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# Metabolic profiling identification of metabolites formed in Mediterranean mussels (*Mytilus galloprovincialis*) after diclofenac exposure

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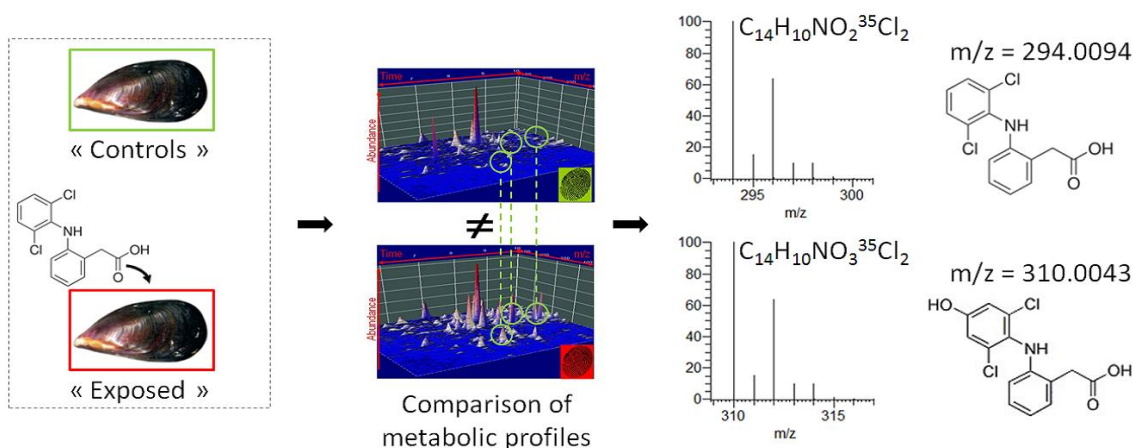
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## Highlights

- Metabolic profiling was applied to study diclofenac metabolism in mussels
- Thirteen compounds were identified as diclofenac metabolites
- 5 diclofenac metabolites are reported for the first time
- This method is relevant to study xenobiotic metabolism in non-target organism

## Graphical Abstract



## Abstract

Despite the growing concern on the presence of pharmaceutically active compounds in the environment, few studies have been conducted on their metabolism in marine organisms. In this study, a non-targeted strategy based on the generation of chemical profiles generated by liquid chromatography combined with high resolution mass spectrometry was used to highlight metabolite production by the Mediterranean mussel (*Mytilus galloprovincialis*) after diclofenac exposure. This method allowed revealing the production of 13 metabolites in mussel tissues. Three of them were phase I metabolites, including 4'-hydroxy-diclofenac and 5-hydroxydiclofenac. The remaining 10 were phase II metabolites, including sulfate and amino acids conjugates. Among all of the metabolites highlighted, 5 were reported for the first time in an aquatic organism exposed to diclofenac.

**Keywords:** NSAID, metabolism, LC-HRMS, non-targeted strategy, aquatic organism

## 1 Introduction

Pharmaceutically active compounds (PhACs) in aquatic environments have become major contaminants of interest over the last decade (Boxall et al., 2012; Fent et al., 2006). Their presence is due mostly to their limited removal rate in wastewater treatment plant effluents, which are considered as the main source of PhACs (Santos et al., 2010). A very small number of them have just been added to the first European Union Water Framework Directive watch list (Directive 2008/105/EC, European Commission, 2015). Diclofenac (DCF) was included on the list with the aim of gathering the first monitoring data.

Marine waters, like surface waters, are concerned by PhAC inputs because of the high population growth rates in coastal areas and the development of sea outfall wastewater treatment plants (Fenet et al., 2014). The extent of coastal PhAC contamination has been studied much less than in continental waters, under the pretext that dilution rates are high in such environments (Maruya et al., 2012). However, lagoons—which are transition zones between continental and marine waters—do not benefit from this dilution and are known to be nursery grounds for early marine fish life stages and a habitat for aquatic shellfish species such as mussels. Relatively few studies have focused on the potential

impact of pharmaceuticals and their bioconcentration in organisms inhabiting coastal environments (Huerta et al., 2012). Recent studies have nevertheless been published on the occurrence and bioconcentration of drug residues in seawater and marine organisms, thus raising questions on associated environmental risks (Alvarez et al., 2014; Arpin-Pont et al., 2016; Moreno-González et al., 2016). Among PhACs found in seawater, DCF concentrations have been reported from few ng/L to approximately 1 µg/L (Gaw et al., 2014).

PhAC exposure may lead to bioconcentration in non-target organisms, particularly mussels because of their limited mobility and filter feeding behavior. Once bioconcentrated, pharmaceuticals can be metabolized by organisms. Recent studies demonstrated that mussels exposed to DCF had a bioconcentration factor (BCF) ranging from 4 to 13, depending on the exposure concentration (Daniele et al., 2016b; Ericson et al., 2010). These results implied a low bioconcentration in mussel tissues, thus raising the question of possible DCF metabolization.

To our knowledge, only a few studies have investigated DCF metabolism in aquatic organisms. Two of them were conducted in bile of rainbow trout (*Oncorhynchus mykiss*) (Kallio et al., 2010; Lahti et al., 2011). The first study (Kallio et al., 2010) was conducted after intraperitoneal exposure to 0.25 mg DCF/100 g fish biomass and the bile samples were collected at 2 days postinjection. The second study (Lahti et al., 2011) was carried out after 10 days of exposure to different DCF concentrations, i.e. an environmental (1–2 µg/L) and a higher (25–50 µg/L) concentration. The results of both studies highlighted the presence of DCF and some of its main metabolites, i.e. 4'-hydroxy-diclofenac and 5-hydroxy-diclofenac, as well as their sulfate and glucuronide conjugates. In both studies, the acyl glucuronide of 3'-hydroxy-diclofenac was also detected. In a third study, DCF metabolite formation was studied in three-spined sticklebacks (*Gasterosteus aculeatus*). The fish were exposed for 6 months to various environmental DCF concentrations (0.05; 0.45 and 4.1 µg/L). Only 4'-hydroxy-diclofenac was detected in fish exposed to the highest concentration (Daniele et al., 2016a). Finally, a last study was conducted on zebra mussels, which to our knowledge is the only study that has been performed on bivalves (Daniele et al., 2016b). Mussels were exposed to three different DCF concentrations (0.05; 0.5 and 5 µg/L) for two different durations, i.e. 3 and 6 months. Only 2-indolone, a DCF

transformation product, was detected in mussel tissues. Note that these four studies were conducted on the basis of a targeted analysis, i.e. a search for already known transformation products, thus limiting the possibility of finding unacknowledged metabolites in organisms such as mussels for which information on PhAC metabolism is limited.

The use of a non-targeted approach, based on the generation of profiles of chemicals detected in organisms exposed and unexposed to a xenobiotic could be a good strategy for studying PhAC metabolism in such organisms. A semi-quantitative mass spectrometry approach based on the comparison of signal intensities detected in both groups of organisms could highlight differential signals corresponding to the administered xenobiotic and its metabolites. This kind of comprehensive approach for characterizing PhAC metabolites has been convincingly applied for drug metabolite detection in rat biological fluids (Plumb et al., 2003). However, it is not yet well applied in ecotoxicology even though it is proven powerful in toxicology (Werner et al., 2008). For example, Southam et al. (2011) demonstrated by such an approach that the main route of fenitrothion (organophosphorus pesticide) degradation in roaches (*Rutilus rutilus*) was O-demethylation based on a metabolomic study performed using Fourier Transform mass spectrometry. This kind of approach could thus be suitable for studying PhACs metabolism in non-target organisms for which data is scarce.

In this context, while very little information is available on the DCF biotransformation in non-target organisms, the goal of the present study was to investigate the potential of such a non-targeted approach for identifying DCF metabolites produced in mussels after exposure. To increase the probability of metabolite detection, mussels were exposed to two DCF concentrations that were higher than those usually found in marine waters. Analyses were performed with liquid chromatography (LC) combined with high resolution mass spectrometry (HRMS). This approach allowed us to putatively identify 13 DCF metabolites in mussel tissues, two of which have yet to be assigned. This is the first time that as many DCF metabolites were reported in an aquatic organism. After metabolite identification, those for which an analytical standard was available were quantified in both tissues and seawater for the two exposure concentrations.

