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Emergence of phenotypic and genotypic resistance in the intestinal microbiota of rainbow trout (*Oncorhynchus mykiss*) exposed long-term to sub-inhibitory concentrations of sulfamethoxazole

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Abstract

Natural waters are contaminated globally with pharmaceuticals including many antibiotics. In this study, we assessed the acquisition of antimicrobial resistance in the culturable intestinal microbiota of rainbow trout (*Oncorhynchus mykiss*) exposed for six-months to sub-inhibitory concentrations of sulfamethoxazole (SMX), one of the most prevalent antibiotics in natural waters. SMX was tested at three concentrations: 3000 µg/L, a concentration that had no observed effect (NOEC) on the in vitro growth of fish intestinal microbiota; 3 µg/L, a theoretical predicted no effect concentration (PNEC) for long-term studies in natural environments; and 0.3 µg/L, a concentration detected in many surveys of surface waters from various countries including the USA. In two independent experiments, the emergence of phenotypic resistance and an increased prevalence of bacteria carrying a sulfonamide-resistance gene (*sul*1) were observed in SMX-exposed fish. The emergence of phenotypic resistance to1000 mg/L SMX was significant in fish exposed to 3 µg/L SMX and was in large part independent of *sul* resistance genes. The prevalence of bacteria carrying the *sul*1 resistance gene increased significantly in the culturable intestinal microbiota of SMX-exposed fish, but the *sul*1-positive population was in large part susceptible to 1000 mg/L SMX, suggesting that the gene confers a lower resistance level or a growth advantage. The increased prevalence of *sul*1 bacteria was observed in all groups of SMX-exposed fish. Overall, this study suggests that fish exposed long-term to waters contaminated with low levels of antibiotics serve as reservoir of antimicrobial resistant genes and of resistant bacteria, a potential threat to public health.

Keywords

Antibiotics; Sub-inhibitory; Water contamination; Long-term exposure; Fish; Antimicrobial resistance

Declarations

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Introduction

Natural waters are frequently contaminated with antibiotics all around the world (Batt et al. 2016; Fatta-Kassinos et al. 2011; Fram and Belitz 2011; Kolpin et al. 2002; Kummerer 2009; Pochodylo and Helbling 2017). Factors contributing to this phenomenon include human and animal excretion of unmetabolized antibiotics, the variable effectiveness of wastewater treatment plants in eliminating antibiotics (Rizzo et al. 2013), run off from antibiotics used in agriculture, inadequate sewage management, and improper disposal of unused antibiotics. Considering that antibiotic resistance is a major global health problem, the potential role of the aquatic environment in the spread of antimicrobial resistance should not be underestimated.

Even though antibiotics are found in trace concentrations in natural waters, exposure of bacteria to levels of antibiotics that are too low to inhibit growth (sub-inhibitory levels) promotes the emergence of resistant bacteria and induces changes in bacterial gene expression. Selection of resistant bacteria has been observed at concentrations of antibiotic that are 4 to 100-fold lower than the Minimum Inhibitory Concentration (MIC) assessed in vitro (Andersson and Hughes 2014; Gullberg et al. 2011; Liu et al. 2011). In addition, sub-inhibitory concentrations of antibiotics cause mutagenesis, horizontal gene transfer, as well as modulating bacterial metabolism and virulence, including promoting biofilm formation (Andersson and Hughes 2014; Bernier and Surette 2013; Froehner et al. 2000; Knudsen et al. 2016; Laureti et al. 2013). As these observations were derived from in vitro systems, it seems imperative to test the consequences of long-term exposure to sub-inhibitory levels of antibiotics in live aquatic systems.

In the present study, we selected the antibiotic sulfamethoxazole (SMX) to assess the consequences of long-term exposure in fish. SMX is a bacteriostatic sulfonamide antibiotic that is commonly used in human and veterinary

74 medicine, and close SMX analogs are approved by the FDA for aquaculture (https://www.fda.gov/animal-
75 veterinary/aquaculture/approved-aquaculture-drugs). In the United States in 2018, over 8.4 million prescriptions of
76 sulfamethoxazole/trimethoprim were prescribed to humans
77 (https://clincalc.com/DrugStats/Drugs/SulfamethoxazoleTrimethoprim). SMX is a structural analog of para-
78 aminobenzoic acid that inhibits folate biosynthesis by competitive binding to dihydropteroate synthase encoded by
79 the gene *folP* (Skold 2000). The lack of folic acid impairs bacterial growth but does not kill the bacteria. Resistance
80 to SMX is common, including in salmonid aquaculture (Capkin et al. 2015; Dominguez et al. 2019; Duman et al.
81 2017). Resistance mechanisms to sulfonamides include chromosomal mutations of the *folP* gene and the acquisition
82 of plasmids carrying *sul* genes (primarily *sul*1 and *sul*2, with lower prevalence of *sul*3 and *sul*4) that encode drug-
83 insensitive variants of *folP* (Sanchez-Osuna et al. 2018; Skold 2000). *Sul* genes appear to have an ancient
84 evolutionary history, however, there are no known naturally occurring sulfonamides, and therefore the impetus for
85 the evolution of *sul* genes is unknown (Sanchez-Osuna et al. 2018).

86 SMX is one of the most common antibiotics detected in natural waters all around the world. It has been detected at
87 concentrations between 0.001 and 2 µg/L and at frequencies reaching up to 89% of sampling (Batt et al. 2016; Fatta-
88 Kassinos et al. 2011; Fram and Belitz 2011; Hossain et al. 2018; Hu et al. 2018; Kolpin et al. 2002; Kummerer 2009;
89 Na et al. 2019). In the US, 78% (141/182) of stream water samples tested between 2008-2010 were positive for the
90 presence of SMX (Batt et al. 2016). Moreover, SMX and resistance genes are detected in higher concentrations in
91 waters proximate to wastewater treatment plants (Marti et al. 2018). The high frequency of SMX detection is related
92 to both its high stability in water and frequency of use (Al-Ahmad et al. 1999; Lam et al. 2004). Exposure of
93 biofilms from wastewater treatment plants to SMX levels as low as 0.5 µg/L modulates the transcription levels of
94 DNA and RNA polymerase-coding genes and of cell envelope-related genes (Yergeau et al. 2010). Sub-inhibitory
95 concentrations of SMX have also been associated with changes in expression of nitrate reduction, carbon utilization,
96 and virulence genes (Johansson et al. 2014; Uhlich et al. 2018; Underwood et al. 2011). However, the long-term
97 effect of environmental concentrations of SMX on the aquatic environment has not yet been tested experimentally
98 (Al-Ahmad et al. 1999; Bengtsson-Palme and Larsson 2016; Straub 2016; Yan et al. 2016).

