Running head: Triclosan affects 16S composition of marine periphyton

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Title of article: Triclosan changes community composition and selects for specific bacterial taxa in marine periphyton biofilms in low nanomolar concentrations

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ABSTRACT

The antibacterial agent Triclosan (TCS) is an ubiquitous environmental contaminant due to its widespread use. Sensitivity to TCS varies substantially among eu- and pro-karyotic species and its risk for the marine environment remains to be better elucidated. In particular, the effects that TCS causes on marine microbial communities are largely unknown. In this study we therefore used 16S amplicon rDNA sequencing to investigate TCS effects on the bacterial composition in marine periphyton communities that developed under long-term exposure to different TCS concentrations. Exposure to TCS resulted in clear changes in bacterial composition already at concentrations of 1 to 3.16 nM. We conclude that TCS affects the structure of the bacterial part of periphyton communities at concentrations that actually occur in the marine environment. Sensitive taxa, whose abundance decreased significantly with increasing TCS concentrations, include the *Rhodobiaceae* and *Rhodobacteraceae* families of *Alphaproteobacteria*, and unidentified members of the Candidate division OD1. Tolerant taxa, whose abundance increased significantly with higher TCS concentrations, include the families *Erythrobacteraceae* (*Alphaproteobacteria*), *Flavobacteriaceae* (*Bacteroidetes*), *Bdellovibrionaceae* (*Deltaproteobacteria*), several families of *Gammaproteobacteria*, and members of the Candidate phylum BD1-5. Our results demonstrate the variability of TCS sensitivity among bacteria, and the importance of extending the ecotoxicological assessment of antimicrobial chemicals, such as TCS, to non-cultivable bacteria and natural communities.

Keywords: amplicon sequencing, metabarcoding, rRNA, marine toxicity tests, microbial toxicology, ecological risk assessment

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INTRODUCTION

Triclosan (TCS, 5-chloro-2-(2,4-dichloro-phenoxy)-phenol, CAS 3380-34-5) is an antibacterial agent commonly used in personal care products (PCP), household cleaning products, textiles, and plastics. The annual usage of TCS in Europe and USA has been estimated at 300 tons in 2005 (Halden and Paull 2005), increasing to 450 tons in 2010 (SCCS 2010). Approximately 85% of the TCS production is used in PCPs (SCCS 2010), and the compound is therefore discharged continuously into the aquatic environment. TCS has become an ubiquitous pollutant, occurring in all environmental compartments (Bedoux et al. 2012). As reviewed by Bedoux and colleagues (2012), TCS concentrations of up to 0.024, 0.047 and 0.1 nM have been reported for coastal waters in Europe, USA, and China, respectively. Furthermore, 0.036 nM was detected in the coastal waters outside Singapore (Bayen et al. 2013), 0.55 nM was measured at the Swedish west coast (Remberger et al. 2002), and a concentration as high as 1.1 nM was detected in Cadiz Bay in Spain (Pintado-Herrera et al. 2014). Given this widespread occurrence, von der Ohe (2012) identified the compound as a priority pollutant in freshwater ecosystems, and Maruya et al. (2015) labeled TCS a contaminant of emerging concern for the marine environment, based on sediment core data in which TCS concentrations increased from the early 1970s to 2007. The environmental risk of TCS has been assessed with conflicting results. A probabilistic risk assessment by Capdevielle et al. (2008) concluded that the risks from TCS at environmental concentrations were negligible, whereas several other studies indicated clear environmental hazards and risks (Brausch and Rand 2011; 2009; Reiss et al. 2002; Wilson et al. 2003; von der Ohe et al. 2012). In a recent global assessment Guo and Iwata (2017) calculated ratios of exposure and hazard (risk quotients) between 0.49 – 9.5 for the aquatic environment, differing between countries. It should be pointed out, that those assessments largely fail to assess risks to the marine environment, due to a lack of adequate data, in particular for marine bacteria.

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The mechanism of action of TCS in bacteria has been identified as the inhibition of type II fatty acid synthesis through binding to the enoyl-acyl carrier protein (enoyl-ACP) reductase (McMurry et al. 1998). Different bacterial species have different conformations of the TCS binding site in the enoyl-ACP reductase which affects the affinity to TCS and thereby TCS sensitivity (Pidugu et al. 2004). Johnson et al. (2009) also report a broad range of bacterial sensitivities to TCS, ranging from 100 nM to 300 µM. Although the inhibition of fatty acid synthesis is a well-described mechanism of action, Escalada et al. (2005) concluded that the toxicity of TCS to bacteria cannot be explained solely by this mechanism. Studies have also shown that TCS induces cell membrane destabilization (Villalaín et al. 2001), inhibits enzymes in the glycolysis pathway, and uncouples the membrane potential in mitochondria (Newton et al. 2005; Phan and Marquis 2006). The toxicity to different prokaryotic species is thus far from trivial to predict. Basing the hazard estimation of TCS on only a few selected species will likely result in highly biased results that might not be representative of natural bacterial communities.

Previous studies have investigated the effect of TCS on freshwater or estuarine bacterial communities (Drury et al. 2013; Johnson et al. 2009; Lawrence et al. 2009; Lubarsky et al. 2012; Nietch et al. 2013; Proia et al. 2011; Proia et al. 2013; Ricart et al. 2010). Studies of TCS effects on marine bacterial communities are, however, scarce. Johansson et al. (2014) studied effects of TCS on bacterial carbon utilization in marine periphyton communities, in which TCS did not inhibit the carbon utilization and did also not cause changes in bacterial functional diversity at concentrations of up to 10 µM. Eriksson et al. (2015) also studied effects of TCS on carbon utilization in marine periphyton using flow-through microcosms in which TCS did not cause effects at concentrations of up to 1 µM. These studies, however,

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97 focused mainly on gross parameters of bacterial function. They do not provide insights into
98 the impact of TCS on microbial diversity. The present study was implemented to provide such
99 information, in order to improve the mechanistic basis for the risk assessment of TCS in
100 marine ecosystems.
101
102 Amplicon sequencing, also known as metabarcoding, enables the analysis of bacterial
103 communities by analyzing amplicons of marker regions, such as 16S rRNA genes. In contrast
104 to the cultivation of individual strains or metabolic assays such as bacterial carbon utilization,
105 metabarcoding provides an integrative view of a community, including its structure and its
106 individual members (for example Tan et al. 2015). Today, modern sequencing platforms offer
107 massive sequencing depth, which has tremendously increased the sensitivity of amplicon
108 sequencing and allows to detect less and less abundant taxa. Consequently, amplicon
109 sequencing can identify changes in the composition of a bacterial community that would be
110 undetectable with traditional methods such as of microscopy, various molecular fingerprinting
111 techniques (e.g. Terminal Restriction Fragment Length Polymorphism and Denaturing
112 Gradient Gel Electrophoresis), or metabolic assays. There are several examples where
113 metabarcoding has been used to pin-point effects in microbial communities caused by
114 exposure to toxicants (e.g. Chariton et al. 2014; Eriksson et al. 2009; Pascault et al. 2014).
115
116 In this study we used 16S rDNA amplicon sequencing to investigate the ecotoxicological
117 effects of TCS on marine periphyton communities that were established under selection
118 pressure from different concentrations of TCS in a flow-through microcosm experiment. Our
119 results show that community structure and the abundance of specific taxa are significantly
120 affected already at a TCS concentration as low as 3.16 nM. Particularly sensitive taxa include
121 the Candidate division OD1 and the Alphaproteobacterial families *Rhodobacteraceae* and

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Rhodobiaceae. We also identify several highly tolerant taxa, in particular the Gammaproteobacterial families *Alteromonadaceae, Oceanospirillaceae,* and *Thiotrichaceae*, and the Flavobacterial family *Flavobacteriaceae*. Taken together, these results demonstrate that 16S rRNA amplicon sequencing is an effective tool for detecting effects from toxicants in complex bacterial communities.

