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Comprehensive study of sulfamethoxazole effects in marine mussels: bioconcentration, enzymatic activities and metabolomics

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Abstract

Antibiotics accumulation in aquatic organisms may be of great concern from an ecological point of view but also from a human perspective, especially when they are accumulated in edible animals like marine mussels. In this work, mussels (*Mytilus galloprovincialis)* were exposed to sulfamethoxazole antibiotic (SMX) at 10 µg/L during 96 h, followed by 24 h of depuration. The experiment was carried out at summer and winter conditions. SMX showed a bioconcentration factor in mussel of 1.5 L/Kg (dry weight) and 69% of the compound was eliminated from the

organism in 24 h. The metabolomics approach revealed alterations in amino acids levels (aspartate, phenylalanine, valine and tryptophan) pinpointing disturbances in osmotic regulation and energy metabolism. Besides, the levels of some nucleotides (guanosine and inosine) and a carboxylic acid were also affected. However, SMX exposed mussels did not show any significant alteration in the enzymatic activities related to the xenobiotic metabolism and oxidative stress. Moreover, some of the changes observed in mussel's metabolites suggested alterations in mussel's organoleptic characteristics that can affect its quality as seafood commodity. Overall, our results showed that SMX exposure to marine mussels may have ecological implications by provoking sub-lethal effects to exposed organisms. Nevertheless, no risk for consumers derived from mussel ingestion is expected due to the low bioconcentration capacity of SMX and fast depuration in this seafood type.

Keywords:

commercial mussels; antibiotic pollution; bioconcentration; non-targeted metabolomics; ecotoxicology

1. Introduction

Antibiotics are worldwide contaminants of emerging concern. They reach the aquatic environment mainly through waste water treatment plant effluents, among other sources (Fatta-Kassinos et al., 2011). These compounds can pose a risk for the aquatic community chronically exposed to them. Besides, there is a growing concern about the contribution of antibiotic pollution to the development of antibiotic resistant bacteria (Grenni et al., 2018; Kümmerer, 2009). Sulfamethoxazole (SMX) is a bacteriostatic antibiotic, effective against both, gram negative and gram positive bacteria. It is extensively used due to its bactericidal broad spectrum and low cost (Carvalho and Santos, 2016). Sulfamethoxazole reaches the coastal areas mainly through river discharges and due to its use in aquaculture (Shimizu et al., 2013; Zhang et al., 2012). Its presence in coastal waters and particularly estuaries, harbors and lagoons has been widely studied with levels ranging from low ng/L up to few μg/L (Rodriguez-Mozaz et al., 2017). Although SMX is not expected to kill eukaryotic organisms at environmental concentrations, it may produce sub-lethal effects and alter the normal functioning of the organisms in the aquatic ecosystem. To this respect, the analysis of enzymatic activities related to xenometabolism and oxidative stress have been commonly used for the characterization of organisms response to different stress factors, including chemical pollution (Vidal-Liñán, 2015). Furthermore, the analysis of organisms metabolome (through a metabolomics approach) has been used in many fields including ecotoxicology, for the evaluation of sub-lethal alterations in organisms exposed to different contaminants, revealing new ecotoxicological effects and postulating biomarkers of exposure (Álvarez-Muñoz et al., 2014; Serra-Compte et al., 2018a). Metabolomics is also applied in food science (foodomics) for the characterization of

food properties like nutritional value, savor, taste and odor within others, and its changes under different conditions or treatments (Cevallos-Cevallos and Reyes-De-Corcuera, 2012).

Among aquatic organisms, marine mussels are highly valuable for ecological studies and also as food source for human consumption. Their characteristics as sessile and filter-feeding organisms make them prone to bioaccumulate contaminants, including antibiotics, present in their surrounding environment (Álvarez-Muñoz et al., 2015). Therefore, they are extensively used as sentinels for chemical pollution monitoring in natural environments (Hellou and Law, 2003; OSPAR, 2016). Despite several studies showed that changes in environmental conditions (i. e. water temperature) or the annual reproductive cycle of mussels, may influence their response to chemical pollution (Costa et al., 2008; González-Fernández et al., 2016; Maulvault et al., 2016; Serra-Compte et al., 2018b) few studies considered seasonality when assessing bioconcentration of contaminants on these organisms (Claudi and Mackie, 1994; Costa et al., 2008; Galvao et al., 2015). On the other hand, the presence of antibiotics in marine mussels may pose a risk for consumers such as allergy and toxicity (Cabello, 2006). Therefore, maximum residue limits (MRL) for some antibiotics in foodstuff from animal origin have been established by the authorities (including SMX, 100 ng/g, wet weight, European Commission, 2010).

Little is known about the ecotoxicological implications of antibiotics exposure to the aquatic organisms. Furthermore, the evaluation of antibiotics effects in foodstuff from animal origin, such as mussels, is also of great interest from a human perspective. We thereby hypothesize that SMX will induce enzymatic responses and modulate the metabolome profile of marine mussels. In this work, an in-vivo exposure of marine

mussels to the antibiotic SMX was carried out during 96 h at summer (water temperature 16.0 ℃) and winter (water temperature 12.5 ℃) conditions. This was undertaken in order to study potential differences in the response of mussel to chemical pollution due to seasonality and consequently to different state of maturity. The ecotoxicological effects were evaluated using metabolomics and through the analysis of enzymatic activities involved in xenometabolism and oxidative stress responses such as carboxylesterases (CbE), glutathione S-transferase (GSTs), glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT) and lipid peroxidation (LPO) levels. The main objectives of this work were: i) to study bioaccumulation, metabolization and depuration of SMX in commercial mussels, ii) to assess the response of mussels to SMX through changes in xenometabolism and oxidative stress enzymatic activities, and iii) to evaluate alterations in mussel's metabolome using a non-targeted metabolomics approach and postulate potential biomarkers of exposure. This is the first time that a metabolomics approach has been addressed to characterize the response of marine mussels to antibiotics pollution.

2. Material and Methods

2.1 Standards and reagents

The cartridges used for solid phase extraction, OASIS HLB (200 mg, 6 mL), were obtained from Water Corporation (Milford, MA, U.S.A.). HPLC grade methanol and water were purchased from Merck (Darmstadt, Germany). Sulfamethoxazole standard as well as the isotopically-labelled internal standard sulfamethoxazole-d4 were purchased from Sigma-Aldrich (St Louis, MO, USA). Sulfamethoxazole metabolites, N-acetyl sulfamethoxazole, desamino-sulfamethoxazole and glucoronide sulfamethoxazole were purchased from Toronto Research Chemicals (TRC) (Ontario,

Canada). All analytical standards were HPLC suitable and were prepared in methanol at a concentration of 1000 mg/L and stored at -20 ºC. Working standard solutions (1 mg/L) were prepared in methanol before each analytical run.