99 Considering its ubiquitous presence in natural waters and the high level of resistance associated with this antibiotic,
100 SMX was chosen to assess the consequences of chronic exposure of fish to antibiotics that contaminate natural

waters. Rainbow trout (*Oncorhynchus mykiss*) was chosen as the fish model because it is native to cold water tributaries in many regions of the world, including North America, and is a salmonid species of economic importance for aquaculture. It has been used as a model in ecotoxicological investigations, and for studies with suspected carcinogens, heavy metals, and agricultural, industrial, and pharmaceutical chemicals (Laville et al. 2004).

For the present study, juvenile rainbow trout were exposed for six months to SMX concentrations equivalent to 10^{-2} to 10^{-6} MIC, the lowest concentration being equivalent to concentrations detected in natural waters. The acquisition of phenotypic resistance and of resistance genes was assessed. The results indicated that exposure of fish to sub-inhibitory concentrations of SMX resulted in the emergence of bacteria resistant to a high concentration of SMX (1000 mg/L) in the culturable intestinal microbiota of fish. In addition, the incidence of bacteria carrying the *sul*1 resistance gene was significantly increased. We conclude that contamination of natural waters with sub-inhibitory concentrations of antibiotics has the potential to contribute to the spread of antibiotic resistance and should be considered a public health risk.

Materials and methods

Animals and husbandry

All animals were cared for in compliance with the *Guide for the Care and Use of Laboratory Animals* and American Association of Laboratory Animal Science Position Statements, and all procedures were approved by the Institutional Animal Care and Use Committee of Cornell University. Fish were housed in an AAALAC-accredited facility. Approximately two months old (7.5 cm length) juvenile rainbow trout (*Oncorhynchus mykiss*) were graciously provided by the New York State Department of Environmental Conservation Bath Hatchery. This hatchery maintains trout in raceways supplied by an underground source of water. The trout had never been treated with antibiotics.

Fish were maintained in 35L tanks in groups of approximately 15 fish per tank. Each individual tank was equipped with air stones and two mechanical pumps circulating the water through dense floss, activated charcoal, and mesh filters. Fifty percent water changes were performed a minimum of twice a week. Water temperature was regulated

by partly submerging the tanks in flow through living streams maintained at 10-15°C. A subset of fish was periodically weighed to allow feeding at 1% body weight once daily with Finfish Starter Slow Sinking diet 1.0 mm and 2.0 mm (Zeigler Bros., Inc).

Intestinal microbiota sampling

Trout were euthanized by immersion in 300 mg/L tricaine methanesulfonate (MS-222 from Western Chemical, Inc) buffered with 300 mg/L sodium bicarbonate, followed by decapitation and pithing. Euthanasia occurred 2-4 hours post-feeding to minimize effects of feed on the intestinal microbiota. Trout weight and length were recorded. The distal 1-1.5 cm segment of the hindgut was dissected with disinfected instruments, opened longitudinally, placed into a tube containing 400 µl sterile PBS, homogenized for 30 seconds using a pestle mixer (Argos Technologies), and stored on ice until processed.

Broth growth curves

Bacterial growth curves were performed using 96-well flat-bottom microtiter plates and an ELx808 BioTek spectrophotometer. Four trout were used for this assay. Each trout intestinal content was diluted in LBL broth (BD Difco) to obtain a concentration of approximately 4 x 10e5 CFU/ml. Rows of two-fold serial dilutions of SMX were prepared in LBL broth and each well was inoculated with ≈ 4 x 10e4 CFU in a final volume of 200 µl per well. Final concentrations of SMX ranged from 1000 mg/L to 1 mg/L with a no antibiotic control well. Each fish sample was tested in triplicate at each antibiotic concentration. Plates were incubated at 16°C. Absorbance at OD_{600} was measured every 6-12 hours for 60 hours.

Toxicological parameters

The No Observed Effect Concentration (NOEC) and MIC were calculated by a linear mixed effect model using maximum likelihood method at time t=48h modeling optical density as a function of time and antibiotic concentration, with fish ID and replicate number as random effects. NOEC was the largest concentration statistically equivalent to the no antibiotic control. MIC was the smallest concentration statistically equivalent to 1000 mg/L SMX. As data from only one trophic level was available, an assessment factor (AF) of 1000 was assigned (ECHA 2008) to measure the Predicted No Effect Concentration (PNEC = NOEC/AF). A review of the literature revealed

that Measure Environmental Concentrations (MEC) of SMX in natural waters ranged from 0.001 to 2 µg/L (Baran et al. 2011; Batt et al. 2016; Fatta-Kassinos et al. 2011; Fram and Belitz 2011; Hossain et al. 2018; Hu et al. 2018; Kolpin et al. 2002; Kummerer 2009; Na et al. 2019; Segura et al. 2009).

Chronic SMX-exposure studies

Two independent trials were performed on consecutive years. In both trials, trout were acclimated for two to three months prior to experimental manipulation. Tanks were dosed at every 50% water change (occurring 2-3 times weekly) to maintain SMX concentrations of 0.3 µg/L, 3 µg/L, and 3000 µg/L, approximating concentrations detected in natural waters, calculated PNEC, and in vitro determined NOEC (Fig. 1 and Table 1) for trout intestinal microbiota, respectively. An additional group not exposed to SMX served as a control. The stock solutions for SMX were prepared as 10,000 mg/L in 95% ethanol and stored at -20C (trial 1), or 170 mg/L in water, and stored at 4°C for up to one week (trial 2). Trout were harvested at time 0- and 6-months exposure to SMX. At each time point, fish were selected from 2-4 tanks, for a total of 5-7 independent tanks per treatment group over the two trials.

