Toxicity of ciprofloxacin and sulfamethoxazole to marine periphytic algae and bacteria

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Keywords: Periphyton, Microbial biofilms, Antibiotics, Ciprofloxacin, Sulfamethoxazole

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Abstract

Ciprofloxacin and sulfamethoxazole are two antibiotics commonly detected in the aquatic environment, but information on their toxicity towards natural microbial communities is largely absent. In particular no data are available for marine microorganisms. Aim of the current study was therefore to evaluate the chronic toxicity of ciprofloxacin and sulfamethoxazole on natural marine biofilms (periphyton), a complex ecological community comprising a variety of bacterial and algal species. The biofilms were sampled along the Swedish west coast and subsequently exposed over 4 days in a semi-static system to a concentration series of each antibiotic.

Effects on the bacterial part of the periphyton community were assessed using Biolog Ecoplates, reflecting total respiration and functional diversity of the bacterial community. Exposure to either antibiotic resulted in a clear concentration-response relationship with EC10 and EC50 values for the inhibition of total carbon source utilization of 46.1 nmol/L and 490.7 nmol/L for ciprofloxacin, respectively 56 and 1073 nmol/L for sulfamethoxazole. The NOEC for ciprofloxacin was 26 nmol/L, with a minimum significant difference of 19.24%, for sulfamethoxazole it was 140 nmol/L with a minimum significant difference of 14%. Multivariate data exploration of the whole carbon source utilization pattern confirmed these results. The data indicate that sulfamethoxazole leads to a general decrease in carbon source utilization, while ciprofloxacin exposure leads to a re-arrangement of the carbon-utilization pattern in the region of 20-50% effect. This corresponds with the higher specificity of ciprofloxacin for certain bacterial species.

Effects on the algal part of the communities were evaluated by analyzing the amount and composition of photosynthetic pigments, and neither ciprofloxacin nor sulfamethoxazole caused any inhibitory effects up to the maximum tested concentration of 9 000 nmol/L. However, sulfamethoxazole exposure did lead to a significant stimulation (75% above control level) of the total pigment content of the biofilm already at the lowest tested concentration of

5 nmol/L. The stimulation then decreased with increasing concentrations to finally return to control level at 3 000 nmol/L. No shifts in the relative pigment composition were observed, indicating a generally increased algal biomass without major shifts in community composition.

1 Introduction

Antibiotics are frequently detected in the aquatic environment, as a result of their use in human and veterinary medicine. Current knowledge concerning their exposure, fate and ecotoxicology has been reviewed by e.g. Kümmerer (2009a, 2009b) and Santos et al. (2010). Quinolones and sulfonamides are amongst the most commonly used antibiotic classes and are frequently detected in aquatic environments Kümmerer (2009a). However, both classes are mainly monitored in freshwater systems, with only a very few studies having been published on their occurrence in the marine environment.

Similarly, data on the toxicity of the two antimicrobial classes to marine microorganisms, most likely the most sensitive group of marine organisms, are largely absent. The aim of this study was therefore to analyze the chronic toxicity of ciprofloxacin and sulfamethoxazole, two of the most frequently detected quinolone respectively sulfonamide antibiotics, to natural marine microbial biofilms (periphyton).

Periphyton is an aquatic biofilm-forming community that comprises a broad range of autotrophic and heterotrophic species, mainly micro-algae and bacteria. They grow on submerged surfaces in the aquatic environment, fulfilling important ecological roles as primary producers and destruents (Azim et al., 2005). Biofilm inhabiting organisms live in a confined space, competing for nutrients, space and light. Species-dependent changes in ecological fitness due to exposure to toxic compounds hence do not only change the overall physiological activity of the biofilm species, but also affect the biodiversity of the community: toxicant-exposed communities are dominated by more tolerant species (Blanck, 2002). Periphyton biofilms can be established in the natural environment and then transferred to the laboratory where they are exposed to concentration-series of a test compound under controlled conditions, combining high ecological realism with the precision and experimental capacity of laboratory-based studies (Porsbring et al., 2007).

Effects on periphytic bacteria were studied in the present paper as catabolic profiles that inform on gross respiratory activity of the community as well as bacterial functional diversity. Pigment composition was used as a measure that integrates information on biomass, community structure and physiological status of the algal part of the periphyton community (Porsbring et al., 2007).

1.1 The tested antibiotics

The physic-chemical characteristics of ciprofloxacin and sulfamethoxazole are summarized in table 1.

Ciprofloxacin

Ciprofloxacin is a fluoroquinolone antibiotic which is widely used against infections with gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa, Salmonella spp*., *Shigella spp.* and *Haemophilus spp*., but which is also effective against some gram-positive bacteria such as *Staphylococcus aureus* (Davis et al., 1996; Van Bambeke et al., 2005).

Ciprofloxacin's antimicrobial properties are caused by its inhibition of DNA gyrase and topoisomerase IV, enzymes which are essential for the unwinding of the supercoiled DNA strands during replication and transcription in procaryotes (Zhanel et al., 2002). However, the severe neurological and psychiatric side-effects that are described in humans (Tomé & Filipe, 2011) might indicate additional molecular modes and mechanisms of action of relevance for higher organisms. Nevertheless, prokaryotic organisms seem to be the most sensitive environmentally relevant organisms, with chronic EC50-values of ciprofloxacin from standard single species assays falling between 15 – 51 nmol/L for the cyanobacterium *Microcystic aeruginosa* (Halling-Sørensen et al., 2000; Robinson et al., 2005) and 241 nmol/L for the gram-negative bacterium *Pseudomonas putida* (Kümmerer et al., 2000). Maul and coworkers investigated the effects of ciprofloxacin on detrivorous stream microbial communities and macroinvertebrates (Maul et al., 2006) and found that 301.8 nmol/L (indicative values already at 30.2 nmol/L) impacted community catabolic profiles. Näslund et al. (2008) provided one of the few data on effects to marine microorganisms, and demonstrated that 1 700 nmol/L inhibited the degradation of pyrene by bacterial sediment communities by 50%. Biomass and richness of salt marsh microbial communities were affected by concentrations exceeding 6 036 nmol/L (Córdova-Kreylos & Scow, 2007). Denitrification by microbial communities in marine sediments was not affected by ciprofloxacin at concentrations of up to 3 018 nmol/L after 24 hours of exposure (Costanzo et al., 2005), and the authors speculate whether the compound precipitated, reducing its bioavailability.

Eukaryotic microalgae seem to be generally less sensitive than prokaryotes, EC50-values for the green algae *Selenastrum capricornutum* and *Pseudokirchneriella subcapitata* range between 9 000 – 56 000 nmol/L (Halling-Sørensen et al., 2000; Martins et al., 2012; Robinson et al., 2005).

Ciprofloxacin is commonly used in human medicine world-wide and is allowed as a veterinary drug, excluding extra-label use, in the US (Davis et al., 2009). Its prophylactic use in aquaculture seems to be on the rise, especially in developing countries such as China or Chile (Cabello, 2006; Cabello et al., 2013). The resulting emissions into the aquatic environment, and a substantial persistence in sewage treatment plants (removal efficiencies are around 60%) (Gros et al, 2010;

Jia et al, 2012) make ciprofloxacin therefore a commonly detected micropollutant of the aquatic environment. There are, however, substantial regional and local differences in ciprofloxacin concentrations in surface waters around the world, monitoring data range from 0.0018 nmol/L in Brazil (Locatelli et al., 2011) to almost 20 000 nmol/L in India, with a world-wide median concentration of 500 nmol/L (Hughes et al., 2013). Ciprofloxacin concentrations measured in European surface waters span between 0.04 nmol/L (Zuccato et al., 2010) and 0.407 nmol/L (Dinh et al., 2011). Concentrations on other continents are higher in average: between 0.091 (Focazio et al., 2008; Kolpin et al., 2002; Kolpin et al., 2004) and 1.09 nmol/L (Batt et al., 2006) were found in North America, while between 0.20 (Zhang et al., 2012) and 19 617 nmol/L (Fick et al., 2009) were detected in Asian surface waters. The use of ciprofloxacin in aquaculture (Cabello, 2006) might be a contributing factor to these comparatively higher concentrations. Other factors influencing environmental concentrations include variations in natural Fe^{3+}-concentrations which impact the oxidative degradation of ciprofloxacin and sunlight-dependent photolysis (Batchu et al., 2013).

