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## **Metabolomics approach reveals disruption of metabolic pathways in the marine bivalve *Mytilus galloprovincialis* exposed to a WWTP effluent extract**

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### **Abstract:**

Conventional wastewater treatment plants (WWTPs) discharge a highly diverse range of organic contaminants in aquatic environments, including marine waters. The health of marine ecosystems could be threatened by contaminants release. Environmental metabolomics can be helpful to assess the effects of multi-contamination on marine organisms without any *a priori* information since it is able to provide meaningful information on the biochemical response of organisms to a stress. The aim of the present study was to evaluate the potential of metabolomics to highlight key metabolites disrupted by a WWTP effluent extract exposure and then elucidate the biological effects of such exposure on Mediterranean mussels (*Mytilus galloprovincialis*). Exposed male mussels showed numerous metabolites altered in response to WWTP effluent exposure. The highlighted metabolites belong mainly to amino acids metabolism (e.g. tyrosine, phenylalanine, leucine, proline, etc.), neurohormones (dopamine and a serotonin metabolite), purine and pyrimidine metabolism (e.g. adenosine, adenine, guanine, uracil etc.), citric acid cycle intermediates (e.g. malate, fumarate), and a component involved in oxidative stress defense (oxidized glutathione). Modulation of these metabolites could reflect the alteration of several biological processes such as energy metabolism, DNA and RNA synthesis, immune system,

osmoregulation, byssus formation and reproduction, which may lead to a negative impact of organism fitness. Our study provided further insight into the effects of WWTP effluents on marine organisms.

**Keywords:** metabolomics; LC-HRMS; wastewater treatment plant effluent; adverse effects; marine mussel; *Mytilus galloprovincialis*

## 1. Introduction

Conventional wastewater treatment plants (WWTPs) are not yet able to completely remove organic contaminants (Bolong et al., 2009; Deblonde et al., 2011; Loos et al., 2013). Hence, numerous contaminants are discharged in the aquatic environment, including a highly diverse range of pharmaceutically active compounds (PhACs), pesticides, polycyclic aromatic hydrocarbons, plasticizers, etc. (Fent et al., 2006; Kolpin et al., 2002; Loos et al., 2013). However, wastewater volumes have been rising proportionately to the quickly growing global human population (Sato et al., 2013). Due to the high demographic pressure, coastlines are affected by the discharge of contaminants through the development of WWTP sea outfalls and also by the rivers discharging in the area. Marine organisms are then chronically exposed to low concentrations (ng- $\mu$ g/L) of a mixture of contaminants (dos Santos et al., 2018; Vidal-Dorsch et al., 2012). Moreover, the coastal environment, especially transition zones between continental and marine waters, constitute key environments for the breeding of many species and biodiversity conservation. The health of those important ecosystems could be threatened by contaminants release.

So far, scientific evidence has highlighted the bioaccumulation and toxicological effects of contaminants carried through WWTP effluents in the aquatic environments (Dodder et al., 2014; Fent et al., 2006; Mezzelani et al., 2018a; Picot Groz et al., 2014; Sánchez-Avila et al., 2012). WWTP effluents effects are mostly described on freshwater organisms, showing endocrine disruption, impacts on immune and reproductive system, neurotoxicological effects, oxidative stress induction, survival and growth reduction (Bouchard et al., 2009; Gagné et al., 2007, 2004; Gillis et al., 2014; Nobles and Zhang, 2015). New approaches are needed to evaluate these various effects of multi-contamination, without preconceived assumptions about the expected effects.

Recent studies have shown the interest of omics approaches to highlight effects from multi-contamination, either metabolomics (Berlioz-Barbier et al., 2018; David et al., 2017; Simmons et al., 2017), transcriptomics (Ings et al., 2011) or proteomics (Bebianno et al., 2016; Simmons et al., 2017). Among them, metabolomics is an efficient approach to assess the health status of organisms based on the identification of low molecular weight metabolites (50-1500 Da), whose production and levels vary with the physiological, developmental or pathological state of cells, tissues, organs or whole organisms (Courant et al., 2014; Lin et al., 2006). Hence, environmental metabolomics is commonly applied to more deeply investigate and unravel the unknown toxicological effects and mechanisms of single contaminants or mixtures (Lankadurai et al., 2013; Lin et al., 2006; Miller, 2007). As an example of metabolomics application in the case of a multi-contamination, David et al. (2017) identified changes in the exposome and metabolome of roaches (*Rutilus rutilus*) exposed for 15 days to a WWTP effluent. They revealed the bioaccumulation of 31 PhACs and metabolites of biodegradation in plasma and tissues. Meanwhile, they demonstrated that effluent exposure resulted in a reduction in prostaglandin levels, as well as tryptophan/serotonin, bile acid and lipid metabolism disruption in fish. Such studies will likely advance our understanding of the link between exposure to complex mixtures of contaminants and their adverse effects, by its ability to screen alterations of multiple metabolic pathways. Moreover, it could constitute a first step forward by highlighting key events that may result in adverse outcomes which could be further used in risk assessment and environmental monitoring (Bundy et al., 2009; Martyniuk, 2018; Van Aggelen Graham et al., 2010).

In marine ecotoxicology and biomonitoring, *Mytilus* spp. mussels are extensively used as wild species to assess environmental health, especially the impact of chemical contamination in estuarine and coastal ecosystems (Farcy et al., 2013; Fasulo et al., 2012). This is due to their wide geographical distribution, sessility, ability to tolerate a range of environmental conditions and suitability for caging experiments at field sites (Fasulo et al., 2012). Furthermore, mussels can tolerate and accumulate xenobiotics in their tissues at levels higher than those present within the aquatic environment (Faggio et al., 2018). In studies conducted in field, it is difficult to establish causal relationships between effects and particular contaminants, because of the multitude of interacting environmental factors (abiotic factors, food availability, predation pressure, etc.). Laboratory experiments are then



sometimes necessary for first reducing confounding factors and understand how organisms respond to contaminants.

The aim of the present study was to evaluate the potential of metabolomics to highlight key metabolites disrupted by WWTP effluent exposure and then elucidate the biological effects of such exposure on Mediterranean mussels (*Mytilus galloprovincialis*). Mussels were exposed for 7 days to a WWTP effluent extract corresponding to an environmental dilution of 5%. Metabolic fingerprints were generated from control and exposed organisms by liquid chromatography combined with high resolution mass spectrometry (LC-HRMS) and processed to highlight potential metabolic alterations. Disrupted metabolites along with their metabolic pathways were then linked to biological effects on this organism. Moreover, the WWTP effluent extract was chemically characterized to better understand these observed effects, based on the screening of 80 contaminants.

## **2. Materials and methods**

### **2.1 Chemicals**

Ultrapure water was generated by a Simplicity UV system from Millipore (Bedford, MA, USA) with a specific resistance of 18.2 M $\Omega$ .cm at 25°C. Pesticide analytical-grade solvents (methanol, dichloromethane and ethanol) and LC/MS grade solvents (water, acetonitrile, formic acid 99%) were from Carlo Erba (Val de Reuil, France). Analytical pure standards used for identification at level one (Sumner et al., 2007) were obtained from the four following suppliers: Sigma-Aldrich (now part of Merck), Santa Cruz Biotechnology, Toronto Research Chemicals and LGC Standards.

### **2.2 WWTP effluent extract preparation**

24-hour composite samples were collected over 3 days for a total of 15 L of effluent from an urban WWTP (470,000 population equivalent) in Hérault (France). Since PhACs consumption has been rising during the last decades, they may constitute a large class of contaminants present in the WWTP effluent. The following WWTP effluent preparation was focused on the extraction of dissolved hydrophilic compounds.

After filtration through GF/C filters (1.2  $\mu\text{m}$ ), effluent (15 L) was concentrated on 30 SPE cartridges (Oasis HLB 500 mg, 6 cc) which allow extracting soluble substances from medium to high polarity. The cartridges were conditioned with 5 mL methanol and 5 mL distilled water. 500 mL of WWTP effluent was deposited on each cartridge. The cartridges were then washed (5 mL Milli Q water), dried and eluted each with  $2 \times 5$  mL of methanol. The eluate of all cartridges were evaporated until 3 mL of concentrated extract was obtained. In addition, two cartridges were also prepared in the same condition, substituting effluent by 500 mL Milli Q water deposited on each cartridge, to get an analytical blank.

The solution (for exposure experiment) was prepared extemporaneously every day from 20  $\mu\text{L}$  of concentrated extract to which 80  $\mu\text{L}$  of methanol was added. In order to obtain a final effluent dilution of 5% in the aquarium, 100  $\mu\text{L}$  of the solution was added to 2 L of seawater during exposure.