Verification of SMX concentrations in aquariums

Water samples were collected from the rainbow trout tanks at multiple time points. All water samples were stored frozen in the dark until processed. SMX concentration was assessed in duplicate by ELISA (Sulfamethoxazole Plate Assay Kit, Abraxis) as per the manufacturer's instructions. Samples were diluted with double distilled water as applicable to fall within the concentration range of the assay (0.015 – 1 µg/L). Absorbance at OD_{400} was read using a ELx808, BioTek spectrophotometer and analyzed by online software (www.elisaanalysis.com) using 4-parameter logistic regression.

Assessing culturable microbiota for resistance

Total CFU per intestinal sample was assessed by plating 50 µl of 10-fold serial dilutions of homogenized trout intestinal segments onto LBL agar plates supplemented with 2,500 mg/L amphotericin B to prevent the growth of fungi, which interfered with counting of CFU. Resistance was assessed by plating undiluted and 1:10 dilutions onto LBL agar plates containing 2,500 mg/L amphotericin B and 1000 mg/L SMX. Plates were incubated at room temperature (≈23°C), and CFU counts were recorded at 3 or 7 days for total or SMX-resistant CFU, respectively.

Bacterial isolation and verification of resistance

For each processed fish, isolated colonies from SMX plates and from non-SMX plates were archived and stored at -80°C. Efforts were made to select phenotypically different colonies. Isolates were re-tested for susceptibility or resistance to 1000 mg/L SMX on LBL agar plates.

Bacteria identification

A subset of archived bacterial isolates was submitted to an accredited veterinary diagnostic laboratory (Animal Health Diagnostic Center [AHDC], Ithaca NY) for identification by MALDI-TOF mass spectrometry (Bruker MALDI-Biotyper) to the closest genus (and species when possible), using 70% formic acid extraction. Protein spectra were compared to a library of known veterinary isolates. Scores >=2.0 were considered acceptable to be reported to genus and species level, and scores of 1.7 to 1.99 were acceptable to be reported to genus level.

Susceptibility testing

A subset of archived colonies (27 SMX-resistant and 7 SMX-susceptible) was submitted to the AHDC for susceptibility to a panel of antibiotics using an automated broth-microdilution technique (TREK Sensititre™ Systems). The antibiotic panel used was the poultry specific panel Thermo Scientific™ Sensititre™ Avian AVIAN1F Plate. Antibiotics tested were: amoxicillin, ceftiofur, clindamycin, enrofloxacin, erythromycin, florfenicol, gentamicin, neomycin, novobiocin, oxytetracycline, penicillin, spectinomycin, streptomycin, sulphadimethoxine, sulphathiazole, tetracycline, trimethoprim/sulfamethoxazole, and tylosin.

Detection of *sul* genes

Archived isolates were grown on LBL agar plates, and isolated colonies were used as a DNA template for PCR. Reactions were performed in 20 µl using the Qiagen *Taq* DNA polymerase kit according to manufacturer's instructions. Primers for detection of the V4 region of the 16S rRNA gene were used as an internal PCR control. Primers and annealing temperatures are given in Supplemental Table 1. A strain of *Salmonella* Typhimurium carrying the *sul*1 gene was provided by Prof. Craig Altier (Cornell University), whereas a DNA extract from bacteria carrying the *sul*2 and *sul*3 genes was provided by Prof. Patrick Boerlin (University of Guelph). These were

used as positive controls for detection of the *sul* genes. PCR that failed to amplify the 16S rRNA gene were excluded from analysis.

Statistical analysis

Statistical analysis was performed using R version 3.5.1 "Feather Spray" (2018-07-02) on a x86_64-w64-mingw32/x64 (64-bit) platform (The R Foundation for Statistical Computing, Vienna, Austria). Resistance was defined as alpha=0.05.

To achieve an arbitrary minimum level of detection of 1 in 10,000, fish with fewer than 10,000 CFU detected on media without SMX were excluded from analysis. The percent ratio of resistance was calculated by dividing the number of resistant CFU by the total number of CFU per fish and multiplying by one hundred.

Resistant fish were defined as any fish that was confirmed to have at least one colony resistant to 1000 µg/ml SMX. The count of resistant fish per treatment group was analyzed by logistic regression using a binomial family. Resistant tanks were defined as any tank in which at least one fish had one colony resistant to 1000 µg/ml SMX. The proportion of resistant tanks, resistant fish, and *sul*1 positive fish were analyzed by logistic regression using a binomial family with post-hoc pairwise comparisons with Tukey adjustment for multiple comparisons.

Results

In vitro susceptibility of culturable trout intestinal microbiota to SMX

The susceptibility of rainbow trout culturable intestinal microbiota to SMX was assessed in broth cultures in 96-well microtiter plates. Each row was supplemented with 2- fold serial dilutions of SMX from 1000 to 1 mg/L, with a no antibiotic control well, and each well was inoculated with ≈ 4 x 10e4 CFU from trout intestinal microbiota. Bacterial growth at 16°C was measured by spectrophotometry at OD_{600} (Fig. 1). The MIC and NOEC were calculated at 48h, during exponential growth, by linear mixed effect modeling. Figure 1 represents the results from trial 1 (n=4). Equivalent results were obtained the following year using trout from trial 2 (data not shown).

Calculated MIC and NOEC for SMX were 250 mg/L and 3.9 mg/L respectively (Table 1). An environmental PNEC of 3.9µg/L was estimated by dividing the NOEC by an AF of 1000.

Chronic SMX-exposure studies

Juvenile rainbow trout were exposed for six months to 0.3, 3.0, or 3000 µg/L SMX to approximate the environmental levels of SMX found in natural waters, the calculated PNEC (NOEC/AF of 1000), and the NOEC determined in vitro (Fig. 1 and Table 1), respectively. A no antibiotic group was added as a control. Two trials were performed on consecutive years with two different batches of fish. SMX concentration was tested in a preliminary experiment to assess stability and at random time points during the two trials. The results indicated that SMX is stable in the aquatic environment for a minimum of 7 days and concentrations were maintained in the targeted range during the trials (data not shown). There was no effect of treatment group on fish standard length, weight, or total intestinal CFU (data not shown) for either trial.