2.2 Experimental design

Mussels and seawater were obtained from a bivalve's distribution plant (Girona, Spain) in two different seasons, summer and winter. Bivalves and seawater were transported to the facilities of Catalan Institute for Water Research (Girona, Spain). The experiment lasted ten days divided in three different periods; an acclimation period (5 days), where mussels were kept in the laboratory experimental conditions but without addition of SMX. Then, half of the mussels were exposed to SMX during 4 days (exposure period of 96 h), while the other half remained in control conditions (without SMX). This short term exposure allowed the evaluation of early ecotoxicological responses of mussels due to antibiotics pollution. After the exposure period, a depuration period without addition of SMX was carried out (during 24 h) at a commercial bivalve's distribution plant. Initially, 300 individuals were acclimated in a 500 L tank of a water recirculation system, equipped with protein skimmers, biological filtration, temperature control and aeration. After the acclimation period, the 300 individuals were randomly distributed in two separate tanks, 150 individuals per tank (500 L) with the same equipment described above. The experiment was carried out two times in order to have two experimental replicates. Specimens were kept under the following conditions: temperature: 16.0 ± 0.7 ºC during the summer trial and 12.5 ± 0.8 ºC during the winter trial. The temperature was set at the same temperature than the one registered in the Mediterranean Sea (Girona coast) during the two sampling trials (June and February). During both trials the pH was 8.0 ± 0.1 units, dissolved oxygen (DO) > 80 %DO/L, salinity 40.4 ± 0.6 ‰ and photoperiod of 12 hours light and 12 hours dark (12L:12D). Ammonia (NH_3/NH_4^+) was

kept below 0.2 mg/L and nitrates (NO_3^-) below 1 mg/L, nitrites were in all analysis below quantification levels (0.004 mg/L). The utilization of water filtration systems (protein skimmer and biological filters) and the large amount of water per tank (500 L), guaranteed the optimal conditions for mussels through the exposure phase (96 h) without the need of water renewal. Besides, low mortality, below 2 %, was observed considering the whole experiment (acclimation, exposure and depuration). Mussels were fed two times per day with a commercial microalgae mix suitable for bivalve molluscs, composed by 2.000×10^6 cells/mL of the following species: *Isochrysis* spp., *Tetraselmis* spp, *Nannochloropsis* spp, *Thalassiosira* spp. (Acuinuga, Spain). Sulfamethoxazole was added at a final concentration of 10 µg/L via water in the exposure tanks. Sulfamethoxazole exposure concentration was higher than the one commonly found in the environment, especially in coastal areas (Rodriguez-Mozaz et al., 2017), but lower than the predicted non effect concentration (PNEC) for many aquatic organisms (Nguyen Dang Giang et al., 2015), in order to evaluate sub-lethal effects of this compound in mussels. Every day, 20 % of the initial concentration of SMX was added to the exposure tanks in order to maintain it constant, and compensate the loss due to degradation and/or adsorption of the chemical in the system. This percentage of loss was measured in a previous experiment carried out for charactering the whole system (aquarium, filter, skimmer, etc.) without presence of organisms. The system was spiked at the target concentration and the compound loss was evaluated in seawater resulting in a loss of 20 % of SMX after 24 h (data not shown). In non-exposure tanks, the equivalent amount of solvent (methanol) was also added. The total amount of solvent added to the tanks represented a 0.05 % of the total water volume. After 96 h of exposure, mussels were transported to the bivalve's distribution plant (approximately 1 h drive in refrigerated conditions) for a depuration period of 24 h

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under real commercial conditions. A depuration period of 24 h in the bivalve's distribution facilities is the usual procedure that commercial mussels follow before being sold in the markets. The depuration system comprises 30 water aquariums distributed in columns of three. The total water capacity is 17 m^3. Seawater was continuously renewed, 6000 L/h in every column of aquariums. Organisms were maintained at the same temperature than in the exposure period as seawater was directly collected from the sea by the bivalve's distribution plant. The system is equipped with decanting pit, skimmer, ozonation and temperature control (Innovaqua S. L.). Therefore, the contribution of this depuration period to the elimination of contaminants, like SMX, that may be accumulated in mussels during farming was evaluated.

For SMX bioaccumulation study, seawater and mussel's soft tissue were sampled at 0, 1, 3, 6, 9, 24, 48, 72 and 96 h of the exposure phase and after 6, 12 and 24 h of depuration phase (from both control and exposure tanks) whereas haemolymph samples were withdrawn from mussel's adductor muscle at 0, 24, 48, 72 and 96 h of the exposure phase and after 24 h depuration. Haemolymph for the metabolomics study, and mussel's digestive gland and gills for enzymatic activity measures, were taken after 96 h of exposure. In all cases mussels were processed individually in order to cope with the biological variation between organisms.

2.3 Target analysis of SMX and its metabolites in mussel's soft tissue, haemolymph and seawater

For SMX accumulation in mussel's soft tissue and haemolymph, four organisms were sampled at each sampling time and treatment (control and SMX exposed mussels). Mussel's soft tissue of each individual separately was taken, snap frozen, freeze-dried and kept at -20 ºC until its analysis. Freeze-dried samples were weighted and extracted

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using pressurized liquid extraction followed by a clean-up using solid phase extraction, applying a method developed by Álvarez-Muñoz et al., (Álvarez-Muñoz et al., 2015). Then, the target analysis of SMX and its related metabolites (N-acetyl-sulfamethoxazole, desamino-sulfamethoxazole and glucoronide sulfamethoxazole) was performed using ultra-performance liquid chromatography coupled to a quadrupole linear ion trap tandem mass spectrometry (UPLC-QqLIT) following the same method (Álvarez-Muñoz et al., 2015). These metabolites were selected because they are some of the most common SMX metabolites in humans and analytical standards are available. Mussel's haemolymph was extracted from the mussel's adductor muscle and diluted with an anticoagulant (Alsever's solution) 1:1; then, it was snap frozen by immersion in liquid nitrogen and kept at -80 ℃ until its analysis. Haemolymph was further diluted 1:1 with methanol before injection in the UPLC-QqLIT. Two replicates of water samples were taken from each tank at each sampling time and they were frozen at -20 ℃ until its analysis. Water samples were directly analysed using the method above indicated based on UPLC-QqLIT. Detailed explanation of sample preparation and UPLC conditions for the target analysis of SMX and its related metabolites in water, haemolymph and mussel's tissue are given as supporting information, as well as the performance of the method, detection limits and recoveries (table S1).

2.4 Enzymatic activities analysis

Six individuals per treatment were sampled after 96 h of exposure. Mussel's digestive gland and gills were weighted and immediately frozen by immersion in liquid nitrogen and kept at -80 ℃ until its analysis. Later on, mussel's digestive gland and gills of each organism were homogenized at a 1:5 (w:v) ratio in 100 mM phosphate buffer pH 7.4 containing 150 mM KCl, 1 mM ethylenediaminotetraacetic acid (EDTA) and 1mM dithiothreitol (DTT) in the case of digestive gland and 50 mM phosphate buffer pH 7.4

containing 1 mM EDTA in the case of gills using a polytron blender. Then, the extracts were centrifuged at 10,000 g for 30 min and the supernatant obtained was used for enzymatic activities analysis. The different assays related with oxidative stress were glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT) and lipid peroxidation (LPO); whereas enzymatic activities related with xenometabolism were glutathione-S-transferase (GSTs) and carboxylesterases (CbE) using different substrates, *p*-nitrophenyl acetate (pNPA), *p*-nitrophenyl butyrate (pNPB), α-naphthyl acetate (αNA) and α-naphthyl butyrate (αNB) were carried out in triplicate at 25 °C in a TECAN Infinite M200 microplate reader. The methodologies for enzymatic determinations, as well as total protein content can be found elsewhere (Dallarés et al., 2018; Solé et al., 2018).