### **2.3 Animals and experimental design**

*Mytilus galloprovincialis* mussels (n=50) were obtained from a Mediterranean Sea mussel culture (Bouzigues, France) in April 2016. Mussels were cleaned and uniformly selected according to their shell size (5.3-7.5 cm). They were randomly distributed in 10 glass aquaria (five mussels per glass aquarium) with 2 L of filtered seawater (which is provided, and already filtered, sterilized and degassed, by IFREMER Palavas, France). During a 7-day acclimatization period, filtered seawater was continuously aerated and renewed daily (static renewal). After 7 days, five aquaria (n = 25 mussels) underwent solvent control exposure (SC, 100  $\mu\text{L}$  absolute methanol) and five aquaria (n= 25 mussels) underwent WWTP solution exposure (20  $\mu\text{L}$  WWTP extract and 80  $\mu\text{L}$  methanol). SC and WWTP solutions were added at the same time after daily water renewal over a 7-day exposure period. Seawater (150 mL) was sampled in each glass aquarium every day. Aquaria pH, temperature and oxygen concentration were checked daily during the experiment. The temperature was maintained at  $14^{\circ}\text{C} \pm 1^{\circ}\text{C}$  and salinity at 36.5 g/L. Mussels were fed once daily with the marine green alga *Tetraselmis suecica* (Greensea, Mèze, France) at constant density (10,000 cells/mL). On the last day, no dead mussels were recorded. The two groups of mussels were collected at the same time for dissection and sex microscopy determination (for SC exposure n=10 males, n=12 females and n=3

undifferentiated; for WWTP effluent exposure n=15 males and n=10 females). Digestive glands were frozen at -80°C. The present study was performed on male mussels, with 9 having SC exposure and 10 WWTP effluent exposure. Shell size was not significantly different between the two groups (p=0.51; result not shown).

## **2.4 Tissue sample preparation**

The tissue sample preparation technique was previously described by Bonnefille et al. (2018). Briefly, 30 mg dry weight ( $\pm$  0.25 mg) of sample digestive gland tissue was extracted in two steps according to the biphasic mixture methanol/dichloromethane/water in a volume ratio of 16/16/13 respectively. First step: 240  $\mu$ L of methanol + 75  $\mu$ L of water were added into each sample and vortexed for 60 sec. Second step: 240  $\mu$ L of dichloromethane + 120  $\mu$ L of water were added and then vortexed again. After a 15 min rest at 4°C, the samples were vortexed and then centrifuged (2,000 g; 15 min; 4°C). 50  $\mu$ L of supernatant (polar phase) was collected in a glass tube. The extracts were evaporated to dryness under a nitrogen stream and reconstituted in 200  $\mu$ L acetonitrile/water (5/95; v/v) and filtered directly into an analysis vial using a 0.20  $\mu$ m PTFE syringe filter (Minisart SRP 4, Sartorius). A quality control (QC) sample was prepared by pooling 10  $\mu$ L of each sample extract included in the experiment.

## **2.5 Metabolic fingerprint LC-HRMS analysis**

The injections were randomly performed on an Exactive Orbitrap LC-HRMS (Thermo Fischer Scientific), equipped with a heated electrospray ionization probe (HESI) source. For LC separation, a reverse phase PFPP analytical column (100 mm  $\times$  2.1 mm; 3  $\mu$ m particle size; Sigma Aldrich) was used. Each sample (5  $\mu$ L) was loaded onto the column with a full loop injection. Two separate mobile phases were used: water was used as solvent A, acetonitrile as solvent B, and both were modified with 0.1% formic acid. The flow rate was 200  $\mu$ L/min according to the following gradient system (A/B): 95/5 from 0 to 3 min, 60/40 at 8 min, 50/50 at 9 min, 30/70 at 13 min, 5/95 from 15 to 18 min, followed by a re-equilibration period (95/5) from 21 to 31 min (total run time, 31 min).

The Exactive HRMS was turned to a mass resolution of 50,000 (FWHM,  $m/z$  200) with a mass spectrum range of 50-1000  $m/z$ . Samples were analyzed in both positive and negative electrospray ionization modes (ESI+ and ESI-). The analyses, respectively in ESI+ and ESI-, were completed with a spray voltage of 3.5 [kV] and 3.40 [kV], a capillary voltage of 45 V and -50 V, a tube lens voltage of 90 V and -120 V, and a skimmer voltage of 26 V and -25 V. The capillary and heater temperature was 250°C.

## **2.6 Quality control**

A quality control (QC) sample corresponding to a pool of 10  $\mu\text{L}$  collected from each sample extract was injected several times at the beginning of the analytical sequence to equilibrate the column. QC injections were repeated every ten samples to control analytical repeatability and sensitivity. Relative standard deviation (RSD) was calculated for each feature detected in the replicated QC injections to assess the former. A repeatability characterized by 70% of features with a RSD <30% (Want et al., 2013) was considered acceptable. Only features with a RSD < 30% were retained for further data processing and annotation.

## **2.7 Data processing and statistical analysis**

To facilitate understanding of the data processing and the subsequent identification strategy, a schematic workflow is presented in Supplementary Data (Figure S1).

### **2.7.1 Data processing**

The raw data were converted into mzXML files with MSConvert freeware (ProteoWizard 3.0, Chambers et al., 2012). Then data were processed using the XCMS package (Smith et al., 2006) in the R environment. Optimized XCMS parameters were implemented:  $m/z$  interval for peak picking was set at 0.01, the signal-to-noise ratio threshold was set at 3, the group bandwidth was set at 8, and the minimum fraction was set at 0.5. A multi-step strategy was applied in the data processing using the XCMS package, as detailed in Courant et al. (2009). First, peak alignment is applied for ensuring that

each ion  $[m_i/z_i]$  of the MS fingerprint appears at identical retention time ( $rt_i$ ) across all the analyzed samples. As a second step, peak picking is carried out to report the feature abundance observed for each ion  $[m/z; rt]_i$  in each of the analyzed samples (i.e. XCMS extracts each specific ion chromatogram corresponding to each  $m/z$  value and integrates the corresponding chromatographic peaks). During a third step, the software compares feature abundances observed for identical ions in two groups of samples based on a Welch  $t$ -test, to highlight metabolites presenting intensities significantly different in the two groups, and ranks these metabolites according to the  $p$ -value associated with the observed difference. Finally, XCMS returns a table containing peak information (i.e.  $m/z$  and retention time) and feature abundances (i.e. area) in all samples. Visual inspection of raw data for all the features reported by XCMS was performed to ensure that each integrated signal 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). If not, features were discarded from the dataset.

### 2.7.2 Statistical analysis

A Welch  $t$ -test was applied using the XCMS package to highlight features presenting intensity modulations between the two groups of exposed and control samples. The Welch- $t$  test is an adaptation of the Student- $t$  test for comparing the mean of two groups with unequal variances. Features with a  $p$ -value lower than 0.1 were selected for annotation and identification. The rationale for choosing such a threshold was based on the opportunity to reveal a biological modulation trend due to the exposure. Considering single metabolites separately is somewhat a reductionist view of metabolic alterations involving complex interconnected metabolic pathways. Indeed, a trend ( $p < 0.1$ ) toward 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 ( $p < 0.05$ ). As a consequence, processed data were then exported to SIMCA 13.0.3 software (Umetrics, Sweden) for multivariate analysis in order to reduce the dataset dimensionality and highlight relevant metabolic patterns with respect to the WWTP effluent exposure. Datasets were log transformed and Pareto scaled. Mussels individuals were considered independent since no clustering

related to glass aquarium was observed using Principal Analysis Components (PCA) or hierarchical clustering analysis (results not shown).

## **2.8 Metabolite annotation and identification strategy**

The annotation strategy was performed on features with a difference of amplitude higher than 30% between the control group and the WWTP exposed group and with  $p < 0.1$ . Metabolite annotation was completed by mass-matching with 0.002 Da precision using the online Human Metabolome Database tool (HMDB; <http://www.hmdb.ca/>). Metabolite annotation was based on two levels of confirmation according to Sumner et al. (2007). Level 1 corresponds to annotation confirmed by injection of the analytical standard on the same analytical platform under the same conditions (validation based on both accurate mass and retention time). Level 2 was characterized by metabolite putatively annotated in agreement with the physicochemical properties and on the basis of public databases. In addition, to add more power to some annotations of level 2, we used the external laboratory results reported by Boudah et al. (2014) using the same chromatographic column. As the gradient used in Boudah et al. (2014) was slightly different, this aspect was taken into account by considering the water/acetonitrile ratio at which the molecules were eluted rather than their retention time. The identification levels are reported in Table 2. Identified and annotated metabolites were assigned to metabolic pathways according to the Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.kegg.jp>) and HMDB. Pathway analysis was then performed using MetaboAnalyst 4.0 (Xia and Wishart, 2010) in order to highlight significantly impacted metabolic pathways (Figure S2).

## **2.9 Characterization of the WWTP effluent extract**

Characterization of the WWTP effluent extract aimed to get a better picture of the chemical responsible for exposure effects. The extraction, performed on SPE HLB with methanol, was chosen to elute mainly hydrophilic compounds such as PhACs and pesticides to the detriment of polycyclic aromatic hydrocarbons or polychlorinated biphenyls. The characterization of the WWTP effluent

extract was based on the screening of 80 organic contaminants belonging to PhACs and pesticides (Table S1), based on the comparison with analytical standards injected in the same analytical conditions.