Data on environmental occurrences of Ciprofloxacin in the marine environment are extremely rare and focus largely on Asia, often within an aquaculture setting. Ciprofloxacin concentrations near Hanoi shrimp aquaculture facilities were under the limit of quantification (0.015-0.030 nmol/L), although lomefloxacin, norfloxacin and ofloxacin / levofloxacin were detected in several samples, and ciprofloxacin was detected in concentrations of up to 3 nmol/L near pig farms in Thailand (Takasu et al., 2011). Ciprofloxacin was detected in all analyzed sediment samples (between 0.007 and 0.002 nmol/g) while norfloxacin was detected at concentrations up to 20 nmol/L in the Chinese coastal environment by He and coworkers (He et al., 2012).

Sulfamethoxazole

The sulfonamide antibiotic sulfamethoxazole is a bacteriostatic broad-spectrum antibiotic effective against gram positive and negative bacteria which is commonly used in human medicine (Baran et al., 2011). It affects bacteria by binding competitively to dihydropteroate synthetase, thereby inhibiting the conversion of para-aminobenzoate to dihydropteroate, the precursor of tetrahydrofolic acid which is essential for the synthesis of nucleic acids. Additionally, sulfonamides block the cross-membrane transport of glutamic acid, another essential component in the folic acid synthesis (Baran et al., 2011).

The acute bacterial toxicity of sulfamethoxazole is low, in concordance with its biosynthesis-related mechanisms of action: the EC50 of the marine bacterium *Vibrio fischeri* after short-term exposure is >395 nmol/L, the acute EC50 for the soil bacterium *Arthrobacter globiformis* is >500 000 nmol/L (Białk-Bielińska et al., 2011). Surprisingly, no studies have been published, to the best of our knowledge, on how environmentally relevant bacterial species are affected by chronic exposure to sulfamethoxazole. The chronic toxicity towards microalgae ranges from

100 nmol/L to 9 500 nmol/L (Białk-Bielińska et al., 2011; Eguchi et al., 2004; Ferrari et al., 2004; Isidori et al., 2005; Yang et al., 2008). The sensitivity differs between various algal groups in the following order: blue-green algae (*Synechococcus leopoliensis*) > green algae (*Pseudokirchneriella subcapitata* > diatoms (*Cyclotella meneghiniana*).

Freshwater biofilm communities react to an exposure of 1.97 nmol/L sulfamethoxazole with changes of the transcriptional activities of genes involved in replication and transcription as well as genes related to structural elements of the cell envelope and outer membrane (Yergeau et al., 2010). In a follow-up experiment, cyanobacterial activity in biofilms exposed to 1.97 nmol/L was shown to decrease significantly and photosynthesis-related transcripts were reduced substantially (Yergeau et al., 2012).

The median concentrations of sulfamethoxazole detected in chemical monitoring surveys in the freshwater aquatic environment was 0.32 nmol/L (Hughes et al., 2013). Concentrations reaching 5.6 nmol/L have been measured in the surface waters downstream a STP (Charmoise River, France) (Dinh et al., 2011). Even higher values have been measured in the Hanoi City Canal with up to 17 nmol/L (Hoa et al., 2011) and in the Nairobi river basin with approximately 79 nmol/L (K'oreje et al., 2012). Sulfamethoxazole concentrations of up to 0.32 nmol/L were detected in the Chinese marine environment (Zhang et al., 2012; Zheng et al., 2012).

2 Material and Methods

2.1 SWIFT periphyton test system

Toxicant effects were studied in a slightly modified version of the SWIFT periphyton test system, described by Porsbring et al. (2007). Two independent experiments were carried out between April and June 2010 at the Sven Lovén Centre for Marine Sciences at Kristineberg, located at the Gullmars fjord at the Swedish west coast. Periphyton communities were established on submerged glass discs (1.5 cm^2) that were mounted on polyethylene racks (Blanck and Wängberg, 1988) over eight days at a depth of approximately one meter in the bay of Kalvhagefjorden (long 11.4, lat 52.23).

Prior to use, the glass discs were washed in simmering concentrated nitric acid and rinsed in deionized water. Immediately before submersion the discs were rinsed with 70% ethanol.

After eight days the glass discs, now covered by a thin biofilm, were transported ashore while shielded against sunlight. Discs with non-typical and uneven biofilms were discarded. The discs were then gently cleaned using a Kleenex to remove any biofilm on the sides and back. Eight discs were then distributed into each glass test vessel, together with 200 mL nutrient amended seawater, with or without toxicant. The periphyton was then incubated for 72 respectively 96 hours, after which the periphyton was sampled for bacteria respectively algae. Incubation took

place on shakers (52 strokes/min) in growth chambers (FiTotron 800 H, Weiss-Gallenkamp Ltd., Loughborough, UK) with a day-light cycle illumination (36W, Lumilux de lux Daylight, Osram AB, Haninge, Sweden) of approximately 125 µmol photons m^{-2} s^{-1}. The light rhythm (15-18 hours light, 8-6 hours darkness) and temperature (7-15 °C) was set to correspond to the conditions at the sampling site.

2.2 Preparation of test solutions

Twelve hour prior to use, test medium for the SWIFT assay was prepared in 250 mL pyrex flasks, consisting of filtered (GF/F, Whatman plc) seawater amended with nutrients (0.7 mmol/L PO_4^{2-} and 8 mmol/L NO^{3-}) (Porsbring et al., 2007). Seawater was collected from the sampling site one day prior to the start of the experiment, filtered and stored in the dark at 4°C until use.

Final nominal concentrations were 5 to 9 054 nmol/L for ciprofloxacin as well as sulfamethoxazole, a concentration span that was designed to cover the whole span between 0 and 100% effect on the bacteria of the biofilm, following the results of previous rangefinding tests. Sulfamethoxazole stock solutions were prepared in methanol at a concentration of 10 mmol/L, i.e. 1 000 times the highest tested concentration. A dilution series was then prepared in methanol using positive displacement pipettes (Microman ®, Gilson). 200 µl aliquots of each dilution was then added into individual 250 mL pyrex flasks, after which the methanol was let to evaporate completely prior to the addition of 200 mL nutrient-amended GF/F filtered seawater. Ciprofloxacin stock solutions and dilution series were prepared in diluted sodium hydroxide (0.2 mol/L) at a maximum concentration of 1 mmol/L, i.e. 100 times the highest tested concentration. 2 mL of the stock solution was then added into 250 mL pyrex flasks. 200 mL GF/F filtered seawater was then added directly, after which the pH was adjusted to the original pH (8.0) of the sampled seawater with HCl. All toxicant dilutions were stirred for 12 hours in the dark prior to use.

New dilution series were prepared every 24 hours and were used for changing the test medium in the periphyton incubation vessels, in order to ensure constant toxicant and nutrient concentrations.

2.3 Biolog Ecoplates

Three glass discs were sampled after 72 hours incubation from each test vessel and pooled in order to harvest a sufficient amount of bacterial biomass. The discs were transferred to glass scintillation vials containing 20 mL medium which consisted of 25% GF/F filtered natural seawater, 75% NaCl solution (2.5% (w/v), the salinity of the seawater at the time of sampling) amended with 0.7 mmol/L PO_4^{2-} and 8 mmol/L NO^{3-} and the corresponding toxicant concentration. The use of a mix of 25% natural seawater and 75% NaCl solution is necessary in order to avoid precipitation of the tetrazolium dye in the Ecoplates. Scintilliation vials with the three glass discs and the medium were then sonicated three times (Bransonic® Ultrasonic

Cleaner 2510E-MT) over 15 seconds followed by rigorous shaking over 15 seconds. Afterwards, the suspension was filtered through sterilized paper (Kimcare, Kimberly-Clark Professional) into a sterile plastic petri dish from where 150 µl were pipetted into each well of a Biolog EcoplateTM using a multipipette.

The Biolog Ecoplates were finally incubated at 22 °C at 100% relative humidity over 96 hours. The optical density at 595 and 700 nm was recorded using a BioTek µQuant™ Microplate Spectrophotometer after 42, 48, 66, 72, 90 and 96 hours of incubation.

2.4 Pigment profiling

After 96 hours of incubation in SWIFT, five glass discs were collected and pooled in order to sample a sufficient amount of biomass. They were transferred into scintillation vials containing 2 mL of ice-cold extraction media (30% methanol, 30% acetone, 30% DMSO (all HPLC grade), 10% MilliQ-Water). The samples were shielded from light and stored at -18℃ until HPLC analysis which was performed 2-3 weeks after sampling.

Samples were extracted by repeatedly sonicating the scintillation vials in an ultrasonication bath (Bransonic® Ultrasonic Cleaner 2510E-MT) over 15 seconds followed by rigorous shaking over 15 seconds. The samples were then filtered through 0.45 µm syringe filters into HPLC vials.

