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Accumulation of antibiotics and antibiotic resistance genes in freshwater crayfish – Effects of antibiotics as a pollutant

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1. Introduction

The extensive use of antibiotics and the inefficient elimination through wastewater treatment plants (WWTPs) lead to the pharmaceuticals presence in the environment [1]. This release of antibiotics into the environment has and will cause further increase in bacteria which are resistant to antibiotics [2]. Hughes et al. [3] compiled data from 41 countries regarding the occurrence of 203 different pharmaceuticals in freshwater environments. Globally, antibiotics were the second most frequent detected pharmaceuticals in freshwaters followed by painkillers. Comparatively very high concentrations of these antibiotics were observed in Asian countries and in another global study pharmaceuticals were found in the environment in 71 countries and notable was that sixteen substances were found in drinking, ground and surface waters in all tested regions worldwide [2]. Also, in Sweden antibiotics and other pharmaceuticals were frequently detected in the three largest lakes and the river systems associated with these lakes [4].

Sulfonamides such as for example Sulfamethoxazole (SMX) are among the most commonly used antibiotics for urinary tract infections around the world, and thus SMX is one of the antibiotics found in lakes and streams. In the compilation of data from 41 countries mentioned above, SMX had a median concentration of 83ng/L and the max concentration recorded was 11 920 ng/L [3]. Sörengård et al. [5] also demonstrated that SMX is inadequately removed from water in the WWTPs (removal was only 8%) and the remaining concentration of SMX is released with the effluent water. Fluoroquinolones belong to another group of common antibiotics used for urinary tract infections, as well as other infections, and among the most common is Ciprofloxacin (CIP). This antibiotic is also frequently found in water, and for example, the median concentration for CIP found by Hughes et al. (2013) was 163 673.5 ng/L and the maximum concentration was 6 500 000 ng/L.

The presence of anthropogenic antibiotics in lakes and watercourses poses a risk for an increased presence of antibiotic resistance genes (ARGs) in the environment. Even very low levels of antibiotics in water could lead to a high incidence of ARGs. Muziasari et al. [6] for example, showed that the gut contents of four different fish species caught in fish farms in the Norden Baltic Sea contained several different ARGs, despite the fact that no antibiotics were used in the fish farms around this area. The presence of ARGs in lakes and watercourses has been demonstrated, and in a recently published study the presence of 296 different ARGs in areas around four cities in Sweden was investigated using high-throughput quantitative PCR arrays [7]. The authors investigated the occurrence in watercourses upstream and downstream of WWTPs and found that the concentrations of ARGs were generally greater downstream of the WWTPs. The highest levels of ARGs were found for resistance to β-lactams and sulfonamides [7]. A similar study in ten different European countries showed an enrichment of ARGs after WWTPs, and among the most common resistance genes detected was *Sul1* giving resistance towards sulfonamides, such as SMX [8].

Not only antibiotics used for treatment of human diseases are a source of increased levels of ARGs in aquatic environments. A large source in many countries comes from the use of antibiotics in animal husbandry and not least in aquaculture, especially in Asia [9]. Crustacean aquaculture is of enormous economic importance in China and South East Asia, and the routine use of antibiotics in intensive shrimp, crab and crayfish farms poses a major risk for the spread of ARGs [9,10]. Thus, feeding antibiotics to the oriental river prawn, *Macrobrachium nipponense* increased the concentration of ARGs in the intestinal tract of the prawn when compared to feeding with antibiotic free food [10].

We have previously shown that the microflora in the gut of crustaceans varies a lot, and especially depending on the feed the animals are given [11]. The large individual variation means that it can be difficult to draw correct conclusions about how the microflora of crustaceans affects the surrounding water environment. In this study, the goal was to investigate what effects environmentally relevant concentrations of the two common antibiotics (SMX and CIP) can have for the presence of antibiotic-resistant bacteria in the gut flora of the freshwater crayfish *Pacifastacus leniusculus*, and more specifically on the possible accumulation of antibiotics and resistance genes in this crayfish after a relatively short exposure to these antibiotics. To circumvent the large individual

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variation, and instead of isolating gut contents, we have in this study collected faeces from individual crayfish before and after exposure to a mixture of SMX and CIP. The reason for using this mixture is because of their presence in Swedish watercourses [4].