## 2 Material and Methods

### 2.1 Chemicals

DCF ( $\geq 98\%$ ) and diclofenac-d4 (DCF-d4,  $\geq 98\%$ ) were purchased from Sigma-Aldrich (Steinheim, Germany). 4'-hydroxy-diclofenac (4'OHDCF,  $\geq 97\%$ ) and 5-hydroxy-diclofenac (5OH-DCF,  $98\% \pm 2\%$ ) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Stock standard solutions of individual compounds were prepared at 1000 mg/L concentration in methanol for DCF, DCF-d4, 4'OH-DCF and 5OH-DCF. Subsequent stock standard dilutions were prepared with methanol. All standard solutions were stored at  $-20\text{ }^{\circ}\text{C}$ .

Ultrapure water was generated by a Simplicity UV system from Millipore (Bedford, MA, USA) with a specific resistance of  $18.2\text{ M}\Omega\text{cm}$  at  $25\text{ }^{\circ}\text{C}$ . Pesticide analytical-grade solvents (methanol) and LC/MS grade solvents (water, acetonitrile) were from Carlo Erba (Val de Reuil, France). Formic acid (98%) was obtained from Fisher Labosi (Elancourt, France). Dispersive Oasis HLB™ SPE cartridges (30 mg, 1 cm<sup>3</sup> and 200 mg, 6 cm<sup>3</sup>) were obtained from Waters (Mildford, MA, USA). 30 mg cartridges were used for sample preparation of tissue while the 200 mg cartridges were used for seawater sample preparation.

### 2.2 Animals and experimental design

*Mytilus galloprovincialis* mussels were purchased in November from Mediterranean mussel suppliers (Bouzigues, France) and immediately transported to the laboratory (b1 h). After they were cleaned, the mussels were acclimatized in aerated (9 mg/L) filtered (GF/F, Whatman) natural seawater (salinity of 55 g/L, pH=7.8) for 7 days before the experiment. During the acclimation and exposure periods, the seawater was renewed every day (static renewal), the room temperature was regulated at  $18\text{ }^{\circ}\text{C}$ , and the mussels were fed *Tetraselmis suecica* (Greensea, Mèze, France) at constant density (10,000 cells/mL). 72 mussels (shell length 6–8 cm) were randomly distributed in 24 glass aquaria at a density of 3 mussels per liter. Four groups were constituted: control (C), solvent control (SC, absolute ethanol) and two groups exposed at nominal concentrations of around 100 and 600  $\mu\text{g/L}$  of DCF, respectively. Each group consisted of 6 replicates, i.e. 6 aquaria of 3 mussels each. During the exposure period, the DCF concentrations were reestablished after daily water renewal. Seawater was

sampled every day to quantify DCF and its hydroxylated metabolites. After 7 days of exposure, no mussel mortality was observed in each group. All mussels were dissected: gills and digestive glands (not within the scope of this study) were frozen at  $-80\text{ }^{\circ}\text{C}$  for further analysis, and the remaining soft tissues were frozen at  $-80\text{ }^{\circ}\text{C}$  before freeze drying (Heto Power dry LL 3000, Thermo) and analysis.

## **2.3 Sample analysis**

### **2.3.1 *Non-targeted analysis of DCF and its metabolites***

#### **2.3.1.1 *Tissue sample preparation***

50 mg dry weight ( $\pm 1$  mg) of tissue samples were extracted twice with 480  $\mu\text{L}$  acetonitrile and 20  $\mu\text{L}$  formic acid using ultrasound (10 min each). After each extraction, samples were centrifuged (2000 g, 15 min,  $4\text{ }^{\circ}\text{C}$ ). Combined supernatants were collected in a glass tube, evaporated to dryness under a nitrogen stream, and reconstituted in 1 mL of water and 200  $\mu\text{L}$  of methanol. A cleaning step was carried out using 30 mg SPE Oasis HLB preconditioned with 2 mL of methanol and 2 mL of ultrapure water. After loading, the cartridges were washed with  $2 \times 1$  mL methanol/water (5/95; v/v). Elution was performed using  $3 \times 1$  mL of methanol/water (80/20; v/v). The extracts were evaporated to dryness under a nitrogen stream and reconstituted in 400  $\mu\text{L}$  acetonitrile/water (20/80; v/v) and filtered directly into an analysis vial using a 0.20  $\mu\text{m}$  PTFE syringe filter (Minisart SRP 4, Sartorius).

#### **2.3.1.2 *LC-MS analysis***

For LC separation, a reverse phase PFPP analytical column (100 mm  $\times$  2.1 mm; 3  $\mu\text{m}$  particle size) (Sigma Aldrich) was used. The LC mobile phases were acetonitrile (A) and water (B), both containing 0.1% formic acid. Optimal separation was achieved using a 200  $\mu\text{L}/\text{min}$  flow rate and the following gradient (A:B, v/v): 5:95 at 0 min, 14:86 at 3 min, 34:66 at 14 min, 45:55 at 18 min, and 95:5 from 20 min to 25 min. The injection volume was 5  $\mu\text{L}$  using full loop injection. An Exactive LC-HRMS (Thermo Fischer Scientific) equipped with a heated electrospray ionization probe (HESI) source was used for analysis. The HESI parameters in positive/negative mode were as follows: sheath gas, 55 arbitrary units (AU); auxiliary gas, 10 AU; capillary temperature,  $275\text{ }^{\circ}\text{C}$ ; heater temperature  $150\text{ }^{\circ}\text{C}$  and electrospray voltage was set at 4.5/−3 kV. The tube lens was set at 95/−110 V, skimmer at 22/−16 V and capillary voltage at 40/−60 V. Full scan mode with a mass range of  $m/z$  100–800 at a mass

resolving power of 50,000 (FWHM,  $m/z$  200) was used in both ionization modes. Moreover, an “all ion fragmentation” MS/MS mode with a mass range of  $m/z$  50–800 at a mass resolving power of 50,000 (FWHM,  $m/z$  200) was used: 10 eV energy was applied to the high energy collision dissociation (HCD) cell.

### *2.3.1.3 Data processing and DCF metabolite identification strategy*

Chemical profiles from 4 SC samples and 4 exposed samples at 600  $\mu\text{g/L}$  of DCF were generated by LC-HRMS. The analysis gave us \*.raw data which were converted into a more exchangeable format (from \*.raw to \*.mzxml) with the free software MS Convert (ProteoWizard 3.0, (Chambers et al., 2012)). The data were processed without any a priori using the open-source XCMS data processing software (Smith et al., 2006), which integrated each chromatographic peak in each sample analyzed. Every step processed by the XCMS software has been previously described by Courant et al. (2009). XCMS parameters were adapted to acquire relevant information: the  $m/z$  interval for peak picking was set at 0.01, the signal to noise ratio threshold was set at 3, the group band-width was set at 8, and the minimum fraction was set at 0.6. After data processing, peaks were sorted to generate a two-dimensional data table in which rows represented the different ions and columns reported characteristics associated with the detected ions (Courant et al., 2014).

This table allowed us to compare the two groups of samples and determine the presence/absence of the peaks in the SC and exposed samples: only signals present in the exposed samples and missing in the SC samples were considered as likely to correspond to DCF metabolites. The fold change was calculated as the ratio of the mean signal intensity in exposed samples versus the mean signal intensity in the control samples. The higher the fold change, the higher the probability that the signal observed was associated with a metabolite appearing in exposed mussels. Only signals with a fold change of higher than 10 in negative or positive ionization mode were kept and further processed to elucidate the structures associated with the signals. Moreover, extracted ion chromatograms (EIC) were checked for all potential signals associated with metabolites so as to confirm the absence of any signal in the controls. Elemental compositions of unknown metabolites were generated by Thermo Xcalibur Qual



Browser (Xcalibur 3.0, Thermo Fisher Scientific Inc.), and those with the best fits, along with C, H, N, O and Cl compositions that could be related to DCF, are reported in this study.