2.5 Non-targeted metabolomics analysis and suspect screening of SMX related metabolites

Ten individuals were sampled for each treatment after 96 h of exposure for metabolomics and SMX related metabolites analysis. Haemolymph samples were pre-treated separately as explained for the bioaccumulation analysis (section 2.3). The workflow of the non-target metabolomics analysis and the suspect screening analysis for SMX related metabolites is shown in figure 1 and a detailed explanation of the methodology is also provided in supporting information. Briefly, the analysis of mussel's haemolymph samples was performed by High-Performance Liquid chromatography–High-Resolution Mass Spectrometry (HPLC-HRMS) using a LC-LTQ-OrbitrapVelos[TM] from Thermo Fisher Scientific, equipped with electrospray ionization (ESI) operating both in positive and negative mode. For the metabolomics approach, data files generated in the Orbitrap were processed using the Thermo Scientific SIEVE 2.0 software which does background subtraction, component

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detection, peak alignment and differential analysis, figure 1. After, a list of candidate structures was built up by searching the exact molecular weight in open source databases such as Human Metabolome database, Pubchem, Chemspider and METLIN. Regarding the suspect screening approach, used for the detection of SMX related metabolites; a list of suspected SMX related metabolites was built based on databases research and prediction tools for SMX degradation under biological processes (table S2, list of the SMX suspected metabolites). These SMX related metabolites were searched in the chromatogram generated in the LC-LTQ-Orbitrap, figure 1. Explanation of HPLC-HRMS conditions as well as the quality controls used is specified in detail in supporting information.

For confirmation purposes, a second injection in the LC-LTQ-Orbitrap using collision induced dissociation (CID) was performed, by using data-dependent analysis through the MS fragmentation of the significant metabolites previously identified based on their exact mass at 3 normalized collision energies (20, 30 and 35 eV).
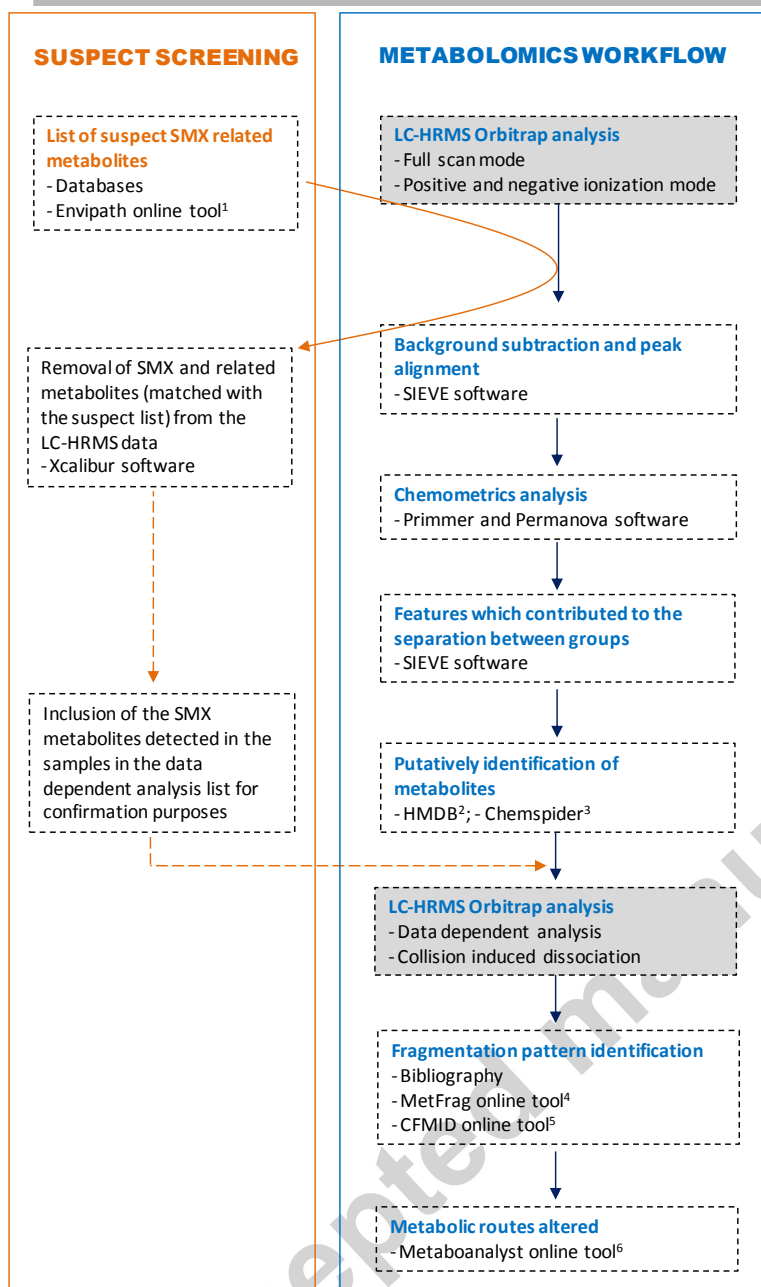
SUSPECT SCREENING

METABOLOMICS WORKFLOW

List of suspect SMX related metabolites
- Databases
- Envipath online tool[1]

LC-HRMS Orbitrap analysis
- Full scan mode
- Positive and negative ionization mode

Removal of SMX and related metabolites (matched with the suspect list) from the LC-HRMS data
- Xcalibur software

Background subtraction and peak alignment
- SIEVE software

Chemometrics analysis
- Primmer and Permanova software

Features which contributed to the separation between groups
- SIEVE software

Inclusion of the SMX metabolites detected in the samples in the data dependent analysis list for confirmation purposes

Putatively identification of metabolites
- HMDB[2]; - Chemspider[3]

LC-HRMS Orbitrap analysis
- Data dependent analysis
- Collision induced dissociation

Fragmentation pattern identification
- Bibliography
- MetFrag online tool[4]
- CFMID online tool[5]

Metabolic routes altered
- Metaboanalyst online tool[6]

Figure 1. Metabolomics and suspect screening of SMX related metabolites workflow. [1] https://envipath.org; [2] https://www.hmdb.ca; [3] http://chemspider.com; [4] https://msbi.ipb-halle.de/MetFragBeta; [5] http://cfmid.wishtartlab.com/predict; [6] http://www.metaboanalyst.ca.

2.6 Data analysis

The bioconcentration factors (in L/kg) were calculated using the following formula:

Bioconcentration factor (L/kg dw) = C_{biota}/C_{water},

where C_{biota} is the SMX concentration in mussels (µg/Kg dw), whereas C_{water} is the SMX concentration in water (µg/L). Bioconcentration factors were calculated for each sampling time during the exposure days.

One-way ANOVA test, followed by Tukey`s post hoc test were performed for the determination of significant differences in SMX bioconcentration in mussel's soft tissue and SMX accumulation in haemolymph along the exposure and depuration period. The normality and variances homogeneity of the data was tested before ANOVAs by using Shapiro-Wilk and Levene's test, respectively. For those compounds that data showed no normality or homogeneity, a Kruskal-Wallis test was performed followed by a Conover post hoc test. These statistical analyses were performed using R software (3.1.0) with a significance level of p-value < 0.05. Principal component analysis (PCA) was undertaken to identify the discriminatory variables for the enzymatic activities belonging to oxidative stress and mussel's xenometabolism, for gills and digestive gland. PCA was also used for the profiling of the metabolome by using all the detected features as variables (after excluding SMX and related metabolites when detected). All PCA were performed using PRIMMER and PERMANOVA software (6.0). Finally, significant differences in metabolites levels comparing control and SMX exposed mussels were determined with a t-test using SIEVE software with a significance level of p-value ≤ 0.05. The fold change and the standard error (SE) of the identified metabolites were calculated according to Motulsky (Motulsky, 1995).

3. Results

3.1 SMX and its related metabolites occurrence in seawater and mussels

3.1.2 SMX concentration in seawater

SMX concentration in water during the exposure and the depuration phase in both trials is shown in supporting information, figure S1. Sulfamethoxazole levels in both trials (summer and winter) were equivalent being the mean concentration during the exposure phase 8.8 ± 0.9 μg/L (mean of summer and winter trials). The daily addition of a 20 % of the initial SMX concentration, allowed a constant SMX concentration along the exposure phase. Sulfamethoxazole was not detected in water from the depuration tanks, with levels always below method detection limit (MDL) (figure S1), nor was detected in water from control tanks (data not shown).