2. Material and methods

2.1. Antibiotics

By dissolving 1 mg Sulfamethoxazole (SMX) (Sigma-Aldrich, CAS: 723-46-6) in 1 mL absolute ethanol a freshly made stock solution was prepared. Also, one mg Ciprofloxacin (CIP)(Sigma- Aldrich, CAS: 85721-33-1) was dissolved in 1 mL 0.01 M HCl to get a stock solution of CIP. Afterwards test solutions were prepared from the stock solutions so that all test solutions had a final concentration of 100 ng/L in the crayfish tanks. The concentration for the test solutions are comparable to concentrations found in freshwater systems in the environment [2–4].

2.2. Animals and exposure to antibiotics

The freshwater crayfish, *Pacifastacus leniusculus* used in this study were originally taken from lake Erken in Sweden and kept in aerated aquaria with oxygenated running tap water at 10–13 °C. Twelve male intermolt crayfish were kept individually separated by plexiglass plates in aerated aquaria with 2 L water, in total six aquaria, i.e. two crayfish separated from each other in each aquaria. Each individual had their own tube shelter. Before starting the 21 days exposure to antibiotics the crayfish were acclimatized for six days. Each aquarium contained 2 L of tap water that was changed two times a week. Six crayfish were exposed to the mixture of antibiotics, and six crayfish were used as controls. Freshly made antibiotic solutions, SMX and CIP were added to the exposure aquarium at the start of the experiment and each time afterwards the water was changed so that the final concentration of each antibiotic was 100 ng/L. Once a week all crayfish were fed 0.2 g potatoes each. The photoperiod was kept at 12 h light/12 h dark. In a second experiment twelve new male intermolt crayfish were kept in six smaller aerated aquaria, two crayfish in each aquarium with a metal net in the middle separating the animals and containing one tube shelter for each individual and with 2 L of water in each aquarium. The exposure was done as above, with SMX + CIP mixture in three experimental aquaria (six crayfish), and three control aquaria (six crayfish).

2.3. Faeces collection, handling, DNA extraction and determination of antibiotic resistance genes (ARGs)

Solid faeces at the bottom of each tank were collected from each individual three days after feeding. First at the beginning of the experiment and then three weeks after SMX + CIP exposure or control exposure, at the end of the experiment. In a second similar experiment faeces were collected at the start of the experiment and then one, two and three weeks after exposure to SMX + CIP or no exposure (control group). All faeces samples were concentrated by centrifugation at 10000×*g* for 15 min at +4 °C, and then the wet weight was determined. Fifty (50) mg of each faeces sample was used for DNA extraction using the QIAamp Fast DNA Stool Mini Kit (Qiagen) according to the instructions by the supplier. Then semi-quantitative PCR was performed using Phusion Plus DNA Polymerase (Thermo Scientific) in a 20 μL reaction mixture containing; 4 mL 5 x Phusion Plus buffer, 1 mL forward primer, 1 mL reverse primer, 0.4 mL 10 mM dNTPs, 2 mL template DNA, 0.2 mL Phusion Plus DNA Polymerase, 11.4 mL H_2O. Then PCR amplification was performed as follows: 98 °C for 30s, 35–40 cycles of 10 s at 98 °C, 10 s at 60 °C and 20 s at 72 °C, followed by a final extension for 5 min at 72 °C and hold at 4 °C. The following primers were used; for the bacterial 16S rRNA gene, forward: 5′AGAGTTTGATCCTGGCTCAG-3′, reverse: 5′GGTTACCTTGTTACGACTT-3’; *Sul1* forward: 5′CTTCGATGAGAGCCGGCGGC-3′, *Sul1* reverse: 5′GCAAGGCGGAAACCCGCGCC-3’; *Qnr1* forward: 5′GGCCATGGATATTATTGATAA-3′, *Qnr1* reverse: 5′GGATCCGGGCAGCACTATTACTCC-3’. The PCR products were analyzed by 1% agarose gel electrophoresis, stained by SYBR Safe DNA Gel Stain. Image analysis was done using Fuji LAS4000 luminescent analyzer, and ImageJ [12] was used to semi-quantify the amount of DNA in the samples.

2.4. Bacteria culture

To be able to quantify the bacteria in the faeces samples, the amount of colony forming units (CFUs) were counted after plating the different samples on Luria-Bertani broth (LB) agar plates in serial dilutions. We collected faeces from each crayfish individual after one, two and three weeks. First the faeces samples were dissolved in 0.15 M NaCl and then diluted so the concentration was 10 mg faeces/mL. Then the faeces samples were incubated in serial dilutions on LB agar plates and incubated at 37 °C overnight. The concentration of bacteria in the faeces samples was determined by counting the number of CFU, and is displayed in Table S1. Two outliers were identified with GraphPad: ROUT Q = 0.1%. Outliers are marked in Table S1.

