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# Metabolomics assessment of the effects of diclofenac exposure on *Mytilus galloprovincialis*: potential effects on osmoregulation and reproduction

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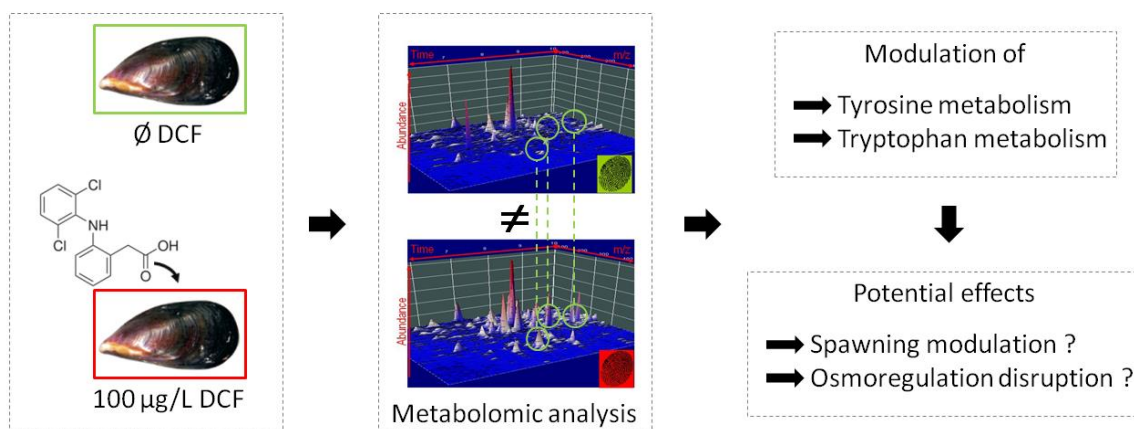
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## Highlights

- A non-targeted approach was applied to gather information on DCF effects on mussels
- Metabolomic study highlighted DCF effects on two main metabolic pathways
- DCF effects on neurotransmitters may be of concern
- DCF exposure could lead to mussel osmoregulation and reproduction damage

## Graphical Abstract



## **Abstract**

The presence of pharmaceutically active compounds in aquatic environments has become a major concern over the past 20 years. Elucidation of their mode of action and effects in non-target organisms is thus now a major ecotoxicological challenge. Diclofenac (DCF) is among the pharmaceutical compounds of interest based on its inclusion in the European Union Water Framework Directive Watch List. In this study, our goal was to investigate the potential of a metabolomic approach to acquire information without any a priori hypothesis about diclofenac effects on marine mussels. For this purpose, mussel's profiles were generated by liquid chromatography combined with high resolution mass spectrometry. Two main metabolic pathways were found to be impacted by diclofenac exposure. The tyrosine metabolism was mostly down-modulated and the tryptophan metabolism was mostly up-modulated following exposure. To our knowledge, such DCF effects on mussels have never been described despite being of concern for these organisms: catecholamines and serotonin may be involved in osmoregulation, and in gamete release in mollusks. Our results suggest potential impairment of mussel osmoregulation and reproduction following a DCF exposure.

**Keywords:** NSAID, non-targeted approach, dopaminergic pathway, serotonin

## **1 Introduction**

Pharmaceuticals have become major environmental contaminants of concern over the last 20 years (Boxall et al., 2012; Heberer, 2002). The presence of pharmaceuticals in waters is mainly due to inputs from wastewater treatment plants (Petrie et al., 2015; Verlicchi et al., 2012). Marine waters are also affected by these inputs due to the development of sea outfall wastewater treatment plants and watershed contributions (Fenet et al., 2014).

There is growing interest in pharmaceuticals and consequently their potential effects on non-target organisms, including marine organisms, is a current research challenge (Segner, 2011). Recent studies have documented the presence of pharmaceuticals in marine organisms (Alvarez et al., 2014; Arpin-Pont et al., 2016; Moreno-González et al., 2016). However, few studies have focused on the potential impact of pharmaceuticals on coastal organisms (Huerta et al., 2012). Among pharmaceuticals found

in seawater, diclofenac (DCF) concentrations from a few ng/ L to 1 µg/L have been reported (Gaw et al., 2014). DCF has recently been added to the first European Union Water Framework Directive Watch List but there is insufficient monitoring data available (Commission Implementing Decision 2015/495, European Commission, 2015). DCF is one of the most widely prescribed non-selective non-steroidal anti-inflammatory drugs (NSAIDs) with the highest environmental concentration and detection rates (along with ibuprofen) (He et al., 2017). Its presence in aquatic ecosystems has led to its detection in wild aquatic organisms, such as mussels, at concentrations of around 10 ng/g along European coasts (Capolupo et al., 2017; Cunha et al., 2017; Mezzelani et al., 2016).

Some effects of DCF on mussels have been documented in the literature. Laboratory exposure of *Mytilus edulis* to high DCF concentrations (1.000 and 10.000 µg/L) led to impaired scope for growth and byssus thread strength (Ericson et al., 2010). Furthermore, different periods of exposure to 250 ng/L of DCF led to oxidative stress in marine mussels (Gonzalez-Rey and Bebianno, 2014). Finally recent study conducted in Mediterranean mussels exposed to 1 and 100 µg/L of DCF during 3 days revealed a prostaglandin E2 synthesis reduction which may be in accordance with both cyclooxygenase COX-1 and COX-2 inhibition, as DCF known mode of action in human (Courant et al., 2017). These studies, conducted by targeted approaches on various endpoints, revealed that DCF can have impacts while also highlighting the necessity of developing one-shot analysis methods capable of detecting diverse effects. Indeed, DCF may have other modes of action beyond inhibition of cyclooxygenase COX-1 and COX-2 activity.

To assess potential undocumented DCF effects on mussels, we investigated the efficiency of a non-targeted approach in order to work without any *a priori* hypothesis. A non-targeted metabolomic approach involves generation and comparison of metabolic profiles acquired from different groups of samples by a suitable analytical technique, such as nuclear magnetic resonance (NMR) or mass spectrometry (Courant et al., 2014). Metabolic profiles provide an overview of all metabolites accessible for analysis and their comparison between different sample groups can provide molecular signatures in certain disease states, physiological conditions or effects of xenobiotic exposure, for example (Courant et al., 2014).