50  $\mu\text{L}$  WWTP effluent extract was evaporated to dryness and reconstituted with 250  $\mu\text{L}$  of acetonitrile/water (20/80; v/v), and filtered into an analysis vial using a 0.20  $\mu\text{m}$  PTFE syringe filter (Minisart SRP 4, Sartorius). The injections (in triplicate) were performed on a Q-Exactive Orbitrap LC-HRMS (Thermo Fischer Scientific). A Zorbax Eclipse XDB-C8 column (3.0 x 150mm 3.5 $\mu\text{m}$ ; Agilent) was used. Each sample (10  $\mu\text{L}$ ) was loaded onto the column with a full loop injection. Two separate mobile phases were used: water was used as solvent A, acetonitrile (ACN) as solvent B, and both were modified with 0.1% formic acid. The flow rate was 400  $\mu\text{L}/\text{min}$  according to the following gradient system: 10% ACN from 0 to 1 min, until 100% at 10 min, 100% ACN at 15 min, followed by a re-equilibration period at 10% ACN from 15.1 to 25 min.

The Q-Exactive HRMS was turned to a mass resolution of 35,000 (FWHM,  $m/z$  200) with a mass spectrum range of 80-450  $m/z$ . Samples were analyzed in both ESI+ and ESI-. The analyses, respectively in ESI+ and ESI-, were completed with a spray voltage of 4.0 [kV] and 3.35 [kV], a S-lens RF level of 50 and a sheath gas flow rate of 55 in both ESI modes. The capillary temperature was 300°C and the heater temperature was 250°C.

## 3. Results

### 3.1 Characterization of the WWTP effluent extract

The screening of 80 contaminants allowed the detection of 38 PhACs and their metabolites from various therapeutic classes, and 4 pesticides, occurring in the WWTP effluent extract (Table 1). In order to evaluate the proportion of each analyzed contaminants, a relative abundance was estimated based on its chromatographic peak area in the analytical standard (prepared at a known concentration) and in the seawater samples. Relative abundance estimated are given in Table 1 and are represented with “+”.

## 3.2 Metabolic fingerprint LC-HRMS analysis

### 3.2.1 Analytical performance and statistical analysis

LC-HRMS analysis of digestive glands of control male mussels (SC) and those exposed to the WWTP effluent extract was carried out in both negative and positive electrospray ionization modes (ESI- and ESI+). Leading to metabolic fingerprints of 4563 features detected in ESI- and 2185 features in ESI+. The percentages of features with a RSD<30% in ESI- and ESI+ were 68% and 65%, respectively. The analytical repeatability was acceptable, as highlighted on the PCA score plot, where the QC samples injected at regular intervals throughout the analytical run were tightly clustered together (Figure 1). PCA score plot (Four principal components for a  $r^2X = 50\%$ ) described a clustering trend for the two groups, i.e. SC and exposed male mussels (Figure 1). However, the latter were split into two sub-groups, one close to the SC group and the other more scattered. An OPLS-DA model is also proposed in Supplementary Data for further information on the structure of the dataset (Figure S3).

Univariate analysis (Welch *t*-test) provided 675 features that differed between the SC and exposed groups in ESI- and 214 features in ESI+ ( $p < 0.1$ ; RSD QC<30%; amplitude difference >30%). Features with  $p < 0.1$  revealed an up- or down-modulation trend in response to exposure. These discriminant features were meaningful, especially if connected in the same metabolic pathway, and may highlight WWTP effluent effects that could be biologically relevant.

### 3.2.2 Metabolite modulation and pathway analysis

Mass-matching analysis using the online HMDB database allowed putative annotation of the features of interest ( $p < 0.1$ ; RSD QC<30%; amplitude difference >30%). Metabolites identified by confirmation with pure analytical standards ( $n=23$ ) and putatively annotated features ( $n=15$ ) belonging to relevant metabolic pathways are reported in Table 2. The Supporting Information (Table S2) reports all features of interest (some with level 2 annotation) which are not presented in Table 2. A negative shift between theoretical and observed mass for low molecular mass compounds was noted in ESI-,



sometimes with a bias of around 14 ppm (Table 2). However, this mass bias was also observed for the pure analytical standard when injected. This suggests lower accuracy in the ESI- data acquisition for our analysis, which was considered for features annotation.

After annotation and identification of main modulated metabolites, twenty-two were assigned to seven amino acid metabolic pathways, six to purine metabolism and four to pyrimidine metabolism. Most of these metabolic pathways were significantly affected as revealed by further pathway analysis from MetaboAnalyst 4.0 (Figure S2), and seem relevant to discuss. Among these, five identified metabolites were significantly down-modulated in the phenylalanine metabolism, including L-phenylalanine (-50%,  $p < 0.05$ ), N-acetyl-L-phenylalanine (-50%,  $p < 0.05$ ), hydroxycinnamate (-31%,  $p < 0.05$ ), phenylethylamine (-81%,  $p < 0.05$ ) and the L-tyrosine amino acid (-41%,  $p < 0.05$ ) (Figure 2). L-tyrosine, is a catecholamine precursor (i.e. dopamine, metanephrine, epinephrine, etc.) directly synthesized from L-phenylalanine. Its down-modulation may lead to a significant decrease in the dopamine neurohormone (-36%,  $p < 0.05$ ) (Figure 2). Other amino acids were down-modulated, including L-methionine (-55%,  $p < 0.05$ ), L-proline (-42%,  $p < 0.1$ ), two branched-chain amino acids (BCAAs) L-isoleucine (-39%,  $p < 0.05$ ) and L-leucine (-35%,  $p < 0.1$ ), as well as L-threonine or its isomer L-homoserine (-29%,  $p < 0.05$ ). Although L-tryptophan was not significantly modulated (results not shown) by the WWTP effluent extract exposure, four metabolites belonging to its metabolic pathway were impacted (Figure 3). We highlighted significant down-modulation of L-kynurenine (-63%,  $p < 0.05$ ), N<sup>1</sup>-formylkynurenine (-55%,  $p < 0.05$ ), 5-methoxyindoleacetate (a serotonin metabolite, -57%,  $p < 0.05$ ) and a decrease in 5-hydrokynurenamine (-63%,  $p < 0.1$ ). WWTP effluent exposure also contributed to purine and pyrimidine metabolism disruption (Figure 4). Purine metabolism was mainly affected through an increase in adenosine (+138%,  $p < 0.1$ ) and adenine (+34%,  $p < 0.1$ ), and down-modulation of guanine (-32%,  $p < 0.1$ ) and xanthine (-78%,  $p < 0.05$ ). Concerning pyrimidine metabolism, uracil (-54%,  $p < 0.05$ ), deoxyuridine (-57%,  $p < 0.05$ ) and deoxycytidine (-28%,  $p < 0.1$ ) were identified as down-modulated metabolites. Two intermediates in the citric acid cycle were down-modulated, including fumarate (-31%,  $p < 0.05$ ) and malate (-24%,  $p < 0.1$ ). Finally, we observed up-modulation of oxidized glutathione (+34%,  $p < 0.1$ ), a biomarker of oxidative stress.

## 4. Discussion

Male mussels exposed for 7 days to a WWTP effluent extract in laboratory conditions showed alterations of their metabolism. These alterations belong to the metabolism of several amino acid (e.g. tyrosine, phenylalanine, BCAAs, proline, etc.), neurohormones (dopamine and a serotonin metabolite), purine and pyrimidine metabolism (e.g. adenosine, guanine, xanthine, uracil, deoxyuridine, etc.), citric acid cycle intermediates (malate and fumarate), and a component involved in oxidative stress defense (GSSG). To our knowledge, our results are the first to highlight a disruption of these metabolic pathways in *M. galloprovincialis* in response to a WWTP effluent exposure. Modulations of these metabolites could reflect potential effects on several of the biological processes discussed below, such as energy metabolism, DNA and RNA synthesis, immune system, osmoregulation, byssus formation and adhesion, and reproduction. WWTP effluents could also activate defense mechanisms, especially against oxidative stress in male mussels. A summary table based on literature, complementary with the discussion, was proposed in order to relate the observed effects with contaminants detected in our WWTP effluent extract (Table 3), although synergistic, antagonistic or additive effects with other chemicals cannot be excluded.

As highlighted by the PCA score plot (Figure 1), male mussels seem to respond differently to WWTP effluent exposure. We believe that the existence of two subgroups in the exposed group is the consequence of inter-individual variability. Although individuals were collected at the same location from a mussel culture, they were still wild organisms with perhaps different life history traits. It can have an incidence on their metabolome. This is a challenge to overcome in environmental metabolomics when working on wild organisms. However, those differences are not due to our experimental conditions during the dissection, sample preparation or analysis. Indeed, those exposed mussels were distributed in different glass aquaria during exposure, they were randomly dissected and the samples were also randomized for extraction as well as for analysis.