3.1.2 SMX concentration in mussels

SMX was not detected with levels below MDL in any sample from control tanks (data not shown). Since no significant differences were observed between summer and winter experiments on SMX concentration accumulated in mussels for most of sampling times (figure S2), the mean SMX values of the two trials per sampling time was used for further discussion (figure 2). Sulfamethoxazole concentration in mussels increased during the exposure phase up to 13.2 ± 0.7 ng/g dw, after 96 h of exposure. After 6 h of depuration the concentration of SMX in mussels reached its highest value being 16.8 ± 1.1 ng/g dw, whereas at the end of the depuration phase (24 h of depuration) SMX concentration in mussels decreased to 4.1 ± 1.1 ng/g (69 % of elimination when comparing the concentration at the end of the exposure phase with the SMX concentration at the end of the depuration phase).
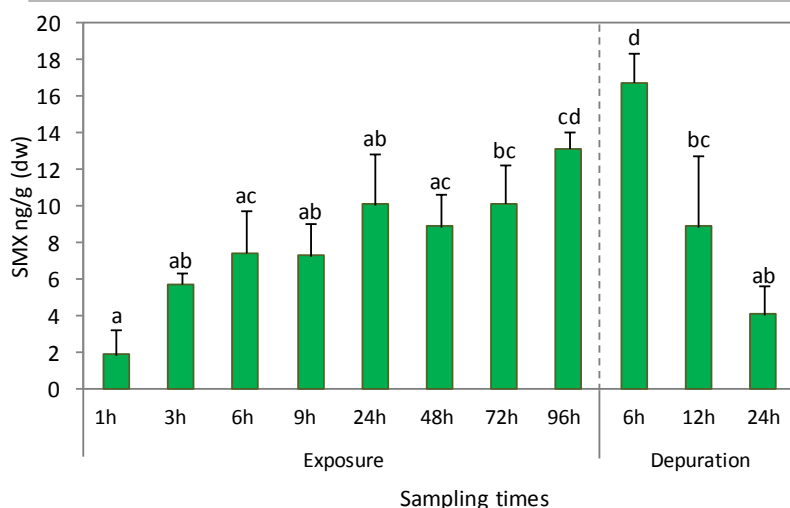
Figure 2. Sulfamethoxazole concentration in mussel's soft tissue from exposed tanks (ng/g, dw) along the exposure and depuration sampling times. Mean concentration of summer and winter trials are presented. Different lowercase letters represent significant differences between the experiment sampling times, significance p value < 0.05. ANOVA test.

The correlation between SMX bioconcentration factor (BCF) (mean of summer and winter trials) and exposure time is presented in figure 3. The highest BCF of 1.5 ± 0.1 L/Kg was achieved after 96 hours of exposure. Sulfamethoxazole uptake by mussels followed a logarithmic pattern with an $R^2 = 0.93$ (figure 3). A stabilization of the BCF was reached after 24 hours of exposure which suggests that the steady state (ss) was achieved after this period. Although a slight increase in the BCF was observed after 96 h of exposure it was not statistically different from the BCFss (ANOVA test p-value < 0.05).
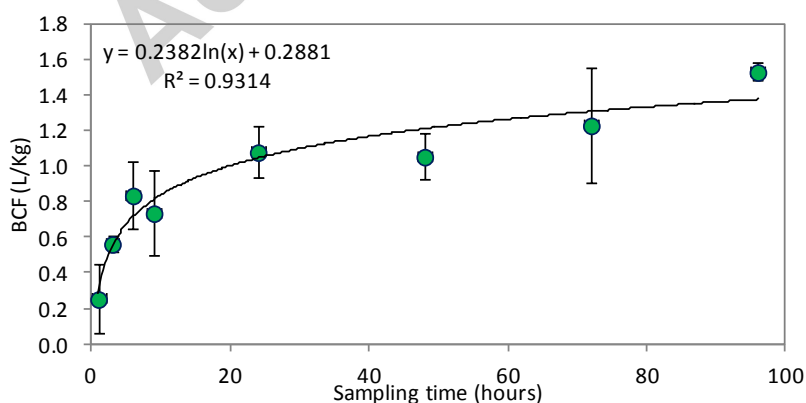

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Figure 3. SSulfamethoxazole bioconcetration factors (L/Kg dw) determined in mussel's soft tissue, mean BCF of summer and winter trials, along the experiment (exposure phase).

The concentration of SMX in mussel's haemolymph is shown in figure 4. Similarly to observations in mussel's soft tissue, no significant differences were seen between the two trials conducted along the exposure phase (figure S3). The highest concentration was found at 48 h of exposure, 3.39 ± 0.48 μg/L. Sulfamethoxazole was not detected in any mussel's haemolymph after 24 h of depuration.
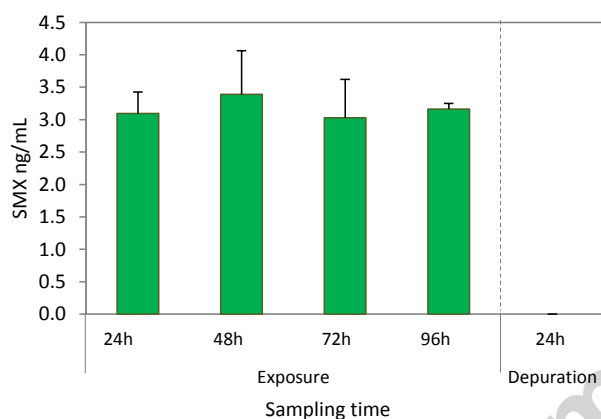

Figure 4. Sulfamethoxazole concentration in mussel's haemolymph (ng/mL) along the exposure and depuration sampling times. Average concentration of summer and winter trials are presented. No significant variations were observed between the experiment sampling times after ANOVA test significance p-value < 0.05.

3.1.3 Target and suspect screening of SMX related metabolites

An analysis of SMX related metabolites with available commercial analytical standards, N-acetyl sulfamethoxazole, desamino-sulfamethoxazole and glucuronide sulfamethoxazole, was targeted in mussel's soft tissue, haemolymph and in seawater of the tanks. None of the target metabolites was detected in any of the samples analysed. Furthermore, a suspect screening approach was performed to investigate the occurrence of other metabolites whose analytical standards are not available in mussel's haemolymph (table S2 shows the list of suspect SMX related metabolites). As in the

case of the target analysis none of the searched SMX metabolites was found in mussel's haemolymph.