This approach has been conclusively applied to assess the effects of various pollutants on fish or crustaceans using liquid chromatography hyphenated to mass spectrometry. A metabolomic study has been conducted following exposure to fenitrothion, an organophosphate pesticide, on roaches and the results highlighted steroid metabolism disruption and hepatic phosphagen system perturbations (Southam et al., 2011). Other metabolomic studies have been conducted to assess the effects of sewage treatment plant effluents on fish, and the findings revealed diverse effects such as prostaglandin or lipid metabolism modulation (David et al., 2017; Samuelsson et al., 2011). However, few metabolomic studies have been conducted to evaluate the impacts of pharmaceuticals exposure on non-target organisms. A metabolomic study of daphnia exposed to carbamazepine and ibuprofen amino acid modulation revealed potential effects of carbamazepine on energy metabolism (Kovacevic et al., 2016). An experiment conducted by Gómez-Canela et al. (2016) on gammarids highlighted the effects of nimesulide and propranolol on protein synthesis, oxidative stress and signaling cascades. These experiments demonstrated that the metabolomic approach is valuable for studying xenobiotics effects on non-target organisms. Metabolomic studies on mussels have mainly been carried out using the NMR approach while being focused on metals, pesticides and other xenobiotics, but no studies have focused on pharmaceuticals to date.

The present study was carried out to assess the potential of metabolomics for highlighting undocumented effects of DCF exposure on Mediterranean mussels. We deliberately exposed mussels to a high DCF concentration for this proof-of-concept. Analyses were performed with liquid chromatography (LC) hyphenated to high resolution mass spectrometry (HRMS). This approach allowed us to highlight two metabolic pathways impacted mainly by DCF exposure. This is the first time that a LC-HRMS metabolomic study has been conducted to assess DCF effects on a non-target organism.

## **2 Material and methods**

### **2.1 Chemicals**

DCF ( $\geq 98\%$ ) was purchased from Sigma-Aldrich (Steinheim, Germany). Stock standard solution was prepared at 1000 mg/L concentration in methanol, as well as subsequent stock standard dilutions. 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 analysis-grade solvents (methanol, dichloromethane) and LC/MS-grade solvents (water, acetonitrile, formic acid 99%) were from Carlo Erba (Val de Reuil, France).

## **2.2 Animals and experimental design**

The experimental design was described elsewhere (Bonnefille et al., 2017). Briefly, *Mytilus galloprovincialis* mussels (6–8 cm shell length) purchased in November from Mediterranean mussel suppliers (Bouzigues, France) were acclimated for 7 days before the experiment. Groups of mussels randomly distributed in glass aquaria were constituted. Among these groups, one was exposed to solvent (ethanol), the vehicle used to expose mussels to DCF (SC group, 3 mussels per aquaria, 6 aquaria), and another one was exposed to a concentration of  $122.6\text{ }\mu\text{g/L}$  ( $\pm 16.3$ ) of DCF (nominal exposure concentration of  $100\text{ }\mu\text{g/L}$ ; 3 mussels per aquaria, 6 aquaria). Daily seawater renewal (static renewal) was carried out for each acclimation and exposure day. During the exposure period, the DCF concentrations were reestablished every day and monitored by LC-HRMS analysis. No mortality was observed during the exposure period. The monitored water parameters were: pH (7.8), salinity (55 mg/L), dissolved oxygen (9 mg/L) and temperature ( $18\text{ }^{\circ}\text{C}$ ). These parameters did not differ among the two exposure groups.

After 7 days of exposure, all mussels were dissected: digestive glands were frozen at  $-80\text{ }^{\circ}\text{C}$  before freeze drying (Heto Power dry LL 3000, Thermo) and analysis. Mature mussels were histologically identified. For each exposure group, only male mussels were studied ( $n = 8$  in each group) due to the insufficient number of female mussels in each group.

## **2.3 Samples analysis**

### **2.3.1 Tissue sample preparation**

Forty-five milligram dry weight ( $\pm 1\text{ mg}$ ) of digestive gland were extracted with a biphasic mixture of water/methanol/dichloromethane (Liebeke and Bundy, 2012; Masson et al., 2010). Briefly, tissues were homogenized with  $360\text{ }\mu\text{L}$  of cold methanol and  $115\text{ }\mu\text{L}$  of cold water by vortex for 1 min. Secondly,  $360\text{ }\mu\text{L}$  of cold dichloromethane and  $180\text{ }\mu\text{L}$  of cold water were added and the samples were

vortexed a second time for 1 min, left on ice for 15 min, and centrifuged at 2000g for 15 min at 4 °C. The methanol/water layer containing the polar metabolites was collected and filtered on modified PES centrifugal devices (VWR, cut off at 10 kDa) at 12000g for 30 min in order to remove high molecular weight proteins. 250 µL of the filtered aqueous phase was evaporated to dryness under a nitrogen stream and reconstituted in 200 µL acetonitrile/water (5/95; v/v). A quality control (QC) sample corresponding to a pool consisting of 50 µL of each sample extract was injected at the beginning of the injection sequence to equilibrate the column. QC injections were repeated throughout the injection sequence to monitor the analytical repeatability and sensitivity during analysis. To assess the injections repeatability, relative standard deviation (RSD) was calculated for each ion detected in the QC injections replicated. Only ions with an RSD lower than 30% were kept for data processing and annotation (Want et al., 2010).

### **2.3.2 LC-MS analysis**

A reverse phase pentafluorophenyl propyl analytical column was used for LC separation (PFPP, 100 mm × 2.1 mm; 3 µm particle size; Sigma Aldrich). The LC mobile phases were 99% acetonitrile and 1% water (A), 99% water and 1% acetonitrile (B), both containing 0.1% formic acid. Optimal separation was achieved using a 200 µL/min flow rate and the following gradient (A:B, v/v): 5:95 during 3 min, 40:60 at 8 min, 50:50 at 9 min, 70:30 at 13 min, and 95:5 from 15 min to 18 min. The injection volume was 5 µL 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 were the following in positive/negative mode: sheath gas, 55 arbitrary units (AU); auxiliary gas, 10 AU; capillary temperature, 350 °C; heater temperature 300 °C and the electrospray voltage was set at |3.5|/|3| kV. The tube lens was set at 90/−90 V, skimmer at 26/−25 V and capillary voltage at 45/−20 V. Full scan mode with a mass range of m/z 50–1000 at a mass resolving power of 50,000 (FWHM, m/z 200) was used in both ionization modes.

