1 **Exposure to Sub-inhibitory Sulfonamide Concentration Promotes the**
2 **Spread of Antibiotic Resistance in Marine Blue Mussels (*Mytilus***
3 ***edulis*)**

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Abstract

Although antibiotic resistance has become a significant and growing threat to public and environmental health, the occurrence and prevalence of this phenomenon in seafood has not been extensively explored. This study aims to evaluate the impact of sub-inhibitory antibiotic concentrations on the spread of antibiotic resistance in mussels. Marine blue mussels were exposed to sulfamethoxazole (SMX) at 100 µg/L; then, the presence of genes conferring resistance to sulfonamides (*sul1* and *sul2*) and the class 1 integron-integrase gene (*int11*), as well as bacterial community composition associated with the gastrointestinal tract were investigated. Results showed that all analyzed genes were present in mussels, even in those non-exposed to SMX. Moreover, an exposure to SMX caused a significant increase in the absolute copy number of *sul1* in mussels, although no significant changes were observed in *sul2* and *int11* genes. Because bacterial community composition was not affected after SMX exposure, the increase of *sul1* may be attributed to its spread within mussel's microbiome due to the pressure exerted by SMX pollution. Overall, our results showed the presence of antibiotic resistance genes (ARGs) in blue mussels, and highlighted the contribution of anthropogenic pollution to the spread of ARGs in aquatic organisms.

1. Introduction

Antibiotic resistance has been classified as one of the biggest global health threats of the 21st century¹. The main reason for the rapid increase of this phenomenon is the overuse and misuse of antibiotics². In fact, an increasing prevalence of antibiotic resistance has been found within a wide range of environments, such as wastewater treatment plants (WWTP) effluents, surface and groundwater, river and lake sediments, and coastal areas³⁻⁹.

The occurrence and prevalence of antibiotic resistance genes (ARGs) have been extensively reported in coastal environments¹⁰⁻¹²; however, the surrounding biota has received less attention. Genes conferring resistance to chloramphenicol and sulfonamides (*cmlA* and *sulI*, respectively) have been detected in farmed shrimps, showing that ARGs can occur in bacteria living within the seafood intestinal tract¹³. Among the organisms of a high interest in aquaculture, marine blue mussels are one of the most consumed seafood worldwide. Mussels for human consumption are produced in coastal areas, which may be chronically exposed to environmental pollutants. Antibiotics accumulation in marine mussels has been previously reported^{14,15}. For instance, sulfamethoxazole (SMX) antibiotic has been found in the marine environment¹⁵, and accumulated in commercial aquatic organisms, up to 245.91 ng/g in *Pangasius* from Thailand¹⁶. Moreover, authorities have established maximum residue limits (MRL) for some antibiotics in foodstuff from animal origin, which include marine blue mussels (i.e., 100 ng/g wet weight for, SMX)¹⁷. Therefore, the analysis of ARGs in highly consumed marine mussel species, as well as the putative contribution of antibiotic pollution to the spread of ARGs in mussels is of great concern in terms of seafood safety.

Given this, the main aim of this work was to evaluate the contribution of antibiotic contamination to the selection of ARGs in bacterial communities located in the mussel gastrointestinal tract. In order to address this objective, mussels were exposed to SMX antibiotic at 100 µg/L, a concentration lower than its minimum inhibitory concentrations (MIC), i.e. 1-16 µg/mL^{17,18}. After the exposure period, the effects of SMX pollution were evaluated on the abundance of genes conferring resistance to sulfonamides (*sul1* and *sul2*) and the class 1 integron-integrase gene (*intI1*), as well as on the bacterial community composition associated with the gastrointestinal tract.

