Long-term effects of the antibacterial agent triclosan on marine periphyton communities

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23 Abstract

24 Triclosan (TCS) is a widely used antibacterial agent that has become a ubiquitous contaminant
25 in freshwater, estuary and marine environments. Concerns for potential adverse effects of TCS
26 have been described in several recent risk assessments. Effects on freshwater microbial
27 communities have been quite well studied but studies addressing effects on marine microbial
28 communities are scarce. Here we describe short- and long-term effects of TCS on marine
29 periphyton (microbial biofilm) communities. Short-term effects on photosynthesis were
30 estimated after 60 – 210 minutes exposure. Long-term effects on photosynthesis, chlorophyll
31 fluorescence, pigment content, community tolerance and bacterial carbon utilization were
32 studied after exposing periphyton for 17 days in flow-through microcosms to 0.316 - 10 000 nM
33 TCS. Results from the short-term studies show that TCS is toxic to periphyton photosynthesis.
34 EC50 values of 1080 and 3000 nM were estimated using $^{14}CO_2$-incorporation and Pulse
35 Amplitude Modulation (PAM), respectively. After long-term TCS exposure in flow-through
36 microcosms photosynthesis estimated using PAM was, however, not inhibited by TCS
37 concentrations up to 1000 nM, but instead increased with increasing TCS concentration.
38 Similarly, the amount of photosynthetic pigments increased after an exposure of 31.6 nM TCS
39 and higher; at 316 nM TCS the pigment amounts reached between 140 and 190% of the control
40 level. Pollution-Induced Community Tolerance (PICT) was observed for algae and
41 cyanobacteria at 100 nM TCS and higher. In spite of the widespread use of TCS as an
42 antibacterial agent, the compound did not have any effects on bacterial carbon utilization after
43 long-term exposure.

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45 Keywords: microbial toxicology, personal care products, mode of action, Pollution-Induced
46 Community Tolerance (PICT), biofilm, irgasan

47

48 INTRODUCTION

49 Triclosan (TCS, 5-chloro-2-(2,4-dichloro-phenoxy)-phenol, CAS-No. 3380-34-5, also known as
50 Irgasan, Aquasept, Sapoderm and Ster-Zac) is used as an antimicrobial compound in a large
51 variety of products, such as personal care products (PCPs) (*e.g.* soap, toothpaste and deodorant),
52 household cleaning products (*e.g.* laundry detergents), textiles and plastics (*e.g.* childrens toys
53 and kitchen utensils) [1, 2]. The TCS production volume in Europe was as high as 450 tons in
54 2006 and since approximately 85% is used as a down-the-drain chemical in PCPs [3], large
55 quantities continuously reach the environment. Therefore, TCS has become a ubiquitous
56 pollutant in all environmental compartments [1].

57

58 The major route of TCS into coastal waters is via wastewater treatment plants (WWTPs) and
59 through rivers and estuaries. Although both degradation and sequestration of TCS to sludge
60 occurs in WWTPs [4], it has been shown to be persistent in wastewater [5]. Mechanisms of biotic
61 and abiotic TCS transformations include the production of methylated TCS, chlorinated
62 dibenzodioxins and chlorinated phenols, some of which are in fact more toxic than TCS itself [1].
63 The occurrence of TCS in WWTPs, rivers and estuaries has been studied extensively, [e.g.
64 reviewed by 1, 6]. Studies of environmental concentrations of TCS in the marine environment
65 are however scarce. As reviewed by Bedoux and colleagues [1] TCS concentrations up to 0.047,
66 0.1 and 0.024 nM have been reported for coastal waters in USA, China and Europe, respectively.
67 At the Swedish west coast concentrations as high as 0.55 nM have been detected in the marine

costal environment [7]. Although TCS is used as an antimicrobial compound, several studies have concluded that the most TCS-sensitive organisms are algae [as reviewed by 1, 2, 8, 9]. TCS is relatively hydrophobic (the log K_{OW} being 4.8 at pH 7) and Coogan and co-authors [10] determined bioaccumulation factors for algae between 900 and 2100.

Several environmental risk assessments of TCS have been published. Capdevielle *et al.* [11] performed a probabilistic risk assessment using a Species Sensitivity Distribution (SSD) with 14 species, including fish, invertebrates, macrophytes and algae. Based on chronic toxicity data these authors calculated a Predicted No Effect Concentration (PNEC) of 5.35 nM (1550 ng/L). Based on estimated Predicted Environmental Concentrations (PEC) for Europe and North America of 0.62 nM (180 ng/L) and 2.94 nM (850 ng/L), respectively, the authors concluded that there were negligible environmental risks of TCS. However, for two algal, respective cyanobacterial species, the toxicity threshold values (NOEC, EC10 or EC25) are actually below the calculated PNEC. This means that although the aim of the risk assessment is to protect 95% of the species to effects of TCS, only 86% of the species in the SSD were actually protected. If TCS selectively eliminates microalgae and cyanobacteria this could also profoundly affect the rest of the community, since these organisms have pivotal roles for e.g. primary productivity. Chalew *et al.* [8] criticized the TCS risk assessment of Capdevielle *et al.* [11] and concluded that if the traditional PNEC determination, i.e. based on the NOEC value of the most sensitive species, is used an environmental risk is indicated. Chalew et al. [8] furthermore showed that environmental concentrations of TCS can exceed acute toxicity threshold values for algae, indicating risk for these organisms. Reiss *et al.* [12] concluded that there are no risks of TCS for fish and invertebrates, but algal species might be at risk. This conclusion was recently re-emphasized in a risk assessment by von der Ohe et al. [13] who showed that the 95th percentile

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of the maximum environmental concentration detected at each site (MEC_{95}) exceeded the PNEC of TCS at more than 75% of the studied sites in the river Elbe, and that the MEC_{95} could exceed the PNEC by a factor of 12 to 41. When ranking the risk of 500 pollutants in this river, TCS came out as the sixth compound of high risk. Furthermore, in their risk assessment of PCPs, Brausch and Rand [6] found that TCS indeed presents a risk in the environment: the risk quotient (ratio of environmental concentration to effect concentration) for algae was 19, based on data for growth of a natural assemblage [14].