## **2.4 Post-acquisition metabolic fingerprint treatment**

### **2.4.1 Data processing and quality control**

Chemical profiles from 8 SC samples and 8 samples exposed to 100 µg/L of DCF were generated by LC-HRMS. The \*.raw data obtained were converted into \*.mzxml data with the freeware MS Convert (ProteoWizard 3.0, Chambers et al., 2012). The data were processed by open-source XCMS data processing software (Smith et al., 2006). Every step processed by XCMS software has been previously described by Courant et al. (2009). The XCMS parameters were as follows: the m/z interval for peak picking was set at 0.01, the signal to noise ratio threshold was set at 6, 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 on which rows represent the different ions and columns report characteristics associated with the detected ions (Courant et al., 2014).

### **2.4.2 Statistical analysis**

Univariate statistical analysis results were obtained from the XCMS treatment using the Welsh test. Multivariate data analyses were performed with SIMCA software (version 13.0, Umetrics, Sweden). Datasets were log transformed and Pareto-scaled, and principal component analysis (PCA) was performed to assess clustering between samples.

### **2.4.3 Metabolite annotation and identification**

The annotation process was conducted on signals with a  $p < 0.1$ . The rationale for choosing such a threshold is based on i) the low number of mussels per group which may lead to a low statistical power and ii) the opportunity to reveal a biological modulation trend due to the exposure. Indeed, a trend ( $p < 0.1$ ) toward the down or up-modulation of several metabolites from the same pathway (highlighting a disruption in the biochemical cascade) may be more biologically relevant than a single marker metabolite ( $p < 0.05$ ). Even if the latter is statistically more powerful, a single metabolite is however not strong enough to reveal a biochemical pathway disruption accurately. Before annotation of those signals, visual inspection of all extracted ion chromatograms was performed to ensure that each selected signals at a defined m/z and retention time corresponded to a metabolite, presenting a Gaussian chromatographic peak (i.e. not a baseline drift or background noise signal). The putative



monoisotopic masses were used for queries in the Human Metabolome Database (HMDB) (<http://www.hmdb.ca/>) with a monoisotopic mass tolerance of 0.002 Da to obtain corresponding possible chemical structures (raw formulas). Among these annotations, only those with a deviation lower than 5 ppm between the observed mass and the theoretical mass were kept. The identity of annotated compounds was confirmed whenever possible with the injection of the corresponding commercially available standard compound on the same analytical system under the same conditions (validation based on both retention time and monoisotopic mass). For compounds for which we lacked analytical standards, when possible a comparison was performed between our data and information available in the literature on the analysis of standard compounds conducted with the same analytical column (Boudah et al., 2014). Levels of annotation are reported in the Table 1 on the basis of the work conducted by Sumner et al. (2007). The level 1 annotated peaks are characterized by a minimum of two independent and orthogonal data relative to an authentic analytical standard analysed under the same analytical conditions (in this study retention time and accurate mass). The level 2 annotated peaks are corresponding to putatively annotated compounds on the basis of the use of literature (public databases) and/or external laboratory results. When several putative annotations were possible for the same signal, they were kept and are reported in Table 1 and Fig. 1. The metabolic pathways presented in this study were drawn up on the basis of human metabolome (KEGG – Kyoto Encyclopedia of Genes and Genomes; <http://www.kegg.jp>) as no information was available on the mussel metabolome.

### **3 Results**

#### **3.1 Analytical performance and statistical treatment**

Extracts of digestive glands from control mussels (SC) and those exposed to DCF were profiled by LC-HRMS in both negative and positive electrospray ionization modes. Analysis in negative ionization mode led to the detection of 8823 signals, and 2372 signals in positive ionization mode. PCA modelling indicated that the profiling methods were repeatable with little analytical variability in both ionization modes. Indeed, the QC samples injected repeatedly during the LC-HRMS analysis were grouped on the score plot (Supporting information, Fig. S1). The percentages of ions with an RSD lower than 30% in negative and positive ionization mode were 80.7% (7120 signals) and 84.4%

(2002 signals), respectively, thus validating the analytical repeatability quality during injections: N70% of the signals should have an RSD of  $\leq 30\%$  (Want et al., 2010).

PCA modelling of both datasets did not show a clear separation between SC and DCF-exposed mussels, revealing a discrete DCF exposure signature. Univariate statistical results revealed 591 signals (differing between SC and exposed groups) with  $p \leq 0.1$  in ESI-, and 161 signals in ESI+. Signals with  $p \leq 0.1$  revealed an up- or down-modulation trend due to exposure. Such signals may be meaningful, especially if connected in the same metabolic pathway, and may highlight DCF effects that could be biologically relevant despite a discrete signature.

### **3.2 Modulated metabolite analysis**

Among the signals that could be annotated (other differential signals are presented in the Supporting information Table S1), many of them were involved in the tyrosine and tryptophan metabolic pathways (Table 1). We highlighted 10 signals for the tyrosine metabolism: catecholamines (dopamine, epinephrine and norepinephrine) and/or their precursors and metabolites were particularly represented (6 annotations). This pathway was mainly down-modulated by DCF exposure (Fig. 1). The down-modulation of these compounds ranged from  $-48$  to  $-25\%$ . Two signals were up-modulated, i.e. norepinephrine-sulfate ( $+27\%$ ), and normetanephrine ( $+54\%$ ). Other tyrosine metabolism signals were gentisate aldehyde ( $-19\%$ ), gentisic acid ( $-39\%$ ), 5,6-dihydroxyindole ( $-34\%$ ), and fumaric acid ( $+12\%$ ). For tryptophan metabolism, 8 signals were highlighted as modulated by DCF exposure. Among them, 5-hydroxy-L-tryptophan (oxitriptan) and 5-hydroxyindoleacetic acid (5-HIAA, a serotonin metabolite) were the compounds most impacted by DCF exposure, with modulation of  $+98$  and  $+275\%$ , respectively (Table 1). Finally, we highlighted 2 signals of the steroid hormone biosynthesis pathway positively modulated by DCF exposure ( $+46$  and  $+50\%$ ). Among these two signals,  $17\alpha$ -hydroxypregnenolone was formally identified by standard injection.