The number of CFUs per mg faeces were then calculated. Similar tests were performed using TCBS agar and Marine agar (Difco).

2.5. ELISA determination of SMX in tissues

In a third exposure experiment, with only SMX eight crayfish were kept individually in aerated aquaria of 2 L for each crayfish. The exposure was done, with SMX 100 ng/L in four experimental aquaria (four crayfish), and four control aquaria (four crayfish). Three weeks after exposure to antibiotics the tail muscles were dissected from four exposed and four control crayfish. The muscle tissues were kept at −80 °C until determination of SMX in the tissues. The concentration of SMX in the tissues were determined using the MaxSignal® Sulfamethoxazole ELISA Kit (PerkinElmer) according to the instructions by the manufacturer. Briefly, after thawing the tissues they were homogenized thoroughly until a paste-like consistency was achieved. Then, 3 mL of ethyl acetate was added to 1.5 g of homogenized tissue and the mixture was vortexed vigorously for 3 min, followed by centrifugation for 5 min at 40000×*g* at room temperature. The supernatants were then transferred to new tubes and dried with a nitrogen evaporator at 40 °C to complete dryness. Two mL of hexane and 1 mL of sample extraction buffer E (kit content) was then added to the samples and after 1 min vortexing the samples were centrifuged for 10 min at 4000×*g* at room temperature. Fifty μL of the lower aqueous phase was then used for the assay in the ELISA 96 well plates provided in the kit and the concentration was calculated by the use of standard solutions provided.

2.6. Statistical analysis

Statistical analysis for the number of CFUs were performed with RStudio version 4.1.2 (R Core Team 2021) and for all other analysis with GraphPad Prism 9.4.1 software. A significant difference was considered true if $p < 0.05$. Statistical analysis of the relative CFU compared to CFU at week 0, the amount of antibiotic resistance genes *Sul1* and the count of total (THC) and differential hemocytes (DHC) was done by parametric paired *t*-test. All results were demonstrated as mean ± standard deviation.

3. Results and discussion

To investigate if the antibiotics SMX and CIP influenced the amounts of bacteria in the gut of *P. leniusculus*, six of the crayfish were kept in tap water containing SMX and CIP together at concentrations often found in the environment (100 ng/L). Six other crayfish were kept as control and were not exposed to the antibiotics. Since our previous study showed that there is a very large individual variation in the intestinal microbial

content in freshwater crayfish [11], we decided to investigate the effect of exposure of antibiotics in each crayfish individually. The only way we could obtain information about how antibiotic exposure affects the intestinal microbial content on an individual basis, was to isolate faeces samples before and after the treatment from the same individual animal. This is also in accordance with the results obtained by Martin et al. [13], who showed that the microbiota of crustacean intestines mainly is localized to the lumen of the gut, inside of the peritrophic membrane. After collecting faeces from each crayfish individually after one, two and three weeks, the results showed the presence of bacteria in all faeces samples, but there was a large variation, as can be seen in Fig. 1. After two and three weeks the bacteria in the group exposed to SMX + CIP were higher compared to the control group, but due to the high individual variation no significant difference in increase of bacterial numbers between exposed and non-exposed crayfish could be observed (Fig. 1). In a previous study we have found that the number of bacteria belonging to *Vibrio* spp. increases after exposure of freshwater crayfish to SMX [11]. Therefore, we also cultured the faeces suspensions on specific *Vibrio* medium, such as TCBS agar, or Marine agar (Difco), but we could not find any colonies on TCBS agar, and the number of CFUs growing on Marine agar were few and did not differ significantly between control and SMX + CIP exposed crayfish (data not shown).

No statistical difference in increase of CFU relative to number of CFU at week zero (that is control values) was found between antibiotic exposed and control group on LB medium or Marine agar. However, there was a large variation, but there is a trend for higher numbers in SMX + CIP exposed group after two and three weeks. Higher numbers of CFU in exposed group could suggest that we have bacteria that have developed resistance towards SMX or CIP.