The higher sensitivity of algae and cyanobacteria to TCS is particularly interesting from two perspectives. 1) TCS is mainly used as an antimicrobial in PCPs and is intended to be acutely toxic to the target organisms (bacteria). However, those seem to be fairly insensitive, whereas some of the non-target organisms (algae and cyanobacteria) are highly sensitive. Moreover, TCS is persistent and is continuously released into the environment and aquatic non-target organisms are therefore continuously exposed. Hence, there is a clear conflict between the use of TCS as an antimicrobial compound in PCPs and the hazards it might pose to non-target organisms in the environment. 2) One mechanism of action of TCS is the inhibition of fatty acid synthesis through binding to the enoyl-acyl carrier protein (enoyl-ACP) reductase (EC 1.3. 1.9) in bacteria [15]. Both algae and cyanobacteria have enoyl-ACP reductase but it is not known whether its inhibition is the most TCS sensitive pathway in these organisms. It is, however, known that different species have different TCS binding sites in the enoyl-ACP reductase, and that this affects the affinity of the TCS binding [16]. It seems reasonable to assume that the similarity of the TCS targets in different species also matters for sensitivity differences between species. In addition, TCS has been shown to have multiple mechanisms of actions. For example,

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it induces cell membrane destabilization [17], inhibits enzymes in the glycolysis pathway and can uncouple membrane potential in mitochondria [18].

Environmental risk assessments of chemicals, including the TCS risk assessments presented above, are commonly based on single species assays. SSD-based approaches capture the differing sensitivities of species to the toxicant of interest, assuming that an adequate dataset is available. However, not even those advanced approaches do account for ecological effects on the level of communities or ecosystems, where toxicants may cause unforeseen, indirect effects at higher levels of biological complexity [e.g. see 19]. One way to overcome this limitation of single-species based approaches in general is to use natural communities for assessing the environmental hazard of the toxicant in question. Although the test capacity and throughput is lower, this approach has a greater ecological relevance [e.g. see 20]. One approach within community ecotoxicology that has already gained considerable interest is the concept of Pollution-Induced Community Tolerance (PICT). As outlined by Blanck [21], differential sensitivity among species results in a Toxicant-Induced Succession (TIS) upon exposure, which leads to an increased abundance of tolerant species and a reduced abundance of sensitive species. In overall, TIS results in an increased average community tolerance which can be detected as PICT. PICT is quantified by comparatively assessing the short-term toxicity in communities after a long-term exposure to the same toxicant. The EC50 values from the short-term assays are then used as descriptors of the average community tolerance. If the short-term EC50 values increase with increasing long-term exposures, the long-term exposure has decreased the average sensitivity of the community, i.e. a PICT is detected, indicating that the community has been re-structured by the long-term exposure to the toxicant in question. Considering the differential sensitivity of algae and cyanobacteria described above, and the

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139 insights PICT can give into structural changes in communities, it is highly relevant to use PICT
140 for microbial phototrophic communities when evaluating the potential environmental hazard of
141 TCS.
142
143 In the freshwater environment a number of studies have described effects of TCS on microbial
144 community structure and function [14, 22-29]. From these studies it can be concluded that TCS
145 exposure can cause effects on community structure and function, and that it can induce
146 resistance in bacteria at relevant environmental concentrations. However, in the marine
147 environment such studies are scarce. One type of marine microbial community that has
148 successfully been used for ecotoxicological testing is periphyton [e.g. see 30, 31, 32]. Periphyton
149 forms a biofilm on underwater surfaces and makes up a food-web of interacting organisms. It
150 contains a huge variety of organisms, such as bacteria, microalgae, protozoans and metazoans
151 from different functional groups and trophic levels [e.g. 33]. Backhaus *et al.* [30] and Johansson
152 et al. [34] used the SWIFT test [31] to study TCS effects on pigment content in marine
153 periphyton. Backhaus et al. [30] detected an EC50 value of 1166 nM and Johansson et al. [34]
154 detected EC50 values of 39.2 and 302 nM in spring and summer experiments, respectively.
155 However, the SWIFT test employs periphyton pre-established in the environment, does not
156 allow immigration of new species during the exposure and is limited to an exposure time of
157 only 96 h. Marine flow-through microcosms can be used to overcome these limitations,
158 resulting in a higher test sensitivity as has been previously shown for the two antifouling
159 compounds tri-butyl-tin and DCOIT [31].
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The aim of this study was to describe short- and long-term effects of TCS on marine periphytic algae and bacteria in ecologically realistic settings. Toxicity to photosynthesis was measured in short-term tests with field-collected periphyton, and we used flow-through microcosms to study long-term effects on photosynthesis, pigment composition, bacterial carbon utilization, and community tolerance (PICT). In the long-term experiment effects from TCS exposure during the entire process of colonization and growth of periphyton were studied.

MATERIALS AND METHODS

Field sampling and microcosm system

Periphyton communities in the microcosms and in the field were established on 1.5 cm^2 round glass discs mounted 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. In the microcosms the polyethylene holders were placed along the long sides of each aquarium. The periphyton communities were then established in the microcosms for 15 to 18 days under toxicant exposure before PAM measurements, sampling for HPLC analyses, inoculation on Biolog Ecoplates and PICT measurements.

For periphyton field sampling the polyethylene holders were placed in a polyethylene rack that was hung from a buoy 1.5 m below the surface at a pristine site in the Gullmar fjord at the Swedish west coast (Lat: 58.250553, Long: 11.443934). The periphyton was allowed to establish itself for 14 days before sampling for short-term toxicity testing using PAM and for 17 days before short-term toxicity testing using ^{14}C-incorporation.

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The microcosm experiment was performed indoors at the Sven Lovén Centre for Marine Sciences, Kristineberg on the west coast of Sweden from 26st of September until 14th of October 2012. The flow-through microcosm system [32], consisted of 24 aquaria and periphyton communities were established over 18 days under 8 different levels of TCS exposure (0.316 - 10 000 nM). Seven microcosms were used as unexposed controls and the TCS concentrations of 3.16, 31.6 and 316 nM were replicated in three microcosms, the concentration of 100 nM was replicated in four microcosms, while the concentrations of 0.316, 1, 10, and 1000 nM were only tested in one microcosm each. This design was chosen as a compromise that allows assessing the statistical significance of treatment effects at the replicated concentrations while still allowing for the possibility to describe concentration-response trends over a broad range of test concentrations.