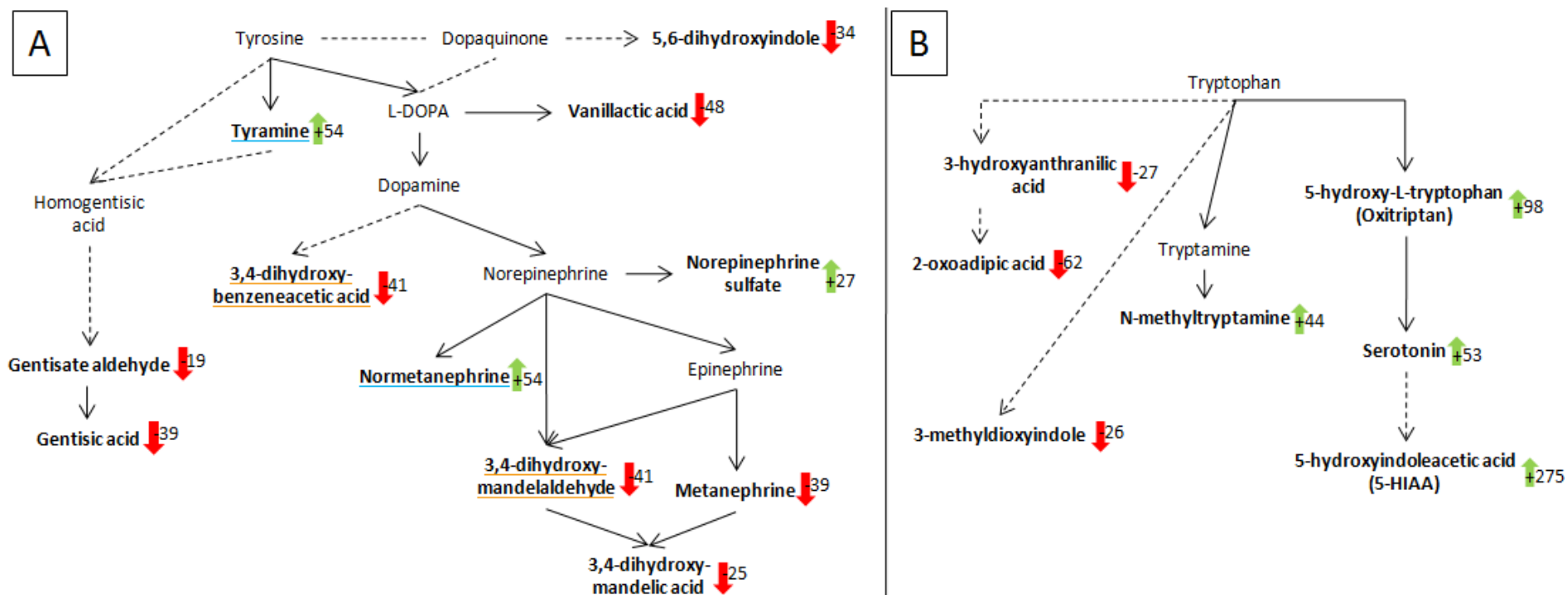


Fig. 1 – Partial maps of the two most impacted pathways following DCF exposure: A/tyrosine metabolism and B/tryptophan metabolism. The detected and annotated metabolites are highlighted in bold on the pathways. The relative modulation of differential metabolites is indicated with arrows. The green arrow corresponds to up-modulation (exposed compared to solvent control) and red arrow to down-modulation (exposed compared to solvent control). Indirect connections between metabolites are indicated with dashed arrows. Metabolites underlined with the same color correspond to two possible annotations for the same signal.

Table 1 – Summary of the main metabolic pathways modulated by exposure to 100 µg/L of diclofenac in mussel digestive glands

Metabolism pathway	Metabolite annotation	Retention time (min)	Molecular ion species detected	Calculated mass	Observed mass	Mass difference (ppm)	Trend	Fold change (%)	p-value	Annotation level ‡
Tyrosine metabolism	5,6-dihydroxyindole	6.82	[M+NH <sub>4</sub> ] <sup>+</sup>	167.0815	167.0809	-3.75	↓	-34	0.002	2
	Vanillic acid	11.93	[M-H <sub>2</sub> O-H] <sup>-</sup>	193.0501	193.0501	+0.81	↓	-48	0.073	2
	3,4-dihydroxybenzeneacetic acid (3,4-dihydroxyphenylacetic acid) or 3,4-dihydroxymandelaldehyde	8.56	[M-H] <sup>-</sup>	167.0350	167.0344	-3.56	↓	-41	0.083	2
	Norepinephrine sulfate	1.65	[M-H] <sup>-</sup>	248.0234	248.0239	+1.74	↑	+27	0.072	2
	Normetanephrine * or Tyramine	9.34	[M-H] <sup>-</sup> or [M+FA-H] <sup>-</sup>	182.0823	182.0818	-2.58	↑	+54	0.052	2
	Metanephrine *	8.37	[M+NH <sub>4</sub> ] <sup>+</sup>	215.1390	215.1384	-2.96	↓	-39	0.078	2
	3,4-dihydroxymandelic acid	8.32	[M-H <sub>2</sub> O-H] <sup>-</sup>	165.0188	165.0187	-0.41	↓	-25	0.052	2
	Gentisate aldehyde Gentisic acid *	8.18 7.18	[M-H] <sup>-</sup> [M-H] <sup>-</sup>	137.0244 153.0193	137.0236 153.0186	-5.69 -4.76	↓ ↓	-19 -39	0.040 0.017	2 2
Tryptophan metabolism	5-hydroxy-L-tryptophan (Oxriptan)	7.66	[M-H] <sup>-</sup>	219.0775	219.0776	0.18	↑	+98	0.100	1
	Serotonin *	11.37	[M+FA-H] <sup>-</sup>	221.0932	221.0931	-0.46	↑	+53	0.072	2
	5-hydroxyindoleacetic acid (5-HIAA)	9.68	[M+H] <sup>+</sup>	192.0655	192.0650	-2.80	↑	+275	0.092	2
	N-methyltryptamine	10.97	[2M+FA-H] <sup>-</sup>	393.2296	393.2293	-0.73	↑	+44	0.077	2
	3-hydroxyanthranilic acid	4.76	[M+FA-H] <sup>-</sup>	198.0408	198.0404	-2.13	↓	-27	0.059	2
	2-oxoadipic acid 3-methyldioxyindole	6.75 7.37	[M-H <sub>2</sub> O-H] <sup>-</sup> [M+H] <sup>+</sup>	141.0188 164.0706	141.0186 164.0700	-1.35 -3.58	↓ ↓	-62 -26	0.092 0.070	2 2
Other compounds of interest annotated - Steroid hormone biosynthesis	17 $\alpha$ -Hydroxypregnenolone	11.18	[M+FA-H] <sup>-</sup>	377.2333	377.2343	+2.49	↑	+46	0.100	1
	18-hydroxycorticosterone or Cortisol	10.68	[M+FA-H] <sup>-</sup>	407.2075	407.2087	+3.00	↑	+50	0.096	2

