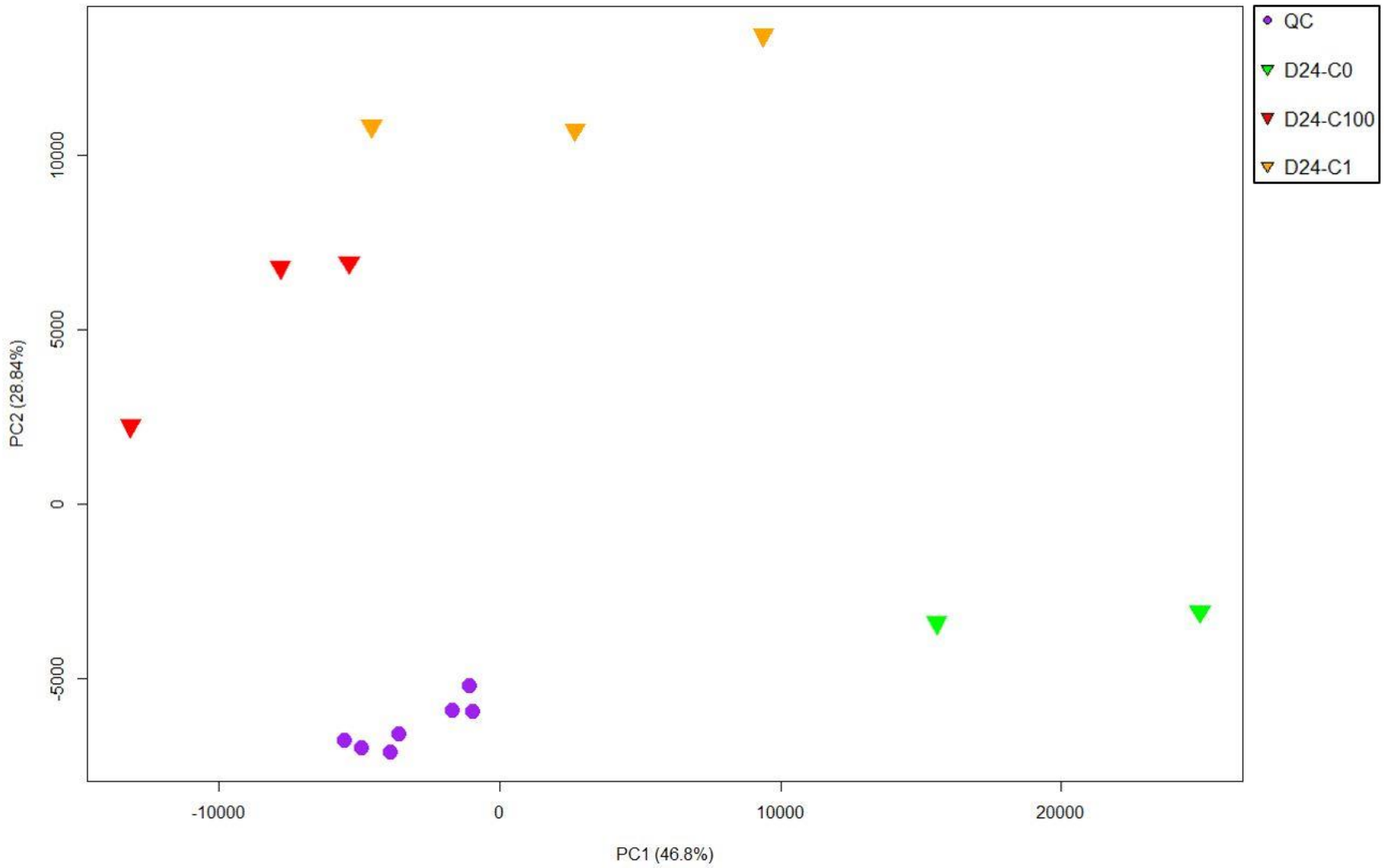

Figure S6. *Principal Component Analysis score plots of male fish after 3 days of depuration (D24) (LC-HRMS performed on the C18-colmun with electrospray ionisation in positive mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.*