Seawater, with its indigenous microbiota, was continuously pumped into the microcosms using an air-driven Teflon-membrane pump (Dominator Maskin AB, Sweden) from 1.5 meters depth in the Gullmar fjord. The volume in each microcosm was 20 L and the seawater flow rate was 220 mL min^{-1} giving a mean residence time of about 90 min. TCS water solutions were pumped into the system at a flow rate of 2 ml/min using a peristaltic pump (Ismatech IPN 26, Ismatech AG, Switzerland). These TCS solutions were renewed every third day. Flow rates of the seawater and TCS water solutions were checked daily and adjusted when deviating more than 1% from the desired values. A nylon net (1 mm mesh) was used to prevent larger organisms from entering the microcosms. Each aquarium had two fluorescent tubes (Osram lumilux Daylight L 18W/12) as light source giving a photon flux density of approximately 200 µmol photons $\cdot m^{-2} \cdot s^{-1}$ at the water surface, and set to the light/dark regime in Sweden at this time of

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year (13.5/10.5 hours). A stirring device in each aquarium ensured thorough mixing of the water.

Triclosan exposure in the microcosms and chemical analyses

TCS stock solutions were prepared in acetone and stored at -20 C°. TCS water solutions were prepared by adding 10 ml stock solution to 10 L of de-ionised water. In order to fully dissolve TCS in the water solutions NaOH was added to a final concentration of 0.3 mM. The same amount of acetone and NaOH was added to the untreated microcosms. The TCS concentrations were analysed two times before the periphyton discs were placed in the microcosms, to ensure that the nominal concentrations were met, two times during the experiment and one time the day after the last measurements. The nominal and analysed TCS concentrations and the number of replicates at the different exposure levels are shown in Table 1.

In order to check nominal concentrations, water sampled from the aquaria was loaded on Isolute ENV+ SPE columns (6 mL, 200 mg, Biotage®) preconditioned with 5 mL methanol and 5 mL MilliQ-water. From aquaria that contained nominal concentrations between 10 and 1000 nM TCS, a total of 10 nmol TCS was loaded on the columns. To avoid loading volumes above 1 L, 3 nmol triclosan was added from aquaria with test concentration of 3.16 nM while 0.3 nmol was added from aquaria with test concentrations of 1 and 0.316 nM. The samples were loaded at a flow rate of 5 mL/min, dried and frozen at -18°C until extraction. For this purpose the samples were washed with 10 mL MilliQ-water and extracted with 3 x 10 mL methanol using a flow rate of 1 mL/min. The three aliquots were analyzed separately on a LC-MS/MS using an Agilent 1260 LC equipped with an Agilent 6410 triple quadrupole detector. The separation was performed on an Agilent Zorbax SB-C18, 2.1 x 50 mm, 1.8 Micron column equipped with a 5 mm guard column. A gradient of water (0.1% formic acid) [A] and acetonitrile (0.1% formic

acid) [B] was applied as follows: 0 min, 5% B; 4 min, 10% B; 15 min, 100% B; 16 min 5% B. The electrospray source of the detector was operated in negative mode with an ionization voltage of 4000 V at 250°C with a nitrogen flow of 11 L/min at 40 psi. The transition from precursor ion 287 *m/z* to 35 *m/z* was used to quantify TCS. The concentrations of TCS in the microcosms were then calculated by comparison to an external standard curve of methanol-dissolved TCS.

Determination of PICT and sensitivity of field-sampled periphyton

PICT in the microcosms, as well as the sensitivity of field-sampled periphyton, was determined in short-term tests as inhibition of photosynthetic activity, measured using ^{14}C-incorporation [32]. One short-term test was performed per tested microcosm. PICT measurements were triplicated for periphyton in the unexposed control microcosms and exposed to 316 nM TCS. For periphyton exposed to 0.316, 1, 10, 100 and 1000 nM the PICT measurements were unreplicated. In addition, one test was performed for unexposed field-sampled periphyton Each short-term test used 8 periphyton-colonised glass discs, which were not exposed, as controls and four discs for each of the 8 TCS concentrations, spanning a concentration range of 3.16 to 10,000 nM TCS. The exposed samples and unexposed controls were incubated in 5 mL test media made of filtered natural seawater (glass microfiber filter, grade GF/F; Whatman), taken from the inflow to the microcosms, in scintillations vials with 0.1 % acetone as co-solvent. The ^{14}C-labeled sodium bicarbonate solution was prepared by diluting a stock solution of 1 mCi mL $^{-1}$ (DHI Lab Products, Hoersholm, Denmark) with filtered natural seawater (glass microfiber filter, grade GF/F; Whatman) taken from the inflow to the microcosms, giving an activity of 1.48 MBq mL $^{-1}$ and a final activity of 0.074 MBq in each sample. Incubation of the samples was done at *in situ* temperature with a photon flux density of approximately 125 mol photons m^{-2} s^{-1}

from fluorescent tubes (Osram Lumilux Daylight L18W/11). The pre-incubation time, i.e. before addition of 50 µL of the ^{14}C-labeled sodium bicarbonate solution to each sample, was 45 minutes, and the subsequent incubation time was 15 minutes. Carbon fixation activity was terminated by adding 50 µL of formaldehyde (37%) to each sample. The samples were then acidified with 1 ml acetic acid, dried at 60°C under a gentle stream of air and 1 ml dimethyl sulfoxide and 8 ml of Ultima Gold scintillation cocktail (PerkinElmer Inc.) were added. The radioactivity of the samples was measured using a liquid scintillation spectrometer (LS 6500 Beckman Inc.). Decays per minute were calculated from counts per min based on the correction factors for sample quench characteristics and machine efficiency. In order to estimate the abiotic ^{14}C fixation 50 µL formaldehyde each was used to terminate the carbon fixation in three samples prior to incubation. The radioactivity of these samples was subtracted from the radioactivity of the other samples in each test.

Photosynthesis Pulse Amplitude Modulation (PAM) measurements

Short- and long-term effects on photosynthesis, as well as long-term effects on *in vivo* chlorophyll *a* concentration, were measured using Pulse Amplitude Modulation (PAM) fluorometry. A Phytopam PAM fluorometer equipped with the Emitter-Detector-Fiberoptics Unit for periphyton measurements was used (Waltz Mess- und Regeltechnik). The Phytopam has four channels with different excitation wavelengths, enabling measurement of the fluorescence signal in photoautotrophic organisms after excitation by 470, 520, 645 and 665 nm. This allows in principal to differentiate between three major algal classes (cyanobacteria, diatoms and green algae) [35]. Such classifications are, however, based on calibration to fluorescence reference spectra of only one representative species from each algal class. The

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communities studied here are much more diverse, their composition can change rapidly, and it is impossible to isolate and culture representative species. Therefore, we used the responses from the excitation channels directly.