### 2.3.2 Targeted extraction of DCF and its hydroxylated metabolites 4'OH-DCF and 5OH-DCF

#### 2.3.2.1 Sample preparation

50mg dry weight ( $\pm 1$ mg) of tissue samples were spiked with DCF-d4 (2 mg/kg). Solid/liquid extraction was conducted using the same protocol as that of non-targeted extraction, except for the washing step which was performed with  $2 \times 1$  mL methanol/water (30/70; v/v). The extracts were evaporated to dryness under a nitrogen stream and reconstituted in 150  $\mu$ L acetonitrile/water (20/80; v/v) before analysis. Histologically identified mature mussels were analyzed (n= 51).

Seawater samples (400  $\mu$ L) were spiked with DCF-d4 (100  $\mu$ g/L) and diluted with milli-Qwater to reach 5mL final volume. Samples were purified and pre-concentrated using 200 mg SPE Oasis HLB cartridges preconditioned with 5 mL of methanol and 5 mL of ultrapure water. After loading, the cartridge were washed with  $2 \times 3$  mL methanol/ water (30/70; v/v) and 1 mL methanol/water (30/70; v/v). Elution was performed using  $2 \times 5$  mL of methanol. The extracts were dried under a nitrogen stream and reconstituted in 200  $\mu$ L acetonitrile/ water (30/70; v/v) before analysis.

#### 2.3.2.2 LC-MS analysis

The above described LC-MS method (§2.3.1.2) was used for water and tissue analysis. Targeted analysis was only performed in negative electrospray ionization mode. Each compound was identified on the basis of the: (1) retention time, (2) mass accuracy of deprotonated molecule  $[M-H]^-$  < ppm in full scan MS mode compared to values obtained for the analytical standard in the same analytical conditions (Table 1).

Table 1 – Retention time and estimated LOD in mussel tissues and seawater (LOD: limit of detection; RT: retention time)

Analyte	[M]	[M-H] <sup>-</sup>	Retention time (min)	LOD in tissues ( $\mu$ g/kg dw)	LOD in seawater ( $\mu$ g/L)
Diclofenac (DCF)	295.0167	294.0094	21.55	1	0.1
Diclofenac-d4 (DCF-d4)	299.0418	298.0345	21.55	1	0.1
4'-hydroxy-diclofenac (4'OH-DCF)	311.0116	310.0043	19.91	1	0.1
5-hydroxy-diclofenac (5OH-DCF)	311.0116	310.0043	19.61	4	1

### *2.3.2.3 Quantification of DCF and its hydroxylated metabolites*

Tissue calibration curves were established in blank mussel tissues by adding a fixed amount of DCF-d4 internal standard and increasing quantities of the target analytes from 0 to 10,000 µg/kg dw for DCF, from 0 to 500 µg/kg dw for 4'OH-DCF, and from 0 to 100 µg/kg dw for 5OH-DCF before extraction.

Seawater calibration curves were established by adding a fixed amount of DCF-d4 internal standard and increasing quantities of the target analytes from 0 µg/L to 600 µg/L for DCF, from 0 µg/L to 50 µg/L for 4' OH-DCF, and for 5OH-DCF in blank seawater.

DCF concentrations in differentiated mussels were measured to evaluate its bioconcentration in the organisms. An apparent bioconcentration factor ( $BCF_a$ ) was calculated as the ratio of the measured concentration in the tissues and the measured concentration in seawater.

### *2.3.2.4 Performance of the method*

Analyte limits of detection were conventionally determined as the concentration inducing a signal to noise ratio of 3 in samples. The repeatability was calculated on the basis of 4 samples spiked with 200 µg/kg dw of each standard for tissues and with 200 µg/L of each standard for seawater. Absolute recoveries were calculated on the basis of analyte signals in samples spiked before extraction compared to analyte signals obtained for samples spiked after extraction. Relative recoveries were calculated as the ratio of the absolute recovery of each target compound to that of DCF-d4.

### *2.3.2.5 Statistical analysis*

Descriptive statistics are given either as means and standard deviation for DCF, 4'OH-DCF and 5OH-DCF. Nonparametric statistical tests were performed since no hypotheses concerning the normal distribution of the 4'OH and 5OH-DCF concentrations in tissue samples were put forward. Differences between groups were evaluated using the one-tailed Mann-Whitney U test. The upper bound approach was preferred: when an analyte was not detected (i.e. below the LOD), its value was expressed as the LOD.

### 3 Results

#### 3.1 Analytical performances

Linearity was found to be satisfactory, with R<sup>2</sup> values better than 0.99 for all target analytes in tissue and better than 0.97 in seawater samples. The average repeatabilities for DCF-d4, DCF, 4'OH-DCF, and 5OH-DCF were around 11% and never exceeded 21% (Table 2). Absolute recovery ranges were higher than 66% for DCF-d4, DCF, 4'OH-DCF, and 5OH-DCF (Table 2).

Table 2 – Linearity range, correlation coefficients, absolute and relative recoveries, and average repeatability of the HPLC-HRMS method

Matrix	Analyte	Linearity range	Correlation coefficient R <sup>2</sup>	Absolute recovery (±SD) (%)	Relative recovery (±SD) (%)	Average repeatability (%)
Tissues	DCF-d4	-	-	68 (± 8)	-	5
	DCF	0 – 10,000 µg/kg	> 0.99	66 (± 8)	97 (± 6)	10
	4'OH-DCF	0 – 500 µg/kg	> 0.99	72 (± 9)	106 (± 6)	13
	5OH-DCF	0 – 100 µg/kg	> 0.98	71 (± 6)	104 (± 9)	9
Seawater	DCF-d4	-	-	106 (± 10)	-	6
	DCF	0 – 500 µg/L	> 0.99	103 (± 7)	97 (± 6)	6
	4'OH-DCF	0 – 50 µg/L	> 0.99	104 (± 9)	98 (± 2)	7
	5OH-DCF	0 – 50 µg/L	> 0.97	81 (± 21)	76 (± 13)	21

#### 3.2 DCF quantification

The DCF concentrations were measured in water to control the exposure of mussels in aquaria. The results are presented in Table 3. DCF was not detected in C and SC aquaria, so the reported concentrations for these conditions correspond to the limit of detection. Exposure concentrations measured in exposed aquaria were quantified at 122.6 and 647.8 µg/L.

Table 3 – Mean concentrations and standard deviations of DCF in water (µg/L), in mussels (mg/kg, dw) and BCF (L/kg) calculated for each exposure group (C: control; SC: solvent control; 100 µg/L: 100 and 600 µg/L: 600)

Group (number of mussels)	C (n=11)	SC (n=11)	100 (n=14)	600 (n=15)
Concentration in water (µg/L)	<0.1	<0.1	122.6 ± 16.3	647.8 ± 108.1
Concentration in mussels (µg/kg dw)	<1	<1	2008 ± 361	7343 ± 1800
Experimental BCF <sub>a</sub> * (L/kg)	-	-	16.5	11.3

\*BCF<sub>a</sub> was calculated on the whole organism tissues exempt of the digestive gland and gills (see §2.3.2.3 for calculation method)

DCF was not detected in C and SC organisms. Mean DCF concentrations in exposed mussels were around 2 mg/kg dw and 7.3 mg/kg dw for exposure at 100 µg/L and 600 µg/L, respectively (Table 3). A low apparent bioconcentration factor ( $BCF_a$ ) was calculated for both exposure concentrations, ranging from 11 to 16 L/kg.

### 3.3 Non-targeted analysis of DCF and its metabolites in tissues

Thirteen metabolites were detected by the applied non-target approach. The non-targeted data treatment revealed more information in negative ionization mode than in the positive mode: 11 potential metabolites were detected in negative mode and 9 in positive mode. Among them, 7 were detected in both ionization modes. These metabolites are described below and are numbered from M1 to M13 according to their increasing retention time. All of them except M13 presented a specific dichlorinated compound pattern, as discussed below for DCF.