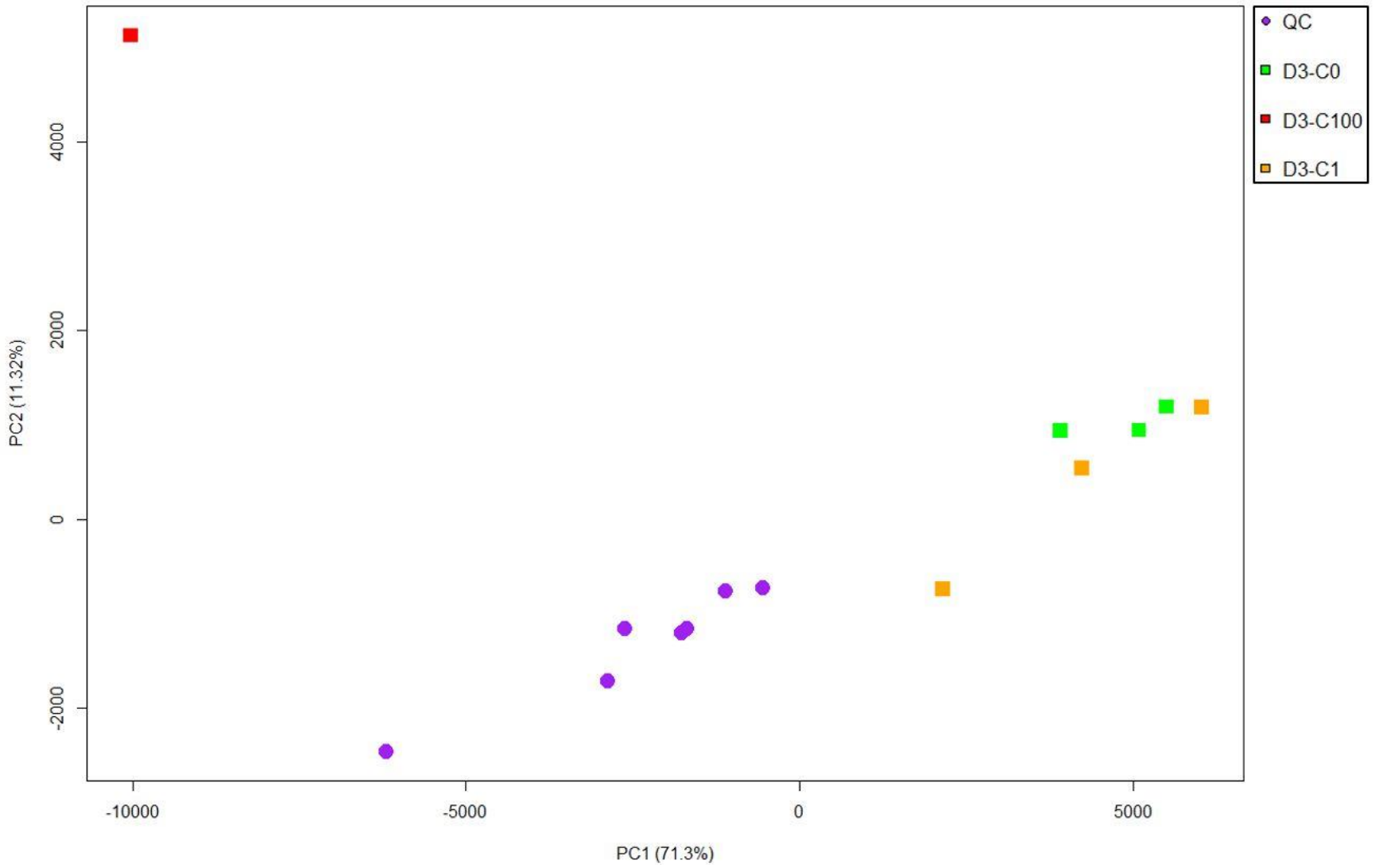

Figure S7. *Principal Component Analysis score plots of male fish after 3 days of exposure (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.*

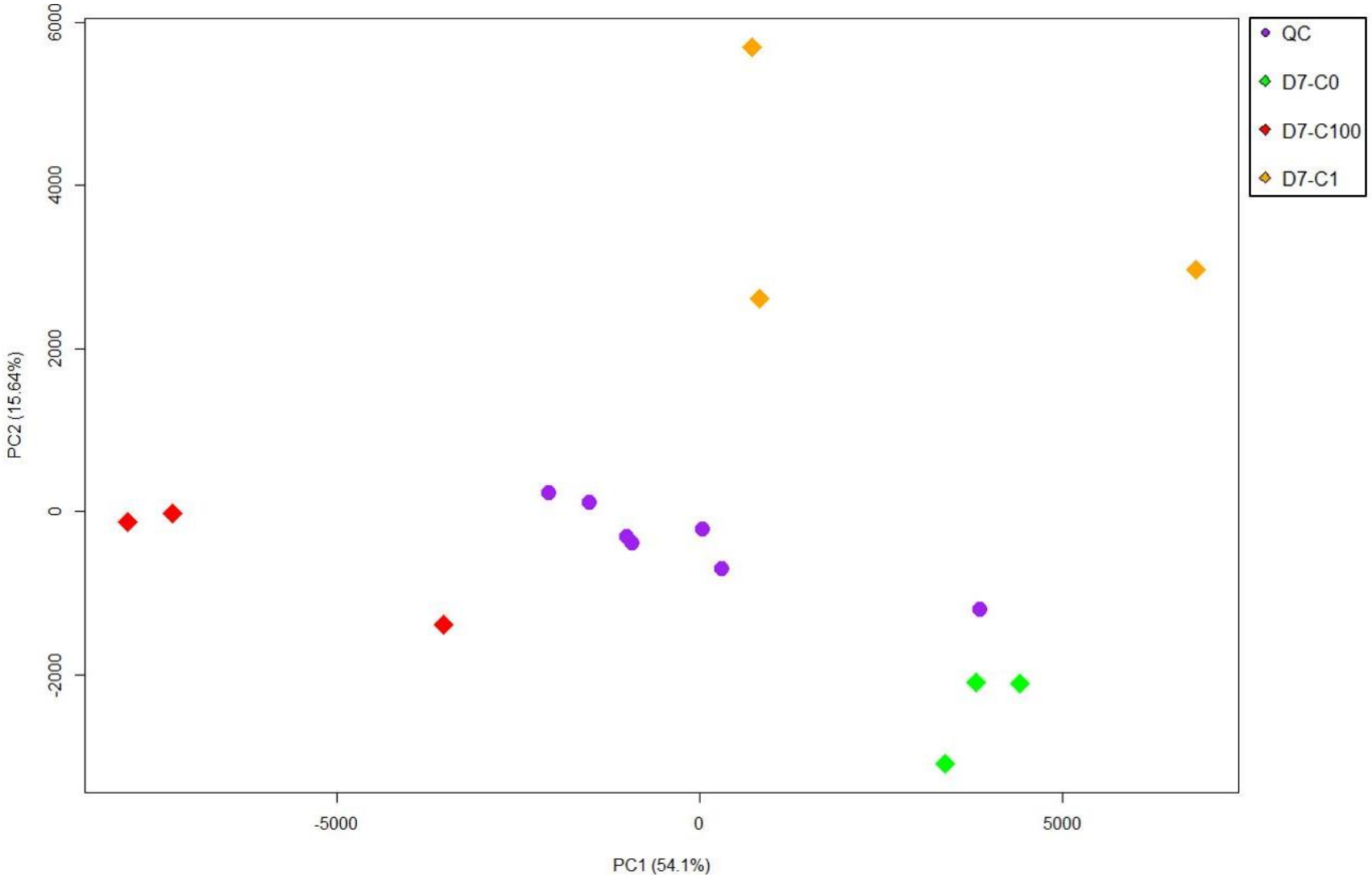

Figure S8. *Principal Component Analysis score plots of male fish after 7 days of exposure (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.*