MATERIAL AND METHODS

Flow-through microcosm experiment

A flow-through experiment was performed at the Sven Lovén Centre for Marine Sciences, Kristineberg on the west coast of Sweden from the 26st of September until the 14th of October 2012. The setup of the microcosm system, the operation and implementation of the triclosan (TCS, 5-chloro-2-(2,4-dichloro-phenoxy)-phenol, CAS-No. 3380-34-5) exposure and the periphyton colonization, as well as the details about the chemical analyses of TCS, the responses of various endpoints (photosynthesis, pigment content, and carbon utilization), are reported in Eriksson et al. (2015). In short, seawater, with its indigenous microbiota, was continuously pumped into 20 L glass aquaria from 1.5 meters depth in the Gullmar fjord. To prevent larger organisms from entering the microcosms, the seawater was filtered through a nylon net with a 1 mm mesh. Periphyton communities colonized and grew on 10.8 cm^2 (1.4 * 7.7 cm) glass slides mounted vertically in polyethylene holders. Prior to periphyton establishment, the discs were boiled for 10 min in concentrated nitric acid, rinsed in de-ionised water, and rinsed again in 70% ethanol. The seawater flow rate in the microcosms was 220 mL min^{-1} giving a mean residence time of approximately 90 min. TCS solutions made in de-ionized water were also pumped into the system creating constant TCS nominal exposures of 0, 0.316, 1, 3.16, 10, 31.6, 100, 316, and 1000 nM. The same amount of de-ionized water without TCS was pumped into the control microcosms. The nominal TCS concentrations were

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close to the analysed TCS concentrations (Eriksson et al. 2015). Hence, nominal concentrations are presented in the following. The exposure concentrations and the number of replicates per treatment are shown in Table 1.

Periphyton sampling and DNA extraction

Periphyton biofilms were scraped off with a scalpel from 17 glass slides (183 cm^2) per microcosm into filter-sterilized water from the respective microcosm. The biofilm material was pelleted by centrifugation at 15000 g for 8 minutes, snap-frozen in liquid nitrogen, and stored at -80 °C. DNA extraction was performed using the FastDNA spin kit for soil (MP Biomedicals, Santa Ana, USA) due to the high extraction yield obtained with this kit (Corcoll et al. 2017). DNA extraction was done following the protocol of the manufacturer. Extracted DNA was quantified by fluorescence with the PicoGreen assay (Quant-iT PicoGreen, Invitrogen). The integrity of the extracted DNA was assessed with a 2200 TapeStation instrument (Agilent Technologies, Santa Clara, USA), and contamination by proteins and carbohydrates was quantified as 260/280 nm and 260/230 nm absorbance ratios, respectively, using a NanoDrop 2000 instrument (Thermo Scientific, Wilmington, USA).

PCR, library preparation, and sequencing

Amplicon 16S rDNA sequences were obtained through a two-step PCR approach as previously described (Sinclair et al. 2015) with some modifications. In short, each sample was first amplified in duplicates using primers targeting the variable 16S regions V3 and V4, equipped with parts of the Thruplex Illumina sequencing adapter. The forward primer: ACACTCTTTCCCTACACGACGCTCTTCCGATCT-NNNN-CCTACGGGNGGCWGCAG and reverse primer: AGACGTGTGCTCTTCCGATCT-

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GACTACHVGGGTATCTAATCC (Andersson et al. 2010) were used. Duplicates were pooled after purification using the Agencourt AMPure XP system (Beckman Coulter) as recommended by the manufacturer. The pooled duplicates were used as templates in a second PCR step using primers equipped with a 7-base index in the Illumina sequencing adapters for multiplexing. The forward primer

AATGATACGGCGACCACCGAGATCTACAC-[i5 index]-ACACTCTTTCCCTACACGACG and reverse primer

CAAGCAGAAGACGGCATACGAGAT-[i7 index]-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT) were used to obtain amplicons with complete Thruplex adapters for Illumina sequencing. After sample purification using the Agencourt AMPure XP kit, and quantification by fluorescence with the PicoGreen assay (Quant-iT PicoGReen, Invitrogen), samples were pooled in equimolar amounts. The pooled samples were sequenced at the SciLifeLab SNP/SEQ next generation sequencing facility (Stockholm, Sweden) using Illumina MiSeq with a 2x300 bp chemistry following the protocols of the manufacturer.

Bioinformatics and statistics

The raw sequence data were analyzed with a pipeline for de-multiplexing and sequence-pair assembly implemented in Python (Sinclair et al. 2015). PANDAseq (Masella et al. 2012) was used to assemble the overlapping paired ends (using default settings). Quality filtering removed any sequences with missing primers or unassigned base pairs (Sinclair et al. 2015). Sequences were then clustered into operational taxonomic units (OTUs) based on a 3% dissimilarity clustering with UPARSE, and singleton OTUs were removed (Edgar 2013). Taxonomic annotation was performed using CREST (Lanzen et al. 2012) and the SilvaMod

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195 database provided by the online resource SILVA (Quast et al. 2012). The raw sequence data
196 were deposited at NCBI under the BioProject accession number PRJNA320539, and with the
197 SRA Experiment accession numbers SRX1744264 - SRX1744266, SRX1744269 -
198 SRX1744273 and SRX1744275 - SRX1744279.

199

200 The Bray-Curtis distance, richness, and evenness were estimated using data rarified to the
201 lowest sequencing depth (n=11,988). Differentially abundant OTUs were identified using the
202 DESeq2 R package. Two types of analyses were implemented: i) pair-wise analysis between
203 the untreated controls and the samples that were exposed to 3.16, 31.6, and 316 nM TCS, and
204 ii) regression analysis between OTU counts and TCS concentration. The resulting p values
205 were adjusted for multiple testing according to Benjamini-Hochbergs false discovery rate
206 (FDR). OTUs with an FDR of less than 0.05 were considered statistically significant. Venn
207 diagrams were used to describe the overlap of the significantly different OTUs between
208 concentrations. Overrepresented taxa among the significant OTUs (FDR<0.05) were tested
209 using Fishers' exact test at the phylum, class, order, and family levels. All analyses were
210 performed in the statistical language R version 3.4 (R Development Core Team 2008).

211

212 **RESULTS AND DISCUSSION**

213 *Results from next-generation sequencing*

214 Sequencing using the Illumina platform resulted in 313,855 16S reads, with an average of
215 24,143 reads per microcosm (Table 1). The sequence reads from all microcosms were
216 clustered into 1,789 OTUs, with an average of 892 OTUs per sample. The number of OTUs
217 from each treatment is presented in Table 1. Taxonomic annotation of the OTUs revealed a
218 high diversity with 31 prokaryote phyla present in all microcosms (Supplementary Table 1).

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219 The phyla *Proteobacteria* and *Bacteroidetes* dominated the communities and constituted 51%
220 and 29% of the OTUs, respectively. These phyla also contained the highest richness with 654
221 and 449 OTUs, respectively (Supplementary Table 1).

222

223 *TCS effects on community composition*

224 TCS exposure clearly changed the OTU distribution of exposed biofilms. The Bray-Curtis
225 dissimilarity between control and exposed communities increased monotonously with
226 increasing TCS concentrations (Fig. 1A). Significant increases in the Bray-Curtis dissimilarity
227 were already observed after an exposure to 1 and 3.16 nM TCS (average difference of 0.21
228 between the treatments and controls, p=0.0279, Welch test). This patterns is confirmed in the
229 Principal Component Analysis (PCA) (Fig. 1B). Moreover, both the OTU richness and
230 evenness of the communities were significantly reduced at 31.6 and 316 nM (Supplementary
231 Figures 1 and 2).