‡ Based on the work of Sumner et al. (2007)

Annotation level 1: Metabolite identity confirmed by injection of the corresponding analytical standard in the same analytical conditions

Annotation level 2: putative annotation, based on the use of public databases

\* In accordance with the retention time observed for the corresponding analytical standard in Boudah et al. (2014) on the same chromatographic column

## 4 Discussion

The main goal of our study was to determine the metabolomics potential to highlight effects of pharmaceuticals in non-target organisms, especially DCF effects on Mediterranean mussels. This approach allowed us to highlight two main metabolic pathways impacted: tyrosine metabolism, particularly compounds in connection with the dopaminergic pathway, and tryptophan metabolism, especially oxitriptan and compounds resulting from its biotransformation (Fig. 1). Signals of the dopaminergic pathway were mostly down-modulated, while those associated with oxitriptan biotransformation were mostly up-modulated (Fig. 1).

Despite the non-detection of dopamine and its metabolite DOPAC (3,4-dihydroxyphenylacetic acid), commonly measured to determine dopaminergic activity, we highlighted the modulation of other compounds connected to catecholamines (e.g. metanephrine, norepinephrine sulfate, Fig. 1). A concentration decrease in dopamine and its metabolite DOPAC were already reported for 21 day exposure at 0.2; 2; and 20 µg/L DCF in fish (Guiloski et al., 2017), in line with the potential down-modulation of the dopaminergic pathway highlighted in our study. Catecholamines are known to be involved in byssus formation (Bandara et al., 2013; Price, 1983). Ericson et al. (2010) highlighted a decrease in byssus thread strength following DCF exposure, which may be due to catecholamine modulation, as supported by our results. This modulation may involve mussel detachment from their support, therefore potentially increasing mortality.

In the tryptophan metabolism, we reported up-modulation of serotonin and 5-HIAA (Table 1). The higher up-modulation of 5-HIAA (+275%) compared to serotonin (+53%) is in line with an increase in serotonergic activity. To our knowledge, this kind of modulation has never been described following a DCF exposure in invertebrates. In vertebrates, Guiloski et al. (2017) studied serotonergic activity modulation following DCF exposure and did not observe any alteration in serotonin and 5-HIAA synthesis, as well as no change in 5-HIAA/serotonin ratio.

Regarding the modulation of both catecholamines and serotonin reported in our study, it may involve an osmoregulation disruption. Indeed, these metabolites are modulated by water salt concentration variations in marine green crab (Zatta, 1987) and freshwater mussels (Dietz et al., 1992), highlighting

their potential role in this function. The DCF potential to affect osmoregulation has already been reported for various aquatic organisms at different observation scales. The osmoregulatory capacity was disrupted in green shore crabs exposed to 10 and 100 ng/L DCF concentrations for 7 days (Eades and Waring, 2010). Other studies conducted in fishes following DCF exposure reported i) structural alterations in gill pillar and chloride cells and ii) modulation in electrolytes ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ ) concentration, each being involved in osmoregulation processes (Saravanan and Ramesh, 2013; Triebkorn et al., 2004). The hypothesis of osmoregulation disruption is also supported by the involvement of PGE<sub>2</sub> in osmoregulatory processes in bivalves (Freas and Grollman, 1980; Saintsing et al., 1983), since PGE<sub>2</sub> is modulated by DCF exposure (Courant et al., 2017). In mussels, osmoregulation disruption may potentially lead to variations in the hemolymph ionic composition and subsequently to cellular homeostasis disruption, while also disrupting nitrogenous compound excretion rates along with disruption of free amino acids involved in cell volume regulation (Gosling, 2003). The loss of free amino acids represents an energy loss for organisms and is suggested to be responsible for stress and even mortality in marine mussels (Hawkins and Hilbish, 1992).

In addition to their involvement in mussel osmoregulation, catecholamines and serotonin are known to be involved in reproduction. They have been described as playing important roles in mussel gamete multiplication and release and in sexual maturation (Lafont and Mathieu, 2007; Mathieu et al., 1991). Serotonin is described as being a gamete release inducer in bivalves, and dopamine as an inhibitor of serotonin activity on gamete release (Fong et al., 1993a, 1993b; Gibbons and Castagna, 1984; Osada et al., 1998). Dopaminergic and serotonergic related compounds modulation, as observed in the present study, may therefore lead to gamete production and release impairment in mussels. Furthermore, PGs, especially PGE<sub>2</sub> and PGF<sub>2</sub> $\alpha$ , are involved in gamete proliferation and release in mollusks, while promoting sensitization to serotonin (Deridovich and Reunova, 1993; Martínez et al., 2000, 1999; Matsutani and Nomura, 1987). Since PGE<sub>2</sub> synthesis is reduced following DCF exposure, this may reinforce the previous hypothesis of gamete production and release impairment (Courant et al., 2017). Our hypothesis that DCF may be involved in reproductive function impairment is in accordance with recent studies. A recent study conducted on marine invertebrates (echinoderms and polychaete worms)

indicated negative impacts on reproductive success due to sperm swimming velocity and motility reduction, negatively affecting fertilization success (Mohd Zanuri et al., 2017). Other studies conducted on vertebrates' aquatic organisms reported imbalances in sex steroids and alteration of gonadotropin and vitellogenin gene expression patterns (Efosa et al., 2017; Gröner et al., 2017).