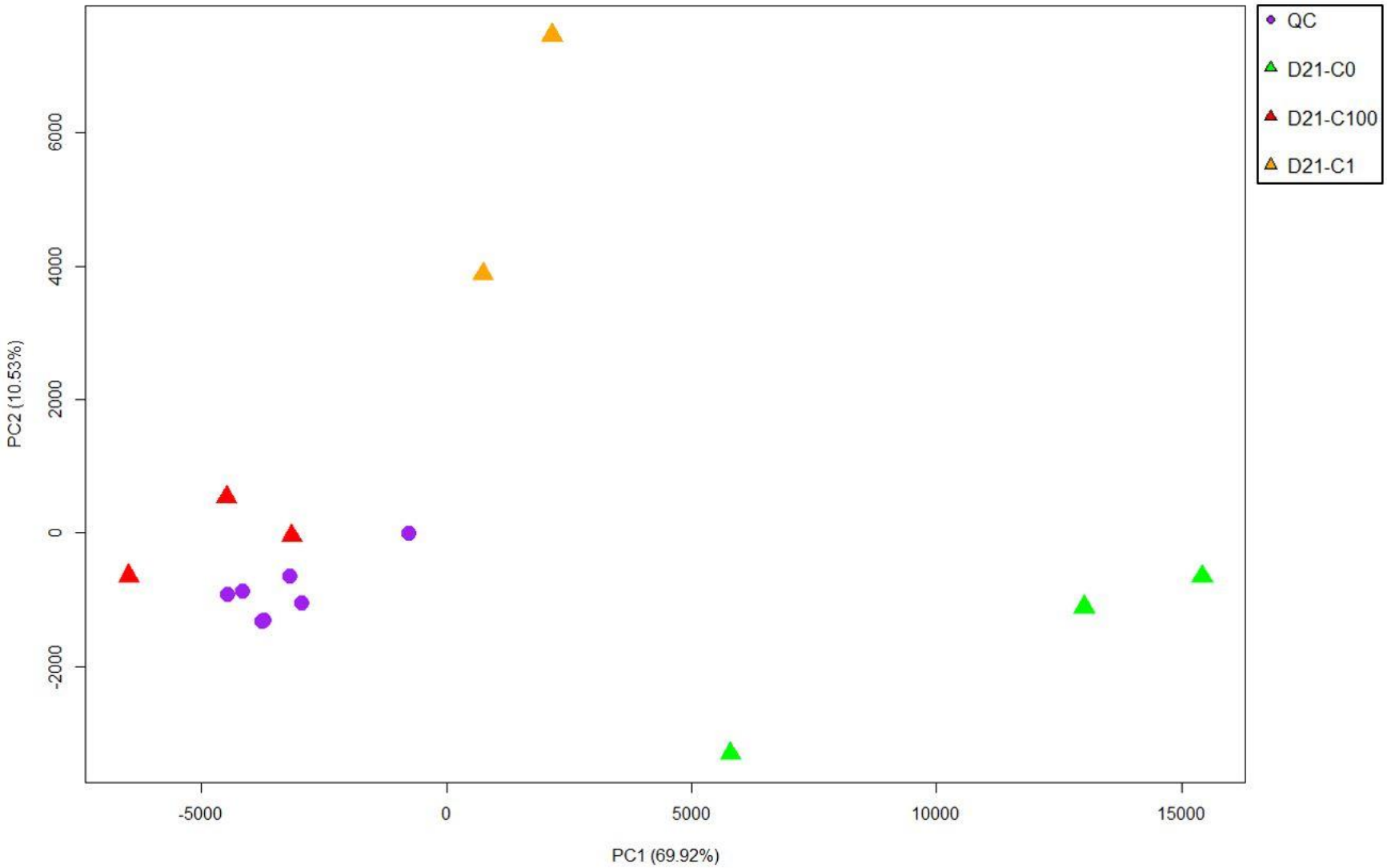

Figure S9. *Principal Component Analysis score plots of male fish after 21 days of exposure (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.*

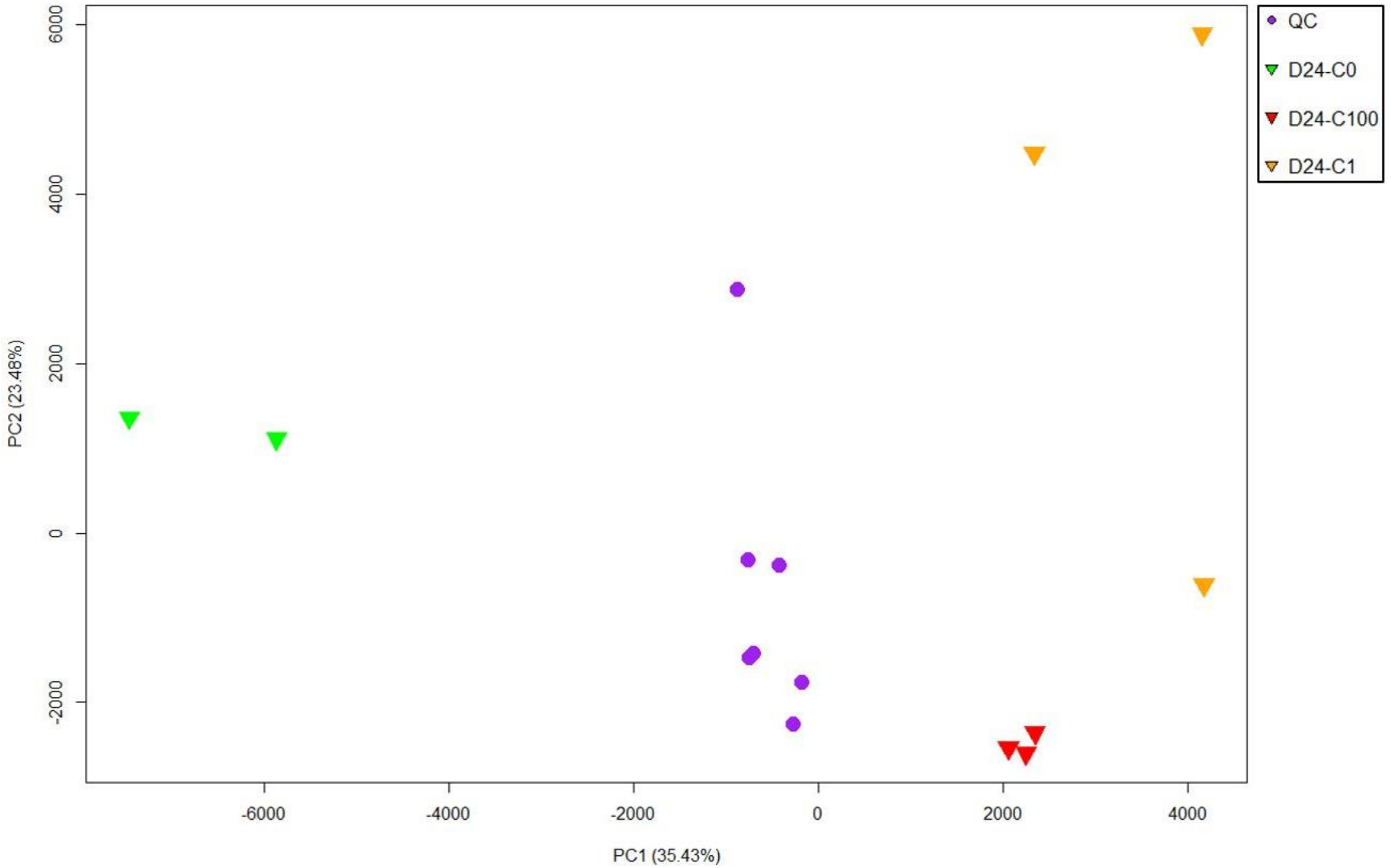

Figure S10. *Principal Component Analysis score plots of male fish after 3 days of depuration (D24) (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.*