232

233 The relative abundance of a total of 164 OTUs was significantly affected (FDR<0.05) by an
234 exposure to 3.16 nM, 31.6 nM or 316 nM TCS (Supplementary Table 3). The number of
235 significantly affected OTUs increased with TCS concentration (Fig. 2A). 10 of the 12 OTUs
236 whose abundance was significantly changed by an exposure to 3.16 nM TCS were also
237 significantly affected at higher exposure levels (Fig. 2B). The abundance of 88 OTUs was
238 significantly affected at both 31.6 nM and 316 nM, but 29 and 53 OTUs showed such
239 difference only in the 31.6 nM and 316 nM treatments, respectively, giving these treatments a
240 distinct community profile. The number of OTUs with a significant increased abundance in
241 the treatments compared to the controls were 2, 46, and 70 for 3.16 nM, 31.6 nM, and 316
242 nM, respectively. The corresponding numbers for OTUs with significant decreased abundance

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in these treatments compared to the controls were 10, 91, and 91, respectively. We also performed regression analysis to identify OTUs whose abundance correlated with TCS exposure. In total 171 OTUs were found to be significantly correlating with TCS exposure (FDR<0.05), of which 83 increased and 88 decreased with increasing TCS concentration (Supplementary Table 2).

Members of the Candidate division OD1 are sensitive to triclosan

TCS effects were visible already at the phylum level, where OTUs of the Candidate division OD1 decreased substantially at a concentration as low as 1 nM (Fig. 3). Fishers' exact test confirmed that the phylum Candidate division OD1 is indeed particularly sensitive, with the abundance of 28% of its taxa showing a significant negative correlation with TCS concentrations ($p=4.0\times10^{-6}$, Table 2). Also in the pairwise comparison between the exposed and the control communities, the Candidate division OD1 was identified as being sensitive, with the abundance of 4.9%, 28%, and 22% of its taxa being significantly reduced after exposure to 3.16 nM ($p=0.029$, Table 3), 31.6 nM ($p=7.4\times10^{-5}$), and 316 nM ($p= 0.0027$) TCS, respectively. The Candidate division OD1, also called Parcubacteria, is a diverse group of bacteria, suggested to constitute a superphylum (Solden et al. 2016). Its members have small genomes and reduced metabolic capabilities, lacking genes for the biosynthesis of cofactors, nucleotides, amino acids and fatty acids. Furthermore, Parcubacteria have been suggested to be symbiotic, commensal, or parasitic organisms (Nelson and Stegen 2015). For example, the bacterium *Candidatus Sonnebornia yantaiens* was found to be endosymbiotic in the algae *Chlorella*, which in turn was endosymbiontic in the ciliate *Paramecium bursaria* (Gong et al. 2014). As periphyton biofilms also harbor a high diversity of eukaryotic organisms, it might be an excellent habitat for such lifestyles. In addition to the TCS-sensitivity demonstrated in this study, Paracubacteria are also sensitive to oil contamination in

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soil (Liao et al. 2015). Conceivably, the streamlined genomes and the reduced metabolic capabilities of these organisms makes them unable to handle the metabolic challenges that toxic exposure might present. It is also possible that their symbiotic or commensal interactions are disturbed when their hosts are exposed to toxic compounds, or that the hosts are eliminated by the exposure.

Proteobacteria can be highly sensitive as well as tolerant to triclosan

In the dominant phyla *Proteobacteria* approximately the same number of taxa were positively and negatively correlated to TCS concentrations (7.8% and 9.2% respectively, Table 2). However, clear patterns in differential TCS sensitivity became evident at lower taxonomic levels, where 19% of the OTUs belonging to *Alphaproteobacteria* were negatively correlated with TCS exposure ($p=1.1\times10^{-12}$, Table 2). Further down in the alphaproteobacterial taxonomy, 57% and 60% of the OTUs belonging to the order *Rhodobacterales* and the family *Rhodobacteraceae*, respectively, were negatively correlated to TCS exposure ($p=9.6\times10^{-24}$ and $p=8.4\times10^{-25}$, respectively, Table 2). The abundance of 8.3% of the OTUs from the family *Rhodobacteraceae* was significantly reduced, already at a TCS concentration of 3.16 nM, (1.8×10^{-4}, Table 3). The family *Rhodobacteraceae* harbors the *genus Roseobacter,* whose members may constitute up to 25% of the bacterial community in marine coastal environments (Wagner-Dobler and Biebl 2006), and who have been shown to be important members of marine biofilms (Doghri et al. 2015; Michael et al. 2016; Sanli et al. 2015). Members of *Roseobacter* can use a large number of metabolic pathways, including anoxygenic phototrophy, denitrification, methylotrophy, and sulfur oxidation (Luo and Moran 2014). The genus *Roseobacter* has thus been indicated as an important contributor to the cycling of nutrients in coastal marine environments. Other TCS-sensitive *Alphaproteobacteria* include the order *Rhizobiales* and the family *Rhodobiaceae*. A full 57%

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293 of the taxa in the family *Rhodobiaceae* was negatively correlated with TCS exposure
294 (p=0.00051, Table 2), and the same percentage was underrepresented at 3.16 nM TCS
295 ($p=3.72\times10^{-8}$, Table 3). *Rhizobiales* are known for their nitrogen fixation in symbiosis with
296 legume plants and have been detected in the marine biofilms before (Sanli et al. 2015).

297

298 *Alphaproteobacteria* also comprise TCS-tolerant taxa. Of the OTUs in the order
299 *Sphingomonadales* and the family *Erythrobacteraceae*, 28% and 44%, respectively, were
300 positively correlated with TCS exposure (p=0.0028 and 0.0011, respectively, Table 2).
301 Bacterial groups in the family *Erythrobacteraceae*, such as *Erythrobacter*, are non-motile,
302 obligate aerobes and are frequently found in coastal environments. They are facultative
303 photoheterotrophs and perform anoxygenic photosynthesis (Koblížek et al. 2003). Yurkov et
304 al. (1996) observed that some *Erythrobacter* displayed resistance to the reactive oxygen
305 species (ROS)-generating compound tellurite, and TCS is well known for its ROS-mediated
306 toxic effects in various organisms (e.g. Li et al. 2018; Pan et al. 2018). It might therefore be
307 hypothesized that TCS exposure selects for *Erythrobacteraceae* because of their superior
308 ability to handle TCS-induced oxidative stress. Our analysis shows that at least for
309 *Alphaproteobacteria*, the class level is too high to analyze differential TCS sensitivity, as the
310 families of *Rhodobacteraceae* and *Rhodobiaceae* were sensitive but the family
311 *Erythrobacteraceae* was tolerant.

312

313 Several *Gammaproteobacteria* were favored by TCS as 13% of its OTUs were positively
314 correlated with TCS exposure ($p=3.8\times10^{-6}$, Table 2) and 7.4% and 15% of its OTUs showed
315 significantly higher abundances at 31.6 nM and 316 nM, respectively, compared to unexposed
316 controls (Supplementary Table 2). However, the gammaproteobacterial families