Resistance to SMX was assessed by plating serial dilutions of intestinal contents on LBL agar with and without 1000 mg/L SMX (four-fold higher than the MIC), and calculating the percentage of resistant to total CFU. At time 0, no resistance was observed (n=12, data not shown). At six months, resistant CFU were observed in both experimental repeats (Table 2 and Fig. 2). Resistance was assessed on the tank level, in the event that fish with resistant bacteria would contaminate their environment and subsequently the other fish in the tank, and on the individual fish level (Fig. 2). On the tank level, all groups exposed to SMX showed an increase in resistance compared to the control group, although the trend was not statistically significant. On the individual fish level, logistic regression analysis reveals an overall significant difference ($p = 1.1E-6$). Significant differences were also revealed by post-hoc pairwise comparisons: 0 vs 3 ($p = 0.01$), 0 vs 3000 ($p = 0.02$), 0.3 vs 3 ($p = 0.01$), and 0.3 vs 3000 ($p = 0.03$) µg/L SMX groups. There were no pairwise significant differences between 0 vs 0.3 and 3 vs 3000 µg/L SMX. The median percentage and limits of SMX-resistant CFU per fish is shown in table 2.

Identification of bacterial isolates resistant and susceptible to SMX

A subset of bacteria resistant or susceptible to 1000 mg/L of SMX (4X MIC) were identified by MALDI-TOF mass spectrometry (Table 3). A total of 234 (90 from trial 1 and 132 from trial 2) isolates from 77 individual fish (27 from

trial 1 and 50 from trial 2) were identified. A different bacterial species was dominant among the resistant isolates for each experimental repeat: *Carnobacterium maltaromaticum* (Trial 1) and *Lelliottia amnigena (*Trial 2). These two species were also found in the subset of susceptible isolates in either trial, including in tanks where no resistant bacteria were found. In trial 2, *Aeromonas* and *Deefgea* species dominated the pool of SMX-susceptible bacteria.

Susceptibility testing of isolates

We performed susceptibility testing for a panel of antibiotics on *L. amnigena* isolates that were susceptible (n=6) or resistant (n=19) to 1000 mg/L of SMX. All *L. amnigena* isolates were resistant to clindamycin, novobiocin, and penicillin. There was no difference in susceptibility patterns for the 18 antibiotics tested. We also performed susceptibility testing of 7 *Pseudomonas* isolates. The *Pseudomonas* isolates had elevated MICs to 8-11 antibiotics: amoxicillin, clindamycin, florfenicol, neomycin, novobiocin, oxytetracycline, penicillin, spectinomycin, streptomycin, sulfathiazole. Two Pseudomonas isolates were additionally resistant to trimethoprim.

Detection of bacteria carrying the *sul1* resistance gene

We aimed to identify the mechanism conferring resistance to SMX in the intestinal isolates of fish exposed to SMX. The presence of *sul*1, the most prevalent resistance determinant to SMX, was assessed by PCR. SMX-resistant isolates carrying the *sul*1 gene were found in five fish total: three out of 35 fish (8.5%) in trial 1 and two out of 103 fish (1.9%) in trial 2 (Fig. 3). The SMX-resistant isolates that were positive for *sul*1 were two *Panotea agglomerans* and three *Pseudomonas species.* SMX-susceptible isolates carrying the *sul*1 gene were found in 85 fish total: six out of 35 fish (17.1%) in trial 1 and 79 out of 103 fish (76.7%) in trial 2 (Fig. 3). In trial 1, fish carrying *sul*1 SMX-susceptible isolates were found in the 3000 µg/L treatment group exclusively. In trial 2, fish carrying *sul*1 SMX-susceptible isolates were found in all groups: 48%, 83%, 89%, and 79% for the 0, 0.3, 3, and 3000 µg/L SMX treatment groups, respectively. When analyzed by logistic regression using a binomial family with post-hoc pairwise comparisons with Tukey adjustment for multiple comparisons, there was a significant difference between groups with p values of 0.005 and 0.001 for trials 1 and 2, respectively. There were no differences between individual groups in trial 1 because of the small sample size. In trial 2, significant differences were present between the control group and the 3 µg/L group with a p value of < 0.01, and between the control group and the 3000 µg/L group with a p value of 0.02.

Discussion

This study shows the emergence of phenotypic resistance to SMX in the microbiota of fish exposed long-term to sub-inhibitory concentrations of this antibiotic. Phenotypic resistance was robust at 1000 mg/L SMX and repeatable in two independent trials in fish exposed to the PNEC of 3 µg/L and the NOEC of 3000 µg/L. In addition, we observed an increased incidence of bacteria carrying *sul*1, a gene associated with resistance to SMX, in fish exposed to as little as 0.3 µg/L SMX, a concentration that has been detected in surface waters in the United States, Europe, and globally (Batt et al. 2016; Fatta-Kassinos et al. 2011; Fram and Belitz 2011; Hossain et al. 2018; Hu et al. 2018; Kolpin et al. 2002; Kummerer 2009; Na et al. 2019). Results of this study underline the importance of in vivo testing to assess the consequences of long-term exposure to sub-inhibitory concentrations of antibiotics on the aquatic life, and the critical role of the aquatic environment in the spread of antimicrobial resistance.

In vitro susceptibility of culturable trout intestinal microbiota to SMX

For this study, we first assessed the limits of susceptibility of the trout microbiota to SMX in broth culture and determined that the MIC (250 mg/L) was 64-fold higher than the NOEC (3.9 mg/L). As with most culture-based systems, these values do not consider anaerobic or otherwise unculturable bacteria which may make up a significant proportion of total bacteria. In addition, these values are population based, whereas, traditionally, antibiotic susceptibility values are determined for monoclonal populations. It is expected that, within a population, the level of susceptibility to SMX will vary among bacterial species. Therefore, the calculated MIC represents the concentration at which the growth of the least susceptible species is inhibited, whereas the NOEC represents the concentration at which the growth of the most susceptible species is not inhibited within this population of culturable intestinal bacteria. Thus, while the MIC and NOEC values are not directly comparable to single species estimations, they are representative of the population dynamics that occur in vivo.