Prior to injection 400 µl of the pigment extracts were diluted with 500 µl MilliQ-water and 100µl of the resulting mixture were injected into the HPLC (ThermoQuest, Thermo Scientific, Labex Instrument AB, Helsingborg, Sweden) equipped with a C18-column (Genetec, Kinetex™ 2.6 µm C18 100A 150x3.00 mm). Pigments were separated using a gradient (supporting information) following the method described by (Porsbring et al., 2007) and detected with a diode array detector (TSP UV6000LP) at 436 nm. The chromatograms were finally analyzed using using LaChrome software (v. 4.0, Thermofinnigan, Thermo Fisher Scientific, Waltham, MA, USA).

The peaks of the chromatograms were then analyzed and six pigments (Chlorophyll C, Fucoxanthin, Diadinoxanthin, Diatoxantin, Chlorophyll A, β-carotene) were identified, while the peaks for all additional pigments were simply described by their retention times. All data were finally documented in MS Excel 2010.

2.5 Data analysis – Carbon Source Utilization

The measured optical densities per Biolog Ecoplate, carbon source, well and incubation time were compiled in Microsoft Excel 2010 and further analyzed in SAS (Vers. 9.2, Calgary, US) according to the following pipeline:

1. Calculation of the background color development *bckgd*, as the median of the three wells without any added carbon source (blanks) per plate.

2. Calculation of the color development (corrected absorbance, *CorrOD*) for each well that contained an added carbon source and for each Ecolog incubation time. This provides a measure of the total catabolic activity per carbon source and is calculated as the optical density at 595 nm (absorbance of the tetrazolium dye after oxidation) corrected for background and turbidity of the sample (absorbance at 700 nm): corrected optical density for each well as:
   CorrOD =(OD595 – OD700)-bckgd
3. Calculation of the average carbon utilization, *AWC*, of each plate and Ecolog incubation time as $AWC = \frac{\sum CorrOD}{93}$ and its percent inhibition as $100\left(1 - \frac{AWC_{Treatment}}{AWC_{Control\ Average}}\right)$.
4. Estimation of the total metabolization of each individual carbon source in the time interval 0-100 hours. This was done by fitting a Weibull model in the form $CorrOD = 1 - \exp - \exp(\theta_1 + \theta_2 \times \log_{10}(time))$ to the data from each carbon source and then estimating the area under the curve, *AUC,* for this interval. Relative *AUC* values of each carbon source , i.e. $auc(rel)_i = auc_i / \sum_{i=2}^{32} auc_i$, were used for analyzing relative shifts in the community metabolization pattern.
5. *CorrOD* values after 72 hours incubation as well as *AUC* values were used for estimating concentration-response curves, which were also based on a Weibull fit, and for the ordination of the data. The latter was done via nonmetrical multidimensional scaling, an ordination method which reduces the multidimensional structure of the data into a 2-dimensional plot in which the distances between the individual samples reflect the multivariate dissimilarity between the original samples, e.g. (Clarke, 1999). We used Manhattan Distances (City-Block Metric), i.e. $MD = \sum_{i=2}^{32} \left| CorrOD_{j,i} - CorrOD_{k,i} \right|$ for estimating the dissimilarity between all pairs of samples *j*, *k*. Calculations were implemented with proc mds of SAS.
6. Significances between control and treatment in order to determine No Observed Effect Concentrations (NOECs) were calculated using Dunnett's test or the Anosim method as suggested by Clarke & Green (1988). Minimum significant differences were calculated following the approach of the US EPA (2010).

2.6 Data analysis – Pigment composition

Effects on pigment composition were calculated as percent inhibition in relation to the average control for total pigments, respectively the individual pigments.

2.7 Visualization

All graphs were plotted using Sigmaplot (Vers. 12.5) and Microsoft Excel 2010.

3. Results & Discussion

3.1. Toxicity of Ciprofloxacin to marine biofilms

Ciprofloxacin proved to be highly toxic to the bacteria in marine biofilm communities. The tested concentrations of 5 – 9 054 nmol/L covered the whole range from non-visible to full effects after a maximum incubation time of 96 hours in the Ecolog plates. All raw data are provided in appendix 1 (supporting information).

The effect of ciprofloxacin on the average well color (AWC), which describes the overall metabolic activity of the biofilm bacteria, is summarized in table 2, where the EC10, EC50 and EC90 values after a fit of the Weibull model are given for an incubation time of 42 to 96 hours in the Ecolog plates, together with the corresponding No Observed Effect Concentrations (NOECs) and Minimum Significant Differences (MSDs).

The assay becomes slightly less sensitive with increasing Ecolog incubation times, the EC50 for example increases from 413 to 600 nmol/L. This is caused by the fact that the metabolization of several carbon sources in the more active samples (controls and lower ciprofloxacin concentrations) comes to an end already after 50-70 hours. Extended incubation times therefore lead to decreasing differences between the controls and treatments, i.e. lower inhibition values.

At the same time the data become more robust as the measured raw color values increase and become less impacted by experimental (technical) noise. This is clearly visible in the decrease of the minimum significant difference (MSD) over time. After 42 and 48 hours the MSD is at approximately 30%, i.e. only an inhibition of 30% or greater would be detected as significantly different from the controls. After an exposure of 90 or 96 hours, the MSD drops to approximately 15%, i.e. the statistical power of the assay more than doubles using the outlined experimental design based on three controls and three treatment replicates.

The data recorded after 72 hours incubation were assessed as a good compromise between assay sensitivity and reliability. The following discussions will hence focus on the AWC data after 72 hours exposure in Ecolog plates (figure 1, fits to the data of the other incubation times are provided in the supporting information). The NOEC for ciprofloxacin (Dunnett's test, α=0.05) after this incubation time is 26 nmol/L, with a MSD of 19.24%. The corresponding regression-based EC10 is 46.1 nmol/L with an approximate 95% confidence interval of 20.2 – 102.2 nmol/L, the EC50 is 490.7 nmol/L (360-663 nmol/L).

This means that the carbon-source metabolization of marine periphyton was a more sensitive endpoint than denitrification, where a LOEC exceeding 3 018 nmol/L was observed for marine

bacterial communities (Costanzo et al., 2005) and microbial pyrene degradation in marine sediments (EC50 = 1 690 nmol/L) (Näslund et al., 2008). The bacteria in the present study are also more sensitive than the salt marsh communities investigated by (Córdova-Kreylos & Scow, 2007), in which ciprofloxacin concentrations exceeding 6 036 nmol/L caused shifts in community function. The same holds true for the study by (Maul et al., 2006), who recorded shifts in community catabolic profiles on stream microbial communities at concentrations of 301.8 nmol/L (indicative values at 30.2 nmol/L).

The catabolic activity in this study was unevenly distributed between the 31 carbon sources present on the Ecolog plates, and not all carbon sources were utilized by the biofilm communities. Seven carbon sources never reached a corrected absorbance (CorrAbs) of 0.05 or higher (C9, C11, C17, C19, C22, C24 and C31, for the chemical identification of the carbon sources see appendix 2 in the supporting information) and were classified as inactive. Maximum activities were observed for C6 and C13 in the controls, which reached CorrAbs values of 0.9 and 0.6, respectively, already after 72hrs incubation. Carbon Sources C14, C15, C25 and C32 were not used by the unexposed control communities at all, but were slightly (CorrAbs between 0.05 and 0.14) metabolized in communities treated with lower concentrations of ciprofloxacin (5- 26 nmol/L). However, this pattern was not present in all replicates and was restricted mainly to longer incubation times.

Time-integrated carbon source utilization was calculated using the area under the curve (AUC), which provides an overall measure of the carbon source utilization during the incubation time of 96 hours. Calculating the inhibition of the AUC in relation to ciprofloxacin exposure depends on a reliable Weibull fit (time vs. CorrAbs) for the untreated control communities, which was possible for 21 of the 31 carbon sources (appendix 3, supporting information). Plotting the average inhibition of the AUC then yields a concentration-response curve that is virtually identical to the AWC inhibition curve (figure 1.)

The CorrAbs(72hrs) values of all carbon sources as well as their AUC data also allow for a multivariate data exploration. The resulting nonmetric multidimensional scaling (nMDS) plot for AUC is given in figure 2. The multivariate Anosim-based NOECs for the CorrAbs and AUC of the 21 different carbon sources were both determined at 140 nmol/L, i.e. one concentration level higher than was previously described as the NOEC for the simple AWC development after 72hrs incubation (table 2).