#### 3.3.1 Diclofenac

Diclofenac (DCF) was the major compound highlighted. The M peak corresponding to the parent compound with molecular mass  $[M-H]^-$  294.0094 in negative electrospray ionization mode (Fig. 1A), was eluted at 21.51 min retention time. In mass spectrometry, the molecular ion region of a monochlorinated compound has two major peaks separated by two m/z units with a 3:1 ratio in peak heights, corresponding to the natural abundance of one chlorine. Indeed, chlorine has two principal stable isotopes, i.e.  $^{35}\text{Cl}$  (75.78%) and  $^{37}\text{Cl}$  (24.22%). In case of dichlorinated compounds, such as DCF, peaks of the molecular ion region (M, M + 2 and M + 4) with gaps of 2 m/z units between them have a 9:6:1 relative peak height ratio. This relative ratio was observed for the diclofenac molecular ions detected in the mussel samples with M ( $[M-H]^- = 294.0094$ ) having an abundance of 9, M + 2 isotopic ions ( $[M-H]^- = 296.0063$ ) having an abundance of 6; and M + 4 isotopic ion ( $[M-H]^- = 298.0032$ ) having an abundance of 1. These abundances are respectively due to the presence of two  $^{35}\text{Cl}$  (M), one  $^{35}\text{Cl}$  and one  $^{37}\text{Cl}$  (M + 2); and two  $^{37}\text{Cl}$  (M + 4) (Fig. 1A). The relative ratio of dichlorinated compound was used as diagnostic isotopic pattern to confirm DCF metabolites identification. The experimental mass  $[M-H]^-$  294.0094 associated with DCF ( $\text{C}_{14}\text{H}_{11}\text{NO}_2\text{Cl}_2$ ) did not differ from the DCF theoretical mass (Table 4). HCD fragmentation

demonstrated the presence of a fragment at  $m/z$  250.0192. This fragment displayed the same 9:6:1 isotopic pattern as that observed for DCF (Fig. 1B). This fragment was associated with a loss of 43.9902 amu (atomic mass unit) compared to the measured DCF  $m/z$  294.0094, corresponding to a neutral loss of  $\text{CO}_2$  in the DCF structure. A second fragment was observed at  $m/z$  214.0419. This fragment corresponded to a loss of 35.9767 relative to the previously observed fragment, relevant with a loss of  $\text{HCl}$ . This  $\text{HCl}$  loss was confirmed by the 3:1 isotopic pattern presented by this fragment, which is specific to a monochlorinated compound (Fig. 1C). The same observations were done in positive ionization mode (Table 4). The DCF identification was confirmed by injection of the analytical standard in the same LC-HRMS conditions.

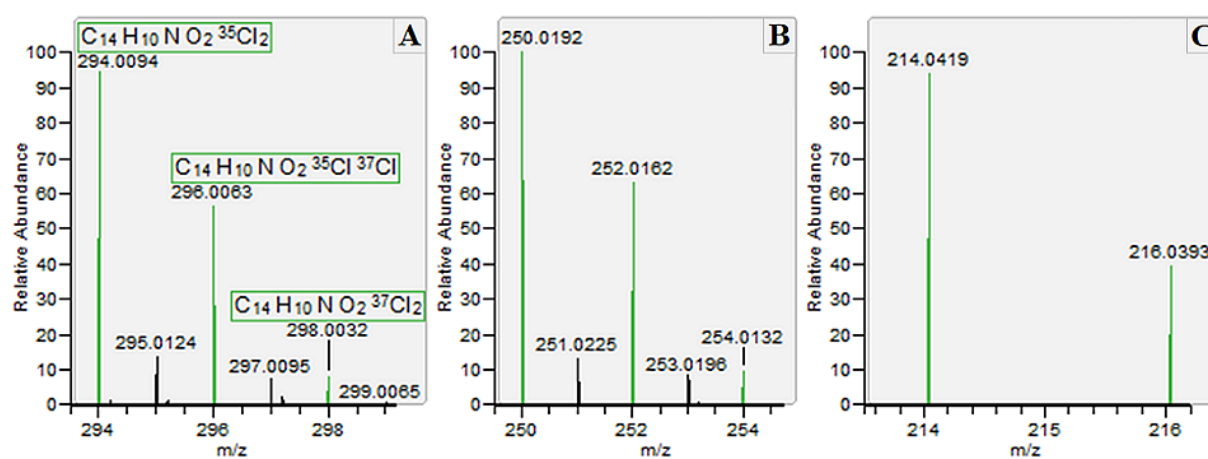


Figure 1 – High-resolution mass spectrum of DCF molecular ion at 50,000 FWHM. The green insets show the diagnostic  $M$  ( $m/z=294.0094$ ) /  $M+2$  ( $m/z=296.0063$ ) /  $M+4$  ( $m/z=298.0032$ ) dichlorinated isotopic distribution (A). High-resolution mass spectrum of molecular of fragments  $m/z$  250.0192 (B) and  $m/z$  214.0419 (C) at 50,000 FWHM

### 3.3.2 Phase I metabolites

#### 3.3.2.1 Oxidative metabolism – hydroxylation

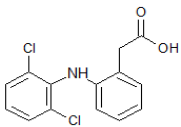
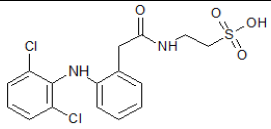
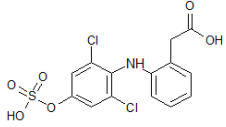
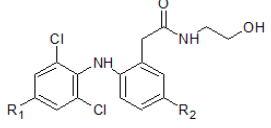
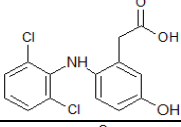
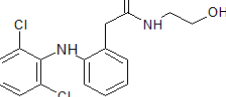
Two metabolites, i.e. M5 and M8, displayed a  $[\text{M}-\text{H}]^-$  of 310.0045 in ESI-. They presented a mass increment of +15.9953 compared to DCF, corresponding to biotransformation of an RH structure into an ROH structure, hypothetically leading to the formation of hydroxy metabolites (Anari et al., 2004; Zhang et al., 2009; Zhu et al., 2006). Mass spectra associated with these putative hydroxy metabolites highlighted the presence of two chlorines in their structure (9:6:1 isotopic pattern) confirming their potential connection with DCF. M5 and M8 exhibited shorter retention times than DCF, thus supporting the hypothesis of DCF hydroxylation, leading to a more polar compound that was eluted

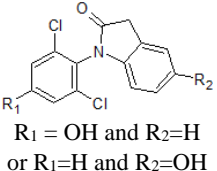
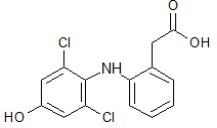
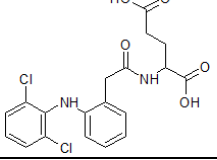
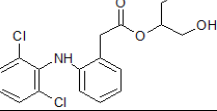
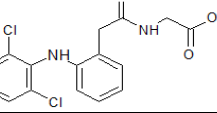
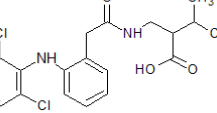
earlier in reverse phase LC (Table 4). For M8, two fragments were highlighted, the first one at  $m/z$  266.0143, corresponding to a  $\text{CO}_2$  loss and a second one at  $m/z$  230.0370, corresponding to an  $\text{HCl}$  loss consecutive to the  $\text{CO}_2$  loss observed for DCF (Table 4). Moreover, these fragments have already been reported for hydroxy metabolites (Sarda et al., 2012). Concerning M5, a fragment at  $m/z$  266.0139 was also observed. The second fragment corresponding to an  $\text{HCl}$  loss consecutive to the  $\text{CO}_2$  loss was not observed, probably because M5 was not sufficiently abundant in mussel tissues. The injection of commercially available hydroxy DCF standards in the same analytical conditions allowed us to allocate the 5OH-DCF structure at M5 and the 4'OH-DCF structure at M8. M5 and M8 were also highlighted in positive ionization mode (Table 4).