Despite the low number of steroid hormones detected in our study,  $17\alpha$ -hydroxypregnenolone was found to be up-modulated following DCF exposure. The role of steroid hormones is poorly understood in mussels (Fernandes et al., 2011; Lafont and Mathieu, 2007). They have been described as promoters of sexual differentiation in mollusks and involved in modulation of PGs and the activity of neurotransmitters, such as catecholamines and serotonin, in bivalves (Cubero-Leon et al., 2010; Deridovich and Reunova, 1993; Dimastrogiovanni et al., 2015; Wang and Croll, 2006).. Hence, steroid hormone modulation in mussels may be related to the modulation of dopaminergic and serotonergic compounds observed and discussed above. From these results, we hypothesize complex modulation of gamete release by steroids hormones and PGs in bivalves, which may be disrupted following DCF exposure and could result in alteration of mussel reproduction success.

In summary, our results suggest another DCF mode of action beyond COX inhibition. Indeed, in human, monoamine oxidase (MAO) is described as being involved in various metabolic pathways, including tyrosine and tryptophan metabolism (Fig. 2) (Glover et al., 1977; Sjoerdsma et al., 1955). MAO is the enzyme involved in dopamine and serotonin oxidation processes. This enzyme has been documented in mussel tissues (Gagné and Blaise, 2003). Our results suggest that MAO activity in mussels may be impacted by DCF exposure and may explain the tyrosine and tryptophan metabolisms modulation observed. This hypothesis of MAO disruption due to DCF exposure has to be assessed and may allow a better understanding of DCF adverse outcome pathway in Mediterranean mussels. From our results and existing knowledge from the literature, we can suggest an adverse outcome pathway of DCF in mussel leading to potential impairment of byssus formation, osmoregulation and reproduction function (Fig. 3).

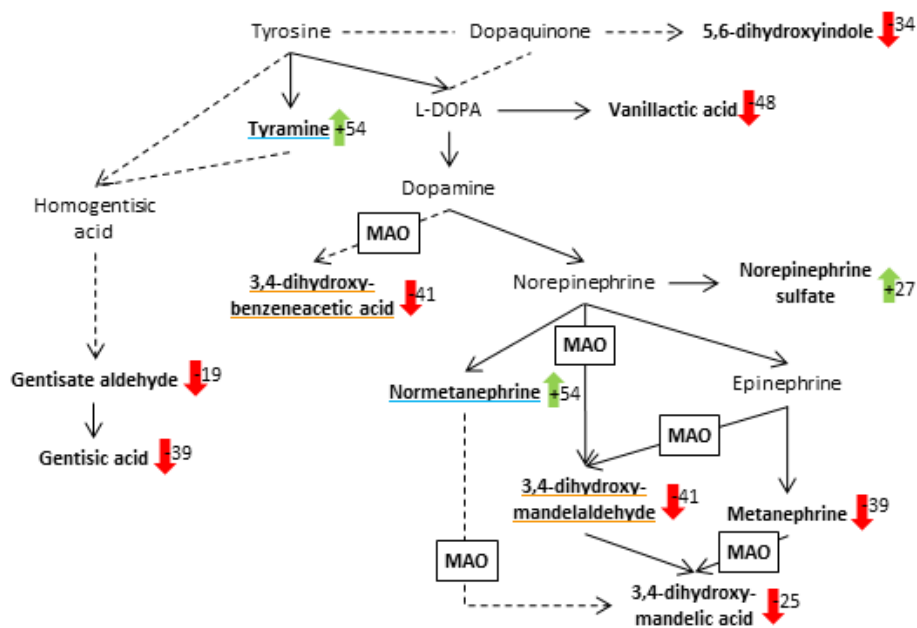


Fig. 2 – Partial map of tyrosine metabolism with the involvement of monoamine oxidase (MAO). The detected and annotated metabolites are highlighted in bold on the pathways. The relative modulation of differential metabolites is indicated with arrows. The green arrow corresponds to up-modulation (exposed compared to solvent control) and red arrow to down-modulation (exposed compared to solvent control). Indirect connections between metabolites are indicated with dashed arrows. Metabolites underlined with the same color correspond to two possible annotations for the same signal.