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317 *Alteromonadaceae*, *Oceanospirillaceae*, and *Thiotrichaceae* were particularly tolerant, as
318 15%, 58%, and 36%, respectively, of their corresponding OTUs increased with increasing
319 TCS concentrations ($p=0.069$, 1.5×10^{-7} and 0.0026, Table 2). These results were confirmed in
320 the pairwise comparisons. At 31.6 nM, the abundance of 46% and 40% of the OTUs in
321 *Oceanospirillaceae* and *Thiotrichaceae* were significantly increased (Supplementary Figure
322 3), and at 316 nM the abundance of 61%, 36%, and 22% of the OTUs in *Alteromonadaceae*,
323 *Oceanospirillaceae*, and *Thiotrichaceae* were significantly increased. These taxonomic
324 groups were favored only at higher concentrations of TCS (Supplementary Table 2,
325 Supplementary Figure 3). Although *Pseudomonas aeruginosa* belongs to *Pseudomonadales*,
326 i.e. a different gammaproteobacterial order, it is worth noting that *P. aeruginosa* is
327 intrinsically resistant to TCS. This resistance is believed to originate from efflux pumps, but
328 Zhu et al. (2010) showed that *P. aeruginosa* carries a TCS-resistant enoyl-ACP reductase
329 isoenzyme, encoded by the *fabV* gene, which results in a 2000-fold increase of the Minimum
330 Inhibitory Concentration (MIC) of TCS. It is, however, currently not known to what extent
331 other *Gammaproteobacteria* also carry a TCS-resistant *fabV* gene. As reviewed by Carey and
332 McNamara (2015), other enoyl-ACP reductase isoenzymes, encoded by the *fabK* and *fabL*
333 genes, can also result in TCS resistance. Furthermore, a combination of resistance
334 mechanisms was induced in the biofilm-forming *Gammaproteobacteria* ***Salmonella enterica***
335 ***serovar Typhimurium*** upon TCS exposure, including upregulation of the genes *fabI*, *micF*,
336 *acrAB*, *bcsA*, and *bcsE*. This resulted in increased production of TCS target sites, reduced
337 influx, increased efflux, and increased production of exopolysaccharides (Tabak et al. 2007).
338 Whether these resistance mechanisms are used by periphyton-inhabiting
339 *Gammaproteobacteria* remains to be clarified.

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341 *Deltaproteobacteria* were less abundant than *Alphaproteobacteria* and *Gammaproteobacteria*
342 (Table 2). Similar to the pattern observed in *Alphaproteobacteria*, approximately the same
343 number of taxa in *Deltaproteobacteria* was positively and negatively correlated with TCS
344 exposure. The deltaproteobacterial family *Bdellovibrionaceae* was clearly favored by TCS,
345 where 25% of its OTUs displayed a significant positive correlation to TCS exposure
346 ($p=0.029$, Table 2). Still, a significant over-representation of taxa only occurred at the highest
347 exposure of 316 nM (Supplementary Figure 3). *Bdellovibrionaceae* predates on other bacteria
348 and was previously thought not to occur in marine waters. However, Kandel et al. (2014)
349 found this family in saline (20 ppt) aquaculture systems, and even showed that
350 *Bdellovibrionaceae* was more abundant in biofilms than in the planktonic phase. Our results
351 thus confirm that *Bdellovibrionaceae* are indeed present in naturally occurring marine
352 biofilms. It actually seems reasonable to assume that predatory bacteria like
353 *Bdellovibrionaceae* should thrive in biofilms due to the high bacterial density in this habitat.
354 This taxon has unique membrane lipid structures (Muller et al. 2011), but whether this
355 characteristic renders them tolerant to the inhibition of fatty acid synthesis from TCS remains
356 to be clarified.

357

358 *Triclosan also affects Bacteroidetes, Candidate division BD1-5, Verrucomicrobia, and*
359 *Actinobacteria*

360 Other examples of bacterial groups clearly favored by TCS were found within the phyla
361 *Bacteroidetes*. The order of *Flavobacteriales* and the family *Flavobacteriaceae* were both
362 significantly overrepresented, having a positive correlation with TCS exposure ($p=1.0\times10^{-6}$
363 and $p=4.2\times10^{-8}$, respectively, Table 2). This, however, was only observed at concentrations of
364 316 nM and higher, but not at lower concentrations. Many periphytic bacteria are known to
365 degrade alginate and other carbohydrates produced by algae (Klindworth et al. 2014; Zozaya-

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366 Valdes et al. 2015). Interestingly, Klindworth et al. (2014) noted that *Flavobacteriaceae*
367 species were the major algal polymer degraders in a diatom bloom, whereas the
368 *Rhodobacteraceae* species exhibited less specialized substrate spectra. If TCS indeed causes
369 mortality in diatom-dominated biofilms, as suggested by the TCS-tolerance pattern of
370 periphytic algae (Eriksson et al. 2015), the fact that *Flavobacteriaceae* are being favored and
371 *Rhodobacteraceae* are being reduced by TCS exposure could be explained by the different
372 substrate spectra of those two groups.

373

374 The phylum Candidate division BD1-5 responded in a similar pattern as *Flavobacteria*, with
375 20% of their OTUs increasing significantly with TCS concentration ($p=0.0016$, Table 2) and
376 only the highest exposure of 316 nM giving a significant over-representation compared to
377 controls. Wrighton et al. (2012) assembled genomes of the Candidate divisions BD1-5 and
378 OD1 from an acetate-amended aquifer and concluded that these organisms have small
379 genomes, are strictly anaerobic, and drive pathways for anoxic carbon, hydrogen, and sulfur
380 cycling similar to those in *Archaea*. In terms of sensitivity to TCS, however, the Candidate
381 divisions BD1-5 and OD1 are not similar, since OD1 was clearly TCS sensitive whereas
382 BD1-5 was tolerant. Hence, small genomes and reduced metabolic capabilities do not seem to
383 determine TCS sensitivity *per se*. The Candidate divisions OD1 and BD1-5 might occupy
384 different ecological niches, and/or have different ecological interactions that are affected by
385 TCS exposure.

386

387 A non-monotonous concentration-response pattern, with significant over-representation at
388 3.16 nM but not at higher exposure levels, was observed for some taxa, for example the
389 family *Rubritaleaceae* in *Verrucomicrobia* (Table 3) and the class *Acidimicrobiia* and the

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order *Acidimicrobiales* in *Actinobacteria* (Supplementary Figure 4). It is possible that ecological interactions changed at intermediate TCS concentrations, favoring these taxa. For example, *Verrucomicrobia* can be symbionts with ciliates (Vannini et al. 2003) and algae (Ferrero et al. 2012), and *Actinobacteria* can be closely associated with marine sponges (Seipke et al. 2012) and marine macroalgae (Hollants et al. 2013), habitats that are similar to periphyton biofilms. If eukaryotic species symbiontic to *Verrucomicrobia*, or associated with *Actinobacteria*, were favored at intermediate TCS concentrations, these bacterial taxa might increase as well.

TCS effects on bacterial communities in marine and freshwater ecosystems

The effects of TCS on the composition of natural bacterial communities have been investigated for both freshwater and marine communities. In the freshwater environment, gel-based methods for separating DNA amplicons and FISH have been used, and TCS concentrations of 10 nM (Johnson et al. 2009), 70 nM (Lubarsky et al. 2012), 35 nM (Lawrence et al. 2009), and 6.2 nM (Lawrence et al. 2015) have been shown to change the composition of freshwater communities. In addition, Drury et al. (2013) used 16S amplicon sequencing to study effects of TCS on freshwater sediment communities in artificial streams. These authors found the taxa *Sphingobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, and *Bacteroidia* to be TCS sensitive, whereas *Anaerolineae* and *Cyanobacteria* were identified as being resistant. In our study with marine biofilms we similarly found some *Sphingobacteria* and some *Deltaproteobacteria* to be TCS sensitive (Table 2), whereas the class *Betaproteobacteria* was not identified as being TCS sensitive.