The PNEC is an estimate of the concentration of antibiotic that would have no deleterious effects on a bacterial population in a complex environment and under chronic exposure (ECHA 2008). A PNEC of 3.9 µg/L was calculated for our model by applying an arbitrary assessment factor (AF) of 1000 to the NOEC (ECHA 2008). SMX has been detected at concentrations between 0.001 and 2 µg/L in many different countries, including Bangladesh, China, France, Germany, India, Luxembourg, South Korea, Spain, and the USA (Batt et al. 2016; Fatta-Kassinos et

al. 2011; Fram and Belitz 2011; Hossain et al. 2018; Hu et al. 2018; Kolpin et al. 2002; Kummerer 2009; Na et al. 2019), and in some studies, at a frequency of 70% and 89% of water samples tested (Batt et al. 2016; Hossain et al. 2018; Hu et al. 2018; Na et al. 2019). These concentrations suggest that a PNEC of 3.9 µg/L SMX would have no effects on the intestinal microbiota of our animal model, the rainbow trout. It is generally accepted that environmental concentrations of SMX pose little toxicological risk to vertebrates, but may be toxic to invertebrates, bacteria, or plants (Baran et al. 2011; Batt et al. 2016; Isidori et al. 2005; Park and Choi 2008; Yan et al. 2016). Ecotoxicological testing provides values for binary measures of toxicity and growth rates, but not necessarily change in phenotype such as antibiotic resistance. Therefore, in addition to theoretical studies, actual exposure experiments, as in this study, are needed to assess more accurate PNEC values.

Emergence of resistance in the intestinal microbiota of fish exposed to sub-inhibitory levels of SMX

The emergence of resistance to 1000 mg/L SMX, a concentration four times higher than the determined MIC, was detected in a percentage of culturable isolates from the intestinal microbiota of trout exposed for six months to SMX at the NOEC determined in vitro (3000 µg/L), the PNEC (3 µg/L), and at a conservative concentration value detected in surface waters (0.3µg/L). Resistance was first analyzed at the tank level with the rationale that any fish within a tank was susceptible to acquire a resistant bacterial clone from any other fish in that tank. A trend toward an increase in the number of tanks with resistant bacteria in SMX-exposed groups was observed, but the difference was not significant. The median percentage of fish with resistant isolates per resistant tank was 55%. However, in the groups exposed to 3 µg/L or 3000 µg/L SMX, every fish in three out of 13 tanks carried resistant bacteria. It is reasonable to speculate that in these three tanks resistant bacterial clones were transmitted orally between fish, but the kinetics of transmission is likely to be time-dependent, increasing as the number of fish carrying and shedding resistant bacteria increases with time. Although the initial acquisition of resistance within a fish intestinal microbiota is random, over time, all fish within a tank are susceptible to colonization by resistant clones shed in the environment. Ultimately, it is not a question of whether a fish will acquire resistant bacteria but rather a question of when.

When resistance data were analyzed at the fish level, the ratio of SMX-resistant bacteria among the culturable microbiota increased in all SMX-exposed groups, and this difference was statistically significant in the groups exposed to 3 µg/L and 3000 µg/L SMX. This difference was present in both trials, but there was variation in the percentage of resistant CFU to total CFU. Variables that might account for this difference include the make-up of the microbiota between the two batches of fish, and the solvent used to prepare the SMX stock solutions. Ethanol was used as a SMX solvent in the first trial, but water was used in the second trial. The concentration of ethanol (V/V) in tank water would have been 0.0017%, 0.0017%, and 0.015% in the 0.3, 3, and 3,000 µg/L SMX groups, respectively. The highest concentration of ethanol is ten to one hundred times lower than the concentrations known to affect transcriptional or metabolic changes in bacteria (Camarena et al. 2010; Ma et al. 1996) and four times lower than the concentration to impair gill ATPases in tilapia (Bhanu and Philip 2011). However, we observed increased particulates in tanks treated with the highest concentration of SMX in ethanol. This difference in water quality might have influenced the experimental outcome due to effects on the microbiota or directly on fish physiology. Yet despite any confounding effects of differing microbiota or vehicles, the phenomenon of emergence of resistance was repeatable in two independent trials carried out in two separate years.

This study was designed to model chronic exposure of fish to sub-inhibitory concentrations of SMX. However, it did not mimic environmental conditions under which actual concentrations of SMX are likely to vary based on inputs such as agricultural use or release from wastewater treatment plants. While SMX is the most commonly detected antibiotic in natural waters, this environment is typically contaminated with multiple antibiotics and other pharmaceutical drugs that may affect bacterial genetic and metabolic functions, contributing to the development of resistance mechanisms or the acquisition of resistance genes (Aubertheau et al. 2017; Batt et al. 2016; Fatta-Kassinos et al. 2011; Fram and Belitz 2011; Hossain et al. 2018; Kolpin et al. 2002; Lam et al. 2004; Na et al. 2019).

Overall, the data indicate that an AF of 1000 to determine the PNEC is an underestimate of the selective pressure from contaminated waters for the emergence of resistance in the microbiota of exposed fish. An AF of 10,000 would be closer to what we observed in our experimental model. We also observed that the acquisition of resistance appears to be more at the individual level with a low incidence of transfer between fish, although a different type of study with traceable resistant clones would be required to verify that observation. Importantly, resistance emerged in two independent trials with different batches of trout and different drug vehicles, demonstrating that chronic

exposure to a concentration of SMX 10-fold lower that the theoretical PNEC reproducibly promotes the emergence of resistance in fish microbiota.

The resistant culturable microbiome is low in diversity

The culturable microbiota from trials 1 and 2 differed extensively, emphasizing the fact that the composition of the intestinal microbiota is plastic and responsive to variables such as genetics and the environment (Egerton et al. 2018). In this study, the fish came from the same hatchery, but the two trials occurred on different years. In each trial, a single species of bacteria predominated the culturable resistant microbiome, arising in multiple independent tanks. The two species, *Carnobacterium maltaromaticum* in trial 1 and *Lelliottia amnigena* in trial 2, were present to a low frequency in the susceptible population but contributed to 92% and 86% of the resistant population, respectively. The overall number of identified genera present in resistant isolates was low: five in trial 1 and three in trial 2.

C. maltaromaticum is a gram-positive facultative anaerobe lactic acid bacteria that has been associated with healthy and diseased salmonids (Leisner et al. 2007). Pathogenic *C. maltaromaticum* has been noted to be resistant to sulfonamides, tetracyclines, and quinolones (Leisner et al. 2007). *L. amnigena*, formerly identified as *Enterobacter amnigenus* (Brady et al. 2013), is a gram-negative, facultative anaerobe. *L. amnigena* is found in the environment, but occasionally causes infection in humans (Leal-Negredo et al. 2017; Stock and Wiedemann 2002). A diverse collection of 18 *L. amnigena* isolates from soil, water, and clinical specimens were tested for susceptibility to various antibiotics; three of the isolates were resistant to 1000 mg/L SMX, whereas the others were susceptible to ≤ 64 mg/L (Stock and Wiedemann 2002). Our results suggest that *C. maltaromaticum* and *L. amnigena* are particularly adapted at evolving resistance to SMX, and/or that an undetectable population of resistant clones in the original microbiome expanded to the detriment of the susceptible population.