Although there are some overlap between the excitation responses for different pigments, the fluorescence from light with the excitation wavelengths of i) 470, ii) 520, iii) 645, and iv) 665 nm foremost represents fluorescence from i) chlorophyll *b*, ii) chlorophyll *c*, fucoxanthin, and carotenoids, iii) allophycocyanin, and iv) chlorophyll *b*, respectively [36]. In order to subtract the background fluorescence a so-called Zero Offset (Zoff) determination was made. The background fluorescence from a clean glass discs and incoming seawater filtrate (0.2 µm, Millipore) was subtracted from the fluorescence signal.

Measurements of minimal fluorescence yield (Fo), maximum quantum yield (ϕ_{IImax}), effective quantum efficiency (ϕ_{II}) and non-photochemical quenching (NPQ) of PS II were conducted. In order to estimate the light intensity to which the periphyton communities were adapted to, a Rapid Light Curve (RLC) was made on a representative sample before the subsequent measurements. The RLC results gives an estimate of the light curve parameter Ik, which is the light intensity above which saturation of photosynthesis becomes dominant. Thus, Ik represents an appropriate light intensity for determining ϕ_{II} and the Fm' value of NPQ. During the measurements of ϕ_{II} and and the Fm' value of NPQ the samples were illuminated at an actinic light intensity just below Ik until the fluorescence reading was stable. Afterwards five saturating pulses, with 2-3 second intervals, were given and the data from the fifth pulse was used in the downstream analyses. This approach was shown to be suitable in pilot studies before the experiment (data not shown).

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296

297 *Short-term toxicity testing using PAM*

298 The short-term toxicity testing using PAM was performed with field sampled, i.e. previously
299 unexposed, periphyton (see Field sampling and microcosm system above). In each short-term
300 test effects of TCS were analysed after 75 and 150 minutes exposure. The exposed samples and
301 unexposed controls were incubated in 15 mL test media made of filtered natural seawater (glass
302 microfiber filter, grade GF/F; Whatman), taken from the intake stream to the microcosms, with
303 0.1 % acetone as co-solvent in small glass beakers. Each beaker contained two periphyton discs
304 for the two different exposure times. For each exposure time five unexposed control discs and
305 four discs for 5 test concentrations of TCS (3.16 to 3160 nM with 0.5 common logarithms in
306 between the test concentrations). Incubation of the samples was done at the same temperature
307 and photon flux density as in the PICT determination tests. Since there were no statistical
308 differences between the controls of the two exposure times, all 10 controls were pooled and
309 used in the concentration repose analyses for the two exposure times. The concentration
310 response relationships were based on the inhibition of ϕ_{IImax} and ϕ_{II}. ϕ_{IImax} reflects the quantum
311 yield of PS II after dark adaptation, whereas ϕ_{II} reflects this variable during illumination and
312 ongoing photosynthesis.

313

314 *Effects on Fo, ϕ_{IImax}, ϕ_{II} and NPQ after long-term TCS exposure in microcosms*

315 Measurements of Fo, ϕ_{IImax}, and NPQ of PS II were conducted via PAM measurements on
316 samples from four unexposed control microcosms, three replicates of the exposure
317 concentrations 3.16, 31.6 and 316 nM TCS, and one replicate of the exposure concentrations

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0.316, 1, 10, 100 and 1000 nM TCS. Measurements of ϕ_{II} were made on four unexposed control microcosms, four replicates of the exposure concentration 100 nM TCS, three replicates of the exposure concentrations 3.16, 31.6 and 316 nM TCS, and one replicate of the exposure concentrations 0.316, 1, 10 and 1000 nM TCS. If the samples have been dark-adapted and no other factor is affecting the photosynthetic electron transport, Fo represents the *in vivo* chlorophyll *a* concentration in the samples. Before the measurements of Fo, ϕ_{IImax} and NPQ the samples were dark-adapted for 40 minutes. Long-term effects on ϕ_{II} were measured after 15 days of exposure in the microcosm system and on Fo, ϕ_{IImax} and NPQ measurements were made after 16 days of exposure in the microcosm system.

HPLC pigment analysis

HPLC analyses were employed to characterize the major photosynthetic pigments in the periphyton communities. Samples were taken from seven unexposed control microcosms, four microcosms with the exposure concentration 100 nM TCS, three microcosms with the exposure concentrations 3.16, 31.6 and 316 nM TCS, and one microcosm each with the exposure concentrations 0.316, 1, 10 and 1000 nM TCS. After an exposure time of 15 days five glass discs from each sampled microcosm were transferred to scintillation vials containing 2 mL ice-cold pigment extraction media (30% methanol, 30% acetone, 30% DMSO and 10% MilliQ-Water, all solvents were of HPLC grade). The samples were stored at -18°C until the HPLC analysis. The pigments were extracted into the media by repeated sonication (3 times 15 seconds) in an ultrasonication bath (Bransonic® Ultrasonic Cleaner 2510E-MT) followed by rigorous shaking. Next, the samples were filtered through a 0.45 µm syringe filter into HPLC vials. Just before injection 400 µL of the sample was diluted with 100 µL water and 100 µL of the resulting mixture

was injected into the HPLC system (UFLC Shimadzu, SIL-10AXL autosampler) equipped with a C-18 column (Phenomenex KinetexTM 2.6 µm C18 100A 150x3.00 mm) heated to 28°C using an UFLC Shimadzu, CTO-10ASvp column oven. The pigments were separated using the gradient listed in Supplementary table 1 and detected with a diode array detector (UFLC Shimadzu, SPD-M20A). Peaks were analyzed at 436 nm.

Community-level physiological profiling

Community-level physiological profiling (CLPP) was performed using Biolog Ecoplates ™. CLPP was analysed as average well colour development as well as the concentration-dependent changes in the utilization of each carbon source over a period of 100 hours, following the strategy presented in [37]. In short, three glass discs were sampled from each microcosm and transferred to scintillation vials with 20 mL medium, consisting of 25% GF/F filtered natural seawater, taken from the inflow to the microcosms, and 75% NaCl solution (0.26 PSU) amended with 0.7 mmol/L PO_4^{2-} and 8 mmol/L NO^{3-} as well as TCS in concentrations of 0.316-1000 nM. The scintillation vials were sonicated 3x15 seconds, followed by rigorous shaking. The suspensions were then filtered through sterilized paper tissue into plastic petri dishes from which 150 µL were pipetted into each well of a Biolog Ecoplate™ (Biolog, Hayward CA). The plates were incubated at 22°C at 100% relative humidity over 96 hours. The optical density was measured at 595 and 700 nm using a BioTek µQuant™ Microplate Spectrophotometer after 42, 48, 66, 72, 90 and 96 hours of incubation. The replication of the Biolog Ecoplates from the microcosms was the same as for the HPLC pigment analyses.