### *Energy metabolism*

In the light of our results, the WWTP effluent extract exposure could affect the energy metabolism. Two plausible hypotheses can be proposed to explain this perturbation. First, environmental stressors can result in elevated ATP demand to fulfil essential maintenance costs and ensure organism survival (e.g. cellular protective mechanisms, detoxification) (Sokolova et al., 2012). Our results highlighted up-modulations of adenine and adenosine. The latter is a constituent of both ADP and ATP, and its up-modulation may serve ATP synthesis. Furthermore, our analysis revealed a down-modulation of glucogenic and ketogenic amino acids (proline, methionine, tyrosine, phenylalanine, isoleucine and leucine). In normal circumstances lipids and carbohydrates are generally used in cellular energy metabolism, while proteins and these amino acids can be consumed extensively in invertebrates under energy deficient conditions (Moyes et al., 1990; Sokolova et al., 2012). Decreases of these amino acids could be due to their over-consumption as energy-generating substrates. Down-modulation of glucogenic and ketogenic amino acids was already observed by Jones et al. (2008) during 4-day exposure of *M. galloprovincialis* to nickel (0.77 mg/L) or chlorpyrifos (4.5 mg/L). Moreover, we observed down-modulation of malate and fumarate, two citric acid cycle intermediates. A change in the citric acid cycle may be consistent with the use of an alternative substrate to produce energy, e.g. amino acids. In response to stress, organisms are able to switch between different metabolic processes involved in energy acquisition and conversion (Sokolova et al., 2012).

A second hypothesis may also be put forward. Our study reveals a dopamine down-modulation and potential modulation of the serotonin pathway (down-modulation of a serotonin metabolite, i.e. 5-methoxyindoleacetate). Both dopamine and serotonin induce contraction or relaxation of the adductor muscles responsible for valves opening or closing (Gies, 1986). In addition, these two neurotransmitters also control siphon activity (Ram et al., 1999) and ciliary movement in various bivalves (Carroll and Catapane, 2007), used primarily for feeding activity. In response to pollution stress, studies have already demonstrated a decrease of feeding activity or a valves closing, probably to avoid the entry of contaminants into the organism (Almeida et al., 2015, 2014; Chen et al., 2014; Oliveira et al., 2017; Solé et al., 2010; Toro et al., 2003; Wang et al., 2005). For example, after 28

days of *M. galloprovincialis* exposure to different carbamazepine concentrations (0.3; 3; 6; 9 µg/L), Oliveira et al. (2017) observed a significantly lower condition index in exposed organisms compared to controls, while the bioaccumulation factor (BCF) showed a decreasing trend with increasing exposure concentrations. In light of the absence of a main carbamazepine metabolism in mussels, they suggested that the decrease in BCF could possibly be due to a decrease in the filter feeding capacity of the mussels at higher exposure concentrations. This means that *M. galloprovincialis* can maintain its valves closed to avoid contaminants bioaccumulation, thus limiting feeding activity. Another PhAC occurring in our WWTP effluent extract, namely propranolol, has also been reported to inhibit the feeding rate of *M. galloprovincialis* exposed for 10 days at 147 µg/L (Solé et al., 2010). Impairment of feeding activity could have repercussion on food intake and subsequently on the energy metabolism. In our study, the decrease in amino acids could also result from disturbed food intake under stress induction.

#### *Osmoregulation*

In bivalves, free amino acids play a crucial role in the regulation of intracellular osmotic pressure to protect these organisms against extracellular osmolarity fluctuations in their environment (Viant et al., 2003; Yancey et al., 1982). As explain in the previous paragraph, the decrease in amino acids reported in our study is probably due to an over-consumption or inversely, a limited food intake. Hence, the unavailability of free amino acids could cause an osmotic pressure imbalance in male mussels. Similar results were also observed in marine green mussels (*Perna viridis*) after 4 weeks of 20 µg/L cadmium exposure (Wu and Wang, 2011). Other contaminants such as tributyltin oxide have an impact on the osmoregulation of the crustacean *Penaeus japonicus* after an exposure at 100-400 µg/L for 48h (Lignot et al., 1998) (Table 3).

In addition to free amino acids, cells use inorganic ions (Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup>) for intracellular osmotic pressure adjustment. In bivalve and crustacean gills, the Na influx was demonstrated to be under control of neurotransmitters such as dopamine and serotonin (Gardiner et al., 1991 ; Sommer and Mantel, 1988). The down-modulation of dopamine and potential disturbance of the serotonin pathway

reported in this study thus may have a repercussion on osmoregulation. Hence, the neurotransmitter modulation associated with the decrease in free amino acids tends to reinforce the hypothesis of an impact of exposure to WWTP effluent on osmoregulation in *M. galloprovincialis*.

#### *DNA and RNA synthesis*

Purine and pyrimidine metabolism were also altered by the WWTP effluent exposure, mainly with a change in levels of nucleic bases such as guanine, xanthine (guanine metabolite), adenine and uracil, and in the nucleosides deoxycytidine and deoxyuridine. Purine and pyrimidine bases are constituents of DNA and RNA. A decrease in these compounds could indicate restriction of substrates involved in DNA and RNA turnover and repair (Duan and Sadée, 1987). These results are in line with those of Gagné et al. (2011a). In a study conducted on freshwater mussels, they showed that PhACs that may occur in WWTP effluents act as inhibitors of key enzymes involved in purine and pyrimidine synthesis. Furthermore, others pollutants have already been reported to disturbed purine and pyrimidine metabolism in mussels and fish (Cappello et al., 2017; Fasulo et al., 2012; Jones et al., 2008; Song et al., 2018; Xu et al., 2015).

#### *Immune system*

Our results show the down-modulation of the two BCAAs L-isoleucine and L-leucine. During protein synthesis in mammalian skeletal muscle, BCAAs play an important role of regulation (Yoshizawa, 2004). Moreover, BCAAs are incorporated into proteins essential for immune human cell lymphocytes, eosinophils and neutrophils (Calder, 2006). Although the role of BCAAs in mollusk remains to be investigated, exposures to the pathogen *Vibrio harveyi* resulted in an increase of BCAAs in hepatopancreas of mussels (Nguyen et al., 2018 ; Liu et al. 2014). These observations support a role of BCAAs in the immune system of mussels as it is the case in mammals. Hence, the down-modulation of BCAAs observed in our study could make the immune system deficient when challenged to a pathogen and reduce the survival of male Mediterranean mussels. Several

contaminants present in our WWTP effluent extract have been reported to affect the immune system of *Mytilus* sp. (Table 3).

#### *Byssus formation and adhesion*

Mussels are able to connect their soft tissues to marine surfaces via adhesive proteins present in their byssus threads. Our results reveal a down-modulation of proline. This amino acid is essential for generating collagen required for byssus formation (Silverman and Roberto, 2007). A decrease in proline could alter the byssus formation. This hypothesis could also be supported by the potential disturbance of L-dopa. Although it was not detected, it is an intermediate in tyrosine transformation into dopamine, both being down-modulated. L-dopa is a component of adhesive proteins contained in high proportion in byssus (Bandara et al., 2013; Lucas et al., 2002). Its modulation could lead to an impairment of the byssus adhesive properties. Our results coincided with observations of Ericson et al. (2010) and Karagiannis et al. (2011) (Table 3). After exposure to 1000 and 10.000 µg/L of diclofenac, ibuprofen and propranolol for 1-3 weeks, *Mytilus edulis trossulus* showed a decrease in byssus strength and byssus thread abundance (Ericson et al., 2010). Similarly, Karagiannis et al. (2011) have shown the impact of atrazine (1000 µg/L) on viability and formation of byssus thread of *M. galloprovincialis* from the tenth day of exposure. Diclofenac, propranolol and atrazine were detected in our WWTP effluent extract. Moreover, another study revealed that *Mytilus trossulus* exposed to untreated sewage produced fewer byssus threads (Moles and Hale, 2003). Hence, the mussels exposure to a WWTP effluent can lead to an alteration of byssus formation and adhesion which would have harmful effects on mussel survival, movement and self-defense (Babarro and Reiriz, 2010).

#### *Reproduction*

Down-modulation of L-phenylalanine and L-tyrosine, i.e. catecholamine precursors, may lead to the decrease in dopamine. In addition, the serotonergic pathway may have been impacted since 5-methoxyindoleacetate, a serotonin metabolite, was also down-modulated. Exposure to wastewater effluents was already reported to be able to disturb dopamine and serotonin levels in several studies

conducted on freshwater *Elliptio complanata* mussels (Gagné et al., 2011c, 2011b, 2004; Gagné and Blaise, 2003). Similar results were as well highlighted by Bonnefille et al. (2018) in *M. galloprovincialis* exposed to 100 µg/L of diclofenac. In bivalves, dopamine (more generally catecholamines) and serotonin are involved in several reproductive functions. Both compounds play roles in sexual maturation, gamete multiplication and release (Gibbons and Castagna, 1984; Lafont and Mathieu, 2007; Martínez and Rivera, 1994). According to (Fong et al., 1993), spawning regulation in bivalves is dependent on the concentration of dopamine and serotonin, with dopamine inhibiting gamete release induced by serotonin. In view of their importance, a modulation of these compounds could significantly impair mussel reproduction.