The general movement of the data from the left to the right side of the plot with increasing concentrations reflects the general decrease in catabolic activity. However, the scatter along the y-axis at intermediate concentrations (140 and 323 nmol/L) indicates that additionally the carbon source utilization is re-arranged, driven by the different impacts of ciprofloxacin exposure on the different carbon sources (figure 2). This intermediate disturbance can be

highlighted by plotting the relative AUC values for the various concentrations (figure 3). Figure 4 exemplifies the different impact of various ciprofloxacin concentrations on the utilization of 4 selected carbon sources that represent different concentration-response patterns. The utilization of C3 slowly and monotonously decreases with increasing ciprofloxacin concentrations, while the utilization of C5 shows a stimulation in the middle concentration range with a subsequent sharp concentration-dependent decrease. C7 follows a similar trend, but is still utilized at high exposure concentrations, while the oxidation of C23 even increases up to an exposure of 300 nmol/L.

Effects on the algal part of the community were investigated in parallel, using chlorophyll *a* content as a first overall indicator for algal biomass, as well as the pigment composition for a more detailed analysis. No effects were visible up to the maximum tested concentration of 9 000 nmol/L. Available literature data for the green algae *Selenastrum capricornutum* and *Pseudokirchneriella subcapitat*a indicates a Lowest Observed Effect Concentration (LOEC) of 6 600 nmol/L and EC50 values between 9 000 and 56 000 nmol/L (Halling-Sørensen et al., 2000; Martins et al., 2012; Robinson et al., 2005), i.e. ciprofloxacin seems to cause toxic effects on eukaryotic algae only at high concentrations beyond those tested in the present study.

3.2. Toxicity of Sulfamethoxazole to marine biofilms

Also sulfamethoxazole, the second of the two tested antibiotics, exerted a clear concentration-dependent toxicity toward periphytic bacteria. As in the case of ciprofloxacin, the studied concentration range of 5 – 9 054 nmol/L gave rise to effects ranging from no visible effects to full effects in the Ecolog plates (all raw data are provided in appendix 1 of the supporting information).

A summary of the effects of sulfamethoxazole on the overall metabolic activity (AWC) of the periphytic bacteria is given in table 3. As in the case of ciprofloxacin, the assay becomes less sensitive with increasing incubation times in Biolog Ecoplates while the statistical power of the assay increases (i.e. the MSD decreases). EC50 values for example increase from 803 – 2 007 nmol/L over the time span of 42 to 96 hours of incubation, while the MSD decreases from 21% to 12%. As in the case for ciprofloxacin, we use the 72 hours values for the subsequent discussion, as a good compromise between both trends. At this time, the NOEC for sulfamethoxazole (Dunnett's test, α=0.05) was 140 nmol/L with a MSD of 14%. The EC10 is determined at 56 nmol/L (22 – 151 nmol/L) and the EC50 is 1 073 nmol/L (727 – 1732 nmol/L).

Out of the 31 carbon sources, five never reached a corrected absorbance (CorrAbs) of 0.05 or higher (C10, C19, C22, C24, C31) and were thus classified as inactive. Five additional carbon sources (C17, C20, C23, C27, C32) were inactive in the controls but metabolized to a low extent (0.051 – 0.265) in the treatments (5-323 nmol/L) mainly after long incubation times and not consistently in all replicates.

The integrated measures (AUC) of carbon source utilization over the 96 hours of incubation in Biolog Ecoplates were possible to calculate for 24 of the 31 carbon sources in untreated control communities (appendix 3 in the supporting information). As in the case of ciprofloxacin, the average inhibition of AUC results in a plot almost identical to the AWC inhibition curve (figure 5).

Multivariate analyses were performed using AUC data of the individual carbon sources and the corresponding MDS plot is shown in figure 6. The main trend in the data is once again a movement from the left to the right side of the graph, following increasing sulfamethoxazole concentrations. For concentrations up to 140 nmol/L there is a cluster of treatments and controls which perfectly agrees with the multivariate Anosim-based NOEC of 140 nmol/L as well as with the NOEC based on AWC data after 72 hours of incubation in Biolog Ecoplates.

No major re-arrangement of carbon source utilization was observed until concentrations well above the AWC-based EC50 of 1 073 nmol/L (figure 7). Between 1 711 and 3 937 nmol/L the utilization of some minor carbon sources stops, but it is not until the highest concentrations that there is a clear shift in utilization pattern. Under the assumption that differences in relative carbon source utilization are indicative of changes in community biodiversity (species composition, physiological activity of each species), one can conclude that sulfamethoxazole affects a broader range of species in a very similar manner, compared to ciprofloxacin, which exerts more specific effects. This is in concordance with the comparatively narrow spectrum of activity that ciprofloxacin shows against human pathogens, where it is predominantely used against gram-negative bacteria, while sulfamethoxazole has a broader spectrum of activity.

Sulfamethoxazole did not inhibit algal development, which corresponds to data from existing studies, which show toxic effects only in concentrations exceeding those tested in this study (see introduction above). In contrast, the content of all the major pigments was significant increased (appendix 4 in the supporting information, figure 8). The maximum stimulatory effect on the total pigment content of the community (75% above control level) was observed at the lowest tested concentration (5 nmol/L), followed by a decrease with increasing sulfamethoxazole concentration (figure 8). At a sulfamethoxazole concentration of 3 000 nmol/L pigment amounts were back to control levels. Multivariate analyses (nMDS) do not provide any indication of concentration-related shifts in relative pigment composition and we hence conclude that the elevated pigment levels are caused by a general increase in algal biomass. Similar stimulatory effects on algal growth (*Pseudokirchneriella subcapitata*) have previously been described by Yang et al. (2008) during sulfamethoxazole exposure, although to a lesser extent. Yergeau et al.(2010) observed increased amounts of transcripts related to cell envelope biogenesis and membranes after an exposure of limnic biofilms to 0.5 µg/L sulfamethoxazole.

The mechanism behind these stimulatory effects of sulfamethoxazole on periphytic algae is currently unknown. Although a smaller follow-up experiment confirmed the phenomenon in principle (data not shown), a full concentration-response curve is still lacking. This would require testing of lower concentrations, which was beyond the scope of the present study, as its concentration spacing was designed to describe the full concentration-response curve on the bacterial part of the community. It would be particularly interesting to investigate whether the stimulation is a direct effect on the exposed algae, or is an indirect effect caused by the inhibition of certain bacterial species, respectively changes in the bacterial metabolism.

3.3. Hazard and risk of ciprofloxacin and sulfamethoxazole to marine microbial communities

First toxic effects became visible at 26 nmol/L respectively 20.25 nmol/L ciprofloxacin (NOEC, respectively lower 95% confidence interval of the EC10) and 140 nmol/L respectively 22.21 nmol/L sulfamethoxazole. The biofilms are hence slightly less sensitive to ciprofloxacin than the cyanobacterium *Microcystis aeruginosa* (Halling-Sørensen et al., 2000; Robinson et al., 2005), with chronic EC50-values between 15 - 51 nmol/L and roughly of equal sensitivity as *Pseudomonas putida* for which an EC50 of 241 nmol/L was determined (Kümmerer et al., 2000). The lower EC50 in the biotest with *Microcystis* can be due to a higher inherent sensitivity of this organism, but is most likely also a result of the increased bioavailability of ciprofloxacin in a test system with a planktonic species that is grown in an inorganic medium. Additionally, ciprofloxacin might also be partly complexed by divalent cations found in the marine waters, further lowering the bioavailability compared to freshwater.

Ferrari and coworkers determined a chronic NOEC for the blue-green alga *Synechococcus leopolensis* for sulfamethoxazole of 23 nmol/L (Ferrari et al., 2004), which roughly corresponds to the sensitivity of periphytic bacteria in the present study. Taking the ecotoxicological data for ciprofloxacin and sulfamethoxazole together, it can be preliminary concluded that the EMA suggested strategy to use blue-green algae instead of bacteria for the environmental risk assessment of antibiotics (European Medicine Agency (EMA), 2006) seems sufficiently protective. It should, however, be noted that no data on the long-term toxicity of neither ciprofloxacin nor sulfamethoxazole to planktonic bacteria is available.