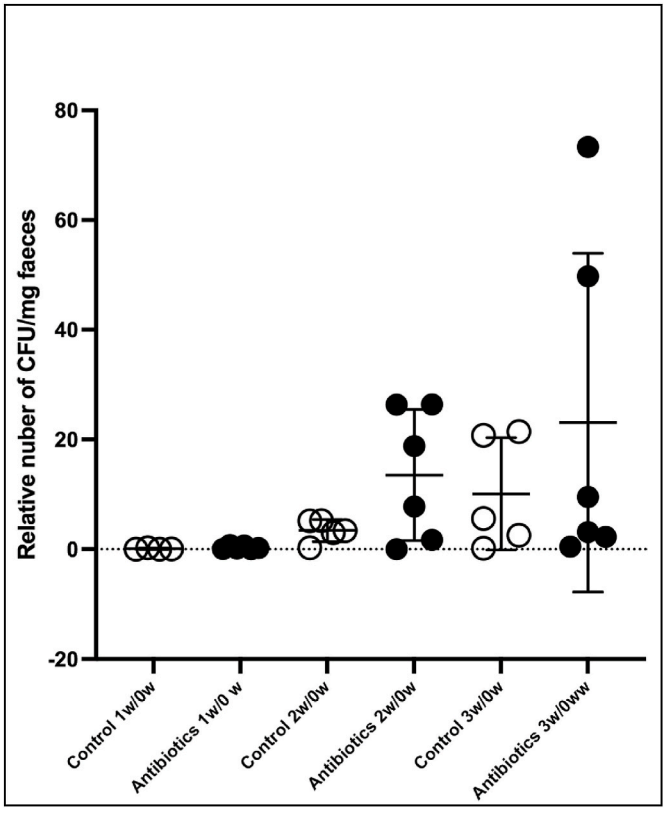

Fig. 1. Relative number of CFUs per mg of faeces from each individual, after one, two and three weeks in the control group and after one, two and three weeks of crayfish group exposed to SMX + CIP 100 ng/L respectively. Two outliers as indicated in Supplementary Table S1 were excluded from the graph. No significant differences between exposed and control group were found (n = 6, P > 0.05).

To investigate if the antibiotic treatment could affect the amount of antibiotic resistance genes in crayfish intestine, faeces samples were analyzed for the presence of two antibiotic resistance genes, one gene for resistance to SMX, *Sul1* [9,14], and one gene for resistance against CIP, *Qnr1* (GenBank: AY675584.1) [15]. However, we were not able to detect any *Qnr1* gene in any sample (data not shown), whereas *Sul-1* was clearly accumulated in the faeces of antibiotic exposed crayfish (Fig. 2).

Thus, it is clear that even exposure to very low concentrations of SMX antibiotics can lead to accumulation of ARGs in the intestine of freshwater crayfish, resulting in that these animals can act as a reservoir for such resistance genes in water ecosystems. Accumulation of ARGs in crustaceans have been shown in several reports to occur in aquaculture ponds where antibiotic use is very common [16–18]. However, here we show that after only three weeks very low but environmentally relevant concentrations of antibiotics may have a large effect on the accumulation of SMX resistance genes in the aquatic environment outside of aquaculture ponds, by release of faeces from freshwater crayfish. In this study, we obtained a more accurate estimation of the effect of antibiotic exposure since we determined the bacterial count and ARGs in the same individuals before and after treatment, which is not possible when intestines are dissected and analyzed for its microbiota [11]. Moreover, the faeces are continuously released into the surrounding water and bottom sediments and therefore contributes substantially to the microbial flora of lakes and rivers.

In our previous studies of SMX exposed crayfish, we have found that this antibiotic could enhance the crayfish tolerance to some pathogenic bacteria [11]. However, we did not find any relevant changes in the host immune defenses, such as the expression of antimicrobial peptides or enhanced activation of the proPO-activating system [19]. Therefore, we hypothesized that the SMX exposure may result in accumulation of antibiotics in tissues of the crayfish and resulting in partial killing effect of infective pathogenic bacteria. Such accumulation of antibiotics in crayfish tissues have been reported to occur, although at low levels, in a few *Procambarus clarkii* samples from the Guadiamar River in Spain [20, 21]. We collected tail muscle tissue from four *P. leniusculus* three weeks after exposure to SMX at 100 ng/L, and tail tissues from four control crayfish. Then we used a commercial ELISA kit for determination of SMX residues in food. As shown in Fig. 3, there was a clear accumulation of SMX in tail tissues in crayfish exposed to this antibiotic for three weeks.