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In marine biofilms, Dobretsov et al. (2007) used T-RFLP and fluorescent *in situ* hybridization (FISH) and found that the overall bacterial density and community composition of 16S in a marine biofilm was affected at a high TCS concentration of 1000 nM, but that the taxa *Alphaproteobacteria* and *Gammaproteobacteria* were affected already at 10 nM. In the present study, we identified Alphaproteobacterial taxa at lower taxonomic levels to be TCS-sensitive (Table 2 and Table 3). However, in contradiction to Dobretsov et al. (2007), we found Gammaproteobacterial taxa to be tolerant to TCS (Table 2). The concentrations in which TCS effects were observed in the current study (1 nM – 3.16 nM) are lower than those of the studies on freshwater communities cited above. It should be underlined that these changes consisted of changes in the relative OTU composition at lower taxonomic levels. Such changes could be missed if techniques are used that are recording effects at high taxonomic levels or if community-level parameters such as bacterial productivity are used. For example, Eriksson et al. (2015) used Biolog Ecoplates to study TCS effects on bacterial carbon utilization using the same samples from which also the material for the amplicon sequencing efforts of the current study was sourced, and no clear effects of TCS were detected. This is most likely a consequence of the functional redundancy of the carbon utilization of the different taxa, due to which subtle shifts in community composition go unnoticed.

Furthermore, it is important to note that we employed an experimental system with a flow-through setting that continuously brings in new bacteria from the environment. This implies that communities were exposed to TCS during the entire lifecycle of the biofilm, including the colonization phase. TCS effects on the early life stages of a biofilm will then be amplified during the course of its succession. It is therefore likely that the experimental design, in combination with amplicon sequencing, facilitated the identification of significant TCS

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effects at comparatively low effect levels and concentrations. In particular, the employed experimental strategy allowed us to identify bacterial species, in an ecologically realistic setting, as either particularly TCS-sensitive or –tolerant.

Conclusions

We identified clear changes in community composition at 10 nM TCS, but effects on specific taxa were seen already at 1-3.16 nM. Our results show that Candidate division OD1 and *Alphaproteobacteria* (primarily *Rhodobacteraceae* and *Rhodobiaceae*) are particularly sensitive to TCS while *Gammaproteobacteria* (primarily *Alteromonadaceae*, *Oceanospirillaceae*, and *Thiotrichaceae*), *Flavobacteria* (primarily *Flavobacteriaceae*), the *Candidate division BD1-5*, the deltaproteobacterial family *Bdellovibrionaceae*, and the alphaproteobacterial family *Erythrobacteraceae* are more tolerant to TCS exposure. The results show that TCS affects marine microbial communities at low nanomolar concentrations, which are actually found in the marine environment (Pintado-Herrera et al. 2014; Remberger et al. 2002). Environmental risk assessments of TCS, such as the recent evaluation published by Guo and Iwata (2017), therefore urgently need to be amended by adequately considering the toxicity of triclosan to environmental bacteria and their natural communities.

Supplementary data

The Supplementary data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx

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468

469 *Data accessibility statement*

470 The raw sequence data are deposited at NCBI under the BioProject accession number
471 PRJNA320539, and with the SRA Experiment accession numbers SRX1744264 -
472 SRX1744266, SRX1744269 - SRX1744273 and SRX1744275 - SRX1744279.

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Figure captions

Fig. 1. Effects of triclosan on the species composition of marine biofilms. (A) Bray-Curtis similarity of the 16S OTU composition plotted against TCS concentration. (B) Principal Components Analysis based on Bray-Curtis similarity indices. All concentrations in nM.

Fig. 2. Number of 16S OTUs affected by triclosan exposure. (A) Number of OTUs with significantly different relative abundances, in comparison to unexposed control communities. (B) Number of co-occurring OTUs with significantly different relative abundances, in comparison to unexposed control communities.

Fig. 3. Average relative abundance of the six most abundant bacterial phyla in relation to triclosan exposure.

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Table 1. Read and OTU count statistics for 16S amplicons from exposed and unexposed microcosms.

Exposure concentration (nM)	Number of replicates (n)	Average (n > 1) or total (n= 1) read count per sample (standard deviation)	Average (n > 1) or total (n= 1) OTU count per sample (standard deviation)
0	4	15,514 (4525)	844 (63)
0.316	1	13,919	789
1	1	22,658	1262
3.16	3	24,286 (15888)	1102 (402)
10	1	45,545	1141
31.6	2	40,628 (1826)	685 (70)
316	2	19,926 (284)	727 (29)

Table 2. Overrepresentation of taxa positively or negatively correlated with TCS concentration.

Taxonomic group *(Phyla)* *(Class)* *(Order)* *(Family)*	Number of OTUs in taxa [a]	Significant increased (+) or decreased (-) taxa	Percent significant increased / decreased taxa (%)	p-value
Bacteroidetes	450		7.1 / 5.3	0.11 / 0.90
Flavobacteria	172	+	15 / 4.1	1.8×10^{-6} / 0.94
Flavobacteriales	167	+	16 / 4.2	1.0×10^{-6} / 0.93
Flavobacteriaceae	97	+	22 / 4.1	4.2×10^{-8} / 0.89
Candidate division BD1-5	40	+	20 / 5	0.0016 / 0.75
Candidate division OD1	47	-	2.1 / 28	0.94 / 4.0×10^{-6}
Proteobacteria	654	+ / -	7.8 / 9.2	0.0049/0.00040
Alphaproteobacteria	222	-	4.5 / 19	0.85 / 1.1×10^{-12}
Rhizobiales	7		0 /12	1 / 0.16
Rhodobiaceae	7	-	0 / 57	1 / 0.00051
Rhodobacterales	53	-	5.7 /57	0.61 / 9.6×10^{-24}
Rhodobacteraceae	50	-	4 / 60	0.80 / 8.4×10^{-25}
Sphingomonadales	18	+	28 / 0	0.0028 / 1
Erythrobacteraceae	9	+	44 / 0	0.0011 / 1
Deltaproteobacteria	145		5.5 / 6.9	0.62 / 0.47
Bdellovibrionales	59		8.5 / 8.5	0.26 / 034
Bdellovibrionaceae	12	+	25 / 0	0.029 / 1
Gammaproteobacteria	237	+	13 / 2.1	3.8×10^{-6} / 1
Alteromonadales	63		11 / 0	0.069 /1
Alteromonadaceae	40	+	15 / 0	0.025 / 1
Oceanospirillales	34	+	35 / 0	1.5×10^{-7}/ 1
Oceanospirillaceae	19	+	58 / 0	7.8×10^{-10} /1
Thiotrichales	21	+	19 / 9.1	0.030 / 0.52
Thiotrichaceae	11	+	36 / 0	0.0026 / 1

[a] The number of OTUs of lower taxonomic levels are included in the number of OTUs of higher taxonomic levels. The OTUs for which lower taxonomic levels couldn't be assigned is included for higher taxonomic levels.

Table 3. Overrepresentation of taxa that are differentially abundant at 3.16 nM TCS compared to controls.

Taxonomic group *(Phyla)* *(Class)* *(Order)* *(Family)*	Number of OTUs in taxa [a]	Significant increased (+) or decreased (-) taxa	Percent significant increased / decreased taxa (%)	p-value
Actinobacteria	46		2.2 / 0	0.055 / 1
Acidimicrobiia	25	+	4.0 / 0	0.030 / 1
Acidimicrobiales	25	+	4.0 / 0	0.030 / 1
Candidate division OD1	41	-	0 / 4.9	1 / 0.029
Proteobacteria	599	-	0 / 1.5	1 / 0.0027
Alphaproteobacteria	204	-	0 / 3.9	1 /5.7×10^{-6}
Rhizobiales	32	-	0 / 13	1 / 3.51×10^{-5}
Rhodobiaceae	7	-	0 / 57	1 / 3.72×10^{-8}
Rhodobacterales	51	-	0 / 7.8	1 / 0.00023
Rhodobacteraceae	48	-	0 / 8.3	1 / 1.8×10^{-4}
Verrucomicrobia	95		1.1 / 0	0.11 / 1
Verrucomicrobiae	64		1.6 / 0	0.076 / 1
Verrucomicrobiales	61		1.6 / 0	0.073 / 1
Rubritaleaceae	26	+	3.8 / 0	0.031 / 1

[a] The number of OTUs of lower taxonomic levels are included in the number of OTUs of higher taxonomic levels. The OTUs for which lower taxonomic levels couldn't be assigned is included for higher taxonomic levels.