Susceptibility testing does not reveal co- or cross-resistance

Co-resistance to multiple antibiotics following the use of a single antibiotic is an interesting phenomenon associated with the acquisition of a plasmid, integron, or transposon that carries multiple resistance genes (Bennett 2008). Cross-resistance is a different phenomenon associated with the acquisition of a single resistance mechanism that

affects the bacterial susceptibility to multiple antibiotics usually, but not exclusively, within the same class. In the present study, a subset of *L. amnigena* and *Pseudomonas* clones resistant and susceptible clones were tested for their susceptibility to a panel of 18 antibiotics. The results suggest that neither co- nor cross-resistance phenomena were associated with the emergence of clones highly resistant to SMX.

Exposure to sub-inhibitory concentrations of SMX selectively promotes the establishment of bacteria carrying the *sul*1 resistance gene

We observed that exposure to SMX caused an increase in the incidence of *sul*1 in the intestinal culturable microbiota but the *sul*2 and *sul*3 genes were not detected in any of the archived isolates. The population of *sul*1 carrying bacteria was comprised in large part of SMX-susceptible clones. In the first trial, SMX-susceptible isolates carrying the *sul*1 gene were detected only in the group of exposed to 3000 µg/L SMX at a level of 50%. The second trial was different as 48% of the unexposed population of fish carried *sul*1 SMX-susceptible bacteria in the intestines. However, the population of fish colonized with *sul*1 bacteria was larger by an average of 75% in all SMX-exposed groups. These results indicate that exposure to sub-inhibitory levels of SMX promotes the selection of *sul*1 carrying bacteria and/or the acquisition of the *sul*1-integron across the population (Bennett 2008).

The fact that the *sul*1 gene was detected primarily in the population of SMX-susceptible isolates suggests that the conditions used for selection of resistant clones were too stringent for the level of resistance conferred by the *sul*1 gene. Indeed, the *sul*1-positive *Salmonella* strain used as a positive control for the PCR assay grew with 500 mg/L SMX but was inhibited by 1000 mg/L SMX, the concentration used in this study to select for SMX-resistant intestinal bacteria. Additional growth assays were performed with a subset of *sul*1 positive fish isolates susceptible to 1000 mg/L, but none of them grew with 500 mg/L SMX (data not shown). These data were inconclusive as the MIC in absence of *sul*1 is not known for any of the tested clones.

Alternative mechanisms exist for the selection of *sul*1 under conditions that are presumably non-selective for SMX-resistant bacteria. Sulfonamides are synthetic compounds with no known natural analogs, yet *sul* genes have an ancient evolutionary past (Sanchez-Osuna et al. 2018). This suggests some fitness benefit or lack of detriment for their evolution, and perhaps the existence of natural sulfonamide analogs. Additionally, the *sul* genes are highly sensitive enzymes, and have Michaelis-Menten constants that are equivalent to or better than the native *folP* in *E.*

coli (Skold 2000; Swedberg et al. 1979). Perhaps the *sul* genes provide fitness benefits by counteracting effects of SMX at low concentrations. The presence of *sul* genes could serve to increase output of the folate pathway, resulting in more available metabolic building blocks. Moreover, exposure to sub-inhibitory doses of sulfonamides have been documented to increase quorum sensing (Deng et al. 2012) and to change expression of genes coding for virulence factors (Moon et al. 2017; Uhlich et al. 2018), outer membrane proteins, and transcription factors (Yergeau et al. 2010). These overall effects of SMX at sub-inhibitory concentrations might have provided a growth advantage within the intestinal microbiota of fish. In summary, long-term exposure to sub-inhibitory concentrations of SMX causes the emergence of *sul*1 carrying bacteria, although the selection pressure for this phenomenon and the potential advantage conferred by the *sul*1 gene within the intestinal microbiota of fish remain to be determined.

Emergence of *sul*-independent resistance to SMX in the intestinal microbiota of fish exposed to sub-inhibitory concentrations of SMX

Less than four percent of SMX-resistant isolates carried a *sul* resistance gene. One of the most common mechanisms of resistance to SMX is mutation in the dihydrofolate reductase gene, *folP* (Skold 2000). Efflux pumps are not a common mechanism of resistance, but Resistance-Nodulation-Division efflux pumps have been shown to efflux sulfonamides, (Li 2016) as well as novel efflux pumps in the AbgT family (Delmar and Yu 2016). It is reasonable to speculate that the SMX-resistant bacteria isolated in this study used more than one mechanism of resistance, independent of the *sul* resistance genes, and that the sum of these mechanisms conferred high resistance to SMX.

Conclusions

This study demonstrates that chronic exposure to SMX at concentrations as low as those found in surface waters across the globe promotes the selection of bacteria carrying the *sul*1 resistance gene and, independently of *sul*1, the emergence of bacterial clones highly resistant to SMX. The development of resistance in the environment with exposure to sub-inhibitory levels of antibiotics has far-reaching consequences on both applied and basic science fronts. Generation of antibiotic resistance and modulation of fish microbiota may have ecological effects on the aquatic habitat. It can potentially expose those in contact with fish, such as anglers, swimmers, or pets, to resistant bacteria. The potential of aquatic animal species as carriers of antibiotic resistant bacteria and the release of these

bacteria in rivers and streams may also pose risk to agriculture, potentially contaminating soils and crops with resistance genes. Further efforts are needed to reduce antibiotic contamination of water.