3.2 Xenobiotic and oxidative stress related enzymatic activities

In order to assess the overall effects of SMX exposure on mussel's xenometabolism and oxidative stress, principal component analysis was undertaken considering all the parameters analyzed at the two trials (figure 5). Figure 5a and 5b show the xenobiotic and oxidative stress related activities measured in gills, whereas figure 5c and figure 5d present the xenobiotic and oxidative stress activities in digestive gland. None of the PCAs showed any clear separation of the groups due to SMX exposure, indicating no significant effects of SMX exposure on mussel's xenobiotic metabolism and oxidative stress. The individual comparisons between control and SMX exposed mussels for each enzymatic activity are shown in supporting information figure S4 and S5 and showed no significant changes for any of the enzymes studied due to SMX exposure in gills or digestive gland.
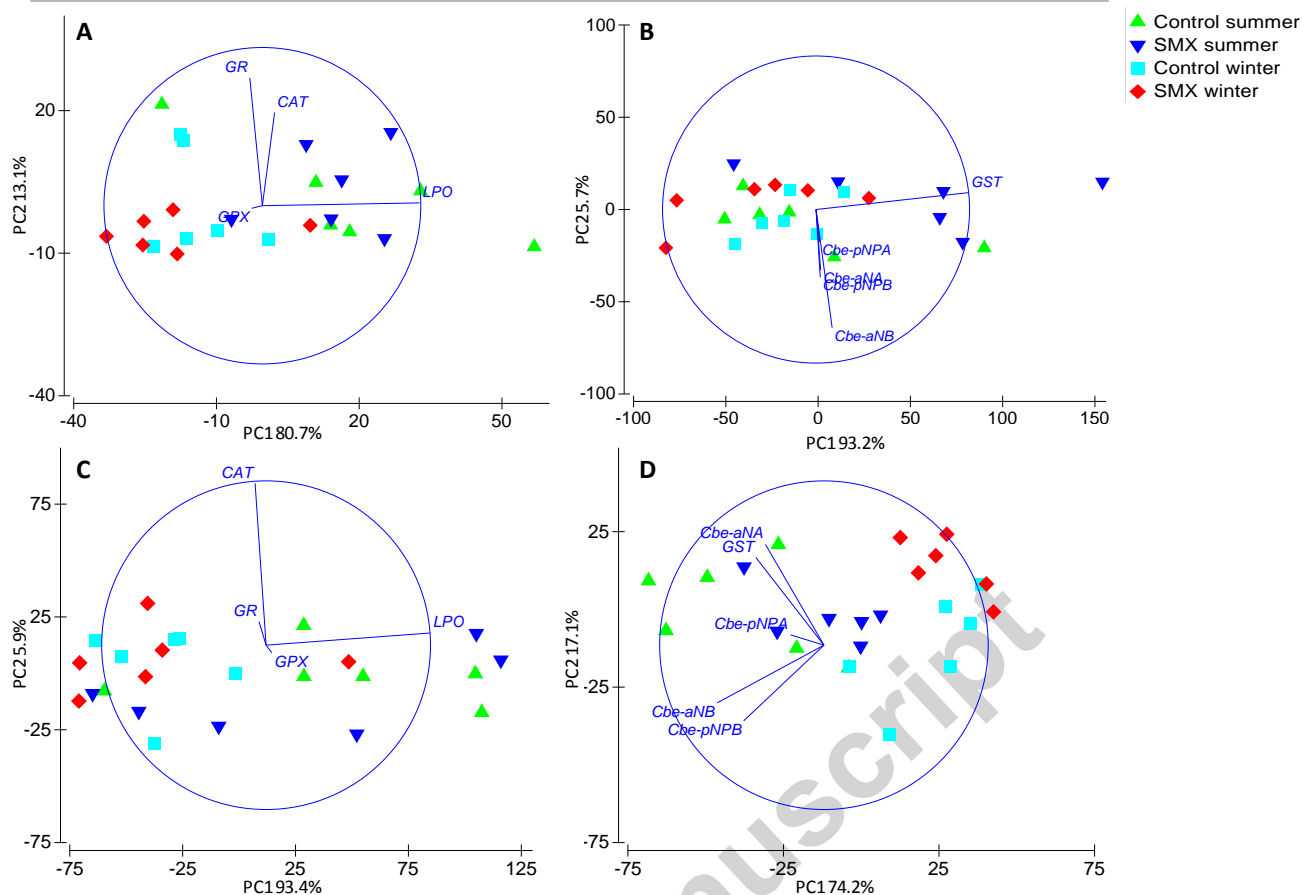
Figure 5. PCA scores plot for the enzymatic activities analyzed. Plots A and B correspond to mussel's gills: A are enzymatic activities related with oxidative stress (CAT, GR, GPX and LPO) and B enzymatic activities related with xenometabolism (GSTs and CbE with different substrates). Plots C and D correspond to mussel's digestive gland: C are enzymatic activities related with oxidative stress and D enzymatic activities related with xenometabolism. The percentages of explained variation for the first two components (PC1 and PC2) are displayed on the relative axes.

3.3 Metabolomics approach

3.3.1 Chemometric analysis

Liquid chromatography coupled to mass spectrometry (LC-LTQ-Orbitrap) analysis led to the detection of 1123 features in summer and 1324 in winter trials (579 features were common in both seasons) in positive and negative ionization modes. The dataset including all features detected in summer and winter (1868 features) was used for the PCA (figure S6). A clear separation was observed along the first axis (95 % of the variation) between those samples from summer compared to samples from winter;

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showing that mussels metabolome highly differed between the two studied seasons. In order to assess the putative effects of SMX on mussel's metabolome, samples from summer and winter trials were analyzed separately, figure 6 (6A summer, 6B winter). Samples from winter showed a separation between the control and SMX exposed treatment along the second axis (20 % of the total variability), figure 6B. However, no separation between control and SMX exposed samples was observed in mussels from summer, figure 6A.
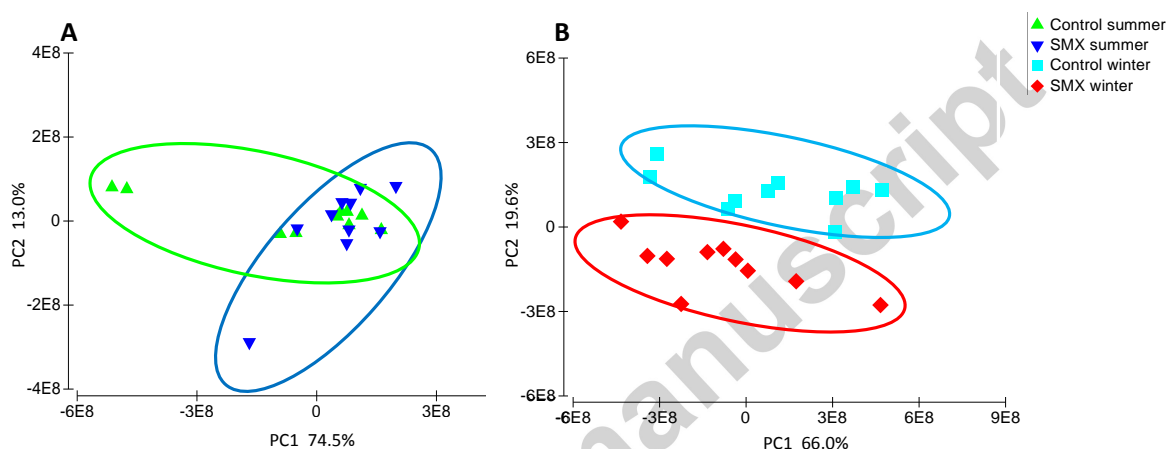

Figure 6. PCA score plots of the metabolomic profiles of mussels. Graphic A, summer trial, graphic B winter trial. Mussel's haemolymph were profiled by LC-LTQ-Orbitrap, with all features detected in +ESI and −ESI mode. The percentages of explained variation for the first two components (PC1 and PC2) are displayed on the relative axes.