### 3.3.2.2 Lactam of hydroxydiclofenac formation

Metabolite M7 was detected at 19.98 min and  $[\text{M}-\text{H}]^-$  291.9940. It presented a characteristic isotopic pattern of a two chlorinated compound. M7 presented a mass shift of 2.0154 with DCF. This mass shift is generally explained by a loss of  $\text{H}_2$ , which could be attributed to five different reactions: i) transformation of the first alcohol to aldehyde; ii) transformation of the second alcohol to ketone; iii) transformation of 1,4-dihydropyridines to pyridines; iv) desaturation; and v) hydroxylation followed by dehydration (Nassar, 2009). The first three reactions were not relevant with respect to a possible DCF metabolite, whereas the last one seemed to be the most suitable for explaining the M7 formation. The lack of specific fragments highlighted after fragmentation in ESI- did not allow us to hypothesize a chemical structure for M7. However, in positive ionization mode, the precursor ion  $[\text{M}+\text{H}]^+$  294.0073 exhibited a fragment at  $m/z$  231.0437, corresponding to a loss of  $\text{CO}$  and  $\text{Cl}$ . A second fragment at  $m/z$  196.0731, corresponding to a loss of  $\text{Cl}$  consecutive to the previous loss of  $\text{CO}$  and  $\text{Cl}$ , was detected. Both of these fragments were similar to those observed for DCF and have already been reported in the literature and associated with a lactame dehydrate of hydroxy DCF (Stülten et al., 2008). Although fragments were observed, we were unable to determine the location of the hydroxyl group, but we hypothesize that the detected M7 was a lactame dehydrate of hydroxy DCF.

Table 4 – Summary of HPLC and mass spectrometric data obtained for non-targeted analysis of DCF and its metabolites in mussel tissues.

Peak ID	t <sub>R</sub> (min)	Assignment	Elemental composition [M]	Chemical structure	Observed [M-H] <sup>-</sup> (Δm)	Fold change	Relevant [M-H] <sup>-</sup> fragment / adduct ions (m/z) (HCD -10 eV)	Observed [M+H] <sup>+</sup> (Δm)	Fold change	Relevant [M+H] <sup>+</sup> fragment / adduct ions (m/z) (HCD -10 eV)	Relative abundance <sup>a</sup>	Reference
M	21.51	Diclofenac	C <sub>14</sub> H <sub>11</sub> NO <sub>2</sub> Cl <sub>2</sub>		294.0094 (0.0 mmu)	Inf.	250.0192 (-0.4 mmu) 214.0419 (-1 mmu)	296.0232 (-0.8 mmu)	Inf.	278.0123 (-1.1 mmu) 250.0173 (-1.2 mmu)	++++	(Botitsi et al., 2006; Huber et al., 2012; Sarda et al., 2012)
M1	13.83	DCF, taurine	C <sub>16</sub> H <sub>16</sub> N <sub>2</sub> O <sub>4</sub> Cl <sub>2</sub> S		401.0134 (-0.1 mmu)	Inf.	ND	ND	-	-	+	(Sarda et al., 2012; Stierlin et al., 1979; Stierlin and Faigle, 1979)
M2	14.66	4'-OH-DCF, sulfate	C <sub>14</sub> H <sub>11</sub> NO <sub>6</sub> Cl <sub>2</sub> S		389.9618 (+0.7 mmu)	Inf.	310.0050 (+0.7 mmu) 266.0151 (+0.6 mmu) 230.0379 (+0.1 mmu) 194.0595 (-1.6 mmu)	ND	-	-	+	(Sarda et al., 2012; Stierlin and Faigle, 1979)
M3	16.57	OH-DCF, ethanolamine	C <sub>16</sub> H <sub>16</sub> N <sub>2</sub> O <sub>3</sub> Cl <sub>2</sub>		353.0460 (-0.5 mmu)	Inf.		355.0607 (-0.4 mmu)	15		+	
M4	16.91	Unassigned	C <sub>19</sub> H <sub>21</sub> NO <sub>6</sub> Cl <sub>2</sub> <sup>b</sup>	N/A	428.0676 (-0.3 mmu) <sup>b</sup>	Inf.	474.0744 (+1.6 mmu) <sup>b</sup>	430.0813 (-0.6 mmu) <sup>b</sup>	30	ND	++	
M5	19.74	5OH-DCF	C <sub>14</sub> H <sub>11</sub> NO <sub>3</sub> Cl <sub>2</sub>		310.0047 (+0.4 mmu)	Inf.	266.0139 (-0.6 mmu)	312.0174 (-1.5 mmu)	Inf.	266.0127 (-0.7 mmu)	++	(Huber et al., 2012; Sarda et al., 2012)
M6	19.98	DCF, ethanolamine	C <sub>16</sub> H <sub>16</sub> N <sub>2</sub> O <sub>2</sub> Cl <sub>2</sub>		337.0513 (-0.3 mmu)	209	373.0283 (0.0 mmu)	339.0657 (-0.5 mmu)	33	361.0476 (-0.5 mmu)	+++	(Cohen et al., 1975)

M7	19.98	OH-DCF, lactam dehydrate	C <sub>14</sub> H <sub>9</sub> NO <sub>2</sub> Cl <sub>2</sub>	 R <sub>1</sub> = OH and R <sub>2</sub> =H or R <sub>1</sub> =H and R <sub>2</sub> =OH	291.9940 (+0.2 mmu)	1037	-	294.0073 (-1.0 mmu)	11	231.0437 (-0.8 mmu) 196.0731 (-2.6 mmu)	+	(Grillo et al., 2003; Stierlin et al., 1979; Stierlin and Faigle, 1979; Stülten et al., 2008)
M8	20.04	4'-OH-DCF	C <sub>14</sub> H <sub>11</sub> NO <sub>3</sub> Cl <sub>2</sub>		310.0045 (+0.2 mmu)	Inf.	266.0143 (-0.2 mmu) 230.0370 (-0.8 mmu)	312.0178 (-1.1 mmu)	Inf.	294.0074 (-0.9 mmu) 266.0124 (-1.0 mmu)	+++	(Huber et al., 2012; Sarda et al., 2012)
M9	20.05	DCF, glutamic acid	C <sub>19</sub> H <sub>18</sub> N <sub>2</sub> O <sub>5</sub> Cl <sub>2</sub> <sup>b</sup>		423.0520 (0.0 mmu) <sup>b</sup>	11	ND	ND	-	-	++	(Steventon and Hutt, 2001)
M10	20.50	DCF, glycerol	C <sub>17</sub> H <sub>17</sub> NO <sub>4</sub> Cl <sub>2</sub>		ND	-	-	370.0602 (-0.5 mmu)	34	352.0497 (-0.5 mmu)	+++	(Giri et al., 2006)
M11	20.78	DCF, glycine	C <sub>16</sub> H <sub>14</sub> N <sub>2</sub> O <sub>3</sub> Cl <sub>2</sub>		ND	-	-	353.0448 (-0.6 mmu)	Inf.	ND	+	(Hutt and Caldwell, 1990; King, 2009)
M12	21.87	DCF, leucine	C <sub>20</sub> H <sub>22</sub> N <sub>2</sub> O <sub>3</sub> Cl <sub>2</sub>		407.0927 (-0.8 mmu)	350	130.0862 (-1.2 mmu)	409.1052 (-2.8 mmu)	Inf.	132.1015 (-0.4 mmu)	++	(Feung et al., 1973)
M13	21.95	Unassigned	C <sub>14</sub> H <sub>10</sub> NO <sub>2</sub> BrCl <sub>2</sub> <sup>b</sup>	N/A	371.9189 (-1.0 mmu) <sup>b</sup>	Inf.	327.9298 (-0.3 mmu) <sup>b</sup>	ND	-	-	++	(Smith, 1968)

**Δm**: observed mass-theoretical mass

**INF**: infinite

**mmu**: milli mass unit

**ND**: not detected

<sup>a</sup> regardless of the ionization efficiency

<sup>b</sup> based on the formula deduced with Thermo Xcalibur Qual Browser



### 3.3.3 Phase II metabolites

#### 3.3.3.1 Conjugation to diclofenac

##### *Conjugation to amino acid*

The M1 metabolite eluted at 13.83 min was highlighted only in negative ionization mode at  $[MH]^-$  401.0134. M1 presented a mass shift of +107.0037 compared to DCF. This mass shift could be associated with conjugation to the taurine amino acid, as observed in a study on indinavir biotransformation by a human hepatic postmitochondrial preparation (Anari et al., 2004; Zhang et al., 2009). This potential conjugation was in accordance with the parity change in mass, i.e. even for DCF and odd for M1, due to the addition of a nitrogen atom in the structure. This mass change was due to the trivalence of the nitrogen atom. Unfortunately, the low intensity of M1 did not allow us to perform a conclusive fragmentation. This compound has already been described by several authors as a DCF conjugation product in studies in dogs (Stierlin and Faigle, 1979) or in mice for DCF (Sarda et al., 2012).