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363 *Statistics*

364 Concentration response curves were modelling using the following Weibull model:

365 $$effect = 1 - \exp - \exp\left(\theta_1 + \theta_2 \times \log_{10}(conc)\right)$$

366 with θ_1, θ_2 being the two model parameters that were estimated in an iteratively reweighted least-
367 square approach [38]. EC50 values were estimated from the corresponding inverse function,
368 confidence values are based on the standard Wald-based estimates, calculated by proc nlin
369 (SAS, Vers. 9.3. Cary, US).

370

371 In order to determine LOEC and NOEC values for short- and long-term effects, one-way
372 analyses of variance (ANOVA) were performed. The homogeneity of variances was checked
373 with Levene's test and statistical significance among treatments was identified using Dunnets
374 post hoc test (IBM SPSS, version 21.0). The PAST software (http://palaeo-
375 electronica.org/2001_1/past/issue1_01.htm, vers. 3.01) was used to create multidimensional
376 scaling (MDS) ordinations of the Bray Curtis similarity index, and statistically significant
377 differences of the compositions was analysed through Analysis of Similarities (Anosim), of
378 square root transformed data of pigment concentrations using the Primer 5 software (version
379 5.2.9).

380

381 RESULTS AND DISCUSSION

382 *Chemical analysis*

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The concentrations of TCS in the flow-through microcosms were analyzed two times before the periphyton discs were put into the microcosms, two times during the experiment (day 3 and 11) and one time the day after the last measurements. The number of samples, the median and the range of analyzed TCS concentrations for the different exposure levels is given in Table 1. The median analyzed concentrations followed the nominal concentrations well (Table 1). The maximum deviations of median analyzed concentrations from the nominal concentrations were 62% and 155%, apart from one sample in which we detected 17 nM TCS at the nominal concentration of 3.16 nM (Table 1). This is likely due to a reduced inflow of seawater into the system just before this specific sampling event. It is however, important to note that this particular sample was taken the day after the last measurements, i.e. this higher exposure concentration most likely did not affect the community prior to sampling. A low background exposure in the controls (0 to 2.7 nM, Table 1) corresponds well to previous marine monitoring studies at the west coast of Sweden [7]. It was hence decided to use nominal concentrations as the exposure descriptor throughout the present study.

Short-term effects of triclosan to field-sampled periphyton

The acute TCS toxicity to the photosynthesis of field-sampled periphyton was studied using both PAM and ^{14}C-incorporation. All four excitation channels of the PAM instrument resulted in similar responses when ϕ_{IImax} (potential photosynthetic activity at PSII) and ϕ_{II} (actual photosynthetic activity of PS II) was measured. Such similarity between the excitation channels was also observed by Schmitt-Jansen and Altenburger when testing the toxicity of herbicides to freshwater periphyton communities [39]. We therefore used the average of the four excitation channels for both endpoints in all analyses.

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406 ϕ_{IImax} and ϕ_{II} were clearly affected after 75 minutes of TCS exposure (Fig. 1), with EC50 values of
407 3760 nM and 3000 nM, respectively. These EC50 values compares well to the EC50 values of
408 3100 – 3800 nM reported for the same variables in freshwater periphyton after 24 hours of TCS
409 exposure [23]. TCS toxicity did not increase when extending the exposure time to 150 minutes
410 (data not shown). Hence, the full effect of TCS on photosynthetic electron transport seems to be
411 achieved within 75 minutes.

412 It can be assumed that, if the photosynthetic electron transport would be the primary site of TCS
413 action in algae, effective quantum efficiency or maximum quantum yield would be amongst the
414 most sensitive endpoints. However, we detected higher TCS toxicity with ^{14}C-incorporation
415 (Fig. 2). The EC50 value from the ^{14}C-incorporation test was 1080 nM after 60 minutes of
416 exposure, which is about 2.5 times lower than the EC50 of 3000 nM for ϕ_{II}. Hence, the results
417 from the short-term tests in this study indicate that TCS does not primarily inhibit
418 photosynthetic electron transport.

419

420 *Long-term effects of triclosan after 17 days exposure*

421 Although short-term exposure to 1000 nM TCS resulted in 18 and 23% inhibition of maximum
422 quantum yield and effective quantum efficiency respectively (Fig. 1), no such effects were
423 observed after an exposure to 1000 nM over 17 days in the flow-through microcosms. In the
424 microcosms, this exposure level instead resulted in a stimulation of maximum quantum yield
425 and effective quantum efficiency by 14 and 8%, respectively (Fig. 3).
426 Effects on phototrophic biomass were assessed with HPLC-based pigment analysis and *in vivo*
427 fluorescence yield (Fo) using PAM. A total of 18 pigments was detected by HPLC, but only 11
428 were included in the subsequent analyses since the peaks for 7 of the pigments were too small to

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429 be unambiguously distinguished from noise. Fucoxanthin, chlorophyll *a* and chlorophyll *c* were
430 present in high amounts. In the control samples these three pigments accounted for 38, 32 and
431 21% of the total pigment content. When the total pigment content and individual pigments
432 (fucoxanthin, chlorophyll *a* and chlorophyll *c*, diadinoxanthin and beta-carotene) were analyzed,
433 a pattern with a small decrease at TCS concentrations of 0.316 – 10 nM and a sharp increase at
434 31.6 – 1000 nM TCS was evident (Fig. 4). The pigment concentration was significantly higher for
435 all these pigments at 100 and 316 nM of TCS exposure, i.e. the NOEC was 31.6 nM. Since the
436 1000 nM treatment was unreplicated no statistical significance could be determined. However,
437 from Fig. 4 it is evident that the pigment concentration was clearly elevated also at this exposure
438 level.
439 These results are in contrast to the results of Backhaus et al. [30] and Johansson et al. [34] who
440 found that TCS exposure lowered pigment concentrations in marine periphyton. However,
441 those data were obtained in a 96 hour exposure system, the so-called SWIFT assay, which in
442 contrast to the flow-through aquaria system employed in the present study, does not allow
443 immigration of new species during the exposure. Periphyton in the flow-through microcosms
444 has a greater adaptability to TCS exposure, since i) the sampling integrates a much larger total
445 biodiversity from a temporally variable environment, ii) immigration allows species
446 replacements in the TCS-exposed biofilms and iii) a longer exposure time allows TIS to develop
447 further. Thus, the differences in the pigment content responses between the SWIFT test and the
448 flow-through microcosms indicate that there is a large reservoir of TCS tolerance elements in the
449 marine environment that the SWIFT test does not incorporate.
450 The phototrophic biomass was also assessed with Fo using PAM. Given that a sample is fully
451 dark-adapted, Fo is thought to be positively correlated to the chlorophyll *a* concentration.
452 Interestingly, there was no stimulation of Fo as was the case for chlorophyll *a*. As seen in Fig. 5