Monitoring data from the marine environment are extremely scarce for both compounds, in particular there are no data available for European coastal environments. Ciprofloxacin concentrations were below detection limits in open coastal water samples in China while sediment concentrations were around 0.002-0.007 nmol/g (He et al., 2012), implying a negligible risk for benthic marine microbial communities. However, ciprofloxacin concentrations near pig farms close to the Thai coastline were 3 nmol/L (Takasu et al., 2011), not even a factor of ten below the NOEC/EC10 as determined in this study. These data clearly

show that the large local and regional differences in exposure concentrations do not allow a general conclusion on the environmental risk of ciprofloxacin, but call for site-specific assessments, in particular in those countries in which ciprofloxacin is used for veterinary purposes in aquaculture. The same holds true for the limnic environment where assessment outcomes also differ fundamentally, depending on the actual exposure situation. Golet et al., (2002) for example concluded a low likelihood for adverse effects on aquatic habitats and on STP degradation processes in the Glatt Valley watershed (Switzerland) and similar conclusions were drawn for Italian STP effluents and receiving waters in the Po Valley (Al Aukidy et al., 2012). In contrast, Halling-Sørensen et al. (2000) estimated that ciprofloxacin might be posing an environmental risk in Europe, based on a PEC/PNEC assessment. Recently publications also concluded that ciprofloxacin might pose a substantial risk to sensitive aquatic organisms in Asian surface waters (Zhang et al., 2012). And finally, Langdon et al. (2010) investigated the risks from bio-solid amended soils and concluded that ciprofloxacin pose a risk for the aquatic environment after run-off events.

Sulfamethoxazole concentrations of up to 0.32 nmol/L were detected in the Chinese marine environment (Zhang et al., 2012; Zheng et al., 2012), which is a factor of 60 below the EC10-based ecotoxicological benchmark that was determined in the present study. Concentrations of 0.13 nmol/L in the Mekong delta (Managaki et al., 2007) and 0.19 nmol/L in the Victoria Harbour in Hongkong (Minh et al., 2009) provide similar values for sulfamethoxazole levels in the marine environment. It would hence depend on the applied assessment factor whether the final risk quotient for sulfamethoxazole would exceed 1. Unfortunately, the EMA guideline on environmental risk assessment of pharmaceuticals focusses exclusively on the freshwater environment. The monitoring data by Zhang et al., for the Bohai Sea and the Yellow Sea (Zhang et al., 2013) measured sulfamethoxazole concentrations of only up to 0.03 nmol/L in the Bohai Sea and the Yellow Sea in China which, based on the available ecotoxicological knowledge, would lead to the conclusion of only negligible risks of sulfamethoxazole to marine benthic microbial communities. However, sulfamethoxazole was detected in 98% of the analyzed samples – which raises the question on whether such individually non-toxic concentrations that occur very frequently might warrant attention during the toxicity assessment of complex pharmaceutical mixtures (Backhaus et al., 2008).

Acknowledgements

Funding by the EU Commission (FP7 project PHARMAS, contract 265346) and the Swedish Research Council (FORMAS) is gratefully acknowledged.

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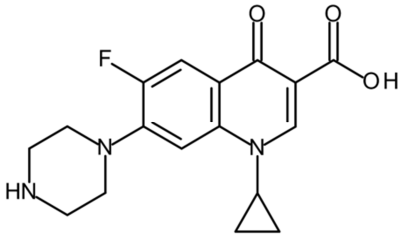
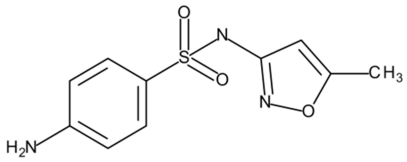
Substance	Cas	M.W (g/mol)	Molecular structure	pKa	logKow (pH=8)	Mode of action
Ciprofloxacin	85721-33-1	331.34		pKa_1 [a]: 5.76 pKa_2 [a]: 8.68	Kow[b]: 0.28	Inhibits the enzyme DNA gyrase and topoisomerase IV, affecting replication and transcription[c].
Sulfamethoxazole	723-46-6	253.28		pKa_1 [a]: 1.97 pKa_2 [a]: 6.16	Kow[b]: 0.89	Inhibits dihydropteroate synthetase (DHPS) which catalyses the conversion of para-aminobenzoate to dihydropteroate, a precursor of folate[d].

References: [a] www.drugbank.ca, acquired 20/11/13; [b] Physprop Database: http://esc.syrres.com/fatepointer/search.asp, acquired 20/11/13; [c] Zhanel et al., 2002; [d] Baran et al., 2011

Table 1 - Physico-characteristics of sulfamethoxazole and ciprofloxacin

Ecolog Time	$\hat{\theta}_1$	$\hat{\theta}_2$	EC10	EC50	EC90	MSD in %	NOEC(AWC)
42	-5.93682	2.12928	53.87 [22.21 – 130.72]	413.09 [283.72 – 604.85]	1513.11 [890.82 – 2460.08]	27.1	140
48	-4.41337	1.59853	22.55 [8.51 - 59.10]	340.11 [227.27 – 502.83]	1917.13 [1051.81 – 3366.52]	25.84	140
66	-5.7431	1.99087	56.80 [27.22 – 119.19]	501.90 [374.06 – 663.34]	2012.05 [1288.32 – 3069.8]	16.91	26
72	-5.30521	1.83539	46.18 [20.25 – 102.81]	490.71 [360.58 - 663.35]	2212.75 [1361.79 – 3492.32]	19.24	26
90	-6.59987	2.24278	86.96 [45.63 – 163.09	601.58 [466.77 - 769.02]	2063.41 [1413.31 – 2957.71]	14.24	26
96	-6.11734	2.06966	73.86 [33.96 – 157.19]	600.66 [441.71 – 812.72]	2284.01 [1439.43 – 3558.87]	13.09	26

Table 2 - Effect on the overall metabolic activity of periphytic bacteria exposed to ciprofloxacin. The activity is described as the inhibition of the average well color (AWC) in Biolog Ecoplates. Estimated parameters of the Weibull fits ($\hat{\theta}_1$, $\hat{\theta}_2$) that were used for estimating EC10, EC50 and EC90 values are given together with their approximate 95% confidence intervals, as well as the No Observed Effect Concentrations (NOECs) and Minimum Significant Differences (MSDs).

Ecolog Time	$\hat{\theta}_1$	$\hat{\theta}_2$	EC10	EC50	EC90	MSD in %	NOEC(AWC)
42	-6.74206	2.19464	111.34 [43.98 – 254.05]	803.63 [582.77 – 1312.99]	2832.00 [1493.65 – 4959.43]	21.47	140
48	-5.78725	1.82771	86.13 [37.25 – 189.08]	924.42 [675.31 – 1362.46]	4194.95 [2283.31 – 7309.75]	13.93	140
66	-5.34104	1.61646	81.66 [35.90 – 182.23]	1195.15 [858.57 – 1797.50]	6608.79 [3556.36 – 7752.14]	13.43	140
72	-4.90305	1.49700	56.16 [22.22 – 151.51]	1072.55 [727.21 – 1731.82]	6708.79 [3303.55 – 7713.37]	14.31	140
90	-5.65470	1.62008	126.28 [45.63 – 335.02]	1837.16 [1132.27 – 3492.28]	10120.17 [4438.54 – 8315.19]	13.22	140
96	-6.66417	1.90685	206.40 [97.27 – 425.92]	2007.50 [1412.96 – 3244.21]	8555.62 [4521.75 – 8239.85]	12.33	140

Table 3 - Effect on the overall metabolic activity of periphytic bacteria exposed to sulfamethoxazole. The activity is described as the inhibition of the average well color (AWC) in Biolog Ecoplates. Estimated parameters of the Weibull fits ($\hat{\theta}_1$, $\hat{\theta}_2$) that were used for estimating EC10, EC50 and EC90 values are given together with their approximate 95% confidence intervals as well as the No Observed Effect Concentrations (NOECs) and minimum Significant Differences (MSDs).