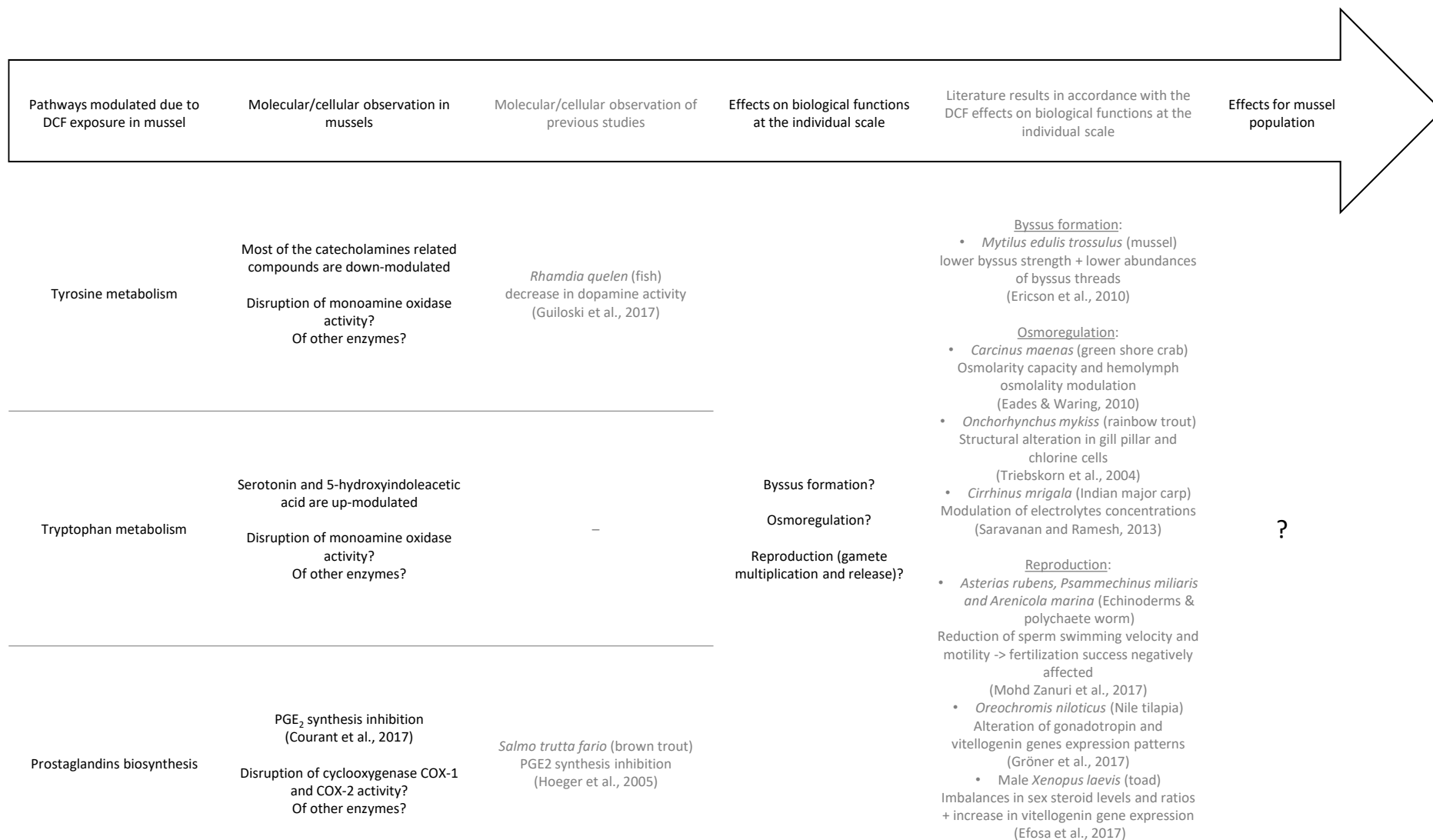


Fig. 3 – Schematic representation of DCF adverse outcome pathway in mussels (with integration of knowledge from other aquatic organisms)

In conclusion, the metabolomic approach was powerfully applied in this study to highlight the potential disruption of important biological functions in mussels following DCF exposure. Our study confirmed the efficiency of metabolomics in highlighting pharmaceuticals effects on non-target organisms. We have reported the effects of DCF on dopaminergic and serotonergic pathways, along with the modulation of prostaglandins already reported for DCF, which may result in impairment of the reproductive function and osmoregulation disruption. Our results, in connection with existing knowledge from the literature, thus demonstrated the existence of key molecular events, while providing new information to identify potential adverse outcome pathway in non-target organisms. Metabolomics may thus be a promising tool to help define adverse outcome pathway for pharmaceuticals (Brockmeier et al., 2017). Further research is needed to confirm the assumptions proposed, as well as to study whether DCF modes of action for exposure to 100 µg/L are similar at lower exposure concentrations.

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## Supporting information

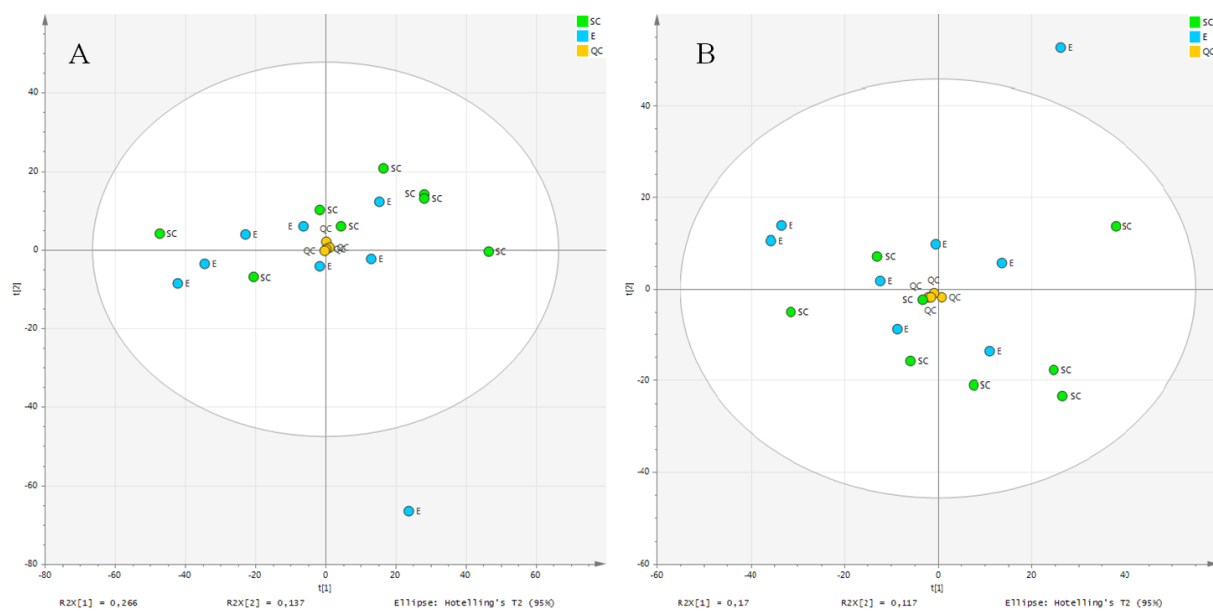


Fig. S1 – Principal component analysis score plots of chemical profiles in male mussels digestive glands exposed to DCF. Extracts were profiled in A/ESI- and B/ESI+ ionization mode. Quality control (QC) samples were used to monitor the analytical performance of the MS platform. The outlier exposed sample highlighted is the same in both ionization modes. This sample was removed before data treatment. QC: quality control – SC: solvent control samples – E: samples exposed to DCF



Table S1 – Summary of the differential signals with p<0.05 modulated by exposure to 100 µg/L of diclofenac in mussel digestive glands. When a putative annotation based on the accurate measured monoisotopic masses is possible, it is reported in the “putative annotation” part of the table.