Figures

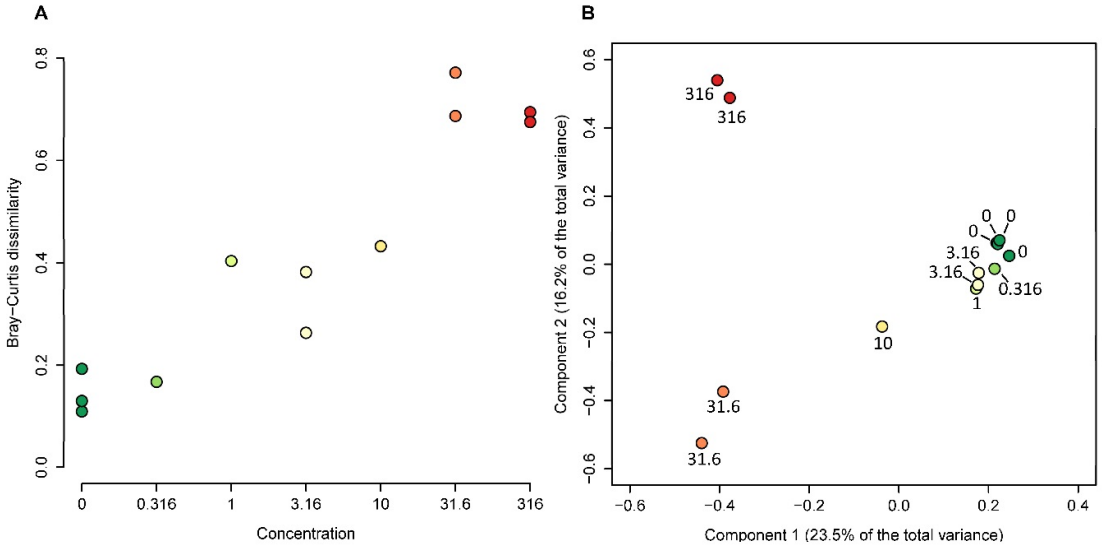


Fig. 1. Effects of triclosan on the species composition of marine biofilms. (A) Bray-Curtis similarity of the 16S OTU composition plotted against TCS concentration. (B) Principal Components Analysis based on Bray-Curtis similarity indices. All concentrations in nM.

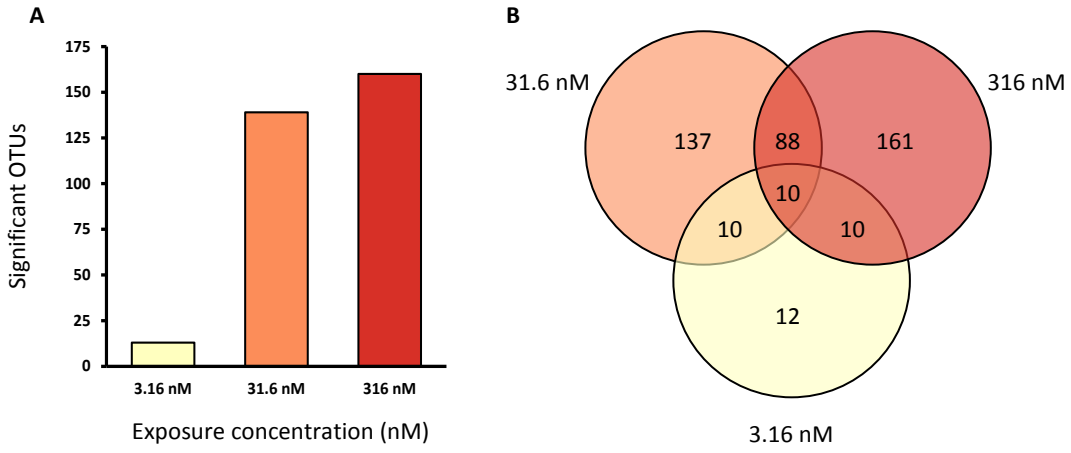


Fig. 2. Number of 16S OTUs affected by triclosan exposure. (A) Number of OTUs with significantly different relative abundances, in comparison to unexposed control communities. (B) Number of co-occurring OTUs with significantly different relative abundances, in comparison to unexposed control communities.

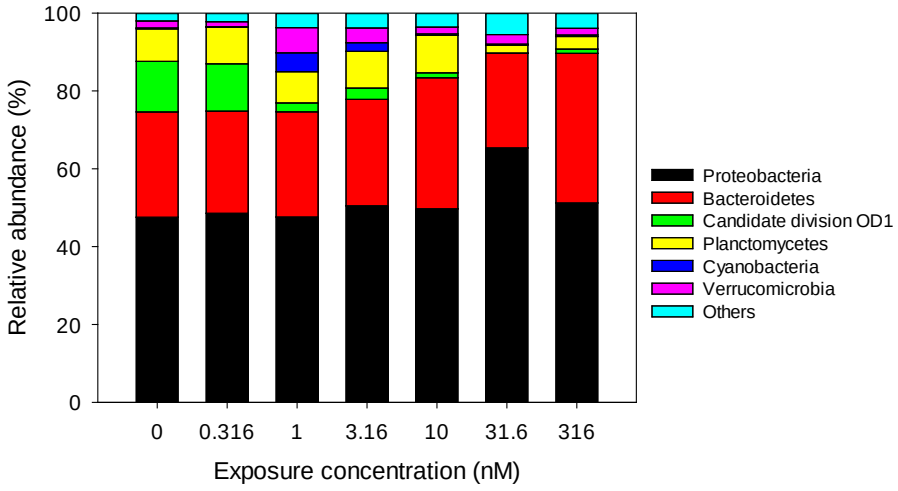


Fig. 3. Average relative abundance of the six most abundant bacterial phyla in relation to triclosan exposure.