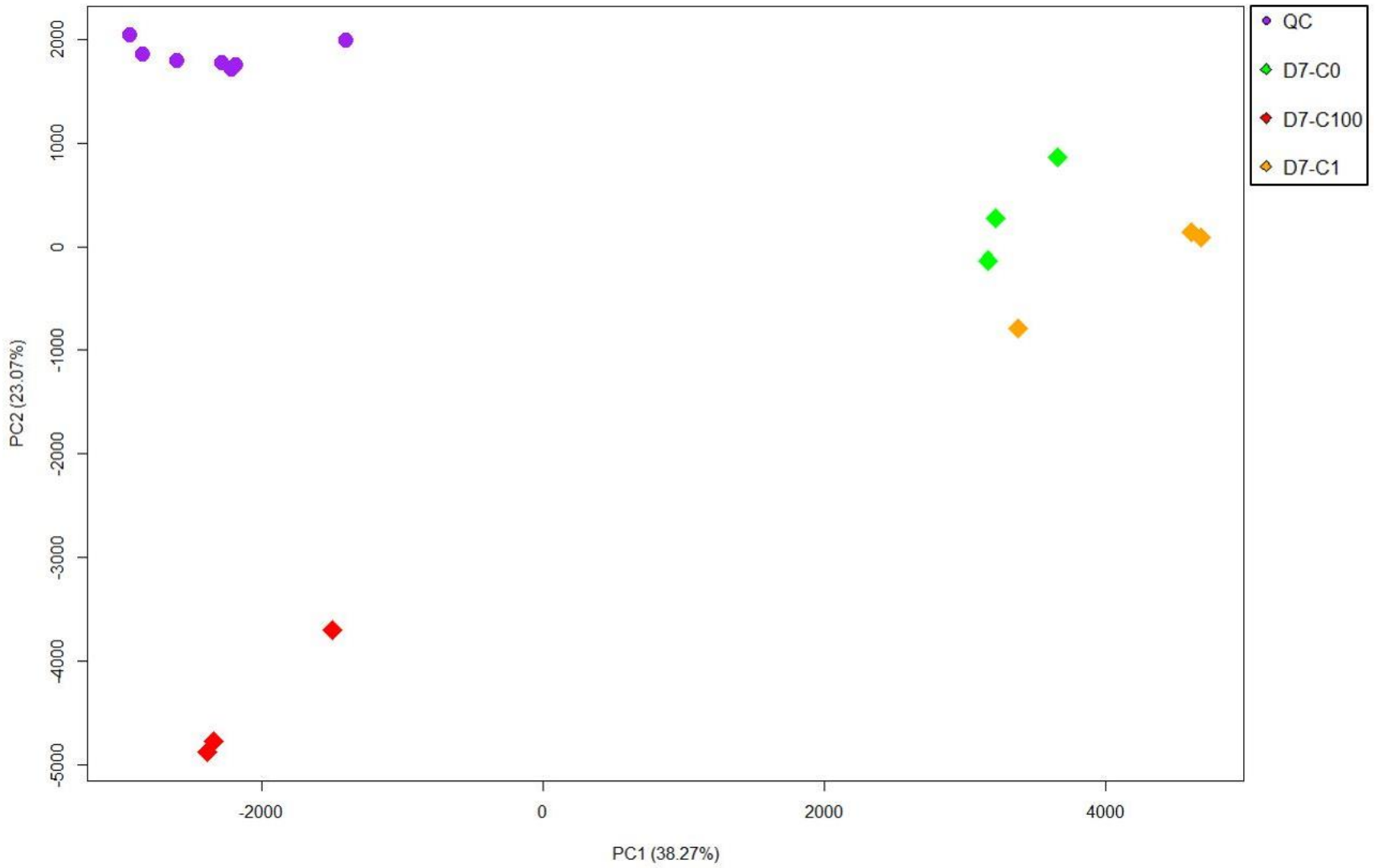

Figure S11. *Principal Component Analysis score plots of female fish after 7 days of exposure (LC-HRMS performed on the C18-column with electrospray ionisation in positive mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.*