In order to identify compound classes of interest, a Van Krevelen diagram was plotted out using the molecular features obtained from winter trial (figure 7) where a clear separation between the groups was observed in the PCA. The Van Krevelen diagram (plotting the ratio H/C versus O/C) regions have been associated to different classes of compounds (Alañón et al., 2015; Minor et al., 2014). In the present work, the majority of the features detected fell into the lipids and amino acids and peptides regions, followed by the condensed hydrocarbons and nucleic acid regions. Only few of them were placed into the amino sugars and carbohydrates regions (figure 7). When control and SMX exposed mussels were compared a higher amount of features in the SMX

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exposed treatment were observed in the amino acids and peptides region, but the abundance of lipids seemed to remain constant between the two treatments (figure 7).
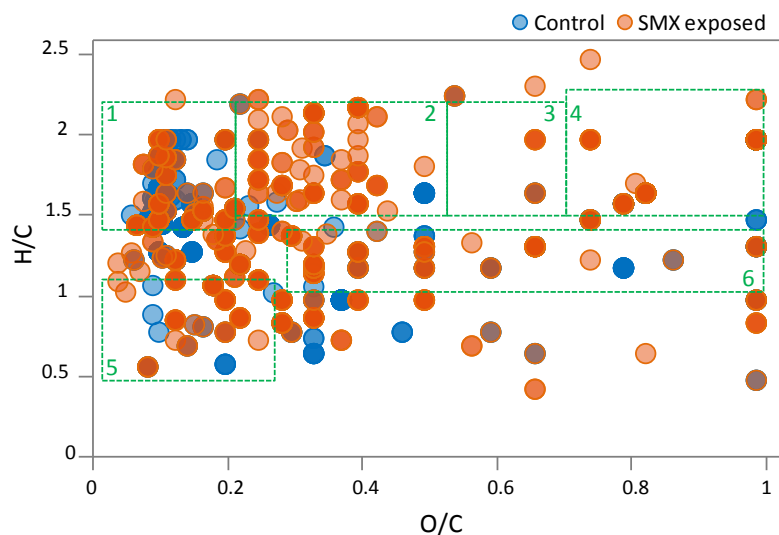

Figure 7. Van Krevelen diagram of molecular features from the metabolomics analysis in mussels from winter season. The rectangles represent the different compound class areas. 1, lipids; 2 amino acids and peptides; 3, amino sugars; 4, carbohydrates; 5, condensed hydrocarbons; 6, nucleic acids.

For the screening of features of interest regarding SMX exposure, the significance of each feature that contributed to the separation between the control and the SMX exposed treatment was evaluated by assessing their p-value ≤ 0.05 (T-test). This resulted in the detection of 378 significant features in winter trial, which were considered for further identification.

3.3.2 Metabolites identification

For identification purposes, the 378 significant features (between control and SMX exposed treatments) from winter trial, were searched in the databases (i. e. Human Metabolomics Database, Chemspider, etc.) on the basis of their molecular exact mass. Features whose exact mass differed less than 5 ppm from the molecular mass of suspect compounds were prioritized and a list of 26 putative metabolites was built up and

considered for confirmation. A second injection of the extracts was thus performed in the data-dependent acquisition mode at different collision energies and the main fragments obtained were also identified through their exact mass with an error below 5 ppm and used for confirmation of the 26 suspects. This approach for a tentative confirmation of metabolites identities is commonly used in metabolomics studies (Serra-Compte et al., 2018a; Villalobos et al., 2013). Seven metabolites were identified: aspartate, benzoate, phenylalanine, valine, guanosine, tryptophan and inosine (table S3 presents their exact mass, retention time, CID generated data and the identification of the main observed fragment ions). Compounds were detected in negative electrospray ionization mode (−ESI) as the [M-H]⁻ ion, or as the [M+H]⁺ in positive electrospray ionization mode (+ESI). The most common fragments observed were the [M-H-18]⁻ fragment, corresponding to the loss of a water molecule from the carboxyl group and the [M-H-44]⁻ fragment corresponding to the loss of a carbon dioxide molecule. These fragments have been previously reported for similar compounds (Serra-Compte et al., 2018a; Villalobos et al., 2013). Explanation of all fragments of the identified compounds is presented in supplementary information, table S3. After the identification of the metabolites they were also searched in samples from summer trial in order to evaluate if they exhibited any change (increase or decrease), and compare their response in the two seasons studied.

3.3.3 Endogenous metabolites altered due to SMX exposure

The metabolites identified in mussels and their significant change due to SMX exposure (increase or decrease) are presented in figure 8. The most altered group of compounds were amino acids (four out of the seven compounds identified), followed by nucleosides (two altered compounds) and one carboxylic acid (table S3). All the metabolites identified (except aspartate) increased their levels in winter trial due to SMX exposure.

Guanosine was the metabolite which presented the highest fold change (x4) within the markers identified. Among the seven metabolites identified in winter trial, aspartate and benzoate also changed significantly during summer trial. Therefore, they can be proposed as biomarkers of SMX exposure in marine mussels. Aspartate showed the same response in both trials (a decrease) whereas benzoate level decreased in summer and increased in winter.
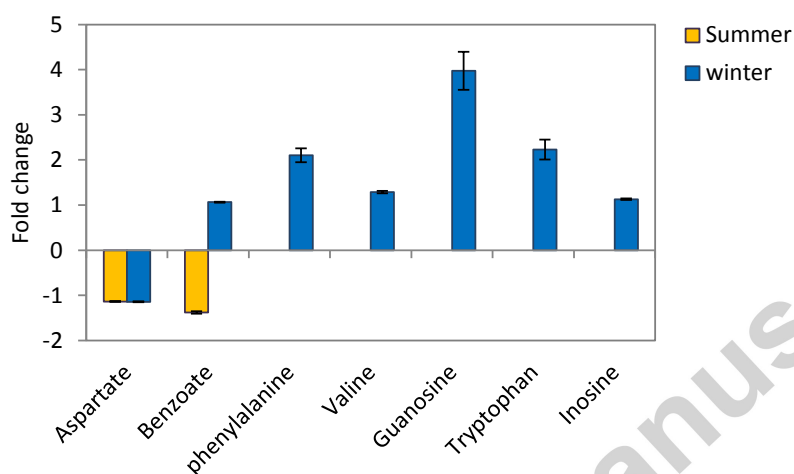

Figure 8. Apparent fold change for the metabolites identified with significant changes between control and SMX exposed mussels.

4. Discussion

4.1 SMX bioconcentration, depuration and risk assessment

Assessing contaminants bioaccumulation in marine organisms is the first step to evaluate their potential risk for the environment and also for human health when accumulating in foodstuff from animal origin. Contaminants accumulation capacity in marine organisms may be determined by both, contaminants physical-chemical properties and the characteristics of the exposed organism. The low bioconcentration capacity of SMX in mussel's soft tissue (up to 1.5 L/Kg, dw) may be related to its

physical-chemical properties; in particular, the low octanol-water partition coefficient LogKow 0.8 units for SMX (Log Dow -0.1 at pH 8). Similar BCFs (between 0 and 10 L/Kg) have been previously reported for SMX in mussels both laboratory and field studies (Klosterhaus et al., 2013; Serra-Compte et al., 2018b).

An increase in SMX concentration in mussel's tissue was observed after 6 h of depuration reaching up to 16.8 ± 1.1 ng/g dw (figure 2). Since SMX was not detected in the water from the depuration tanks, this increase in bioconcentration may suggest that mussels accumulated SMX from the contaminated seawater kept inside the valves while they were transported to the depuration plant. After 12 h of depuration, SMX concentration decreased, although the variability was high (figure 2). This may be attributed to biological differences between organisms facing anoxia and valve closure during transportation. After 24 h of depuration the concentration of SMX was significantly reduced in mussel's soft tissue (69 % of elimination) with a value of 4.1 ± 1.1 ng/g dw.

Despite SMX was not completely eliminated from the mussel's edible tissue after 24 h of depuration, it was completely eliminated from the haemolymph after the same period. This may be explained because ionizable compounds such as SMX, that are transported in the circulatory system, may be removed from the haemolymph stream and bioaccumulated in the organism, by entering inside the cells or by adsorption to the organism's tissues.