SUPPLEMENTARY MATERIAL

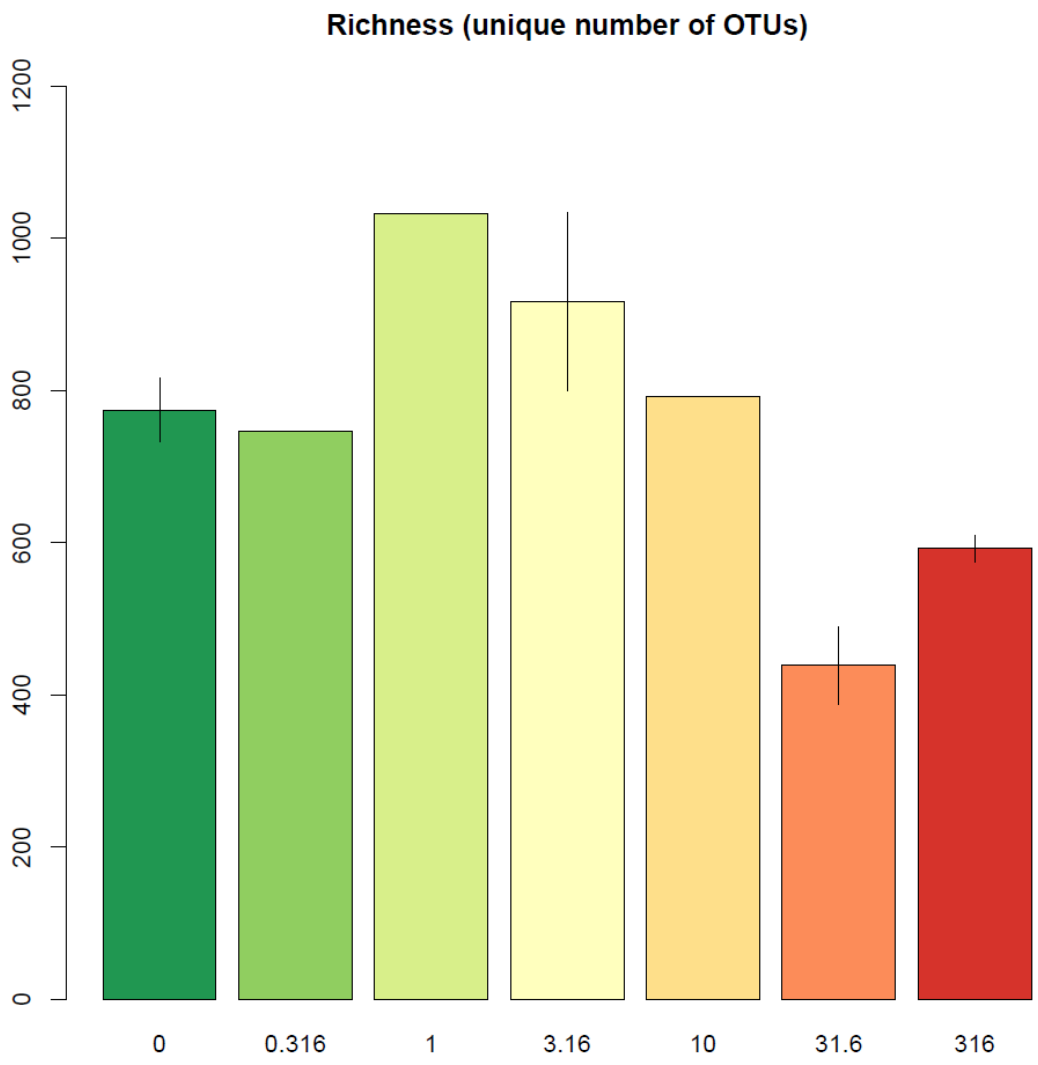
Supplementary Table 1. Total 16S OTU abundance and richness of the detected phyla from all microcosms.

Phyla	Abundance	OTU richness
Proteobacteria	166098	654
Bacteroidetes	90767	450
Planctomycetes	20652	248
Candidate division OD1	12620	47
Verrucomicrobia	8533	100
Cyanobacteria	3312	46
Candidate division BD1-5	2979	40
Actinobacteria	2633	52
Candidate division SR1	2412	14
Chlamydiae	1225	40
Firmicutes	879	10
Chloroflexi	588	19
Lentisphaerae	241	11
Deinococcus-Thermus	192	4
Acidobacteria	167	11
Fusobacteria	94	2
Candidate division TM7	68	7
Gemmatimonadetes	47	5
Fibrobacteres	44	1
Candidate division WS3	29	3
Chlorobi	26	2
Armatimonadetes	25	1
Candidate division BRC1	24	5
Candidate division TM6	23	3
Candidate division WS6	19	2
WCHB1-60	12	1
Nitrospirae	6	2
Spirochaetes	6	2
Candidate Division OP8	2	1
Candidate division OP3	2	1

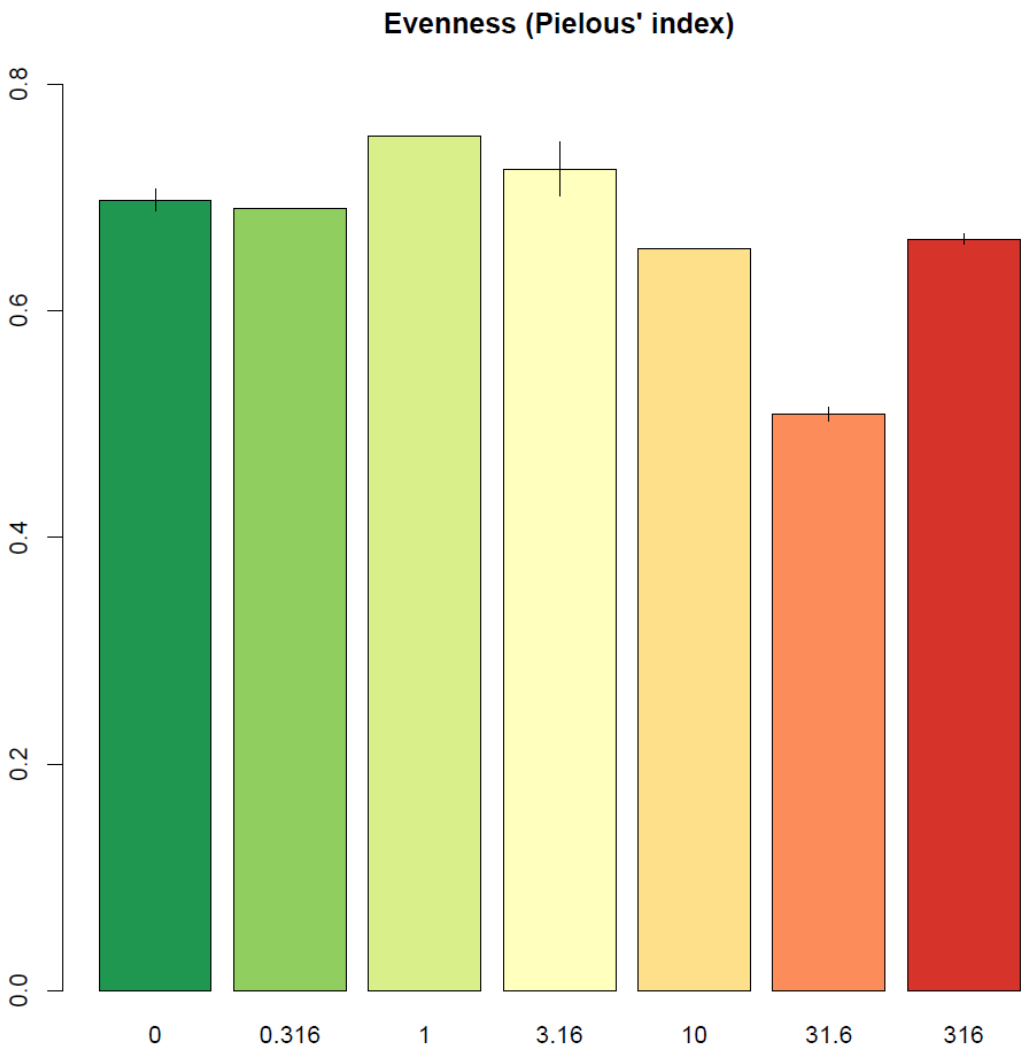
Supplementary Table 2. Taxonomic annotation of OTUs with a significant positive/negative correlations to TCS concentration. Note that the number of OTUs at lower taxonomic levels (e.g. *Flavobacteriaceae*) are included in the number of OTUs at the higher taxonomic levels (e.g. *Bacteroidetes*). Hence, some of the OTUs with positive correlation (in total 83 OTUs) and the some of the OTUs with negative correlation (in total 88 OTUs), are counted several times in the column to the right.