2. Materials and methods

2.1 Experimental design

Blue mussels and seawater were obtained from a bivalve's supply plant (Mediterranean coast, Girona, Spain) and transported to the Catalan Institute for Water Research facilities. Mussels were randomly distributed in four tanks, 5 L each (two tanks per each treatment; namely control and exposure experiment) with a maximum density of one organism per liter of seawater. Animals were acclimatized for 5 days under the following conditions: temperature of 13.5 ± 0.2 °C, which was the same than the one registered in the Mediterranean Sea (Girona coast) during sampling time (November, 2017); pH of 8.0 ± 0.1 ; dissolved oxygen > 90 % DO/L; salinity of 43.5 ± 0.7 ‰ and photoperiod of 12 h light and 12 hours dark (12L:12D). Ammonia, nitrite and nitrate levels were evaluated in the water sampled from the tanks using ionic chromatography. Ammonia and nitrates were kept below 0.5 mg/L and 1 mg/L, respectively, during the whole experiment, acclimation and exposure; whereas nitrites were below quantification levels (0.004 mg/L) in all cases. After the acclimatization period, mussels from the exposure treatment were exposed to sulfamethoxazole (SMX) via water at a nominal

concentration of 100 ng/mL. The exposure period lasted 96 h, which has been shown to be enough to reach the steady state in marine mussels¹⁸. The mussels from the control treatment were held in the same conditions but without addition of SMX, only an equivalent amount of solvent (methanol) being added to control water. The amount of solvent added to the tanks was below 0.05 % of the total water volume. During the entire experimental period, seawater was renewed daily at 80 % of the total volume, and the corresponding amount of SMX (and methanol in the control tanks) was added after each water renewal. Low mortality was observed (below 1 %) only during the acclimatization period. During the whole experiment, mussels were fed two times daily with a commercial algal mix suitable for bivalves (Acuinuga, Spain). The amount of algal mix added to each tank corresponded to 1 % of the estimated wet weight of mussels placed in a tank. In order to evaluate the effects of the SMX exposure, organisms were only sampled after the exposure period (96 h). Six individuals from control tanks and six from exposure tanks were sampled ($n = 6$), the gastrointestinal tracts were collected and kept at -20 °C until DNA extraction. From the same individuals, haemolymph was extracted, immediately frozen by immersion in liquid nitrogen and kept at -70 °C until SMX analysis. Finally, water samples ($n = 4$, per treatment) were collected along the exposure period (0, 24, 72 and 96 h) and kept at -20 °C until SMX analysis.

2.2 DNA extraction and genes quantification

Mussel gastrointestinal tracts were weighted. DNA was then extracted from each individual separately using a commercial kit (DNeasy Power Soil Kit, QIAGEN) and quantified using NanoDrop™ 2000 (Thermo Scientific, Wilmington, DE, USA). The abundance of 16S rRNA, *int11*, *sul1* and *sul2* genes was quantified using real-time PCR

(qPCR) assays following the conditions previously described¹⁹. Previous studies have demonstrated that *sul1* and *sul2* are the most abundant sulfonamide resistance genes in different environments, including marine areas²⁰; therefore, they were selected for being analyzed in this work. The copy numbers of ARGs were normalized to grams (g) of sample (wet weight) in order to obtain the absolute copy number. Experimental details regarding genes quantification are described in the supporting information.

2.3 Sequence analysis and phylogenetic classification

Genomic DNA samples were submitted to BMR Genomics (Padua, Italy) for 16S rRNA gene high-throughput sequencing, whose hypervariable (V3-V4) regions were amplified using universal primers²¹ and sequenced on an Illumina MiSeq instrument (Illumina, San Diego, USA) using 2×300 bp paired-end reads. Sequences were then quality trimmed using the MOTHUR software package²², and aligned using the SILVA reference database²³. Subsequently, sequence libraries were randomly subsampled to contain the same number of sequences (8,008) for α - and β -diversity analyses. The Shannon diversity index (H') and the Chao1 richness estimator were calculated for α -diversity comparisons, whereas the Yue & Clayton index, which measure community structure (number of shared genera and their relative abundances), were calculated for β -diversity comparisons. Analysis of molecular variance (AMOVA) was used to test statistically significant differences between the bacterial communities based on the Yue & Clayton index. Sequences were assigned to operational taxonomic units (OTUs) based on a 97% sequence similarity. The Ribosomal Database Project (RDP) pipeline and Classifier function were used to assign identities at a confidence threshold of 80%²⁴.

2.4 SMX analysis in seawater and mussel's haemolymph

SMX concentration in seawater and haemolymph (diluted 1:1 with methanol) was analyzed by ultra-high performance liquid chromatography coupled to quadrupole linear ion trap tandem mass spectrometry (UPLC–QqLIT), using an adapted method previously described²⁵. Experimental details regarding UPLC-QqLIT parameters are described in the supporting information.