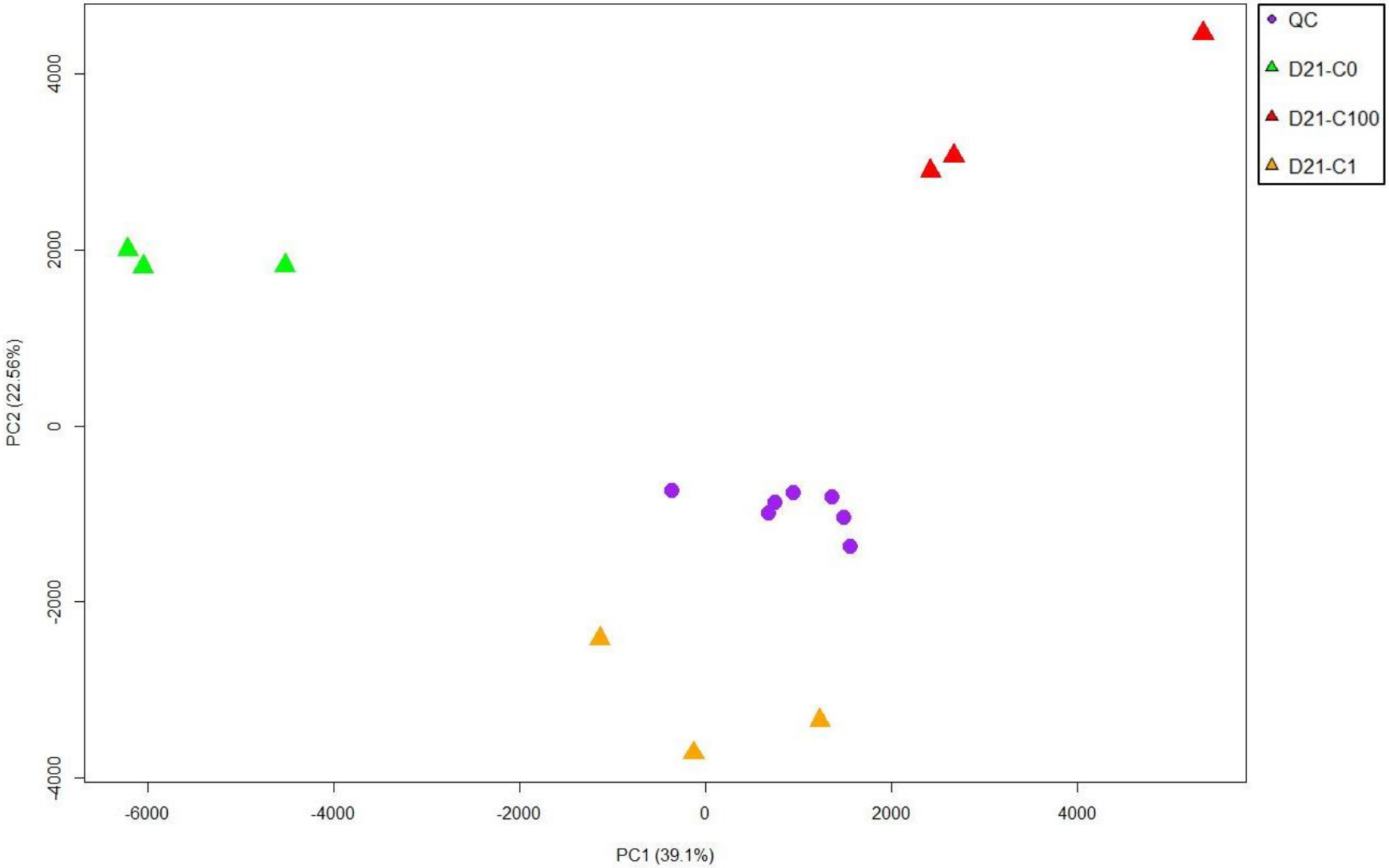

Figure S12. *Principal Component Analysis score plots of female fish after 21 days of exposure (LC-HRMS performed on the C18-column with electrospray ionisation in positive mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.*

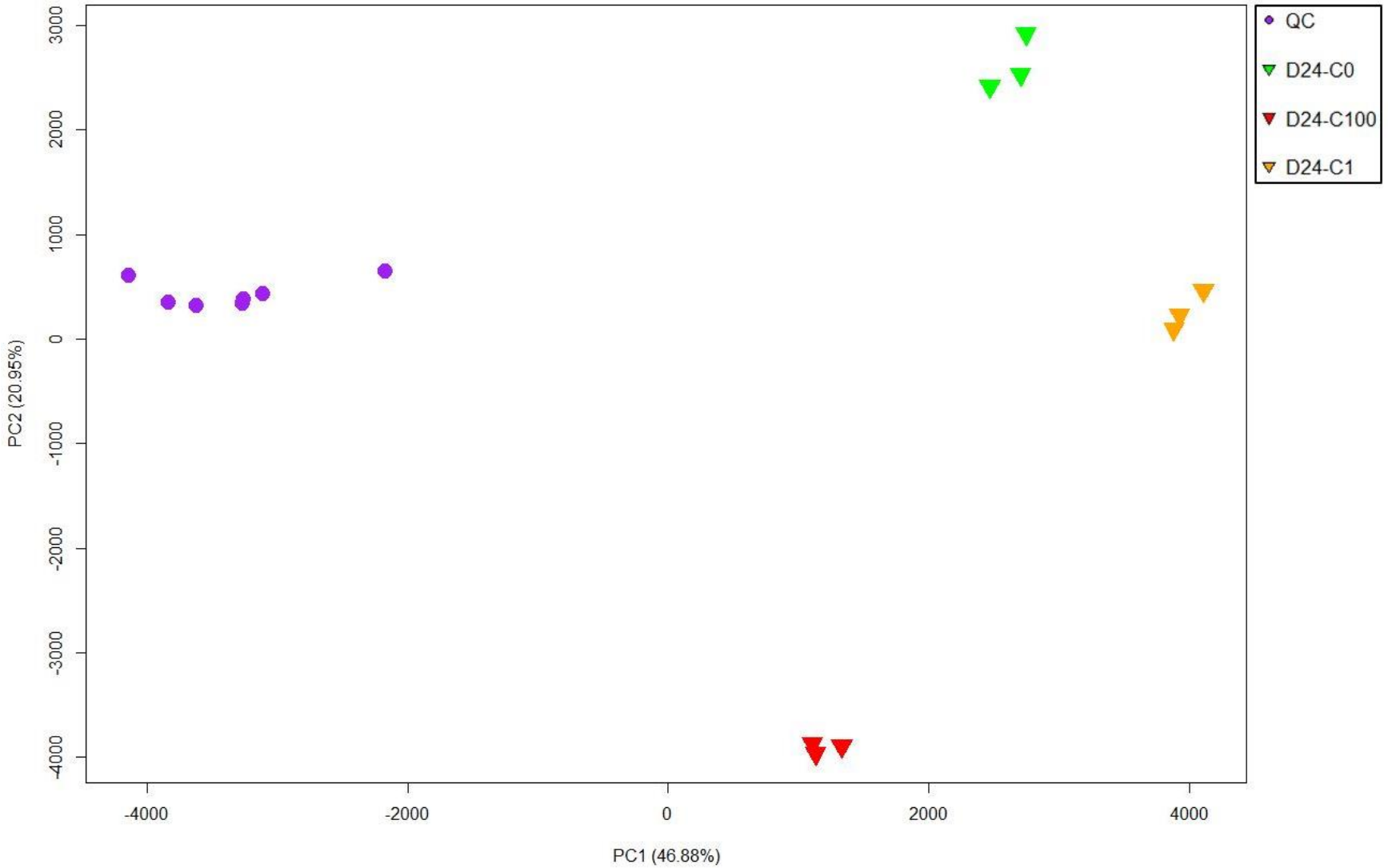

Figure S13. *Principal Component Analysis score plots of female fish after 3 days of depuration (D24) (LC-HRMS performed on the C18-column with electrospray ionisation in positive mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.*