Taxonomic annotation	Number of OTUs in taxa	Number of OTUs with significant positive/negative correlations	Percent OTUs with significant positive/negative correlations
Actinobacteria/Acidimicrobiia/Acidimicrobiales	27	0 / 1	0 / 3.7
Bacteroidetes	450	25 / 18	5.5 / 4.0
Cytophagia	60	0 / 1	0 / 1.7
Flavobacteria/Flavobacteriales	166	20 / 5	12 / 3.0
Cryomorphaceae	31	3 / 1	9.7 /3.2
Flavobacteriaceae	97	17 / 3	18 / 3.1
Unknown	29	0 / 1	0 / 3.4
Sphingobacteria/Sphingobacteriales	164	5 / 8	3.0 / 4.9
Chitinophagaceae	18	0 / 1	0 / 5.6

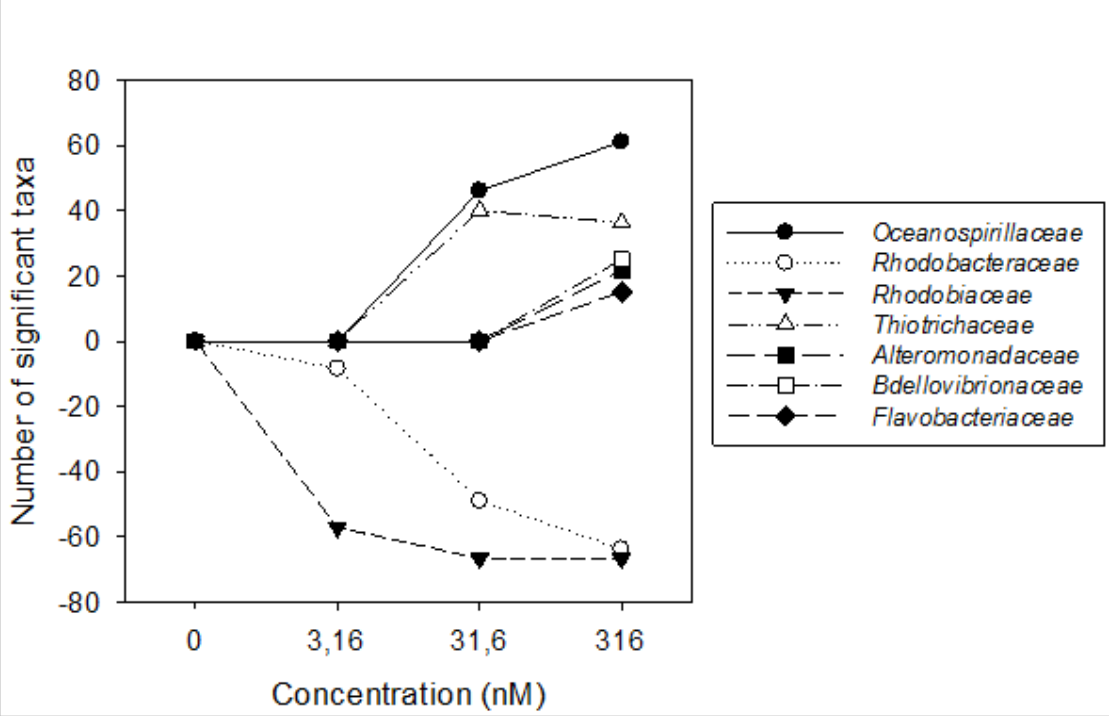
Saprospiraceae	55	3 / 3	5.5 /5.5
Unknown	83	2 / 4	2.4 / 4.8
Candidate division BD1-5	40	8 / 2	20 / 5
Candidate division OD1	47	1 / 10	2.1 / 21
Chlamydiae	40	1 / 0	2.5 / 0
Cyanobacteria	46	2 / 0	4.3 / 0
Deinococcus-Thermus	4	1 / 0	25 / 0
Planctomycetes	248	3 / 8	1.2 / 3.2
Phycisphaerae/Phycisphaerales	55	0 / 5	0 / 9.0
Phycisphaeraceae	24	0 / 2	0 / 8.3
Unknown	31	0 / 3	0 / 9.7
Planctomycetacia/Planctomycetales/Planctomycetaceae	73	0 / 1	0 / 1.4
Unknown	19	3 / 2	16 / 11
Proteobacteria	654	42 / 47	6.4 / 7.2
Alphaproteobacteria	222	6 / 37	2.7 / 17
Rhizobiales/Rhodobiaceae	7	0 / 3	0 / 43
Rhodobacterales/Rhodobacteraceae	50	2 / 25	4.0 / 50
Rickettsiales	26	0 / 2	0 / 7.7
Sphingomonadales	18	3 / 0	17 / 0
Erythrobacteraceae	9	2 / 0	22 / 0
Sphingomonadaceae	8	1 / 0	13 / 0
Unknown	19	1 / 4	5.2 / 21
Deltaproteobacteria	145	8 / 7	5.6 / 4.8
Bdellovibrionales	59	5 / 4	8.5 / 6.8
Bdellovibrionaceae	12	3 / 0	25 / 0
Unknown	33	2 / 4	6.0 / 12
Desulfuromonadales	19	1 / 0	5.3 / 0
Myxococcales	22	2 / 1	9.1 /4.5
Nannocystaceae	11	1 / 0	9.1 / 0
Unknown	6	1 / 1	17 / 17
Unknown	18	0 / 2	0 / 11
Gammaproteobacteria	237	27 / 2	11 / 0.84
Alteromonadales	63	6 / 0	9.5 / 0
Alteromonadaceae	40	5 / 0	13 / 0
Unknown	8	1 / 0	13 / 0
Chromatiales	11	2 / 0	18 / 0
Ectothiorhodospiraceae	1	1 / 0	100 / 0
Granulosicoccaceae	7	1 / 0	14 / 0
Oceanospirillales/Oceanospirillaceae	19	9 / 0	47 / 0
Thiotrichales/Thiotrichaceae	11	4 / 1	36 / 9.1
Unknown	7	6 / 1	86 / 14
Unknown	9	0 / 1	0 / 11
Verrucomicrobia	100	0 / 1	0 / 1



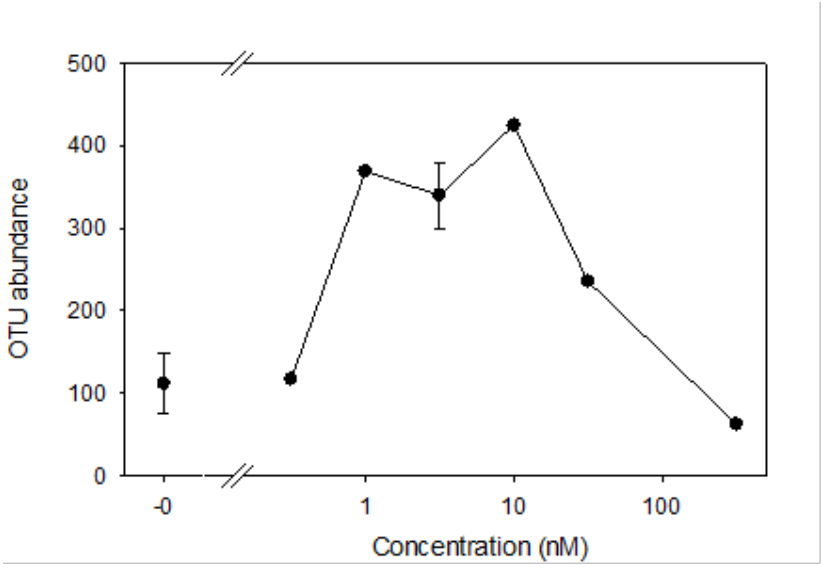
Supplementary Figure 1. Richness, measured as the unique number of OTUs, for each concentration of TCS. For replicated concentrations, the bar represents the standard error. The richness in 31.6 and 316 nM was significantly decreased compared to the controls (p=0.0236 and p=0.0169 respectively).



Supplementary Figure 2. Evenness, measured using Pielous' index, for each concentration of TCS. For replicated concentrations, the bar represents the standard error. The richness in 31.6 and 316 nM was significantly decreased compared to the controls ($p=8.18\times10^{-5}$ and $p=0.0299$ respectively).



Supplementary Figure 3. Percentage of significant over- and under-represented taxa in families from pairwise comparisons between controls and exposure treatments. Only families containing three or more taxa are included. Significance determined as adjusted p-value < 0.05 in Fisher's exact test.



Supplementary Figure 4. Abundance of *Actinobacteria* OTUs at different concentrations of TCS. Error bars denote standards error of the mean.