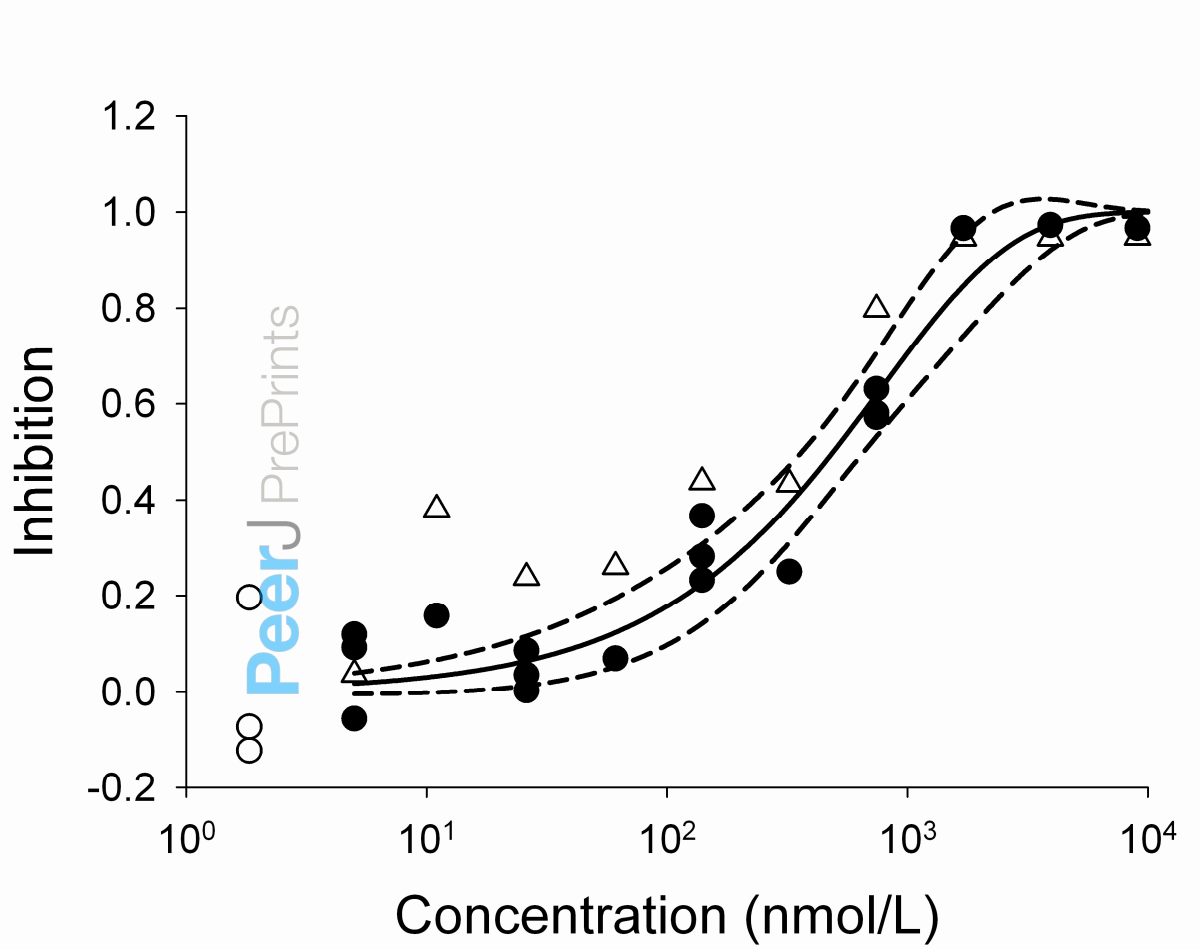


Figure 1

Comparison between concentration-response data after ciprofloxacin exposure for AWC (72 hours) (●) and the area under the curve (Δ). Solid line gives the Weibull fit to the AWC data, dashed lines their approximate 95% confidence intervals.

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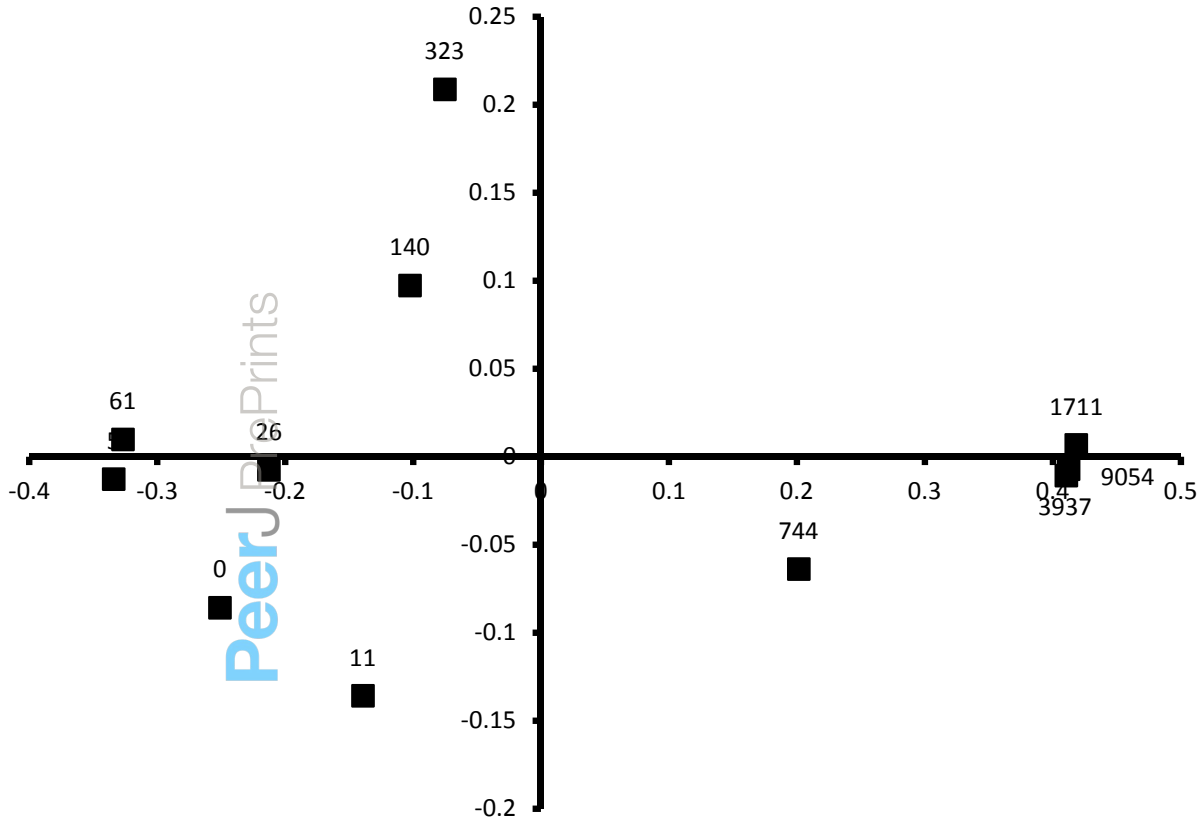

Figure 2

Nonmetric multidimensional scaling (nMDS) showing the effects on metabolic activity (AUC) after ciprofloxacin exposure.

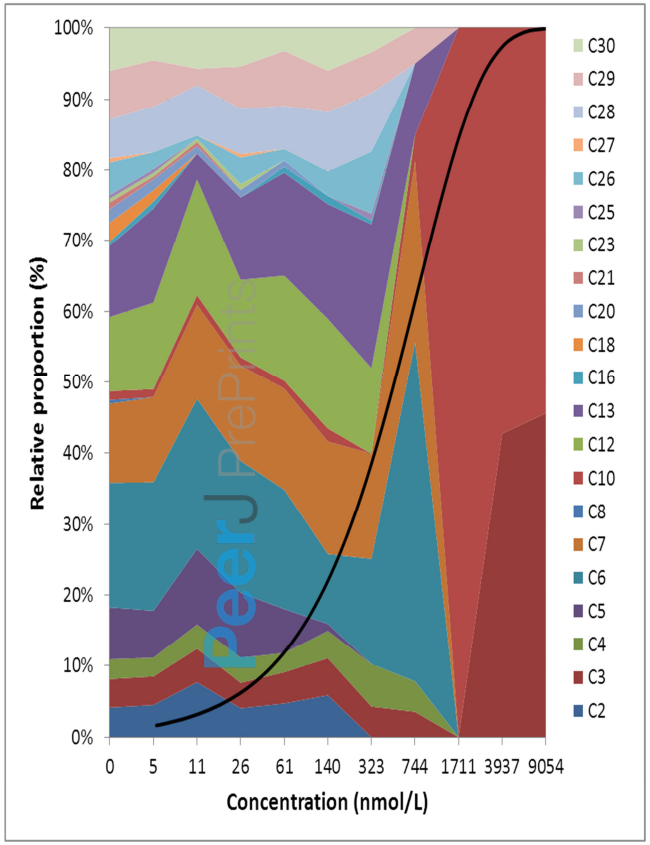

Figure 3

Relative AUC of individual carbon sources and the corresponding AWC (72 hours) Weibull function (continuous line) plotted against ciprofloxacin exposure concentration.