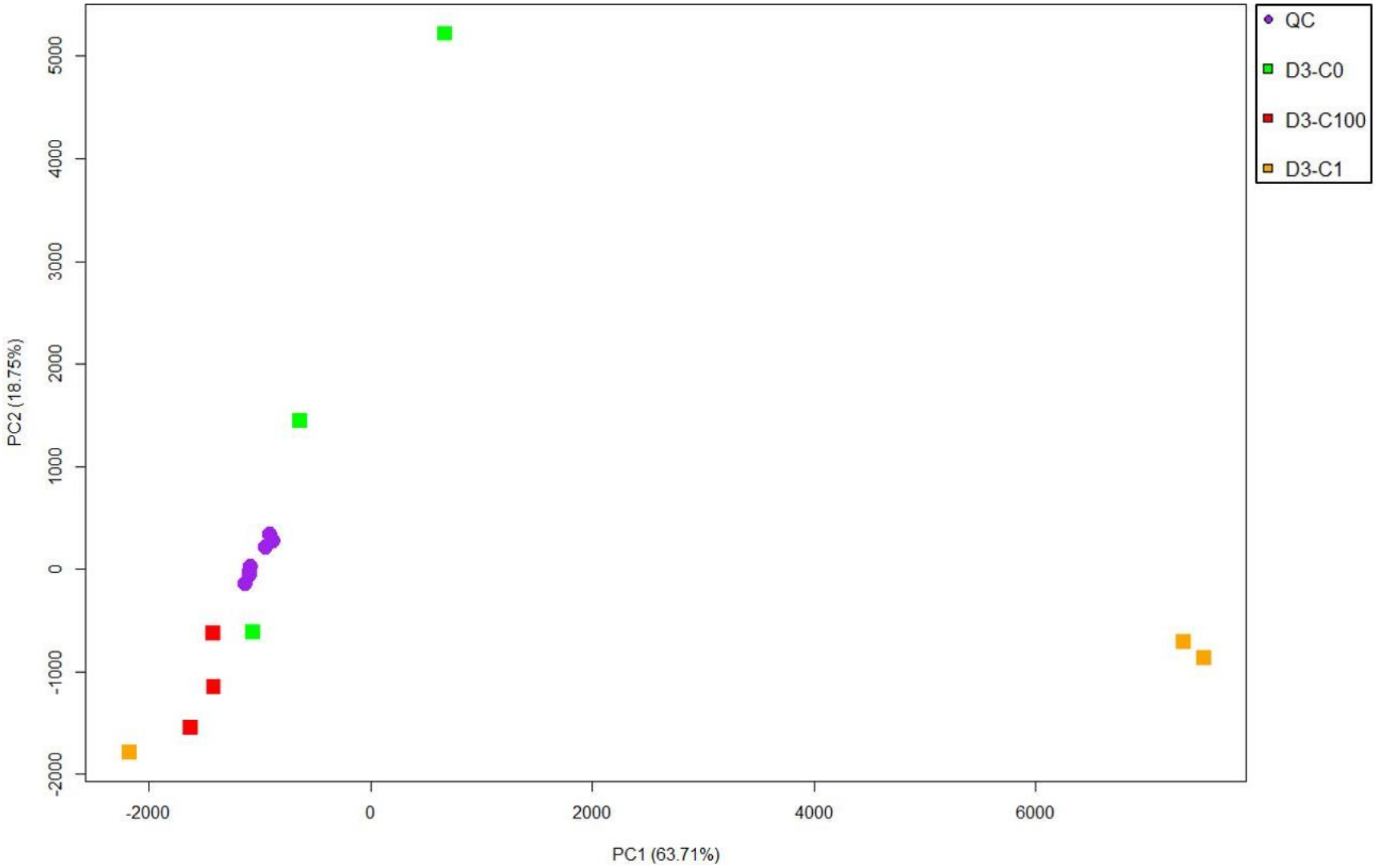

Figure S14. *Principal Component Analysis score plots of female fish after 3 days of exposure (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.*

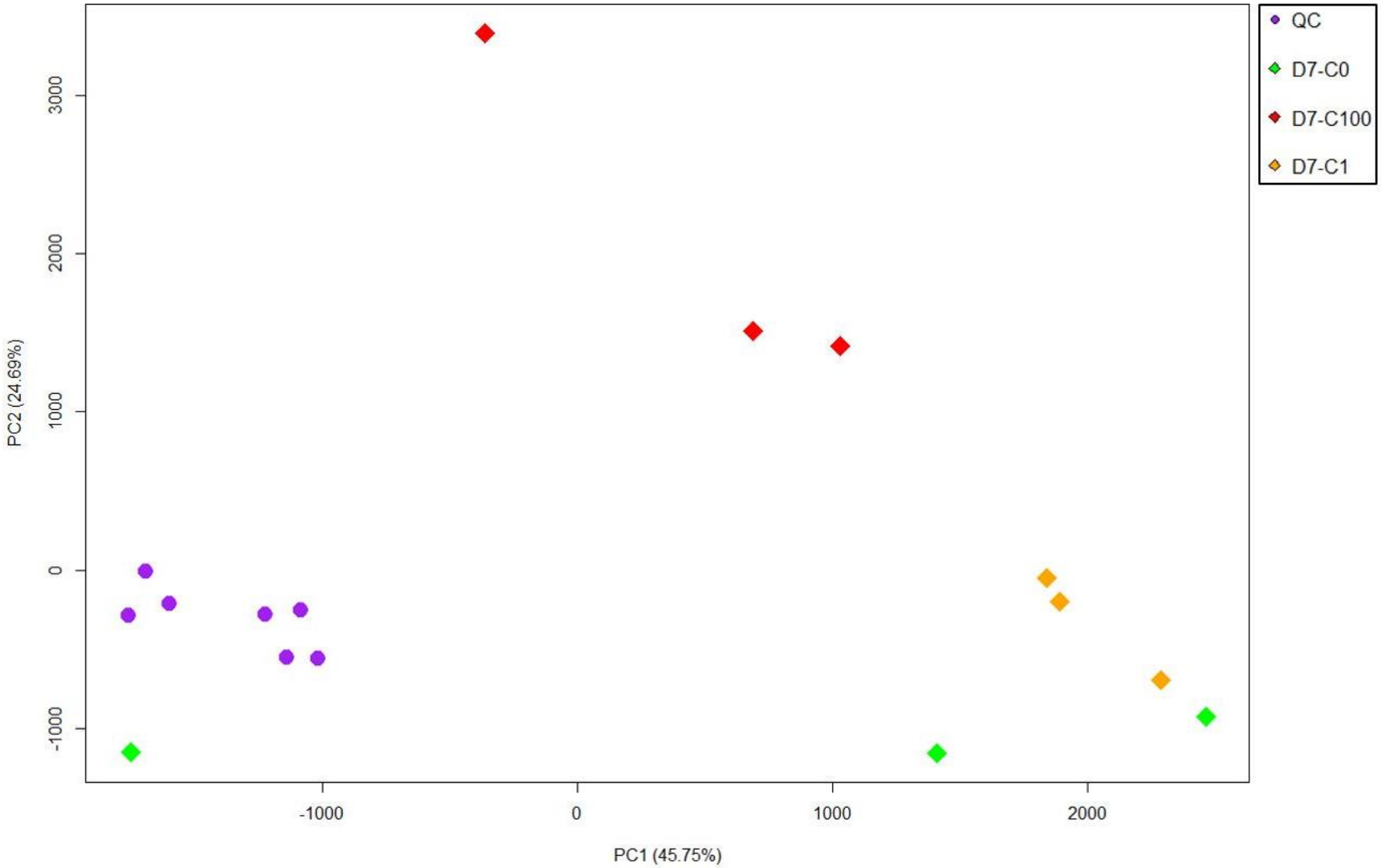

Figure S15. *Principal Component Analysis score plots of female fish after 7 days of exposure (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.*