Ionization mode	Observed mass	Retention time (min)	Trend	Fold change	p-value	Putative annotation					
						Annotation	HMDB code	Molecular ion species detected	Calculated mass	Mass difference (ppm)	Metabolic pathway
+	249.1478	11.27	↓	-47	0.005	Gamma-CEHC	HMDB01931	[M+H] <sup>+</sup>	249.1485	-2.96	Metabolites of Vitamin E
-	121.0285	8.30	↓	-30	0.006	Benzoic acid	HMDB01870	[M-H] <sup>-</sup>	121.0295	-8.15	Phenylalanine metabolism
-	200.0924	3.88	↑	45	0.017	Pantothenic acid	HMDB00210	[M-H <sub>2</sub> O-H] <sup>-</sup>	200.0923	0.78	Beta-Alanine metabolism Pantothenate and CoA biosynthesis Vitamin digestion and absorption
+	340.3564	17.73	↓	-23	0.019	Docosanamide	HMDB00583	[M+H] <sup>+</sup>	340.3574	-2.94	Primary fatty acid
-	157.1226	11.95	↓	-55	0.020				157.1234	-5.00	Lipids
+	160.0963	8.15	↓	-30	0.024	5-acetamidovalerate	HMDB12175	[M+H] <sup>+</sup>	160.0968	-3.04	Lysine degradation
-	158.0816	8.17	↓	-24	0.029	5-acetamidovalerate	HMDB12175	[M-H] <sup>-</sup>	158.0823	-4.02	Lysine degradation
+	162.1118	2.95	↑	44	0.036	L-carnitine	HMDB00062	[M+H] <sup>+</sup>	162.1125	-3.95	Non-essential amino acid
-	127.0391	8.10	↓	-29	0.042	(s)-2-aceto-2-hydroxybutanoic acid	HMDB06900	[M-H <sub>2</sub> O-H] <sup>-</sup>	127.0395	-2.97	Valine, leucine and isoleucine biosynthesis 2-Oxocarboxylic acid metabolism
-	450.2635	12.72	↑	113	0.043	LysoPE (0:0/16:1(9z))	HMDB11474	[M-H] <sup>-</sup>	450.2626	2.07	Glycerophosphoethanolamines
-	574.3162	12.85	↑	63	0.046	LysoPE (0:0/22:4(7z,10z,13z,16z))	HMDB11493	[M+FA-H] <sup>-</sup>	574.315	1.98	Glycerophosphoethanolamines
-	324.2187	12.00	↑	60	0.049	10-nitrolinoleic acid	HMDB05049	[M-H] <sup>-</sup>	324.218	2.05	Lipids
-	256.0864	1.68	↑	105	0.001						
-	189.1280	9.82	↑	325	0.003						
-	282.0099	12.38	↑	356	0.003						
+	250.0498	1.67	↑	99	0.003						
-	196.0644	1.67	↑	85	0.005						
+	197.1167	9.22	↓	-46	0.005						
+	234.0760	1.67	↑	93	0.005						
-	165.0914	8.33	↓	-38	0.007						
-	179.0668	1.63	↑	94	0.010						

-	218.1033	8.47	↑	97	0.011						
-	197.0817	7.12	↓	-42	0.014						
+	265.2111	2.85	↑	84	0.016						
-	229.1809	13.98	↓	-19	0.016						
+	173.0289	3.23	↓	-54	0.017						
-	418.1917	10.70	↑	43	0.017						
-	153.0186	7.18	↓	-39	0.017						
-	159.0656	3.45	↓	-45	0.019						
-	223.1338	12.52	↑	45	0.019						
-	180.0604	1.63	↑	76	0.021						
-	271.2283	15.07	↓	-27	0.024						
-	265.0699	7.58	↓	-25	0.024						
-	263.1656	13.57	↑	25	0.024						
-	178.0634	1.65	↑	76	0.025						
-	329.0807	9.58	↓	-59	0.025						
-	210.1133	10.75	↑	82	0.026						
-	163.1122	9.40	↓	-46	0.026						
-	303.1820	10.45	↑	74	0.027						
+	196.0448	3.23	↓	-56	0.028						
+	130.0648	10.27	↑	85	0.028						
-	240.1244	9.77	↑	67	0.029						
-	166.0221	8.30	↓	-29	0.029						
-	243.1234	6.68	↑	585	0.029						
+	182.1252	12.07	↓	-46	0.029						
-	238.1450	11.60	↑	58	0.031						
-	680.3204	13.25	↑	102	0.031						
-	305.1379	11.33	↓	-54	0.033						
-	195.1023	9.13	↓	-32	0.033						
-	276.0995	9.55	↑	262	0.034						

+	271.0652	3.22	↓	-74	0.034						
-	240.0854	8.67	↑	147	0.036						
-	457.0740	12.97	↑	2851	0.036						
+	116.5499	3.23	↓	-49	0.036						
-	353.0224	3.32	↓	-61	0.037						
-	282.0196	12.45	↑	59	0.037						
-	263.1061	12.22	↓	-53	0.037						
-	383.3539	18.25	↓	-10	0.037						
-	217.0910	6.65	↓	-62	0.038						
-	326.1638	10.97	↑	103	0.039						
-	125.0962	11.85	↓	-20	0.039						
-	298.1126	6.87	↓	-41	0.040						
-	171.1020	11.03	↓	-27	0.041						
-	263.0602	7.22	↓	-39	0.041						
-	359.0637	1.70	↑	67	0.041						
-	494.2136	3.72	↑	130	0.042						
-	198.1132	10.15	↑	69	0.042						
+	226.1541	6.47	↓	-31	0.042						
-	135.0496	3.42	↑	51	0.042						
+	119.0393	3.23	↓	-54	0.043						
-	254.1401	10.70	↑	35	0.045						
+	309.1772	1.63	↑	57	0.045						
-	250.1565	9.02	↑	435	0.046						
-	223.0556	9.05	↓	-67	0.047						
-	197.0302	5.25	↓	-54	0.047						
-	170.0817	8.02	↑	42	0.048						
-	101.0597	7.15	↓	-49	0.048						
+	88.0394	11.08	↑	38	0.049						