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respectively, *E. gergoviae* and *E. pyrinus* into Pluralibacter gen. nov. as *Pluralibacter gergoviae* comb. nov. and *Pluralibacter pyrinus* comb. nov., respectively, *E. cowanii*, *E. radicincitans*, *E. oryzae* and *E. arachidis* into Kosakonia gen. nov. as *Kosakonia cowanii* comb. nov., *Kosakonia radicincitans* comb. nov., *Kosakonia oryzae* comb. nov. and *Kosakonia arachidis* comb. nov., respectively, and *E. turicensis*, *E. helveticus* and *E. pulveris* into Cronobacter as *Cronobacter zurichensis* nom. nov., *Cronobacter helveticus* comb. nov. and *Cronobacter pulveris* comb. nov., respectively, and emended description of the genera Enterobacter and Cronobacter Syst Appl Microbiol 36:309-319 doi:10.1016/j.syapm.2013.03.005

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Table 1. Toxicological parameters used to derive SMX-treatment groups

MIC	250 mg/L	in vitro-derived from trout microbiota – Fig. 1
NOEC	3.9 mg/L	in vitro-derived from trout microbiota – Fig. 1
PNEC	3.9 µg/L	NOEC/Assessment Factor of 1000 (ECHA 2008)
MEC	0.001-2.0 µg/L	(Batt et al. 2016; Fatta-Kassinos et al. 2011; Fram and Belitz 2011; Hossain et al. 2018; Hu et al. 2018; Kolpin et al. 2002; Kummerer 2009; Na et al. 2019)

Table 2. Percentage of intestinal CFU resistant to 1,000 mg/L SMX

	Trial 1		Trial 2	
SMX treatment group (µg/L)	Median[a] (n)[b]	Range[a]	Median (n)	Range
0	N.D.[c]	N.A.[c]	9.01E-5 (1)	N.A.
0.3	N.D.	N.A.	N.D.	N.A.
3.0	4.16E1 (7)	1.39E-5 – 1.00E2	1.87E-3 (10)	3.04E-5 – 2.14E-1
3000	5.71E-1 (9)	1.90E-5 – 5.67E1	1.32E-3 (4)	8.2E-5 – 1.21E-1

[a]Median percentage and range of SMX-resistant CFU per treatment group, excluding fish in which ratio of SMX-resistant CFU were not available

[b] n: number of fish carrying SMX-resistant CFU

[c]N.D.: Not Detected; N.A.: Not applicable

612 **Table 3.** Archived bacterial isolates from trout intestines identified by MALDI-TOF

SMX treatment group (µg/L)	Bacterial ID[a]	Trial 1	Trial 2
0	*Acinetobacter tjernbergiae*	N.D.[b]	S[c]
	Aeromonas species[d]	N.D.	S
	Bacillus subtillis	S	N.D.
	Deefgea rivuli	N.D.	S
	Flavobacterium plurextorum	S	N.D.
	Lelliottia amnigea	N.D.	S
	Micrococcus luteus	S	N.D.
	Pseudomonas species[d]	N.D.	R[c] and S
	Stenotrophomonas maltophilia	N.D.	S
0.3	*Aeromonas species*[d]	N.D.	S
	Bacillus licheniformis	N.D.	S
	Carnobacterium maltaromaticum	S	N.D.
	Deefgea species	N.D.	S
	Lelliottia amnigea	S	S and R
	Staphylococcus warnerii	R	N.D.
	Stenotrophomonas maltophilia	N.D.	S
3	*Aeromonas species*[d]	N.D.	S
	Bacillus species[d]	N.D.	S
	Carnobacterium maltaromaticum	R and S	N.D.
	Deefgea species	N.D.	S
	Lelliottia amnigea	N.D.	R and S
	Panotea agglomerans	R	N.D.
	Pseudomonas chlororaphis	N.D.	R
	Staphylococcus hominis	S	N.D.
3000	*Aeromonas species*[d]	R	S

	Carnobacterium maltaromaticum	R and S	N.D.
	Deefgea species	N.D.	S
	Lelliottia amnigea	N.D.	R
	Microbacterium maritypicum	N.D.	S
	Pseudomonas species[d]	R	R
	Staphylococcus species[d]	S	S

[a]Isolates were identified by MALDI-TOF mass spectrometry to the closest genus or species

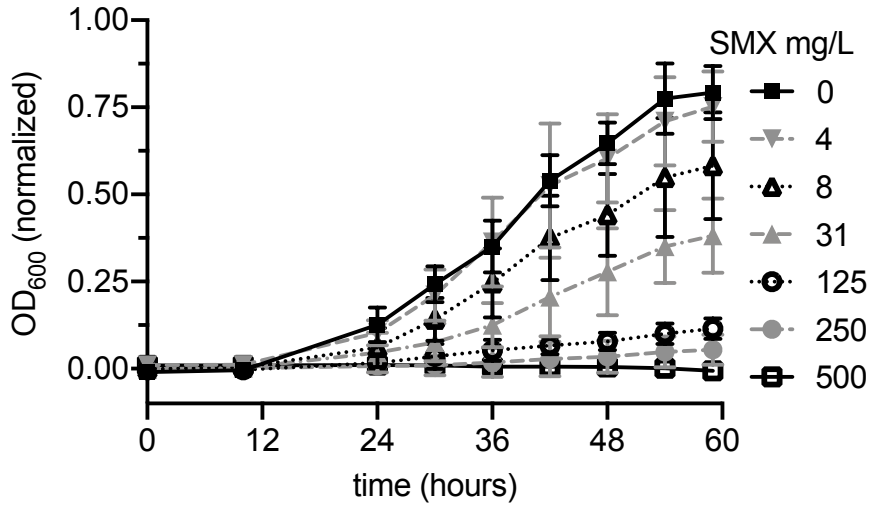
[b]N.D.: Not detected

[c]Susceptible (S) or resistant (R) to 1000 mg/L SMX

[d]Multiple species identified

Supplemental Table 1 Primers used in this study.

Primer name	Sequence (5'-3')	Product size (bp)	Annealing (°C)	Reference
Sul1 forward	TGAGATCAGACGTATTGCGC	406	53	(Rahmani et al. 2013)
Sul1 reverse	TTGAAGGTTCGACAGCACGT	406	53	(Rahmani et al. 2013)
Sul2 forward	GCGCTCAAGGCAGATGGCATT	285	64	(Aarestrup et al. 2003)
Sul2 reverse	GCGTTTGATACCGGCACCCGT	285	64	(Aarestrup et al. 2003)
Sul3 forward	GAGCAAGATTTTTGGAATCG	772	51	(Perreten and Boerlin 2003)
Sul3 reverse	CATCTGCAGCTAACCTAGGGCTTTGGA	772	51	(Perreten and Boerlin 2003)
16S forward (515F)	GTGYCAGCMGCCGCGGTAA	290	53 or 51	(Caporaso et al. 2011)
16S reverse (806R)	GGACTACCGGGGTATCT	290	53 or 51	(Caporaso et al. 2011)



620

621 **Fig. 1** Determination of MIC and NOEC for culturable trout intestinal microbiota. The growth assays were
622 performed at 16°C in broth with SMX concentrations ranging from 0 to 1,000 mg/L. Each intestinal sample was
623 tested in triplicate. Results were normalized by subtracting the average OD_{600} reading at time 0 from all subsequent
624 timepoints for each individual sample. Select concentrations are presented as mean +/-SD, n=4 fish.