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453 the average Fo over the four excitation channels is more or less stable over all tested TCS
454 concentrations, but the chlorophyll a content detected with HPLC increased to approximately
455 160% of controls at exposure levels of 100 nM and above. This clear discrepancy between Fo
456 (PAM) and chlorophyll *a* (HPLC) might originate from methodological differences. In particular,
457 the light used for excitation in the fluorometer might not reach all cells in a thicker biofilm and
458 the emitted fluorescence might also be quenched in a dense biofilm. Although Schmitt-Jansen
459 and Altenburger [35] found quite good correlations between fluorescence yield detected with
460 Phyto-PAM and chlorophyll concentrations detected with HPLC, they noted that fluorescence
461 yield underestimated chlorophyll a concentration for thick biofilms, which corresponds to the
462 results of the present study.
463
464 The multivariate analyses of the pigment profiles revealed that communities exposed to 100 nM
465 and 316 nM TCS had distinct and statistically significantly different pigment composition
466 compared to the controls (Anosim, p=0.05, Supplementary Fig. 1), confirming the NOEC for TCS
467 on pigment composition of 31.6 nM. The highest exposure level of 1000 nM was unreplicated
468 and hence no multivariate statistical analysis was carried out. However, the placement of the
469 1000 nM treatment in the multi-dimensional scaling (MDS) plot is clearly separated from control
470 and communities exposed to low TCS concentrations (Supplementary Fig. 1). These changes
471 mainly originate from a general increase in pigment content, since the content of all major
472 pigments increased at 100 nM and above (Fig. 4).
473
474 The pigment concentration and composition in an exposed algal community reflects
475 physiological responses, biomass and changes in species composition. Hence, TCS stimulated
476 pigment synthesis, phototrophic biomass and/or selected for species with higher pigment

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content. Stimulation of phototrophic biomass could also be an indirect effect, if grazers on these organisms were more TCS sensitive than the algae and cyanobacteria who would hence be relieved from grazing pressure. However, neither Nietch [26] nor Lawrence [24] detected any TCS effects on grazing in freshwater periphyton. Increased pigment content has previously been shown in TCS exposed freshwater periphyton [26], but only at lower exposure levels of 0.35 – 3.5 nM. At such low exposures the marine periphyton communities studied here actually had a slightly lowered content of the major pigments (Fig. 4).

One factor that affects TCS lipophilicity, and hence its bioaccumulation and toxicity, is pH, as the compound is a weak acid with a pKa of 8.1. As expected, Orvos et al. [2] could demonstrate that the unionized, more lipophilic form, which dominates at lower pH, has a greater toxicity. Differences in pH could hence at least partly explain the lower toxicity of TCS to marine compared to freshwater periphyton, as TCS is present at only roughly 50% in its unionized form at the typical pH level of marine coastal environments (8.0 – 8.1 in the present study). Periphyton photosynthesis in the microcosms will increase the pH even more, which will further reduce the fraction of unionized TCS.

A fundamental difference between the experiments with short- and long-term exposure is that long term exposure allows for the selection of tolerant species, whereas short term exposure does not. The differences in toxicity to photosynthesis after short-term and long-term exposure hence reflects the absence and presence of a toxicant induced succession (TIS), respectively. In Fig. 8 it is evident that community tolerance to TCS increased during the long-term exposure to 100 nM and above. In the short-term PICT detection tests, in which the uptake of ^{14}C was used

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as the endpoint, the average EC50 of the unexposed control communities was 560 nM. After a long-term exposure to 100 or 316 nM TCS the short-term EC50 increased more than tenfold to 5950 and 5850 nM, respectively, indicating a substantial tolerance development. The EC50 values of the communities exposed to 316 nM were significantly different from the EC50 values of the unexposed control communities ($p=0.05$). Unfortunately we were unable to accommodate replicated concentration-response curves from the microcosms with the long-term exposure of 100 nM, and from microcosms with the long-term exposure of 3.16 and 31.6 nM. Assuming that the variability of the EC50s is the same at the long-term exposures of 316 and 100 nM, the LOEC of PICT is 100 nM. Since the EC50 determined for periphyton exposed to 10 nM TCS is within the 95% confidence limits of the unexposed control microcosms, it seems reasonable to assume that the NOEC of PICT to TCS would be between 10 to 100 nM. At the highest long-term exposure of 1000 nM community tolerance to TCS increased even further to a short-term EC50 of 7840 nM.

As reviewed by Blanck [21] many authors have shown that an increased community tolerance reflects elimination of sensitive species. In general, physiological processes also contribute to PICT, but over longer time scales differences in physiological capabilities between species will likely lead to changes in species composition. The differences in short-term and long-term toxicity to photosynthesis, as well as the TCS-induced changes in pigment profiles in the communities, are consistent with the PICT results. A scenario in which TCS eliminated sensitive species and selected for tolerant species with higher pigment content and thus a higher photosynthesis capacity seems likely.

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The long-term concentration of >10 to 100 nM that induced PICT in the present study is slightly higher than published EC50 values for the highly sensitive green algae and cyanobacteria, which were estimated at 2.4 – 15 [2], 6.6 [23], 12 [40] and 16 nM [41]. It is, however, known that marine periphyton in general contains only very few green algae and is to a large extent dominated by diatoms and cyanobacteria [31, 42]. In freshwater periphyton, Nietch et al. [26] also noted that cyanobacteria were more sensitive than diatoms. However, other authors have observed the reversed sensitivity pattern between these two groups [22, 24]. The fluorescence yield response pattern of this study does not indicate sensitivity differences between cyanobacteria and diatoms (Fig 5).