Apart from the accumulation of the parent compound in organisms, the presence of its related metabolites in biota is becoming a topic of great interest, as some antibiotic metabolites can be biologically active and it also provides information about the metabolization degree of the compounds. In this study, two different approaches were

used for the analysis of SMX related metabolites in mussels. A target analysis of some of the most common SMX metabolites described in humans, for which commercial analytical standards are available, and a suspect screening analysis based on databases research and theoretical prediction of SMX biotransformation products. None of the two approaches allowed for the detection of SMX related metabolites in mussels. This suggests that mussels might not be able to metabolize this compound at the tested conditions. Despite the fact that mussels are able to metabolize other organic compounds, including pharmaceuticals (Boillot et al., 2015; Dallarés et al., 2019), and that SMX is highly metabolized in humans (around 60 % is excreted as metabolites, mainly as N4-acetylated metabolite form), the set of enzymes responsible of the xenobiotic metabolism in mussel is more restricted than in humans, and to the best of the authors knowledge there is no evidence in the literature of SMX metabolization by bivalves. This suggests that metabolization of pharmaceuticals by mussels may be not only limited but also compound dependent. In line with this, in previous studies carried out with marine mussels exposed to pharmaceutical compounds, SMX and carbamazepine metabolites were not detected while venlafaxine was metabolized since metabolites were detected (Serra-Compte et al., 2018b), as well as, metabolites were detected when exposed to the antiviral Tamiflu (Dallarés et al., 2019).

Regarding the putative risk for consumers, the highest SMX concentration detected in mussels was 16.8 ± 1.1 ng/g dw after 6 h of depuration, or 3.05 ng/g ww, when converting to wet weight by applying a conversion factor of 5.5, commonly used for the conversion of shell-free dry weight to wet weight in bivalves (Ricciardi and Bourget, 1998). The SMX concentration measured in mussels was much lower than the MRL established at 100 ng/g ww by the authorities for sulfonamides residues in foodstuff from animal origin (European Commission, 2010). As this experiment was performed at

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SMX concentrations higher than the ones normally found in the marine environment, no risk for consumers is foreseen in commercially available marine mussels. Besides, a depuration period of 24 h under existent commercial practices, significantly reduced SMX levels in edible mussels even more. Nevertheless, as sulfonamides antibiotics may be ingested through other food sources apart from seafood, such as meat or eggs (Mehtabuddin et al., 2012), an aggregated assessment would be recommended. On the other hand, as even higher levels of SMX were measured after mussels transportation to the bivalve's distribution plant compared to the end of the exposure phase of the experiment, the analysis of mussels collected in the markets final destination (after its transportation) and not only at the aquaculture facility, is recommended to correctly assess the potential risk for shellfish consumers.

4.2 Metabolomics and enzymatic activities analysis

Two different approaches were used in the present work in order to characterize the ecotoxicological effects of SMX exposure to marine mussels, the analysis of enzymatic activities related to mussel's xenobiotic metabolism and oxidative stress, and a non-targeted metabolomics approach in order to detect changes in mussel's endogenous metabolites. None of the enzymatic activities possibly related to mussel's xenobiotic metabolism were significantly altered due to SMX exposure. Carboxylesterases are key enzymes involved in phase I metabolism of xenobiotics (Xu et al., 2016), whereas glutathione S-transferase is involved in phase II metabolism. Alteration of these activities in mussel due to an exposure of contaminants may be indicative of a detoxification mechanism for different contaminants including pesticides and pharmaceuticals (Dallarés et al., 2019; Solé and Sanchez-Hernandez, 2018). The no alteration of enzymatic activities related to xenometabolism activity in the present work

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supports the hypothesis of no metabolization of SMX by mussels at the tested conditions, as previously discussed in section 4.1.

Regarding oxidative stress related enzymatic activities, no changes were observed in mussels due to SMX exposure. In agreement to our findings, other authors found slight alterations in mussel's oxidative stress when they were exposed to antibiotics. As example, (Matozzo et al., 2016) observed slight alterations in mussels GSTs but not in LPO when mussels were exposed to high concentrations (100, 200 and 400 ug/L) of the antibiotic amoxicillin, after seven days of exposure. Considering other pharmaceutical compounds such as gemfibrozil and diclofenac (Schmidt et al., 2011) found alterations in mussel's enzymatic activities like GSTs when exposed at 1 µg/L and 100 µg/L during 24 h and 96 h. On the other hand, Oliveira et al., 2017, did not observe an oxidative stress induction, when Mediterranean mussels were exposed to carbamazepine at concentrations up to 9 µg/L, for 96 h (acute) and 28 days (chronic) exposures. These results pinpoint low induction of oxidative stress enzymes in mussels due to pharmaceuticals and especially to antibiotics after short term exposures.

The main ecotoxicological alterations in mussels due to SMX exposure were encountered through the metabolomics approach. Mussel's metabolome seems to respond differently to SMX pollution in a season-dependent manner, being more responsive in winter than in summer. These differences may be attributed to the different physiological status of the organisms, since a clear difference in mussel's metabolome was observed between organisms from summer and winter (figure S6). Furthermore, enzymatic activities also differed between the two studied seasons, especially those related with oxidative stress in gills and those related with oxidative stress and xenometabolism in digestive gland (figure 5a, 5c and 5d). This highlights the different physiological state of mussels between the two different seasons. Previous

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studies showed different sensitivity of mussels to pesticides (molluscicidals) depending on the season. This was related to an increased filtration rate in mussels during summer season (which may in turn increase contaminants accumulation) (Costa et al., 2008), or to mussels physiological status, mainly due to the spawning/resting cycle (Claudi and Mackie, 1994). In the present work, the bioaccumulation of SMX was similar in the two seasons. Therefore, the differences observed in the mussel's response to SMX exposure are most likely due to different physiological status of the organisms (mainly related to reproduction) rather than an increase in filtration rate. Higher sensitivity to pollutants after mussels spawning due to physiological fatigue has been previously reported (Claudi and Mackie, 1994; González-Fernández et al., 2016) and in the case of *Mytilus galloprovincialis* the spawning occurs mainly at the end of winter, January-February (Da Ros et al., 1985), when the winter trial was conducted in our study. Therefore, mussel's metabolome seems to be more affected due to SMX exposure in winter experiments compared to summer experiments, though some of the identified metabolites were also altered during summer (figure 8).