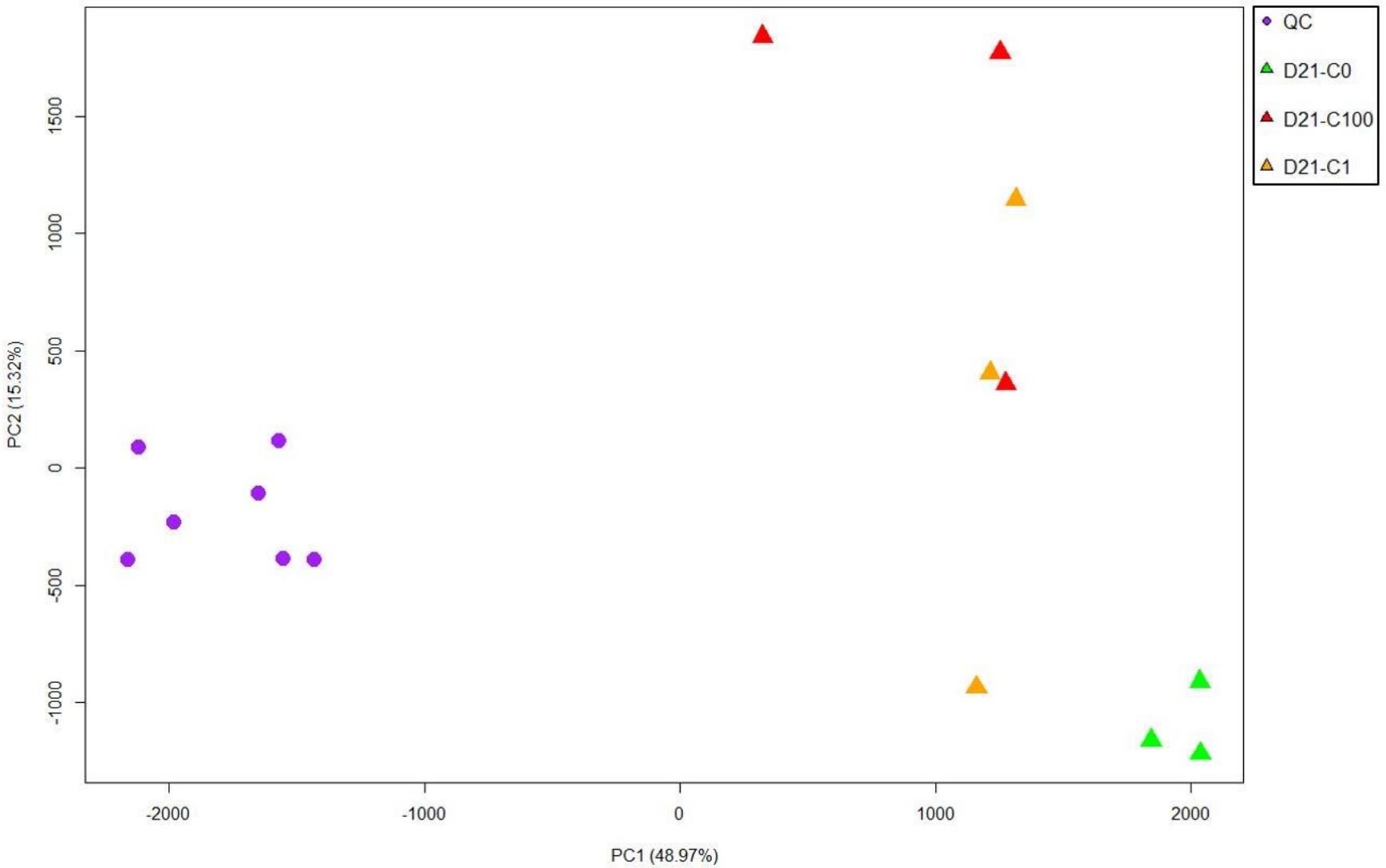

Figure S16. *Principal Component Analysis score plots of female fish after 21 days of exposure (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.*

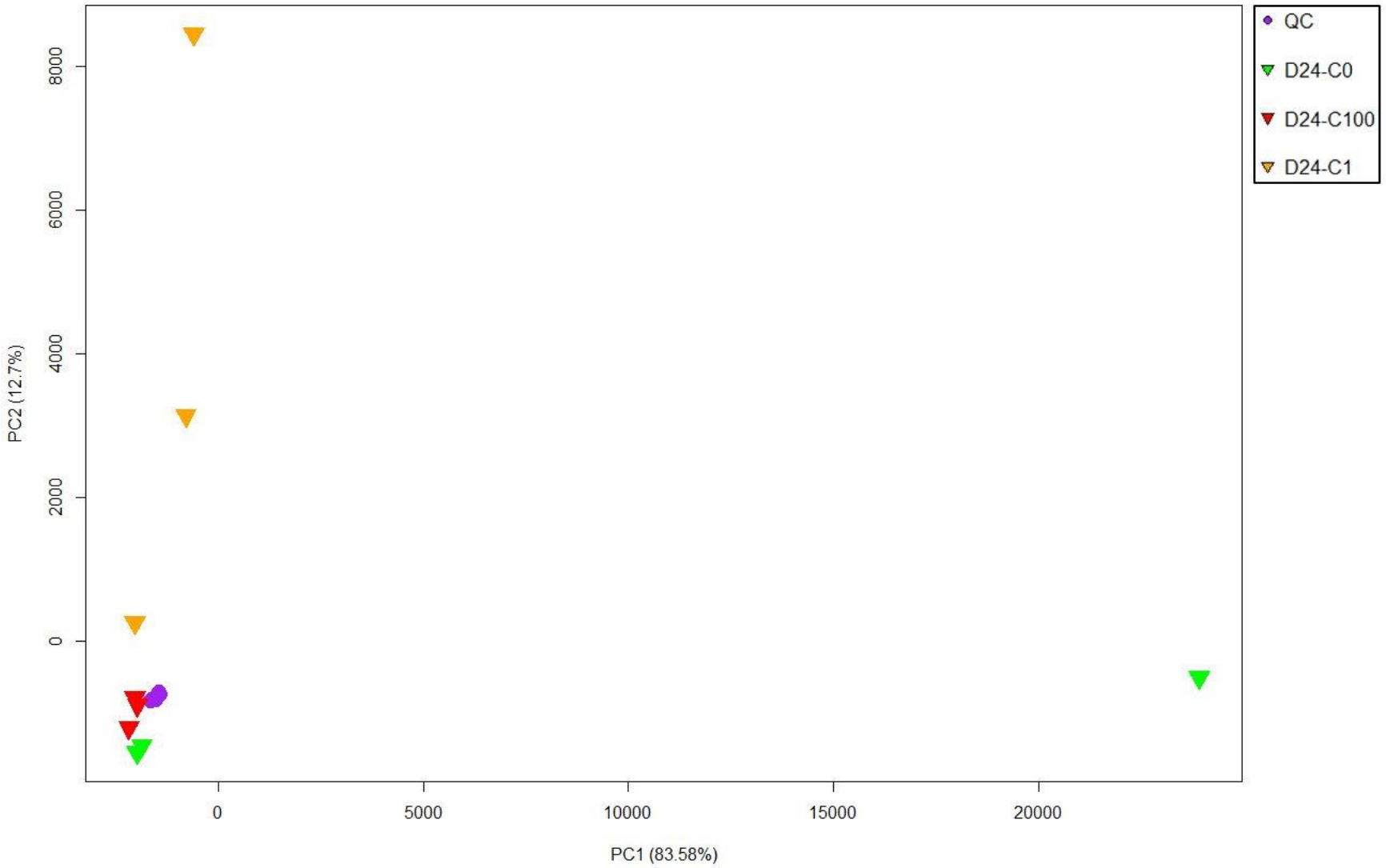

Figure S17. *Principal Component Analysis score plots of female fish after 3 days of depuration (D24) (LC-HRMS performed on the HILIC-column with electrospray ionisation in negative mode). C0 : controls, C1 : 1 µg/L, C100 : 100 µg/L; QC : control quality.*