2.5 Data analysis

A *t*-test was used to compare the ARG concentrations between exposed and non-exposed (control) mussels to SMX, whose differences were considered significant at $p < 0.05$. All statistical analyses were performed using R software (3.1.0).

3. Results and Discussion

3.1 SMX occurrence in seawater and accumulation in mussel's haemolymph

SMX was not detected in any control seawater sample or haemolymph samples from control mussels (data not shown). SMX concentration in seawater from SMX exposure treatments had similar values throughout the exposure phase (figure S1); its concentration at 96 h of exposure was $125.5 \pm 9.2 \mu\text{g/L}$, whereas the concentration in mussel's haemolymph was $81.3 \pm 18.2 \mu\text{g/L}$, after the same exposure time.

3.2 Quantification of ARGs

ARGs (*sul1* and *sul2*), *intI1* and the 16S rRNA genes were quantified by qPCR in the gastrointestinal tract of marine mussels. High R^2 values > 0.992 and high efficiencies $> 90\%$ (except *sul2* which was 80%), were obtained, indicating the validity of these quantifications. The total copy numbers of the 16S rRNA gene was consistent in all samples (no differences were observed between control and SMX exposed mussels, $p>0.05$). The concentrations of *intI1*, *sul1* and *sul2* genes normalized to grams of sample (mussel gastrointestinal tract) are shown in Figure 1, whereas the concentrations normalized to 16S rRNA copies and ng of DNA are shown in supporting information (Figure S2 and S3, respectively). Similar results were obtained for all analyzed genes using the three different normalizations (Figure 1, S2 and S3).

Figure 1. Absolute concentration of ARGs in gastrointestinal tract samples from mussels exposed and non-exposed (control) to SMX. Asterisk (*) indicates significant differences ($p<0.05$).

3.3 Abundance of *sul1*, *sul2* and *intI1* genes in SMX non-exposed marine mussels

The analysis of ARGs in control mussels revealed the occurrence of *sul1*, *sul2* and *intI1* genes in these organisms, despite the absence of antibiotic pressure in water. The *intI1* gene presented the highest concentration with a mean value of 6.8×10^9 copies / g, followed by *sul1*, 5.5×10^5 copies / g and *sul2*, 1.1×10^5 copies / g (Figure 1, Table S1). These values are in the same range than the ARGs concentrations (including *sul1* and *sul2* among others) found in the intestinal tract of aquaculture shrimps, ranging from 1.26×10^4 to 1.74×10^7 copies/g¹³. The presence of ARGs in control mussels (non-exposed to SMX) demonstrates that mussels can act as reservoirs of ARGs (e.g. *sul1*, *sul2* and *intI1* genes); to the best of the authors' knowledge, this is the first time that *sul1*, *sul2* and *intI1* genes have been detected in marine blue mussels. The presence of *intI1* gene has already been reported in bacteria isolated from other aquatic organisms, such as fish (rainbow trout) in Australia²⁶. Besides, the *sul1* and *sul2* genes have also been detected in aquatic organisms such as fish^{27,28} and shrimps¹³. Our results confirm *sul1* as a predominant sulfonamide resistance gene in mussels when compared to *sul2*. Moreover, the occurrence of *sul1*, *sul2* and *intI1* genes in marine blue mussels highlighted the importance of monitoring ARG in these organisms in order to evaluate the potential risks not only for the environment but also for humans after the ingestion of organisms carrying these ARGs. The ingested bacteria may occasionally survive the human gastric barrier²⁹, and then enter in contact with the human microbiota. In fact, human intestinal microbiome has been postulated as a reservoir of ARGs³⁰. This resistance can then be transferred through horizontal gene transfer (HGT) to other endogenous human bacteria, including potential human pathogens³¹.