M9 was only detected at  $[M-H]^-$  423.0520. M9 eluted earlier than DCF (20.05 min) and was therefore more polar. The M9 parity mass change supported the hypothesis that there is one more nitrogen atom in its structure compared to DCF. No fragments were highlighted for M9. The calculated theoretical formula for M9 was  $C_{19}H_{18}N_2O_5Cl_2$  which could correspond to conjugation to N- or O-acetylserine, or glutamic acid. Among these compounds, to our knowledge, no O-acetylserine conjugation has yet been described for xenobiotic metabolism. N-acetylserine conjugation to a reactive metabolite of paracetamol, the N-acetyl-p-benzoquinoneimine, was investigated in vitro, and no evidence of conjugation was highlighted in the work of Madsen and coworkers (Madsen et al., 2007). However, conjugation of different xenobiotics such as benzoic acid or 3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid to glutamic acid has already been reported in bats and cows, and for other compounds in other organisms (Steventon and Hutt, 2001), so it therefore seems likely to occur in mussels.

M11 was only detected in positive ionization mode with at  $[M+H]^+$  of 353.0448 and presented a shorter retention time than DCF (20.78 min). The mass shift between M11 and DCF was +57.0216. This mass shift could be associated with conjugation to the glycine amino acid (Anari et al., 2004; Zhang et al., 2009). This conjugation was confirmed by the odd mass observed. Although we did not observe any M11 fragments, we are confident in this putative identification: glycine conjugation has already been well described for PhACs metabolism (Hutt and Caldwell, 1990; King, 2009), although not yet reported in mussels.

The M12 metabolite was detected at  $[M-H]^-$  407.0927, with a retention time of 21.87 min. M12 presented an odd mass and a mass shift of +113.0833 with DCF. The mass shift could be associated with conjugation to the leucine amino acid (or isoleucine). The hypothesis of leucine (or isoleucine) conjugation was in line with the observation of a fragment detected at  $m/z$  130.0862, corresponding to leucine loss. Moreover, the detection of M12 at  $[M+H]^+$  409.1052 in positive ionization mode, with a fragment at  $m/z$  132.1015, also supports this hypothesis. Leucine conjugation has already been described in plants exposed to the herbicide 2,4-dichlorophenoxyacetic acid (Feung et al., 1973).

#### *Conjugation to other compounds*

M6 measured at  $[M-H]^-$  337.0513 and its adduct  $[M-Cl]^-$  at  $m/z$  373.0283 eluted at 19.98 min. M6 also presented an odd mass that could be associated with the addition of a nitrogen atom in its structure. A mass shift of +43.0416 compared to DCF was observed and indicated conjugation with a  $C_2H_7NO$  compound. Because of the minor intensity of M6, no fragments were detected in negative ionization mode. Although the M6 compound was detected in positive ionization mode at  $m/z$  339.0657, no fragments were observed in this ionization mode either. In this ionization mode, an Na adduct was detected at  $m/z$  361.0476. The molecular mass observed in both negative and positive ionization mode corresponded to the chemical formula  $C_{16}H_{16}N_2O_2Cl_2$ . Conjugation to a  $C_2H_7NO$  compound was in agreement with conjugation with ethanolamine, which has already been observed in human urine after exposure to the anesthetic halothane (Cohen et al., 1975).

The M10 metabolite was only detected in positive mode at  $[M+H]^+$  370.0602 and eluted at 20.50 min. A fragment at  $m/z$  352.0497, corresponding to a loss of  $H_2O$ , was observed but did not allow us to determine the chemical structure of M10. The mass shift between M10 and DCF was equal to +74.0370. The attributed theoretical chemical formula of this compound was  $C_{17}H_{17}NO_4Cl_2$ , suggesting possible conjugation with a  $C_3H_8O_3$  compound, putatively glycerol. Conjugation with glycerol has already been reported in mouse urine for arecoline, an areca nut constituent, which has been used medicinally as an antihelmintic (Giri et al., 2006).

### 3.3.3.2 Conjugation to diclofenac phase I metabolites

M2 was only detected in negative ionization mode at  $[M-H]^-$  389.9618 at 14.66 min. The M2 retention time was shorter than that of DCF, 4'OH-DCF and 5OH-DCF. M2 showed a mass shift of +79.9570 compared to the OH-DCF metabolite, characteristic of sulfate conjugation during biotransformation (Anari et al., 2004). Moreover, different fragments were observed, i.e. one fragment at  $m/z$  310.0050 corresponding to a sulfate loss, one fragment at  $m/z$  266.0151 corresponding to a  $CO_2$  loss, and two other fragments at  $m/z$  230.0379 and  $m/z$  194.0595, corresponding to the consecutive loss of one or two chlorines, respectively (Table 4). The observed mass corresponded to sulfate conjugation to an hydroxy DCF metabolite, as already described in mouse urine and feces (Sarda et al., 2012). The observed M2 fragments have been described as specific to a 4'OH-DCF sulfate compound in the literature (Sarda et al., 2012).

M3 was measured in negative ionization mode at  $[M-H]^-$  353.0460 and in positive ionization mode at  $[M+H]^+$  355.0607. This compound eluted at 16.57 min. A mass shift of +43.0414 compared to OH-DCF metabolite is observed. This mass shift is described above as corresponding to a putative conjugation to ethanolamine. As noted for other low intensity metabolites highlighted in this study, such as M1, M6 or M11, no fragments were detected for M3 in both ionization modes. The lack of observed fragments did not allow us to determine the location of the hydroxyl group in the M3 structure.

### 3.3.4 *Unassigned metabolites*

Among the 13 potential DCF metabolites, 2 of them have yet to be structurally identified (Table 4).

M4 was detected at  $[M-H]^-$  428.0676, with its formic acid adduct at  $m/z$  474.0744 in negative ionization mode, and at  $[M+H]^+$  430.0813 in positive ionization mode. M4 eluted at a shorter retention time than DCF (16.91min), supporting the hypothesis of a more polar compound than DCF. The mass shift between M4 and DCF was +134.0579 and did not correspond to any known metabolic reaction. No fragments were detected for M4, which did not allow us to hypothesize a structure for this compound (Table 4). The theoretical chemical formula calculated for M4 was  $C_{19}H_{21}NO_6Cl_2$ . We were unable to putatively identify this compound, in accordance with current literature on xenobiotic or PhAC metabolism.

M13 was only detected in negative ionization mode at  $[M-H]^-$  371.9189 and eluted at a higher retention time than DCF (21.95 min). A fragment corresponding to a  $CO_2$  loss was observed at  $m/z$  327.9298. The mass shift of +77.9095 compared to DCF did not to our knowledge correspond to any known metabolic reaction. The formula calculated for M11 was  $C_{14}H_{10}NO_2BrCl_2$ . The M13 observed mass spectrum was close to the theoretical one determined for the  $C_{14}H_{10}NO_2BrCl_2$  formula (Fig. 2). Few studies report bromination of halogenated and phenol compounds in dogfish or fungus, but this reaction has never been observed for PhACs (Smith, 1968).