In order to assess the PICT response in the flow-through microcosms, we compared the sensitivity of periphyton in the microcosm controls to that of field sampled periphyton. Here, it is important to note that the field-sampled periphyton was established during the same period as the microcosm experiment and collected very close (approximately 7 meters) to the water intake of the microcosms, and hence there were no differences in water chemistry or TCS bioavailability. This comparison thus specifically tests if the short-term sensitivity to TCS differs between the natural environment and the microcosms. From Fig. 2 it is evident that the sensitivity of the field sampled periphyton was only slightly lower than the average of the control microcosms. For two of the three control microcosms, the 95% confidence values around the EC50 overlaps with the corresponding confidence values for the unexposed field sampled periphyton (data not shown). This shows that the TCS sensitivity of the periphyton in the microcosms is representative for the TCS sensitivity in the natural marine environment. A

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544 similar good agreement between the sensitivities of field-sampled periphyton and periphyton in
545 such microcosms was also noted by Dahl and Blanck [43]

546

547 TCS is known to inhibit lipid synthesis in bacteria by binding to the enoyl-ACP reductase[15]
548 and in the higher plant *Arabidopsis* [44]. Hence, this mechanism of action might also be of
549 relevance for microalgae. Another possible mechanism of action is uncoupling of
550 photophosphorylation. Franz *et al.* [23] noted that an uncoupling mechanism of action fitted well
551 to their recorded TCS toxicity to diatom photosynthesis. It has been shown that substituted
552 phenols uncouple photophosphorylation in photosynthetic membranes of purple bacteria
553 Rhodobacter sphaeroides [45, 46]. Moreover, Escher *et al.* [45] suggested that bulky substitution
554 groups in the *ortho*-position dramatically increase the uncoupling potency, which also applies to
555 TCS with its 2,4-dichlorophenoxy group bound in the *ortho*-position. Interestingly, Ricart et al
556 [29] reported that non-photochemical quenching (NPQ) was a very sensitive endpoint for effects
557 of TCS in freshwater periphytic algae. NPQ describes all quenching processes of PS II
558 chlorophyll fluorescence not related to photochemistry and includes, for example, the build-up
559 of the pH gradient over the thylakoid membrane. It is also known that uncoupling compounds
560 reduce NPQ in diatoms [47]. Although Ricart and co-authors suggested that reduced NPQ
561 might indicate damage the photosynthetic apparatus, their data can actually be interpreted as
562 support of uncoupling as a mechanism of action for TCS in algae. Unfortunately, our data on
563 long-term effects of TCS on NPQ were irregular with high variance, and hence neither support
564 nor falsify the hypothesis of TCS acting as an uncoupler in marine algae.

565

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566 However, one might speculate that the increased pigment content we detected as a TCS
567 response might mitigate the uncoupling by TCS. Pigment content increased at the same
568 exposure level as PICT was induced, and elevated pigment levels would harvest more light
569 energy, which in turn would uphold the pH gradient over the thylakoid membrane and mitigate
570 the uncoupling effect of TCS. In this scenario, the increased pigment content seen in this study is
571 a tolerance mechanism for TCS. Supporting this hypothesis is the observation of increased
572 chlorophyll a concentrations as a response to uncoupling compounds in *Chlorella vulgaris* [48]
573 and in a microalgal consortium [49].
574
575 Although TCS is used as an antimicrobial compound, several studies have shown that algae are
576 more sensitive to TCS than bacteria [1, 2, 8, 9, 40, 41]. However, Ricart and co-workers [29]
577 reported that TCS caused larger mortality on bacteria than algae in freshwater periphyton.
578 Again, it needs to be noted that freshwater, as well as marine, periphyton is dominated by
579 diatoms, which are known to be less TCS sensitive than green algae. Nietch et al. [26] showed
580 that TCS decreased the bacterial abundance in freshwater periphyton, but also that this pattern
581 roughly followed that of the algae. Proia et al. [27, 28] suggested that the TCS effects on algae
582 could be indirect effects resulting from TCS toxicity to bacteria. However, this suggestion is
583 contradicted by Lawrence et al. (2009) who showed that 35 nM of TCS caused freshwater
584 periphyton to become more heterotrophic.
585
586 In the present study, we performed community-level physiological profiling (CLPP), using
587 Biolog Ecoplates, to detect if TCS changed the capacity of the periphytic bacteria to catabolize

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588 different carbon sources. No changes in carbon utilization pattern or in average well color
589 development were detected at any of the tested long-term concentrations (data not shown).

590

591 When the PICT response reported here is compared to the PNEC value of 5.4 nM calculated by
592 Capdevielle *et al.* [11] it seems as if the PNEC is also protective for photosynthetic organisms in
593 marine periphyton. However, the difference between the PNEC and the lower end of the NOEC
594 estimation for PICT in this study (10 nM) is not even a factor of 2.

595

596 To summarize and conclude, we demonstrate that TCS is acutely toxic to photosynthesis of
597 marine periphyton communities at concentrations exceeding 1000 nM. However, when TCS was
598 tested in an open, more ecologically realistic test system, with the exposure duration prolonged
599 to 17 days, large increases in the amounts of photosynthetic pigments and a PICT response was
600 detected at a long-term exposure of 100 nM and above. Hence, TCS likely eliminated sensitive
601 species and adversely affected the communities at this concentration. The bacterial capacity to
602 catabolize different carbon sources was not affected at the tested concentrations of up to 10,000
603 nM TCS.