#### *Defense mechanisms*

Exposure of mussels to WWTP effluent extract induced defense mechanisms. Our analysis revealed a 34% increase ( $p < 0.1$ ) in oxidized glutathione (GSSG) levels. GSSG up-modulation would indicate the presence of reactive oxygen species. Falfushynska et al. (2014) also revealed an elevated GSSG level in the digestive glands of freshwater mussels exposed to pharmaceutical and personal care products commonly found in WWTP effluents. Moreover, oxidative stress has already been noticed through different biomarkers analyzed in freshwater mussels after exposure to WWTP effluents (Gagné et al., 2011b; Goodchild et al., 2015). The occurrence of oxidative stress in digestive glands is not surprising regarding its functions. This organ accumulates contaminants and actively participates in the xenobiotic metabolism and detoxification (Faggio et al., 2018; Fernández et al., 2012; Porte et al., 2001).

Moreover, methionine acts as a precursor to glutathione (GSH) biosynthesis by providing sulfur atoms in cysteine synthesis (Stipanuk, 1986). The methionine down-modulation observed in this study could be explained by its use for the synthesis of GSH, which is then mobilized in oxidative stress defense, resulting in a GSSG elevation. Methionine can also serve as the principal methyl donor. Methylation is a conjugation pathway in xenobiotic metabolism, producing compounds with decreased biological activity (Silverman and Holladay, 2014).

## 5. Conclusion

Our non-targeted LC-HRMS metabolomics approach effectively highlighted the effects of a multi-contamination in a marine organism. The response of *M. galloprovincialis* to the WWTP effluents extract in this laboratory study was characterized by the presence of a high quantity of modulated metabolites, although only a few of them have been annotated or identified because of the current lack of knowledge on mussel metabolism. Among them, the main impacted metabolites belong to the metabolism of several amino acids, purine and pyrimidine metabolic pathway or citric acid cycle. According to the literature, molecular events observed in this study reflect potential disrupted biological processes such as energy metabolism, DNA and RNA synthesis, immune system, osmoregulation, byssus formation and adhesion, and reproduction. In addition to enriching knowledge about the impact of WWTP effluents in marine environment, our work then supports the relevance of metabolomics approach to highlight key molecular events triggered by chemical exposure, which could lead to negative impacts on individual fitness (growth, survival and reproduction). Metabolomics data, and more generally omics data (genomics, transcriptomics, proteomics), are very helpful in the characterization of contaminants' mode of action leading to an adverse outcome at the individual level. The integration of omics in a multilevel systems biology approach – combining molecular, cellular, tissue, individual and population level data – would represent a powerful and relevant approach, especially in ecological risk assessment. By reflecting specific responses of organisms to counteract potential toxics effects, metabolomics could offer useful information for further environmental monitoring and reveal which contaminant classes are exerting those effects (Jones et al., 2008; Van Aggelen Graham et al., 2010; Viant et al., 2003). Furthermore, effects-based monitoring may complement traditional methods based on chemical residue analysis, by hazard ranking of sites, effluents, etc., for prioritization of in-depth chemical analysis.

This study revealed the potential hazard of contaminants from medium to high polarity, discharged in coastal environment, on marine organisms. The first very promising results obtained in this laboratory study would deserve to be confirmed in a field study. Indeed, natural conditions can



modulate some of the elements highlighted here depending on the trophic status of the environment and environmental conditions. Anyway, efforts must be focused on the removal of contaminants in WWTPs to avoid ecotoxicological risks. Potential of metabolomics to predict adverse outcomes to individual could be exploited and integrated in ecological risk assessment and environmental monitoring.

## **Declarations of interest**

None.

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## **Appendix. Supplementary data**

Workflow of data processing and identification strategy used for the identification of metabolites impacted by a WWTP effluent extract exposure in *Mytilus galloprovincialis* (Figure S1). Pathway analysis of annotated and identified metabolites performed with MetaboAnalyst 4.0 (Xia and Wishart, 2010) (Figure S2). OPLS-DA score plot (1 predictive component and 1 orthogonal component;  $R^2X = 0.367$ ,  $R^2Y = 0.912$ ,  $Q^2 = 0.63$ ) of metabolic fingerprints from control (green circle) and exposed (red triangle) mussels, validated with cross-validation (leave-one-out). The numbers represent the injection order (Figure S3). Screening of 80 pharmaceutically active compounds, pesticides and, their metabolites suspected in the WWTP effluent extract (Table S1). Summary of the differential features with  $p < 0.05$  modulated by exposure to wastewater treatment plant effluent extract in mussel digestive glands (Table S2).

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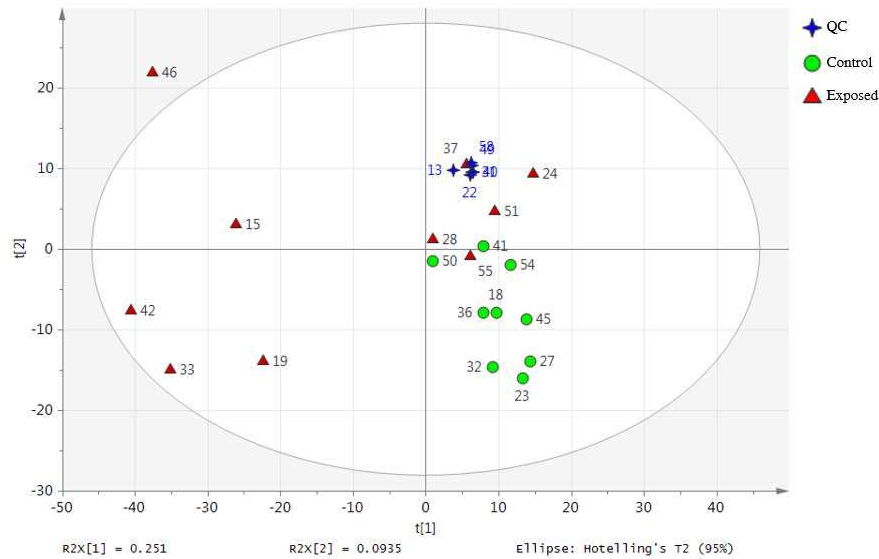
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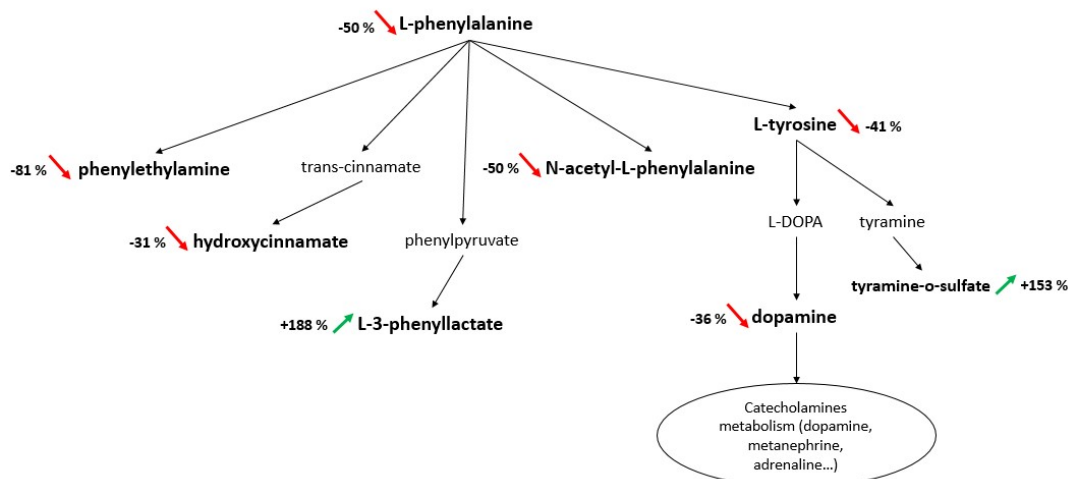
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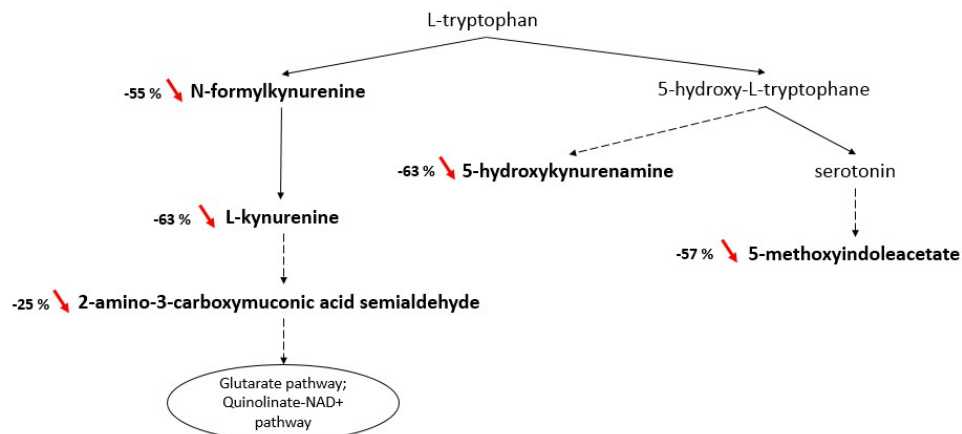
## Figures