The Van Krevelen diagram showed that the highest differences between control and SMX exposed mussels in winter trial were in the amino acids and peptides region; in concordance with these results, four out of the seven compounds identified in this study were amino acids. Apart from them, two nucleotides and one carboxylic acid were identified. The identified compounds are involved in different metabolic pathways that may be altered due to SMX exposure. Using MetaboAnalyst 4.0, the metabolic pathways potentially affected based on the identified compounds are presented in figure 9. MetaboAnalyst asses a p-value of each altered pathway based on the number of metabolites identified belonging to the same pathway and the role of each metabolite in a specific pathway. Taking a threshold limit of $p < 0.05$, eight metabolic pathways were

significantly altered in mussels due to SMX exposure based on the metabolites identified (table S4). Aminoacyl-tRNA biosintyesis (phenylalanine, aspartate, tryptophan and valine are involved in this pathway), nitrogen metabolism (phenylalanine, tryptophan and aspartate are involved in this pathway) and pantathenate and CoA biosynthesis (valine and aspartate are involved in this pathway) were the most altered pathways in mussels due to SMX exposure. The rest of the altered metabolic pathways are involved in the biosynthesis of the identified metabolites, mainly amino acids, such as phenylalanine, tyrosine and tryptophan biosynthesis, phenylalanine metabolism and glycine, serine and threonine metabolism. Besides, alteration in purine metabolism pathway was also significant (figure 9).
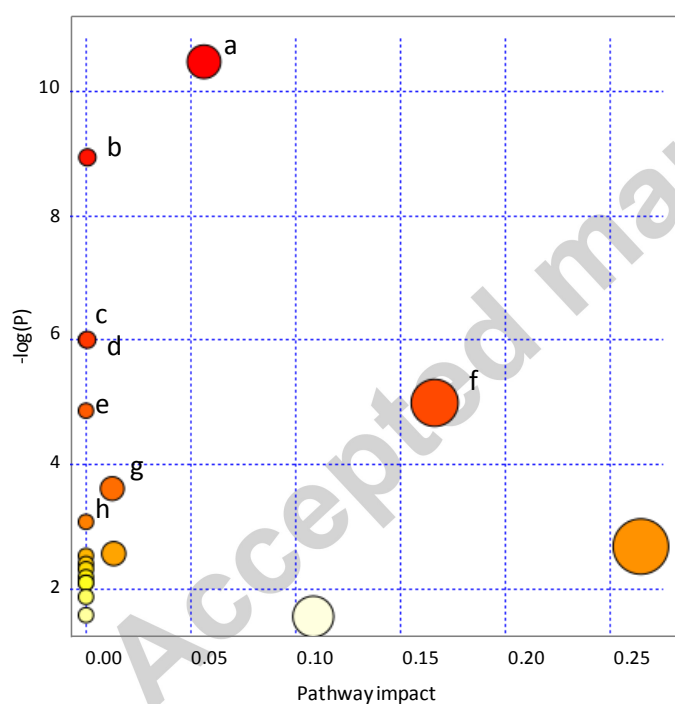

Figure 9. Metabolic pathways matched based on the metabolites identified, using MetaboAnalyst 4.0. The color and size of each circle was based on p-value and pathway impact value, respectively. Only metabolic pathways with a p-value < 0.05 are identified in the figure. (a) Aminoacyl-tRNA biosynthesis; (b) Nitrogen metabolism; (c) Pantothenate and CoA biosynthesis; (d) Phenylalanine, tyrosine and tryptophan biosynthesis; (e) Glycine, serine and threonine metabolism; (f) Phenylalanine metabolism; (g) Purine metabolism metabolism; (h) Cyanomino acid metabolism.

Amino acids have important roles in mussel's metabolism such as osmoregulation, energy metabolism and synthesis of proteins. Alteration in mussel's amino acids levels when exposed to different environmental contaminants has been previously observed (Bonnefille et al., 2017; Sanchís et al., 2018) and they seem to be one of the primary response of mussels to different stressors. Alterations in free amino acids levels in marine mussels have been related to an imbalance of osmoregulation. Kwon et al., 2012 and Liu et al., 2014 found an increase in free amino acids such as valine and phenylalanine in mussels due to heavy metals exposure and *Vibrio harveyi* induction respectively, in both cases it was related to alterations in osmoregulation. Besides, alterations in mussel's nitrogen metabolism has also been related to changes in osmotic regulation when mussels were exposed to stress conditions, such as different concentrations of water salinity (Livingstone et al., 1979). These previous findings support the hypothesis of a possible alteration in the osmotic regulation of mussels due to SMX exposure. Oxidation of amino acids to produce energy was observed in mussels exposed to stressors (*Vibrio harveyi* exposure), as well as, changes in other molecules involved in the energy metabolism such as ATP or glucose (Liu et al., 2014). In the present work, besides the changes found in amino acids, a significant alteration in pantothenate and CoA biosynthesis was detected, which may also suggest disturbances in mussel's energy metabolism. Finally, amino acids are the main constituents of more complex molecules such as proteins. Changes in amino acids levels may lead to changes in protein synthesis (through the aminoacyl-tRNA biosintyesis), and this may have further implications for the metabolism of the exposed organisms (Song et al., 2016).

In addition to the above mentioned metabolites, the metabolomics approach allowed the detection of two nucleotides altered due to SMX, inosine and guanosine. Changes in the nucleotide metabolism has been detected previously in mussels due to different

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occasions (i. e. wild and food limitation conditions), and nucleotides were pinpointed as a biomarker of health status in mussels (Roznere et al., 2014). We here observed that nucleotides may also be indicative of SMX pollution in mussels, but alterations were only observed under winter conditions.

Changes in mussel's metabolites profile can also be discussed from a gastronomic perspective, as changes in endogenous metabolites levels may influence the quality of mussels for its human consumption. Mussels are a highly valuable food source of proteins, lipids, and carbohydrates which have shown to be very beneficial for human health (Grienke et al., 2014). Besides, the concentration of omega-3 polyunsaturated fatty acids (ω-3 PUFAs) is well recognized as health beneficial and nutritional (Fuentes et al., 2009). In the present work, no changes in lipids concentrations were found; pinpointing that the nutritional characteristics of mussels were not affected by SMX exposure. However, as shown in the Van Krevelen diagram (figure 7) the amino acids and peptides region was highly affected by SMX exposure, and this was confirmed after compounds identification where some amino acids were altered due to SMX exposure. Alterations in amino acids levels may affect organoleptic aspects of mussels such as taste, odor, aroma and flavor (Fuentes et al., 2009). Furthermore, the amino acid aspartate, that decreased due to SMX exposure, is one of the most important taste-active compounds in mussels (Cha et al., 1998). These changes in the amino acids profile and especially aspartate, can influence in the mussel's characteristics as food product (Cha et al., 1998; Fuentes et al., 2009). Besides, alterations in amino acids levels may also influence the final protein content or the protein profile of mussels. It is well known that parameters such as seawater conditions, food availability and the gametogenesis cycle influence the meat characteristics of mussels (Fernández et al., 2015; Orban et al., 2002). Here it is suggested that the exposure of mussels to environmental contaminants,

such as antibiotics, may also affect the meat characteristics, influencing mussel's commercial quality and organoleptic properties. However, further research is needed in order to confirm this hypothesis and complemented with food sensory tests would be recommended.

5. Conclusions

Sulfamethoxazole showed low bioconcentration capacity in mussels exposed via water under laboratory controlled conditions. Twenty four hours of depuration under real commercial conditions allowed a significant reduction (69 %) of SMX concentration. No SMX related metabolites were detected, suggesting that mussels may not metabolize this compound at the tested conditions. In line with these findings, SMX exposure did not provoke any change in the enzymes related to xenometabolism nor in those related to oxidative stress. However, the metabolomics analysis did reveal alteration is mussel's metabolome mainly during winter conditions. Amino acids were the most altered group of compounds, which may be related to mussel's osmotic regulation and energy metabolism. The amino acid aspartate and the carboxylic acid benzoate were altered under both conditions and they can be postulated as biomarkers of SMX exposure. On the other hand, due to the low accumulation tendency observed for SMX in mussels, no risk for consumers is expected (according to the MRL established by European authorities). However, changes in mussel's organoleptic characteristics are suggested which may affect their commercial quality.

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Graphical abstract:

- A multidisciplinary approach was used to characterize mussels response to sulfamethoxazole
- No oxidative stress neither xenometabolism was observed
- Non-targeted metabolomics did reveal alterations mainly in amino acids levels
- Osmoregulation and energy metabolism were affected due to sulfamethoxazole exposure
- Mussels organoleptic properties may be altered but no risk to consumers is foreseen