3.4 Impact of sulfamethoxazole on the abundance of ARGs and bacterial community composition

The addition of SMX in the water where mussels grow led to an increase of *intI1* gene about one order of magnitude (from 6.8×10^9 copies / g in control mussels to 6.0×10^{10} copies / g in SMX-exposed mussels; Figure 1). However, this increase was not statistically significant ($p > 0.05$), probably due to the high biological variability between organisms. On the other hand, SMX exposure caused a significant increase ($p < 0.05$) in the absolute copy number of *sul1* in exposed mussels when compared to those non-

Group	No. of reads	Total no. of OTUs	Shannon diversity index	Chao1 richness estimator	exposed to SMX (from

5.5×10^5 copies / g in control mussels to 3.5×10^6 copies / g in SMX-exposed mussels) (Figure 1, Table S1). Regarding the *sul2* gene, it did not show significant changes ($p > 0.05$) when mussels were exposed to SMX compared with control ones (Figure 1): 1.1×10^5 and 5.4×10^4 copies / g were detected in control and SMX-exposed mussels respectively (Figure 1, Table S1).

The bacterial community composition was investigated in control and SMX-exposed mussels. Figure 2 shows the most abundant phyla and genera observed in both, control and SMX-exposed communities. Shannon diversity and Chao richness indices were calculated for both treatments, the values being presented in Table 1.

Table 1. Measures of α diversity in gastrointestinal tract samples from mussels exposed and non-exposed (control) to SMX. Average values $\pm$ standard deviation are shown ($n = 6$). OTUs were defined at a threshold of 97 % similarity.

Control	8,008	226±27	2.64±0.09	589±104 ²³³
Exposed	8,008	233±27	2.49±0.26	689±175 ²³⁴

No significant differences ($p>0.05$) were observed in bacterial diversity and richness between control and SMX-exposed mussels. Moreover, the AMOVA test (as implemented by MOTHUR) did not show significant differences ($p>0.05$) in bacterial community structure of both treatments. These results confirm that a SMX exposure under its MIC did not cause alterations in the microbiota associated with the mussel gastrointestinal tract. However, despite no alterations were seen in the bacterial community composition, an increase in *sulI* abundance was observed, as indicated above. This increase could be attributed to gene mobilization through HGT. In fact, previous studies have shown that the exposure of antibiotics at sub-lethal concentrations can promote HGT³². Moreover, the increase (despite not statistically significant) observed in the absolute copy number of *intI1* in SMX-exposed mussels may support gene mobilization through HGT. The *intI1* is a gene capture platform, where *sulI* has reported to be linked with. The *intI1* gene is also located in mobile genetic elements (MGE), which can be mobilized under anthropogenic pollution with pesticides, heavy metals, pharmaceuticals and/or personal care products, among others³³. In contrast, *sul2*, not reported to be located in MGE, did not change after SMX exposure. Therefore, ARGs location on MGE is shown to be a key factor influencing their spread in different environments³⁴ including the bacteria living within the mussel gastrointestinal tract.

Figure 2. Relative abundance of major bacterial genera found in gastrointestinal tract samples from mussels exposed and non-exposed (control) to SMX. Average values for control and exposed communities are shown ($n = 6$). Only values higher than 0.5 % are shown.

Our results demonstrated the presence of sulfonamide resistance genes, *sul1* and *sul2*, in marine blue mussels. The occurrence of all investigated resistance genes in control mussels (without antibiotic occurrence in water) highlighted that mussels can act as reservoirs of ARGs in the marine environment. Furthermore, SMX exposure caused a significant increase in the absolute copy number of *sul1* in marine mussel's gastrointestinal tract, suggesting that the presence of antibiotics in the marine environment where mussels grow is a key factor influencing the abundance of ARGs in these organisms. These results may have further implications regarding the spread of ARGs, as an exposure to a single antibiotic can promote the development and spread of unrelated ARGs via plasmids and transposons³⁵. Given this, monitoring of antibiotics in the aquatic environment and the implementation of strategies to reduce their amount,

especially in seafood producing areas, becomes crucial to mitigate the occurrence and prevalence of ARGs and the associated risks for human and animal health.

Supporting information

The supporting information is available free of charge on the ACS Publications website. Additional information about DNA extraction and SMX analysis in seawater and mussels haemolymph. Results obtained in SMX quantification in seawater (Figure S1) and quantification of ARGs (Figure S2, S3 and Table S1).

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