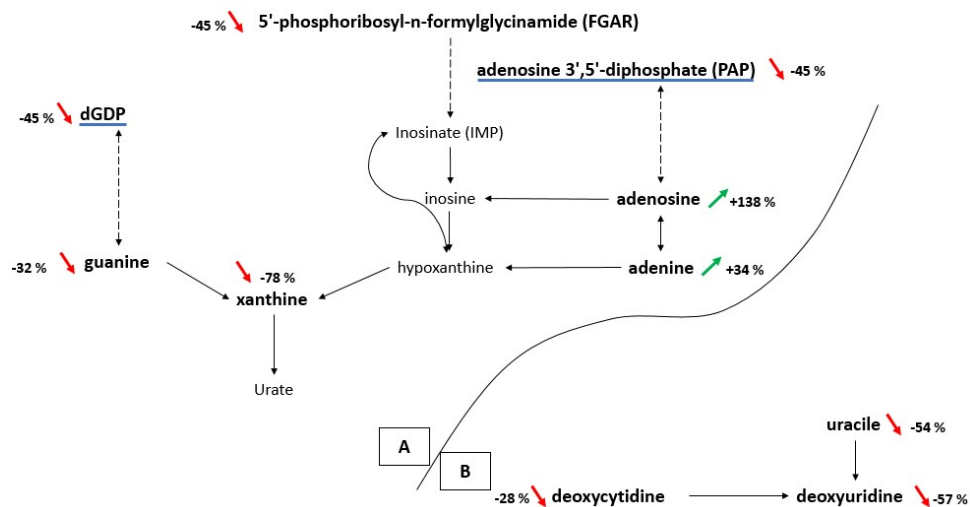
**Figure 1:** PCA score plot of metabolic fingerprints from control (green circle) and exposed (red triangle) mussels showing a clustering pattern between these two conditions. In blue (cross), QC are clustered. The numbers represent the injection order.



**Figure 1:** Partial map of phenylalanine and tyrosine metabolism in the response of *M. galloprovincialis* to a WWTP effluent exposure extract. Metabolites modulated by exposure are shown in bold, while metabolites not detected or not modulated are not in bold. The red arrow corresponds to down-modulation and the green arrow to up-modulation (exposed compared to control). Direct connections between metabolites are indicated with full arrows.



**Figure 3:** Partial map of tryptophan metabolism in the response of *M. galloprovincialis* to a WWTP effluent extract exposure. Metabolites modulated by exposure are shown in bold, while metabolites not detected or not modulated are not in bold. Red arrows correspond to down-modulation and green arrows to up-modulation (exposed compared to control). Direct connections between metabolites are indicated with full arrows while indirect connections are shown with dashed arrows.



**Figure 4:** Partial map of purine metabolism (A) and pyrimidine metabolism (B) in the response of *M. galloprovincialis* to a WWTP effluent extract exposure. Metabolites modulated by exposure are shown in bold, while metabolites not detected or not modulated are not in bold. Red arrows correspond to down-modulation and green arrows to up-modulation (exposed compared to control). Direct connections between metabolites are indicated with full arrows, while indirect connections are shown with dashed arrows. Underlined metabolites of the same color correspond to several possible annotations for the same feature.

**Table 1:** Detected pharmaceutically active compounds and pesticides in the WWTP effluent extract.

Class	Compounds	Molecular ion species detected	Calculated mass	Observed mass	Mass difference (ppm)	Retention time WWTP extract (min)	Standard retention time (min)	Relative abundance <sup>a</sup>
<b>Analgesics</b>	4-acetylaminoantipyrine (Noramidopyrine metabolite)	[M+H] <sup>+</sup>	246.1237	246.12401	1.3	5.75	5.72	++
	4-formylaminoantipyrine (Noramidopyrine metabolite)	[M+H] <sup>+</sup>	232.10805	232.10829	1.0	5.78	5.76	++
	Acetaminophen	[M+H] <sup>+</sup>	152.07061	152.07079	1.2	3.63	3.55	+++
	Antipyrine	[M+H] <sup>+</sup>	189.10224	189.10255	1.6	6.8	6.78	++
	Codeine	[M+H] <sup>+</sup>	300.15942	300.15982	1.3	3.88	3.86	+++
	Ketoprofen	[M+H] <sup>+</sup>	255.10157	255.10204	1.8	9.8	9.8	+++
	Mefenamic acid	[M+H] <sup>+</sup>	242.11756	242.11794	1.6	11.52	11.52	+
	Mepivacaine	[M+H] <sup>+</sup>	247.18049	247.18048	0	6.35	6.29	+
	Amoxicillin	[M+H] <sup>+</sup>	366.11182	366.11243	1.7	6.2	6.19	+++
	Flumequine	[M+H] <sup>+</sup>	262.0874	262.0881	2.7	9.02	9.02	+
<b>Antibiotics</b>	Metronidazole	[M+H] <sup>+</sup>	172.07167	172.07185	1.0	3.8	3.7	+++
	Ofloxacin	[M+H] <sup>+</sup>	362.15106	362.1514	0.9	5.98	5.94	+++
	Sulfamethoxazole	[M+H] <sup>+</sup>	254.05939	254.05974	1.4	7.92	7.91	+++
	Sulfapyridine	[M+H] <sup>+</sup>	250.06447	250.06476	1.2	6.01	5.98	+++
	Trimethoprim	[M+H] <sup>+</sup>	291.14517	291.14545	1.0	5.9	5.82	+++
	Cyclophosphamide	[M+H] <sup>+</sup>	261.0321	261.03232	0.8	7.89	7.89	+
	Ifosfamide	[M+H] <sup>+</sup>	261.0321	261.03214	0.2	7.78	7.78	+
	Oxazepam	[M+H] <sup>+</sup>	287.05818	287.05865	1.6	8.96	8.94	+++
	Atenolol	[M+H] <sup>+</sup>	267.17032	267.17059	1.0	2.3	2.31	+++
	Metoprolol	[M+H] <sup>+</sup>	268.19072	268.19092	0.7	6.66	6.62	++
<b>Beta-Blockers</b>	Nadolol	[M+H] <sup>+</sup>	310.20128	310.20175	1.5	5.61	5.55	++
	Propranolol	[M+H] <sup>+</sup>	260.16451	260.16501	1.9	7.58	7.5	++
	Sotalol	[M+H] <sup>+</sup>	273.12674	273.12714	1.5	2.69	2.66	+++
	Salbutamol	[M+H] <sup>+</sup>	240.15942	240.15974	1.3	2.24	2.24	++
<b>Antineoplastic agents</b>	Cyclophosphamide	[M+H] <sup>+</sup>	261.0321	261.03232	0.8	7.89	7.89	+
	Ifosfamide	[M+H] <sup>+</sup>	261.0321	261.03214	0.2	7.78	7.78	+
<b>Benzodiazepines</b>	Oxazepam	[M+H] <sup>+</sup>	287.05818	287.05865	1.6	8.96	8.94	+++
	Atenolol	[M+H] <sup>+</sup>	267.17032	267.17059	1.0	2.3	2.31	+++
<b>Bronchodilators</b>	Metoprolol	[M+H] <sup>+</sup>	268.19072	268.19092	0.7	6.66	6.62	++
	Nadolol	[M+H] <sup>+</sup>	310.20128	310.20175	1.5	5.61	5.55	++
	Propranolol	[M+H] <sup>+</sup>	260.16451	260.16501	1.9	7.58	7.5	++
	Sotalol	[M+H] <sup>+</sup>	273.12674	273.12714	1.5	2.69	2.66	+++
<b>Bronchodilators</b>	Salbutamol	[M+H] <sup>+</sup>	240.15942	240.15974	1.3	2.24	2.24	++

Table 1: (continued)

Class	Compounds	Molecular ion species detected	Calculated mass	Observed mass	Mass difference (ppm)	Retention time WWTP effluent extract (min)	Standard retention time (min)	Relative abundance <sup>a</sup>
<b>Corticosteroids</b>	Methylprednisolone	[M+H] <sup>+</sup>	375.2166	375.2171	1.3	8.52	8.5	+++
<b>Lipid regulators</b>	Fenofibric acid	[M+H] <sup>+</sup>	319.07316	319.07376	1.9	10.83	10.83	+++
<b>Non-steroidal anti-inflammatory drug</b>	Diclofenac	[M-H] <sup>-</sup>	294.00941	294.00955	0.5	10.86	10.86	+++
<b>Psychiatric-drug</b>	10,11-epoxycarbamazepine (Carbamazepine metabolite)	[M+H] <sup>+</sup>	253.0971	253.09749	1.5	7.9	7.9	++
	Amitriptyline	[M+H] <sup>+</sup>	278.19033	278.19089	2.0	8.18	8.27	+
	Carbamazepine	[M+H] <sup>+</sup>	237.1022	237.10254	1.4	8.7	8.7	+++
	Citalopram	[M+H] <sup>+</sup>	325.17107	325.17157	1.5	7.86	7.78	+
	Primidone	[M+H] <sup>+</sup>	219.1128	219.11346	3.0	6.9	6.89	++
	Venlafaxine	[M+H] <sup>+</sup>	278.21146	278.21158	0.4	7.25	7.19	+++
<b>Ulcer healings</b>	Omeprazole	[M+H] <sup>+</sup>	346.12199	346.12244	1.3	6.95	6.95	++
	Ranitidine	[M+H] <sup>+</sup>	315.14854	315.14902	1.5	2.51	2.46	+
<b>Stimulants</b>	Caffeine	[M+H] <sup>+</sup>	195.08765	195.08794	1.5	5.32	5.25	++++
	Nicotine	[M+H] <sup>+</sup>	163.12298	163.12311	0.8	1.54	1.46	++
	Paraxanthine (Caffeine metabolite)	[M+H] <sup>+</sup>	181.072	181.07208	0.4	3.35	3.29	++++
<b>Herbicides</b>	Atrazine	[M+H] <sup>+</sup>	216.10105	216.10129	1.1	9.65	9.64	+
	Simazine	[M+H] <sup>+</sup>	202.0854	202.08592	2.6	8.77	8.79	+
	Terbutryn	[M+H] <sup>+</sup>	242.14339	242.14378	1.6	9.14	9.1	+
<b>Insecticides</b>	Fipronil	[M-H] <sup>-</sup>	434.93143	434.93134	-0.2	11.61	11.61	+

<sup>a</sup> Relative abundance was calculated based on their chromatographic peak area. The more “+” there are, the higher the proportion.