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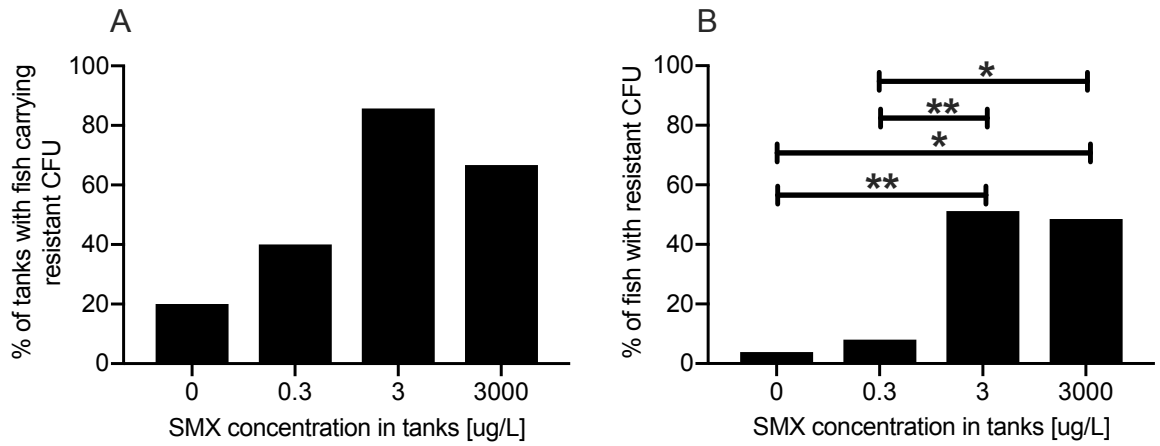

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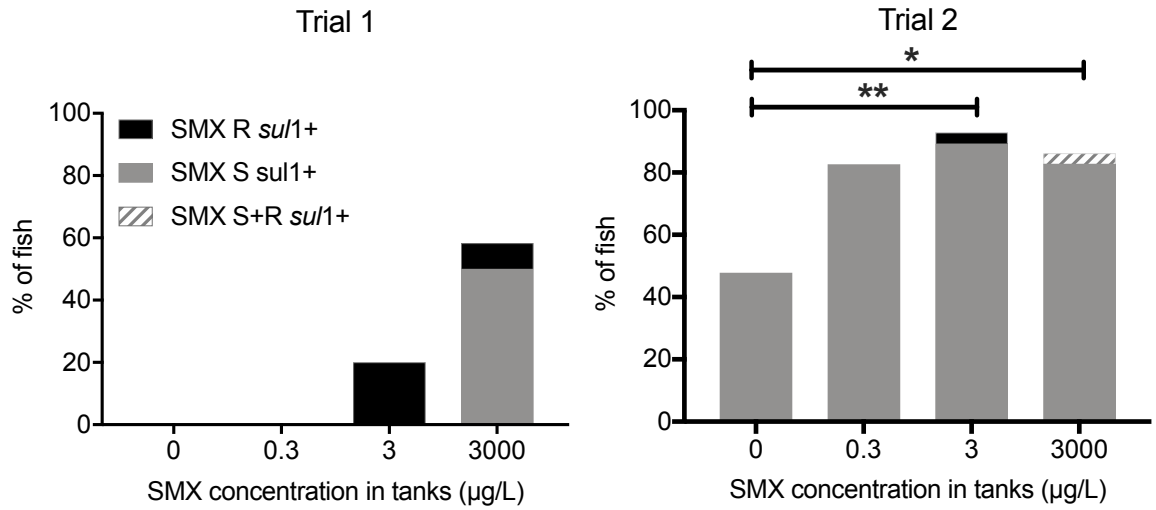
628 **Fig. 2** Emergence of CFU resistant to 1000 mg/L SMX in the intestinal microbiota of trout exposed to SMX for six
629 months. Trials 1 and 2 are combined. A: percentage of tanks with fish carrying SMX-resistant CFU for each
630 experimental group. Total number of tanks per experimental group was 5, 5, 7, and 6 for the 0, 0.3, 3, and 3000 µg/L
631 SMX groups, respectively. B: percentage of fish carrying SMX-resistant CFU for each experimental group. Total
632 number of fish tested per experimental group was 26, 25, 41, and 35 for the 0, 0.3, 3, and 3000 µg/L SMX groups,
633 respectively. Data were analyzed by logistic regression followed by post-hoc pairwise comparisons. On the
634 individual fish level, logistic regression analysis reveals an overall significant difference ($p = 1.1E-6$). * and **
635 indicate p values of ≤ 0.03 and 0.01, respectively, for pairwise comparisons.

636



637

638 **Fig. 3** Prevalence of fish colonized with *sul*1 positive (+) bacterial isolates. Isolates from individual fish were
639 tested by PCR for the presence of *sul*1. In black are fish carrying *sul*1+ isolates resistant (R) to 1,000 mg/L SMX. In
640 grey are fish carrying *sul*1+ isolates susceptible (S) to 1,000 mg/L SMX. One fish in trial 2 in the group exposed to
641 3,000 µg/L SMX had *sul*1+ R and S isolates. The treatment groups are indicated along the X axis and the number of
642 fish tested for *sul*1 were: 6, 6, 10, and 12 in trial 1, and 23, 23, 28, and 29 in trial 2 for the 0, 0.3, 3, and 3000 µg/L
643 SMX groups, respectively. Data were analyzed by logistic regression followed by post-hoc pairwise comparisons.
644 Logistic regression analysis reveals an overall significant difference between groups with p values of 0.005 and 0.01
645 for trials 1 and 2, respectively. * and ** indicate p values of 0.02 and < 0.01, respectively, for pairwise comparisons.