4. Conclusion

This study shows that antibiotics in low concentrations in lakes or rivers will result in an accumulation of this antibiotic in a crustacean even after a short exposure time of three weeks. Even more concerning was that one resistance gene was produced in these SMX exposed animals and this gene was then released by the faeces into the surroundings. This must be considered as a concerning discovery and should urge authorities to consider decreasing the release of antibiotics into the external environment. One important difference between this investigation and previous reports about the effects of antibiotics on intestinal microflora and antibiotic resistance is that we have examined each animal at the individual level by analyzing the contents of the faeces before and after exposure.

Author contribution statement

Methodology, Formal analysis, Investigation RH and IS, Conceptualization RH, KS and IS, Writing - original draft RH and IS, Writing – review & editing RH, KS, and IS, Funding acquisition, IS.

CRediT author statement

Methodology, Formal analysis, Investigation RH and IS, Conceptualization RH, KS and IS, Writing - original draft RH and IS, Writing – review & editing RH, KS, and IS, Funding acquisition, IS.

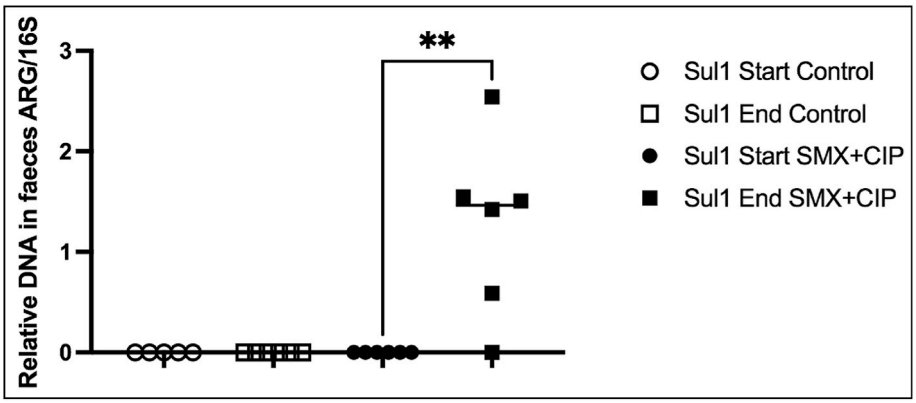

Fig. 2. Amount of the antibiotic resistance gene *Sul-1* DNA relative to total bacterial 16S DNA in faeces samples from freshwater crayfish before and after three weeks of exposure to SMX + CIP 100 ng/L respectively (n = 6, $p < 0.01$).

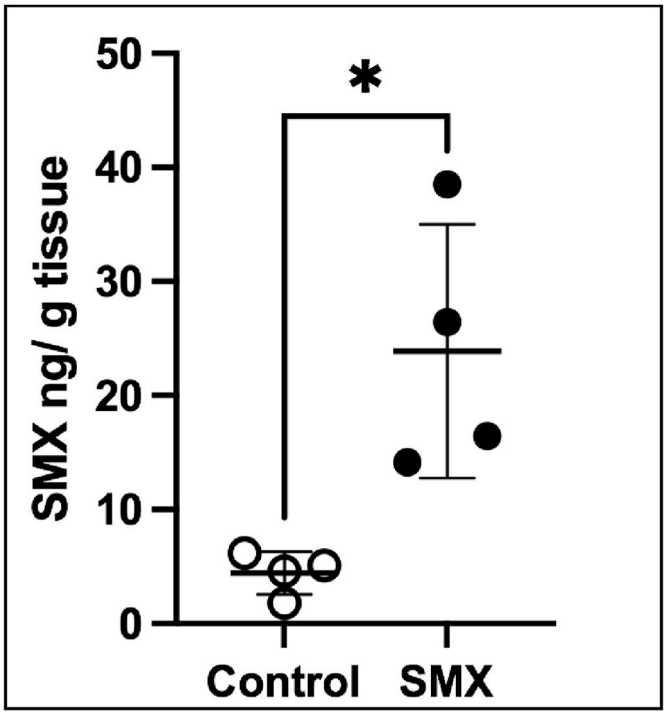

Fig. 3. Amount of SMX detected in tail muscle tissues of freshwater crayfish exposed to SMX at 100 ng/L for three weeks compared to unexposed crayfish (* = $p < 0,05$, n = 4). The detection was made using the MaxSignal® Sulfamethoxazole ELISA Kit.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2023.108836.

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