**Table 2:** List of the main metabolic pathways modulated by exposure to WWTP effluent extracts in male mussel digestive glands, considering also metabolites with a p-value < 0.1.

Metabolism pathway	Metabolite	Retention Time (min)	Molecular ion species detected	Calculated mass	Observed mass	Mass difference (ppm)	RSD QC <sup>c</sup> %	RSD SC <sup>c</sup> %	RSD Exposed <sup>c</sup> %	Difference amplitude %	p-value	Annotation level <sup>a</sup>
	<i>L-tyrosine</i> <sup>†</sup>	3.82	[M-H] <sup>-</sup>	180.0666	180.0654	7	33	19	74	↘ -41	0.01745	1
	L-phenylalanine	6.90	[M+H] <sup>+</sup>	166.0863	166.0860	2	3	17	54	↘ -48	0.00042	1
		6.92	[M-H] <sup>-</sup>	164.0717	164.0704	8	3	30	61	↘ -50	0.00215	1
<b>Phenylalanine metabolism</b>	N-acetyl-L-phenylalanine	9.40	[M-H] <sup>-</sup>	206.0823	206.0812	5	3	20	29	↘ -50	0.00002	1
	4-hydroxycinnamic acid	9.50	[M-H] <sup>-</sup>	163.0401	163.0388	8	8	37	22	↘ -31	0.04103	1
	Phenylethylamine	10.92	[M+H] <sup>+</sup>	122.0964	122.0963	1	4	79	94	↘ -81	0.01519	1
	L-3-phenyllactic acid	9.30	[M-H] <sup>-</sup>	165.0557	165.0544	8	17	74	77	↗ +188	0.02734	2
		Dopamine	4.95	[M-H] <sup>-</sup>	152.0717	152.0703	9	10	31	58	↘ -32	0.06220
<b>Tyrosine metabolism</b>		4.97	[M+H] <sup>+</sup>	154.0863	154.0860	2	11	34	52	↘ -36	0.03397	1
		3.82	[M-H] <sup>-</sup>	180.0666	180.0654	7	33	19	74	↘ -41	0.01745	1
	Gamma-glutamyltyrosine	5.68	[M-H] <sup>-</sup>	309.1092	309.1088	1	7	52	73	↘ -51	0.02850	2
	Tyramine-o-sulfate	8.02	[M-H] <sup>-</sup>	216.0336	216.0326	5	2	17	81	↗ +153	0.04239	2
		L-kynurenine	7.6	[M-H] <sup>-</sup>	207.0775	207.0765	5	9	70	71	↘ -63	0.02887
<b>Tryptophan metabolism</b>		7.58	[M+H] <sup>+</sup>	209.0921	209.0917	2	3	71	77	↘ -63	0.03020	1
		7.27	[M-H] <sup>-</sup>	235.0724	235.0716	3	8	63	75	↘ -55	0.03902	1
	5-hydroxykynurenamine	3.67	[M+NH4] <sup>+</sup>	198.1237	198.1233	2	29	73	177	↘ -63	0.06585	2
		10.10	[M+NH4] <sup>+</sup>	223.1077	223.1073	2	4	44	64	↘ -57	0.00513	2
	<b>Valine, leucine and isoleucine biosynthesis</b>	L-isoleucine	3.90	[M-H] <sup>-</sup>	130.0874	130.0859	12	8	25	73	↘ -39	0.03391
L-leucine		4.37	[M-H] <sup>-</sup>	130.0874	130.0859	12	11	23	71	↘ -35	0.05334	1
		1.45	[M-H2O-H] <sup>-</sup>	85.0290	85.0280	12	14	24	25	↘ -32	0.00450	2

**Table 2:** (continued)

Metabolism pathway	Metabolite	Retention Time (min)	Molecular ion species detected	Calculated mass	Observed mass	Mass difference (ppm)	RSD QC <sup>c</sup> %	RSD SC <sup>c</sup> %	RSD Exposed <sup>c</sup> %	Difference amplitude %	p-value	Annotation level <sup>a</sup>
Arginine and proline metabolism	<i>L-proline</i>	1.57	[M+H] <sup>+</sup>	116.0706	116.0707	1	7	46	77	↘ -42	0.05913	1
	N-acetyl-L-alanine	2.22	[M-H] <sup>-</sup>	130.0510	130.0495	12	10	24	32	↘ -49	0.00016	1
Cysteine and methionine metabolism	L-methionine	2.57	[M-H] <sup>-</sup>	148.0438	148.0423	10	19	35	97	↘ -55	0.00773	1
	Glycine, serine and threonine metabolism	1.50	[M-H] <sup>-</sup>	118.0510	118.0494	14	6	15	48	↘ -29	0.03384	2
	L-threonine or homoserine	1.47	[M+H] <sup>+</sup>	120.0655	120.0653	2	5	20	60	↘ -31	0.05461	2
	<i>Adenosine</i>	3.28	[M+H] <sup>+</sup>	268.1040	268.1035	2	5	66	85	↗ +127	0.07510	1
3.28		[M+FA-H] <sup>-</sup>	312.0950	312.0944	2	7	61	82	↗ +138	0.05656	1	
<i>Guanine</i>	2.42	[M-H] <sup>-</sup>	150.0421	150.0407	9	9	45	36	↘ -32	0.0866	1	
	Xanthine	1.87	[M-H] <sup>-</sup>	151.0261	151.0247	9	5	37	86	↘ -78	0.00011	1
Purine metabolism		5'-phosphoribosyl-n-formylglycinamide or 2-oxo-4-methylthiobutanoic acid	6.95	[M-H <sub>2</sub> O-H] <sup>-</sup>	295.0331	295.0325	2	7	13	55	↘ -45	0.00087
	<i>Adenine</i>	2.80	[M-H] <sup>-</sup>	134.0472	134.0459	10	8	36	31	↗ +34	0.07383	1
dGDP or Adenosine 3',5'-diphosphate		3.63	[M+FA-H] <sup>-</sup>	472.0276	472.0277	0	29	16	58	↘ -45	0.00174	2

**Table 2:** (continued)

Metabolism pathway	Metabolite	Retention Time (min)	Molecular ion species detected	Calculated mass	Observed mass	Mass difference (ppm)	RSD QC <sup>c</sup> %	RSD SC <sup>c</sup> %	RSD Exposed <sup>c</sup> %	Difference amplitude %	p-value	Annotation level <sup>a</sup>
	Uracil	1.72	[M-H] <sup>-</sup>	111.0200	111.0185	14	7	25	49	↘ -53	0.00018	1
<b>Pyrimidine metabolism</b>		1.72	[M+H] <sup>+</sup>	113.0346	113.0345	1	22	28	60	↘ -54	0.00058	1
	<i>Deoxyvitidine</i>	2.93	[M+FA-H] <sup>-</sup>	272.0888	272.0887	0	5	32	43	↘ -28	0.06584	1
	Deoxyuridine	2.18	[M-H] <sup>-</sup>	227.0673	227.0666	3	29	66	73	↘ -57	0.03682	1
	<i>(r)-b-aminoisobutyric acid</i>	3.37	[M+H] <sup>+</sup>	102.0561	102.0546	14	13	32	87	↗ +158	0.05324	2
<b>Pyruvate metabolism</b>	<i>Malate</i>	1.55	[M-H] <sup>-</sup>	133.0142	133.0128	11	8	20	44	↘ -24	0.07911	1
	Fumarate	2.57	[M-H] <sup>-</sup>	115.0037	115.0022	13	25	31	47	↘ -31	0.04040	1
	<i>S-acetyldihydroipoamide</i>	1.95	[M+H] <sup>+</sup>	250.0930	250.0946	6	12	30	32	↘ -46	0.00148	2
	<i>S-lactoylglutathione</i>	2.75	[M-H] <sup>-</sup>	378.0977	378.0976	0	29	69	60	↗ +87	0.05693	2
	<i>Oxidized glutathione</i>	2.40	[M-H] <sup>-</sup>	611.1447	611.1443	1	31	25	33	↗ +34	0.09481	1
<b>Glutathione metabolism</b>	Cysteineglutathione disulfide	1.50	[M-H] <sup>-</sup>	425.0806	425.0802	1	17	26	66	↘ -44	0.00797	2
	Cysteinylglycine <sup>b</sup>	4.38	[2M-H] <sup>-</sup>	355.0752	355.0742	3	16	24	86	↘ -51	0.00556	2
<b>Analogue to GSH</b>	Noropthalamic acid	1.60	[M-H] <sup>-</sup>	274.1045	274.1040	2	17	13	35	↘ -39	0.00022	2

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.