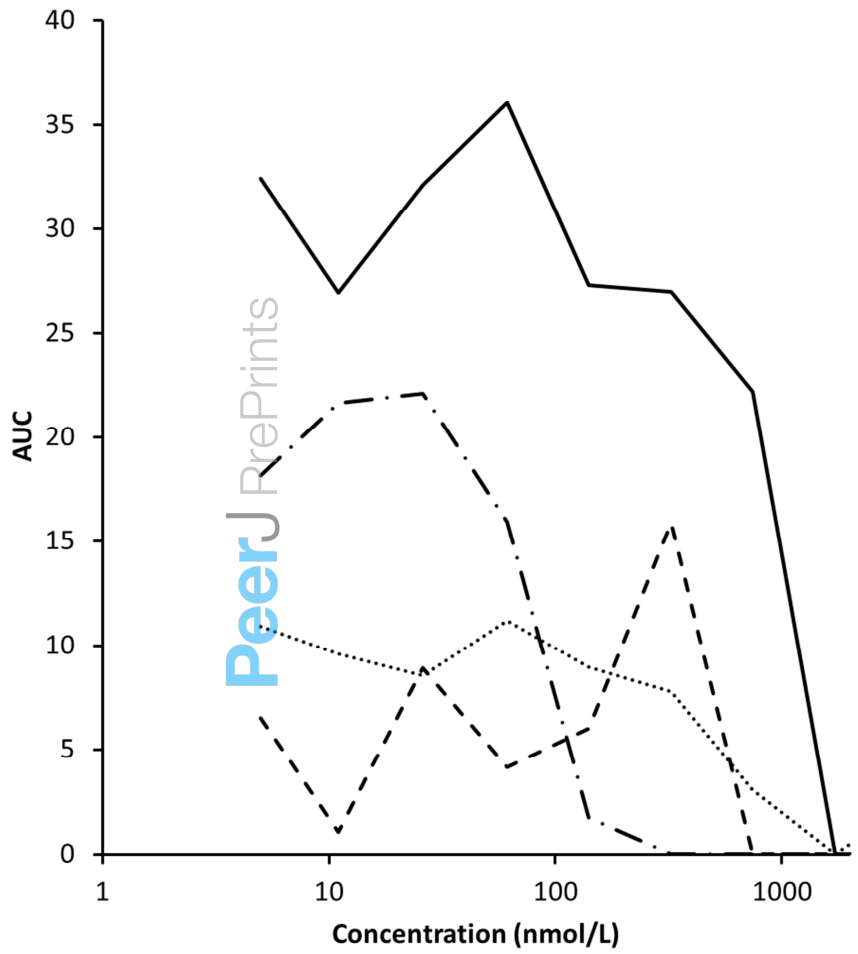

Figure 4

Development of the relative AUC with increasing concentrations for the individual carbon sources C3 (•••), C5 (—•—), C7 (——) and C26 (- - -).

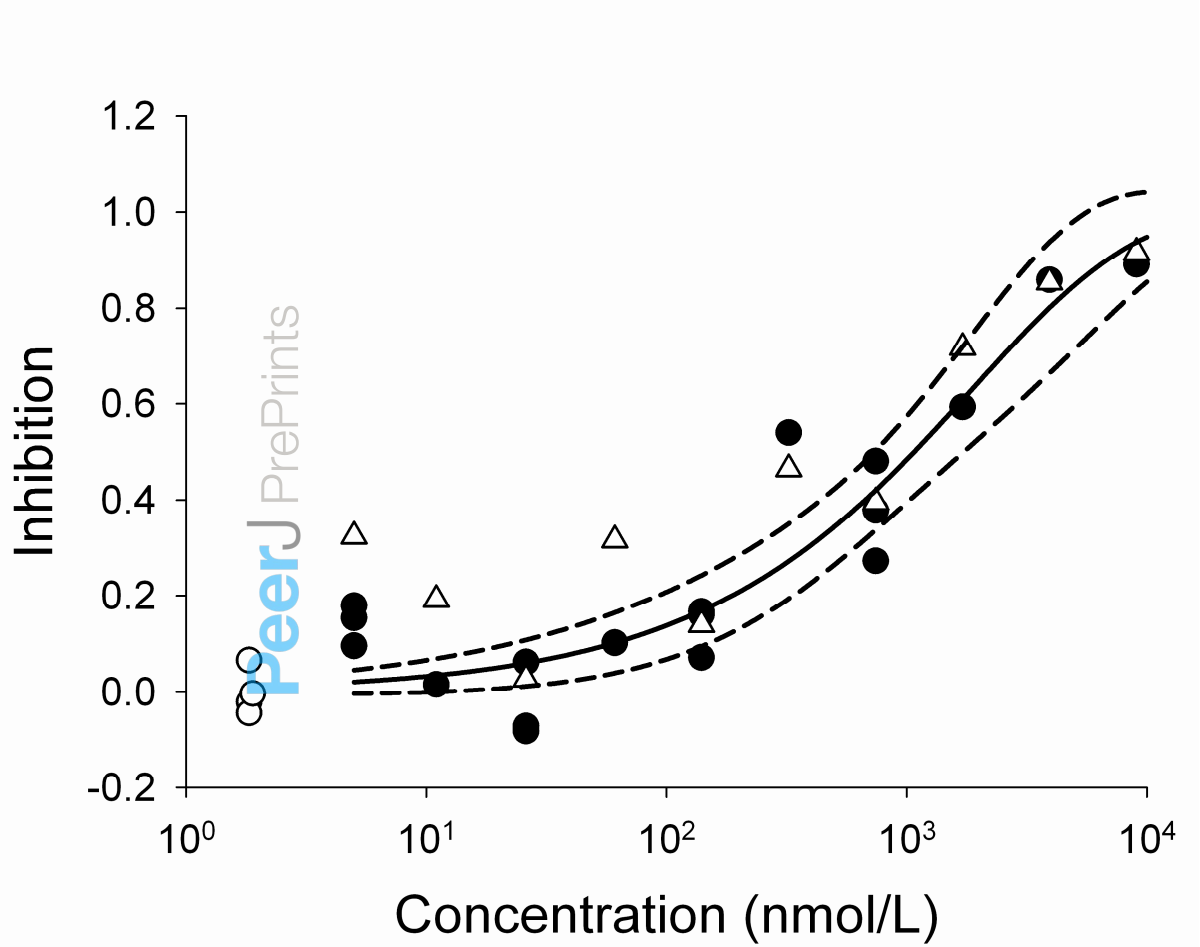

Figure 5

Comparison between concentration-response data after sulfamethoxazole exposure for AWC. (72 hours) (●) and the area under the curve (Δ) and average well colour with corresponding AWC (72 hours) controls (○).Solid line gives the Weibull fit to the AWC data, dashed lines their approximate 95% confidence intervals.