Table S1. *Labeled internal standards employed and various adducts used during the untargeted LC-HRMS method.*

Compounds	Chemical group	Supplier	Purity (%)	Formula	Adduct	Exact mass	LC-HRMS conditions	R_T (min)
^{15}N-Valine	Amino acid	Sigma-Aldrich	98.0	$C_5H_{11}{}^{15}NO_2$	$[M+H]^+$ 119.0833 $[M-H]^-$ 117.0676	118.0760	RPLC ESI+ HILIC ESI-	1.1 8.0
^{13}C-Glycine	Amino acid	Sigma-Aldrich	99.0	$^{13}CCH_5NO_2$	$[M-H]^-$ 75.0270	76.0354	HILIC ESI-	8.9
D-Lysine	Amino acid	Sigma-Aldrich	98.0	$D_4C_6H_{10}N_2O_2$	$[M+H]^+$ 151.1379	150.1306	RPLC ESI+	0.7
D-Methionine	Amino acid	Sigma-Aldrich	98.0	$D_3C_5H_8NO_2S$	$[M-H]^-$ 151.0615	152.0699	HILIC ESI-	7.7
^{13}C,^{15}N-Leucine	Amino acid	Sigma-Aldrich	98.0	$^{13}C_6H_{13}(^{15}N)O_2$	$[M-H]^-$ 137.1034	138.1118	HILIC ESI-	7.4
D-Octanoyl-L-carnitine	Peptide	Sigma-Aldrich	97.0	$D_3C_{15}H_{26}NO_4$	$[M+H]^+$ 291.2358 $[M+Na]^+$ 313.2177 $[M-H]^-$ 289.2201	290.2285	RPLC ESI+ RPLC ESI+ HILIC ESI-	10.3 10.3 8.3
D-Biotine	Vitamin	Sigma-Aldrich	98.0	$D_2C_{10}H_{14}N_2O_3S$	$[M-H]^-$ 245.0923	246.1007	HILIC ESI-	4.4
D-β-estradiol	Hormone	CDN isotopes	99.5	$D_2C_{18}H_{22}O_2$	$[M+H]^+$ 275.1975	274.1902	RPLC ESI+	13.0
D-Testosterone	Hormone	CDN isotopes	99.7	$D_2C_{19}H_{26}O_2$	$[M+H]^+$ 291.2288	290.2215	RPLC ESI+	13.3
D-Progesterone	Hormone	CDN isotopes	98.5	$D_9C_{21}H_{21}O_2$	$[M+H]^+$ 324.2883	323.2811	RPLC ESI+	14.3
^{13}C-Mannose	Sugar	Sigma-Aldrich	99.0	$^{13}CC_5H_{12}O_6$	$[M-H_2O-H]^-$ 162.0478 $[M-H]^-$180.0584	181.0667	HILIC ESI- HILIC ESI-	2.6 2.6

Table S2. *Biometric parameters (mean ± SD) measured in males and females three-spined stickleback exposed to diclofenac (DCF) during 21 days followed by 3 days of depuration (D24). Controls (ctrl): n = 20, DCF 1 and DCF 100 : n = 10; HSI = 0.1*

Time	**D3**			**D7**			**D21**			**D24**		
Condition	**Ctrl**	**DCF 1**	**DCF 100**	**Ctrl**	**DCF 1**	**DCF 100**	**Ctrl**	**DCF 1**	**DCF 100**	**Ctrl**	**DCF 1**	**DCF 100**
Weight (g)	2.5 ±0.6	2.6 ±0.6	2.3 ±0.6	2.8 ±0.5	2.6 ±0.6	2.5 ±0.5	2.7 ±0.7	2.6 ±0.6	2.7 ±0.6	2.6 ±0.5	2.7 ±0.4	2.7 ±0.4
Size (mm)	55.0 ±4.6	55.3 ±5.2	53.8 ±5.4	58.3 ±2.5	55.3 ±4.1	55.2 ±3.5	56.9 ±4.1	56.4 ±4.7	55.6 ±3.5	55.55 ±4.1	56 ±2.7	56.9 ±3.1
Fulton index (K)	1.5 ±0.1	1.5 ±0.2	1.5 ±0.2	1.4 ±0.2	1.5 ±0.2	1.5 ±0.2	1.4 ±0.2	1.4 ±0.1	1.6 ±0.3	1.5 ±0.2	1.5 ±0.2	1.5 ±0.1

Table S3. *Annotated metabolites, not significantly impaired, detected in males and females three-spined sticklebacks exposed at 1 and 100 µg/L of diclofenac (DCF) for 21 days followed by 3 days of depuration.*

Metabolism pathway	**Level of the identification confidence**	**Annotation/Identification**	**Sex**	***m/z***	**Error mass (ppm)**	**R_T (min)**	**Molecular ion species detected**
Cysteine and methionine metabolism	Level 2	Homoserine	♂	120.0656	4.16	0.82	$[M+H]^+$
Arginine and proline metabolism	Level 3	Glutamate; methyl aspartate	♂	148.0605	3.38	0.82	$[M+H]^+$ $[2M+H]^+$
			♀	148.0606	2.70	0.82	$[M+H]^+$ $[M-H_2O+H]^+$
	Level 3	Valine; amino pentanoate	♂	118.0864	3.39	1.15	$[M+H]^+$
			♀	118.0864	3.39	1.13	
Pyrimidine metabolism	Level 2	Uracil	♂	113.0346	4.42	1.56	$[M+H]^+$ $[M+Na]^+$
			♀	113.0346	4.42	1.55	$[M+H]^+$
Purin metabolism	Level 2	Hypoxanthine	♂	135.0313	4.44	2.61	$[M-H]^-$
			♀	135.0314	5.18	2.61	$[M-H]^-$ $[2M-H]^-$
	Level 2	Adenine	♀	134.0474	5.22	2.75	$[M-H]^-$