<sup>a</sup> Based on the work of Sumner et al. (2007).

<sup>b</sup> In accordance with the retention time observed for the corresponding analytical standard in Boudah et al. (2014) with the same chromatographic column.

<sup>c</sup> RSD: Relative Standard Deviation calculated for each feature detected in Quality Control (QC) sample injected throughout the analytical sequence (analytical variability), in solvent control (SC) group and exposed group (biological variability).

<sup>†</sup> Belong to several metabolic pathways

Metabolites marked in italic show a trend in modulation (p<0.1 and/or modulation amplitude < 30 %) or are analytically less restrictive (RSD QC < 35 %). Nevertheless, they may represent biologically relevant metabolites.

**Table 3:** Summary table relating observed effects with contaminants detected in the WWTP effluent extract.

Impacted metabolites and metabolic pathways	Impacted metabolites are implied in these biological functions	Contaminants already reported as altering these biological functions
<p><b>Amino acids metabolism:</b> isoleucine, leucine, methionine, phenylalanine, proline, tyrosine</p>	<p><b>Energy metabolism</b> Sokolova et al., 2012</p>	<p><b>Caffeine:</b> 0.5, 3 and 18 µg/L, alterations on energy reserves of <i>Ruditapes philippinarum</i> (Cruz et al., 2016); 0.2, 0.3 and 0.4 mM, inhibition of glycogen phosphorylase in <i>Mytilus galloprovincialis</i> (Serrano et al., 1995).</p>
<p><b>Citric acid cycle:</b> malate, fumarate</p>		<p><b>Carbamazepine:</b> 0.3, 3 and 6 µg/L, decrease of glycogen and protein content in <i>Scrobicularia plana</i> and <i>Diopatra neapolitana</i> (Freitas et al., 2015); 50 µg/L, alteration of siphoning behavior in <i>Corbicula fluminea</i> (Chen et al., 2014).</p>
<p><b>ATP components:</b> adenine, adenosine</p>		<p><b>Diazinon or endosulfan:</b> 0.47 and 0.60 µg/L or 0.44 and 0.61 mg/L respectively, impact of filtration and ingestion rate of <i>Daphnia magna</i> (Fernández-Casalderrey et al., 1994).</p>
		<p><b>Ketoprofen:</b> 2.5 µg/L, changes in Acyl CoA oxidase activity which catalyzes the first reaction of β-mitochondrial oxidation of fatty acids in <i>Mytilus galloprovincialis</i> (Mezzelani et al., 2018b).</p>
		<p><b>Propranolol:</b> 147 µg/L, inhibition of feeding rate of <i>Mytilus galloprovincialis</i> (Solé et al., 2010).</p>
		<p><b>Simazine or terbutryn:</b> 4, 20 and 50 µg/L or 0.2 and 2 µg/L, increase activity of transaminase enzymes and lactate dehydrogenase and increase blood glucose in <i>Cyprinus carpio</i> (Velisek et al., 2011, 2009).</p>
		<p><b>Atrazine:</b> <math>4.6 \times 10^{-5}</math> M, increase the death of hemocytes (mussel immune cell) of <i>Mytilus edulis</i> (Feng et al., 2017).</p>
<p><b>Branched chain amino acids:</b> isoleucine, leucine</p>	<p><b>Protein synthesis and immune system</b> Calder, 2006; Liu et al., 2014</p>	<p><b>Carbamazepine:</b> 0.01, 0.1, 1, 10 and 100 µg/L, impact hemocytes of <i>Mytilus galloprovincialis</i> (Tsiaka et al., 2013).</p>
		<p><b>Diazinon:</b> 7830 µg/L, cause immunotoxicity in <i>Oreochromis niloticus</i> (Girón-Pérez et al., 2007).</p>
		<p><b>Ibuprofen or ketoprofen:</b> 2.5 µg/L for both, decrease of granulocytes / hyalinocytes ratio and inhibition of phagocytosis capacity in <i>Mytilus galloprovincialis</i> (Mezzelani et al., 2018b).</p>



**Table 3:** (continued)

Impacted metabolites and metabolic pathways	Impacted metabolites are implied in these biological functions	Contaminants already reported as altering these biological functions
<p><b>Free amino acids:</b> isoleucine, leucine, methionine, phenylalanine, proline, tyrosine</p>	<p><b>Osmoregulation</b> Yancey et al., 1982</p>	<p><b>Clofibrac acid or diclofenac:</b> 1, 10 and 100 µg/L for both, disturbance of ionoregulatory responses of <i>Cirrhinus mrigala</i> (Saravanan and Ramesh, 2013).</p> <p><b>Diclofenac:</b> 10 and 100 ng/L, impairment of osmoregulatory ability of <i>Carcinus maenas</i> (Eades and Waring, 2010).</p> <p><b>Tributyltin oxide:</b> 100, 200, 300 and 400 µg/L, impact of osmoregulation of <i>Penaeus japonicus</i> (Lignot et al., 1998).</p>
<p><b>DOPA synthesis:</b> tyrosine, dopamine</p> <p><b>Collagen components:</b> proline</p>	<p><b>Byssus formation and adhesion</b> Silverman and Roberto, 2007; Lucas et al., 2002</p>	<p><b>Atrazine:</b> 1000 and 2000 µg/L, impact viability and formation of byssus thread of <i>Mytilus galloprovincialis</i> (Karagiannis et al., 2011).</p> <p><b>Diclofenac, ibuprofen or propranolol:</b> 10,000 µg/L for each, lower strength and abundance of byssus thread of <i>Mytilus edulis trossulus</i> (Ericson et al., 2010).</p>
<p><b>Catecholamines and their precursors:</b> dopamine, norepinephrine sulfate, phenylalanine, tyrosine</p>	<p><b>Reproduction</b> Lafont and Mathieu, 2007</p>	<p><b>Atrazine:</b> 4.6x10<sup>-7</sup> and 4.6x10<sup>-5</sup>M, reduction of mussel sperm motility and down-regulation of androgen receptor expression in target Leydig cells (Feng et al., 2017).</p> <p><b>Carbamazepine:</b> 200 µg/L, decrease of the reproduction rate and of the number of molts in <i>Daphnia magna</i> (Oropesa et al., 2016).</p> <p><b>Diazinon:</b> 0.25 and 0.3 µL, disturbance of reproduction in <i>Daphnia magna</i> (Fernández-Casalderrey et al., 1995).</p> <p><b>Ibuprofen:</b> 250 ng/L, induction of reproductive biomarkers (vitellogenine, alkali-labile phosphates) in <i>Mytilus galloprovincialis</i> (Gonzalez-Rey and Bebianno, 2012).</p>
<p><b>Serotonin metabolite:</b> 5-methoxyindoleacetate</p>		<p><b>Propranolol:</b> 100 and 250 µg/L, disturbance of reproduction in <i>Hyalella azteca</i> and <i>Ceriodaphnia dubia</i> (Huggett et al., 2002).</p>

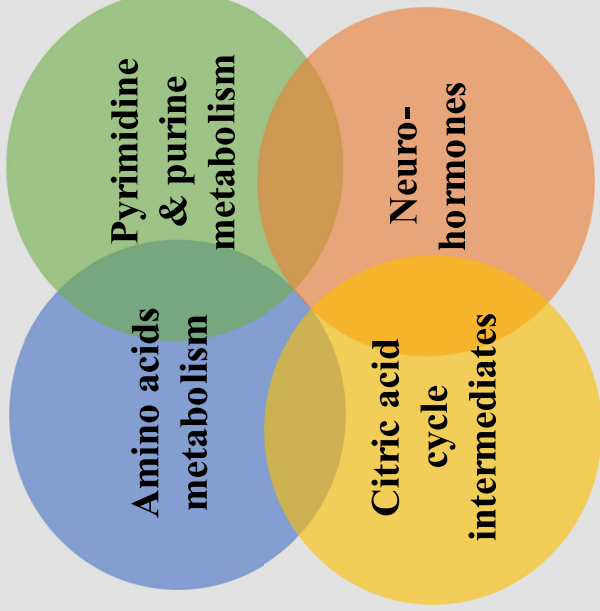
Underlined contaminants refer to contaminants occurring in our WWTP effluent extract, while not underlined contaminants were not targeted in the analysis.

## WWTP effluent extract

7-days exposure



## Impacted metabolites



## Biological process potentially altered

- Energy metabolism
- Osmoregulation
- DNA & RNA synthesis
- Immune system
- Byssus formation
- Reproduction

U n t a r g e t e d   m e t a b o l o m i c s   a p p r o a c h