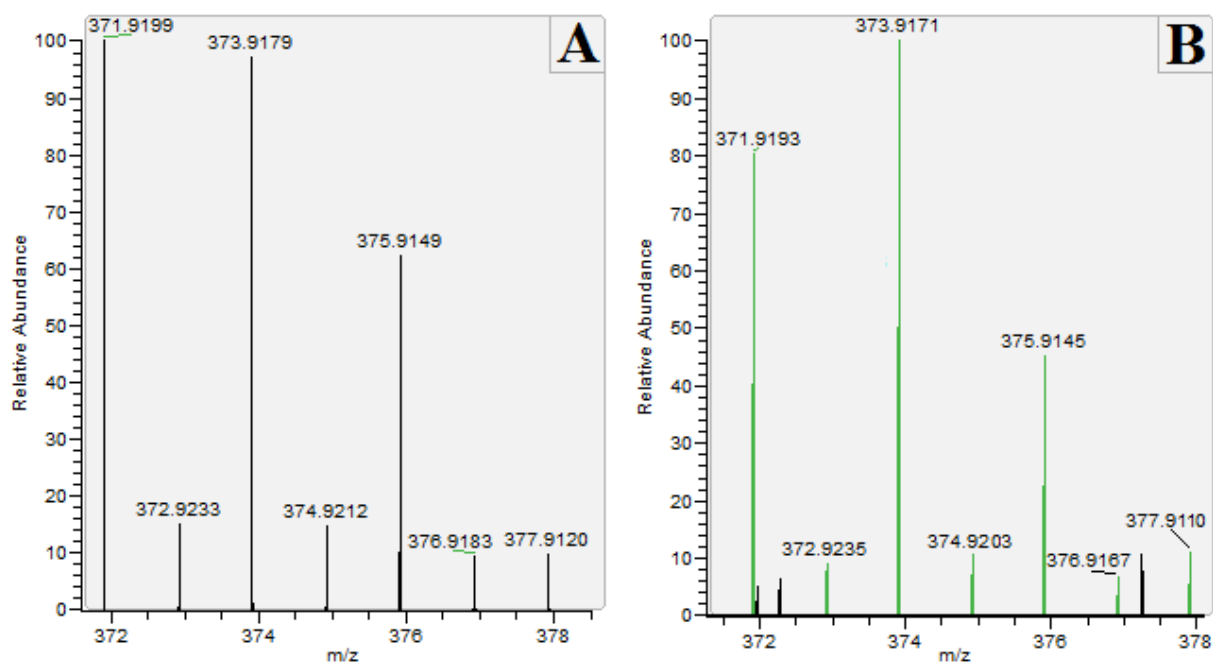


Figure 2 – Theoretical high-resolution mass spectrum of C<sub>14</sub>H<sub>10</sub>NO<sub>2</sub>BrCl<sub>2</sub> molecular ions in negative ionization mode (A), and high-resolution mass spectrum of molecular ions of M13 at 50,000 FWHM in negative ionization mode (B)

### 3.4 Quantification of hydroxy diclofenac metabolites

Both DCF hydroxy metabolites previously highlighted in mussel tissues have been quantified for the two exposure concentrations. Mean concentrations of 4'OH-DCF ( $89.0 \pm 42.7$  and  $178.6 \pm 79.8$   $\mu\text{g}/\text{kg}$  dw) and of 5OH-DCF ( $10.7 \pm 4.7$  and  $23.2 \pm 17.3$   $\mu\text{g}/\text{kg}$  dw) were measured in tissues exposed to 100 and 600  $\mu\text{g}/\text{L}$  of DCF, respectively. We can conclude that 4'OH-DCF was prevalent in tissues compared to 5OH-DCF. Moreover, both hydroxy metabolites were searched in seawater, which was sampled every 24 h before renewal of control and exposed aquaria. 5OH-DCF concentrations were under the limit of detection in all aquaria, 4'OH-DCF was detected at mean concentrations of 0.5 and 2.2 ( $\pm 1.6$ )  $\mu\text{g}/\text{L}$  in aquaria exposed to 100 and 600  $\mu\text{g}/\text{L}$  of DCF, respectively. As these two metabolites have not been described as DCF photolysis products (Agüera et al., 2005; Bartels and von Tümpling Jr., 2007), we concluded that DCF metabolism was triggered in <24 h in mussels.

## 4 Discussion

The observed BCF (11.3 and 16.5 for DCF exposure at 100 and 600  $\mu\text{g}/\text{L}$ , respectively) were in the same range as those already obtained for mussels exposed to DCF. In a first study, the BCF reported for Baltic Sea mussels exposed to 1 mg/L of DCF was of 10 (Ericson et al., 2010). A second study

conducted in zebra mussels reported a BCF ranging from 4 to 13 following exposure to DCF environmental concentrations (0.05, 0.5 and 5 µg/L) for 3 and 6 months (Daniele et al., 2016b). Based on the obtained values, we concluded that there was little DCF bioconcentration in mussel tissues.

This low bioconcentration associated with the presence in mussels of cytochrome P450 (CYP 450) complexes involved in xenobiotic metabolism supported the hypothesis of DCF metabolic transformation in mussels (Livingstone et al., 1989).

The non-targeted analysis of mussel tissues allowed us to identify 3 phase I metabolites, and 10 phase II metabolites, none of which have to date been described for DCF metabolism in mussels. These 13 above described metabolites allowed us to propose a partial metabolic pathway for DCF metabolism in mussels (Fig. 3).

Previous studies were conducted to identify DCF metabolites in humans and baboons, dogs, rats and mice (Sarda et al., 2012; Stierlin et al., 1979; Stierlin and Faigle, 1979). CYP2C9 and CYP3A4 enzymes have been reported as being involved in the formation of 4OH-DCF and 5OH-DCF, respectively, in humans (Leemann et al., 1993; Shen et al., 1999). However, the presence of these enzymes in mussels has yet to be formally documented, but some CYP450 enzymes have been described in several mollusk species, including *M. galloprovincialis* (Livingstone et al., 1989). More recently, CYP3-like genes have been identified in *M. edulis* mussels. The CYP3-like sequences identified were described as sharing 36 to 39% identity with human CYP3A4, which is involved in 5OH-DCF formation (Zanette et al., 2013), so the observation of DCF hydroxylated metabolites in mussels is relevant. Other hydroxy metabolites have been previously described in baboon, human and rat urine and bile, including 3'-hydroxy-diclofenac and 4',5-dihydroxy-diclofenac (Stierlin et al., 1979; Stierlin and Faigle, 1979). These other hydroxy metabolites were not detected in our study, nor in another study conducted in mussels (Daniele et al., 2016b). Three assumptions could be put forward to explain this: i) these compounds were not concentrated enough to be detected in our study, ii) the sample preparation was not adapted to extract them from mussel tissues, or iii) these metabolites were not generated during DCF transformation in mussels. Hydroxy-diclofenac lactame dehydrate (M7) was previously described as an artifact formed during sample preparation of urines of different species

exposed to DCF (Stierlin et al., 1979). However, this hypothesis was challenged by Grillo and coworkers. They hypothesized that lactame dehydrate derivative (also called indolinone derivative) formation may occur via two pathways: diclofenac-S-acyl-glutathione degradation or intramolecular cyclization of diclofenac-S-acyl-CoA thioesters, i.e. intermediary products which are both unstable (Grillo et al., 2003). More recently, the hydroxyl-diclofenac lactame dehydrate was described as a human urinary metabolite after exposure to DCF (Stülten et al., 2008). The sample preparation applied to urine samples in that study was not the same as that used by (Stierlin et al., 1979), thus invalidating the assumed artifact formation during the sample preparation. Therefore, the hypothesis of M7 formation in mussels could be considered realistic.

Some of the phase II metabolites have already been described in the literature. The DCF taurine conjugate was described as the major metabolite detected in dog urine (Stierlin et al., 1979), while also being described in mouse urine (Sarda et al., 2012). Other OH-DCF taurine metabolites have also been reported in mouse urine (Sarda et al., 2012), but only taurine conjugation to the parent compound (M1) was observed in our study. Although amino acid conjugation to pharmaceuticals has been well described in many species — but not in mussels to our knowledge — we reported for the first time glutamic acid (M9), glycine (M11) and leucine (M12) conjugated to DCF. Leucine conjugation to an xenobiotic is less common. Although the formation of such a metabolite was described elsewhere (Feung et al., 1973), leucine conjugation has not been described in the literature for marine organisms. No information is available concerning leucine conjugation formation, so the mechanism involved in M12 formation in mussels remains unclear and did not allow us to confirm or refute our hypothesis.

Sulfate conjugate of 4'-hydroxy-diclofenac (M2) has already been described in rats (Stierlin and Faigle, 1979) and mice (Sarda et al., 2012). Other sulfate conjugates of DCF metabolites have also been described in these organisms (i.e. 5OH-DCF sulfate or 4'-OH-DCF glucose sulfate), but these metabolites were not detected in this study. The same assumptions than those described for the hydroxylated metabolites can be made concerning the non-detection of sulfate conjugates.