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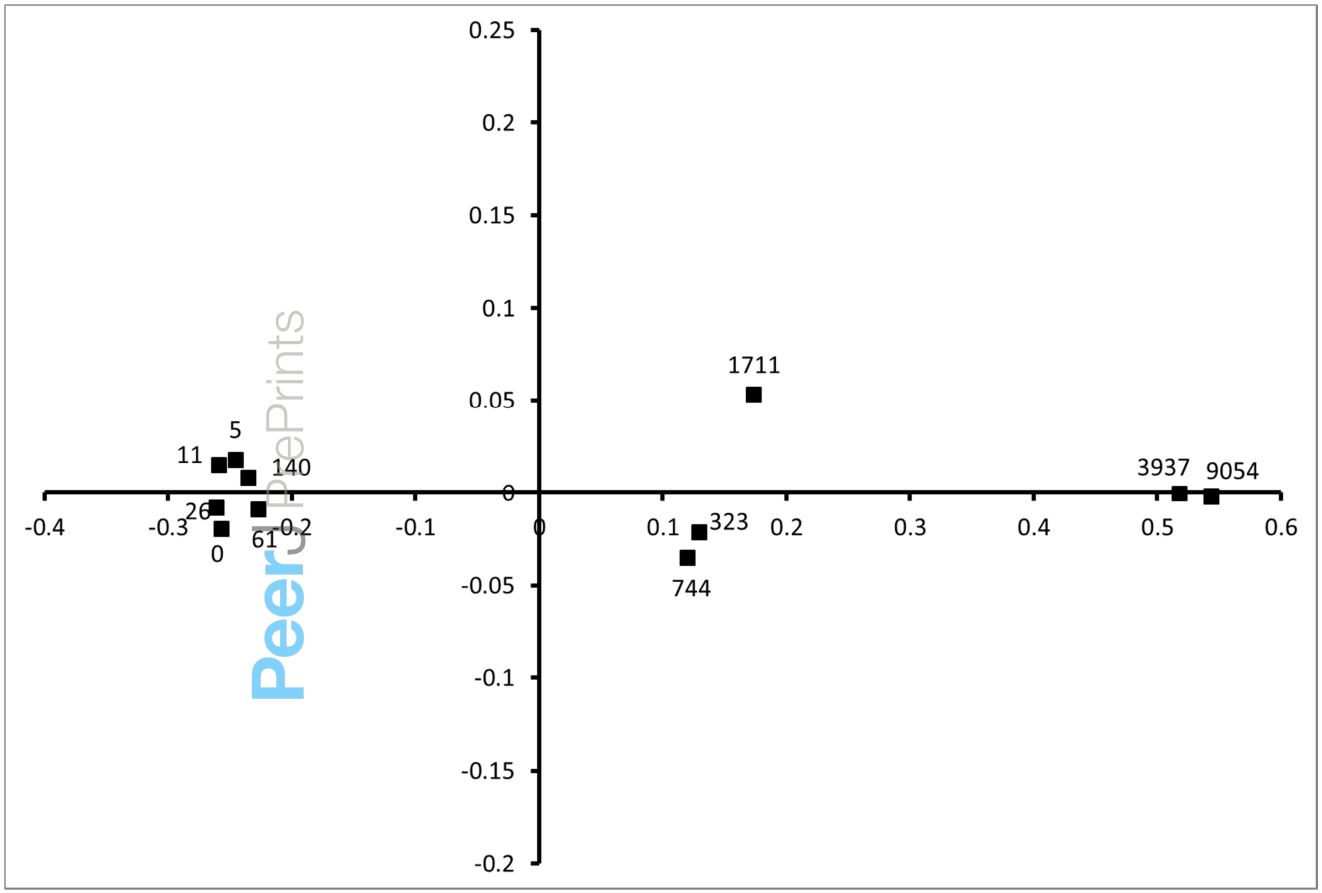

Figure 6

Nonmetric multidimensional scaling (nMDS) showing effects on metabolic activity (AUC) after sulfamethoxazole exposure.

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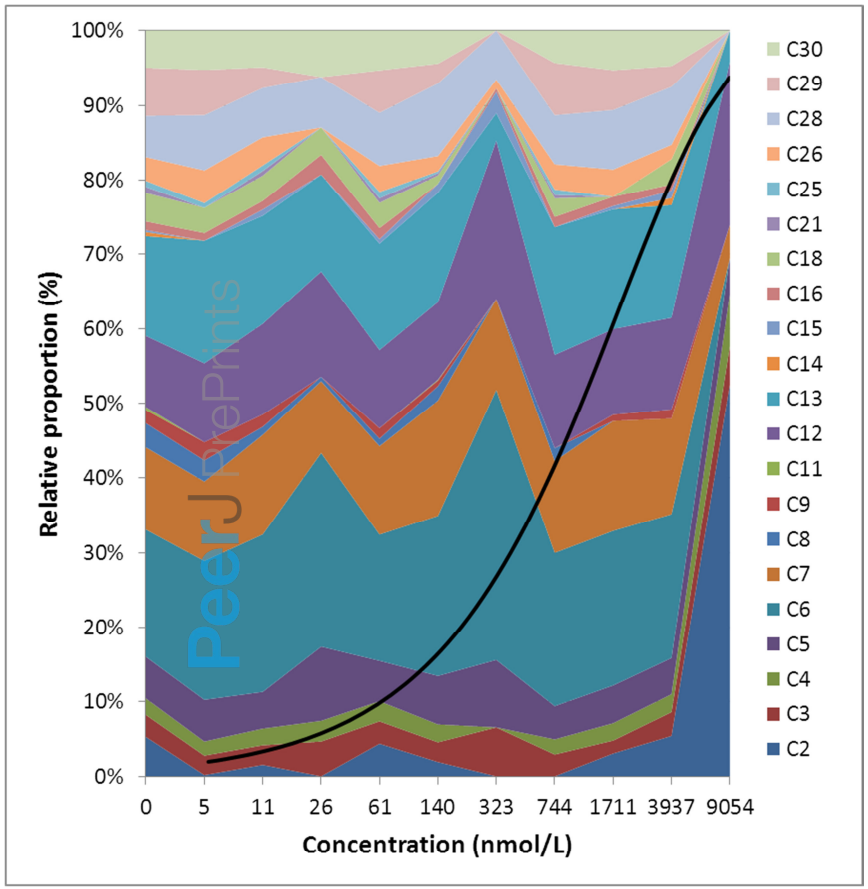

Figure 7

Relative AUC of individual carbon sources and the corresponding AWC (72 hours) Weibull function (continuous line) plotted against sulfamethoxazole exposure concentration.

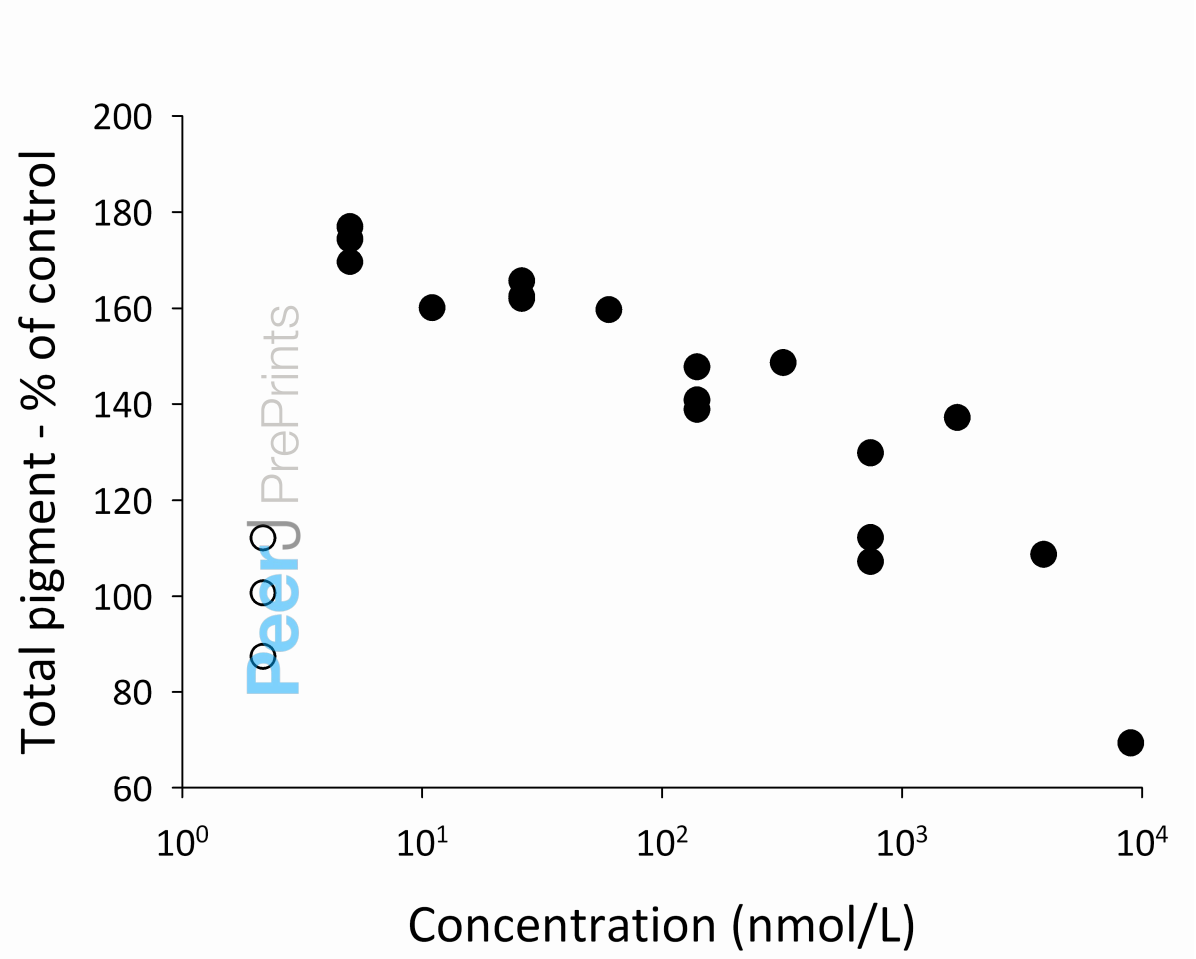

Figure 8

Total pigment content in sulfamethoxazole treated (●) periphyton communities compared to controls (○).