Glucuronide conjugates of DCF, OH-DCF and OH-DCF conjugates have been reported in fish (Kallio et al., 2010; Lahti et al., 2011). These glucuronide conjugates were not observed in mussel tissues

(present and former studies). This lack was not associated with any inability of xenobiotic conjugation to glucuronide in mussels: *in vitro* experiments carried out by exposing mussel digestive glands to aminofluorene showed glucuronide conjugate formation (Kurelec and Krča, 1989). However, glucuronidation seems to be less common than glucosidation in invertebrates (James, 1987), which could explain the absence of detection of such metabolites. The previous hypothesis put forward for hydroxylated and sulfate metabolites are also possible.

2-indolone was observed in mussel tissues (Daniele et al., 2016b), which was not detected in the present study. Two hypotheses could be put forward to explain this: i) our sample preparation did not allow us to recover 2-indolone, or ii) 2-indolone was formed following DCF photolysis (Bartels and von Tümpling Jr., 2007), but note that the former study on mussels was conducted in a mesocosm. Nevertheless, 2-indolone has also been described as an intermediate in the formation of hydroxy-diclofenac lactam dehydrate in rat bile (Grillo et al., 2003). This suggests that this intermediate was too reactive to be observed in our study.

Concerning the less common conjugates observed, such as glycerol or ethanolamine, few studies have reported their production in organisms exposed to xenobiotics. As far as we are aware, very few studies have reported such conjugation to PhACs in mammals and none of them refer to marine organisms.

In humans, hydroxy metabolites and glucuronide conjugates are the main metabolites detected in plasma and urine (Riess et al., 1978; Tang, 2003). However, the quantification of both hydroxy metabolites in tissues indicated 5OH-DCF (M5) and 4'OH-DCF (M8) represented about 0.4% and 3.4% of the DCF concentration measured in tissues, respectively. In these conditions, M8 seemed to be predominant in relation to M5 and was one of the most abundant metabolites detected (when not considering the ionization efficiency). Daniele and coworkers did not detect any DCF metabolites in their study, except 2-indolone. However, considering that: i) 4'OH-DCF has a low formation rate in mussels, and ii) the highest exposure concentration in the study of Daniele was 5 µg/L, whereas that in our study was 120-fold higher (600 µg/L), 4'OH-DCF (and other metabolites) were probably close to their method's limit of quantification. Another hypothesis is that the higher exposure concentration



applied in our work may trigger different and/or more metabolic pathways. Both of these hypotheses may explain the higher number of metabolites observed in the present study. Our results tends to confirm that high concentrations are more suitable for studying PhAC metabolism in organisms for which little published information is available. Nevertheless, considering the low formation rate of all the metabolites detected in the present study, we probably did not identify all metabolites formed after DCF exposure, possibly because of unsuitable sample preparation, or due to the poor ionization efficiency of those omitted metabolites in the mass spectrometry analyses. Further research using other techniques (e.g.  $^{14}\text{C}$ -labelling) could help to overcome this limitation.

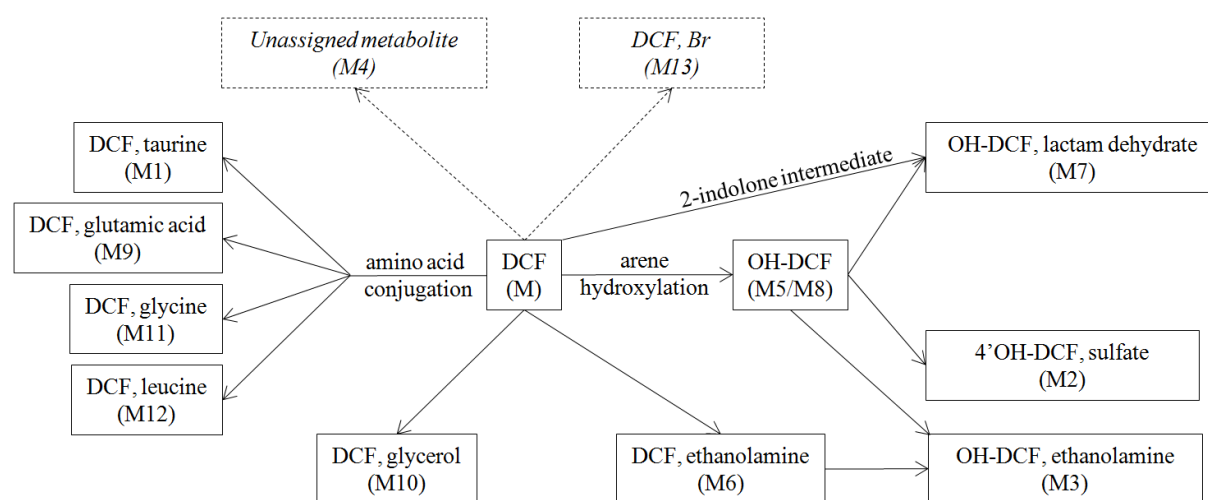


Figure 3 – Proposed metabolic pathway of diclofenac in mussel tissues.

--- Unassigned metabolites  
 — Assigned metabolites

Knowledge on pharmaceuticals metabolism in non-target organisms is a matter of increasing concern. The xenobiotic (bio)transformation product potential activity has been pointed out as a significant issue in studying pharmaceuticals environmental impact (Escher and Fenner, 2011; Huerta et al., 2012; Schmitt-Jansen et al., 2007; Schulze et al., 2010). Nonsteroidal anti-inflammatory drugs activity in human is known to be associated with the inhibition of both cyclooxygenases (COX)-1 and (COX)-2, leading to the inhibition of prostaglandins synthesis which are involved in inflammation processes. This mode of action was confirmed in aquatic organisms such as fish or mussels exposed to ibuprofen (Bhandari and Venables, 2011; Gagné et al., 2005; Morthorst et al., 2013) or to DCF (Mehinto et al., 2010; Courant et al, in prep.). However, very little information is available concerning the activity of

diclofenac metabolites. 4'OH-DCF was described as able to inhibited the cyclooxygenase-2 activity, leading to the prostaglandin E2 synthesis inhibition in human cell (Yamazaki et al., 1997) and it has been suggested as more potent than DCF (Menassé et al., 1978). Some other DCF metabolites are well known to generate (toxic) effects in organisms. In human and rats, both 4'OH-DCF and 5OH-DCF are known as potential protoxicants because of their potential biotransformation in quinone imines and arene oxides DCF metabolites. Moreover, the common glucuronide conjugates are also described as potentially reactive, particularly by means of protein adducts formation (Boelsterli, 2003). None of these metabolites (glucuronide conjugates, arene oxide or quinone imines) were observed in this study. As suggested above it does not mean that these metabolites were not formed in mussels but probably we were not able to highlight them. Further studies would be necessary to study the potential formation of such reactive DCF metabolites in mussels.

In summary, this study revealed that different pathways were involved in DCF metabolism in mussels — the description of the different metabolites formed was in accordance with the low apparent BCF calculated in the studied tissues. Perspectives would be to study the effects after DCF exposure (at environmental concentrations) in mussels. Metabolomics and particularly the MS-chemical profiles generated through this approach could help to generate information: i) on biotransformation products of a particular compound (which was within the scope of this study for DCF metabolites), and ii) on variations in endogenous metabolites that could occur following exposure and that would correspond to the physiological response of the organism to this exposure. In this latter case, the same approach based on a comparison of signal intensities detected in profiles collected before and after exposure to a pollutant, could highlight down- or up- regulation of some metabolites, corresponding to perturbations of particular metabolic signalization pathways. This may help to gain insight into the possible adverse effects of pollutants in non-target organisms.

## **5 Conclusion**

The use of a non-targeted approach was powerfully applied for screening DCF transformation products in Mediterranean mussels. It allowed us to detect the parent compound and 13 DCF metabolites, 3 of which were phase I metabolites, and 10 were phase II metabolites. Non-targeted

analysis, carried out without any a priori, enabled us to detect metabolites that we might have overlooked in a targeted analysis because of their general lack of mention in the literature. We conclude that in ecotoxicology the non-targeted approach is effective for studying xenobiotic metabolism in non-target organisms, particularly those for which little is known regarding their metabolism.

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