604

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610

611 References

612 1. Bedoux, G., Roig, B., Thomas, O., Dupont, V., Le Bot, B., Occurrence and toxicity of
613 antimicrobial triclosan and by-products in the environment. *Environ. Sci. Pollut. Res.* 2012, *19*, (4),
614 1044-1065.
615 2. Orvos, D. R., Versteeg, D. J., Inauen, J., Capdevielle, M., Rothenstein, A., Cunningham, V.,
616 Aquatic toxicity of triclosan. *Environ. Toxicol. Chem.* 2002, *21*, (7), 1338-1349.
617 3. SCCS. 2010. Opinion on triclosan (antimicrobial resistance). Scientific Committe on Consumer
618 Safety: 22 June 2010.
619 4. Singer, H., Müller, S., Tixier, C., Pillonel, L., Triclosan: Occurrence and Fate of a Widely Used
620 Biocide in the Aquatic Environment: Field Measurements in Wastewater Treatment Plants,
621 Surface Waters, and Lake Sediments. *Environ. Sci. Technol.* 2002, *36*, (23), 4998-5004.
622 5. Mezcua, M., Gómez, M. J., Ferrer, I., Aguera, A., Hernando, M. D., Fernández-Alba, A. R.,
623 Evidence of 2,7/2,8-dibenzodichloro-p-dioxin as a photodegradation product of triclosan in
624 water and wastewater samples. *Analytica Chimica Acta* 2004, *524*, (1–2), 241-247.
625 6. Brausch, J. M., Rand, G. M., A review of personal care products in the aquatic environment:
626 Environmental concentrations and toxicity. *Chemosphere* 2011, *82*, (11), 1518-1532.
627 7. Remberger, M., Sternbeck, J., Strömberg, K., Screening av triclosan och vissa bromerade
628 fenoliska ämnen i Sverige. *IVL Report* 2002, *B1477*,
629 (http://www.ivl.se/download/18.7df4c4e812d2da6a416800071681/1350484216659/B1477.pdf).
630 8. Chalew, T. E. A., Halden, R. U., Environmental exposure of aquatic and terrestrial biota to
631 triclosan and triclocarban. *J. Am. Water Resour. Assoc.* 2009, *45*, (1), 4-13.

9. Dann, A. B., Hontela, A., Triclosan: environmental exposure, toxicity and mechanisms of action. *J. Appl. Toxicol.* 2011, *31*, (4), 285-311.

10. Coogan, M. A., Edziyie, R. E., La Point, T. W., Venables, B. J., Algal bioaccumulation of triclocarban, triclosan, and methyl-triclosan in a North Texas wastewater, treatment plant receiving stream. *Chemosphere* 2007, *67*, (10), 1911-1918.

11. Capdevielle, M., Van Egmond, R., Whelan, M., Versteeg, D., Hofmann-Kamensky, M., Inauen, J., Cunningham, V., Woltering, D., Consideration of exposure and species sensitivity of triclosan in the freshwater environment. *Integrated Environmental Assessment and Management* 2008, *4*, (1), 15-23.

12. Reiss, R., Mackay, N., Habig, C., Griffin, J., An ecological risk assessment for triclosan in lotic systems following discharge from wastewater treatment plants in the United States. *Environ. Toxicol. Chem.* 2002, *21*, (11), 2483-2492.

13. von der Ohe, P. C., Schmitt-Jansen, M., Slobodnik, J., Brack, W., Triclosan-the forgotten priority substance? *Environ. Sci. Pollut. Res.* 2012, *19*, (2), 585-591.

14. Wilson, B. A., Smith, V. H., Denoyelles, F., Larive, C. K., Effects of three pharmaceutical and personal care products on natural freshwater algal assemblages. *Environ. Sci. Technol.* 2003, *37*, (9), 1713-1719.

15. McMurry, L. M., Oethinger, M., Levy, S. B., Triclosan targets lipid synthesis. *Nature* 1998, *394*, (6693), 531-532.

16. Pidugu, L. S., Kapoor, M., Surolia, N., Surolia, A., Suguna, K., Structural Basis for the Variation in Triclosan Affinity to Enoyl Reductases. *Journal of Molecular Biology* 2004, *343*, (1), 147-155.

17. Villalaín, J., Mateo, C. R., Aranda, F. J., Shapiro, S., Micol, V., Membranotropic Effects of the Antibacterial Agent Triclosan. *Archives of Biochemistry and Biophysics* 2001, *390*, (1), 128-136.

656 18. Phan, T.-N., Marquis, R. E., Triclosan inhibition of membrane enzymes and glycolysis of
657 Streptococcus mutans in suspensions and biofilms. *Canadian Journal of Microbiology* 2006, *52*, (10),
658 977-983.
659 19. Daughton, C. G., Ternes, T. A., Pharmaceuticals and personal care products in the
660 environment: agents of subtle change? *Environmental health perspectives* 1999, *107*, (6), 907-938.
661 20. Geiszinger, A., Bonnineau, C., Faggiano, L., Guasch, H., Lopez-Doval, J. C., Proia, L., Ricart,
662 M., Ricciardi, F., Romani, A., Rotter, S., Munoz, I., Schmitt-Jansen, M., Sabater, S., The relevance
663 of the community approach linking chemical and biological analyses in pollution assessment.
664 *Trac-Trends Anal. Chem.* 2009, *28*, (5), 619-626.
665 21. Blanck, H., A critical review of procedures and approaches used for assessing pollution-
666 induced community tolerance (PICT) in biotic communities. *Human and Ecological Risk*
667 *Assessment* 2002, *8*, (5), 1003-1034.
668 22. Drury, B., Scott, J., Rosi-Marshall, E. J., Kelly, J. J., Triclosan Exposure Increases Triclosan
669 Resistance and Influences Taxonomic Composition of Benthic Bacterial Communities. *Environ.*
670 *Sci. Technol.* 2013, *47*, (15), 8923-8930.
671 23. Franz, S., Altenburger, R., Heilmeier, H., Schmitt-Jansen, M., What contributes to the
672 sensitivity of microalgae to triclosan? *Aquat. Toxicol.* 2008, *90*, (2), 102-108.
673 24. Lawrence, J. R., Zhu, B., Swerhone, G. D. W., Roy, J., Wassenaar, L. I., Topp, E., Korber, D. R.,
674 Comparative microscale analysis of the effects of triclosan and triclocarban on the structure and
675 function of river biofilm communities. *Sci. Total Environ.* 2009, *407*, (10), 3307-3316.
676 25. Lubarsky, H. V., Gerbersdorf, S. U., Hubas, C., Behrens, S., Ricciardi, F., Paterson, D. M.,
677 Impairment of the Bacterial Biofilm Stability by Triclosan. *PLoS One* 2012, *7*, (4).

678 26. Nietch, C. T., Quinlan, E. L., Lazorchak, J. M., Impellitteri, C. A., Raikow, D., Walters, D.,
679 Effects of a chronic lower range of triclosan exposure on a stream mesocosm community.
680 *Environ. Toxicol. Chem.* 2013, *32*, (12), 2874-2887.
681 27. Proia, L., Morin, S., Peipoch, M., Romani, A. M., Sabater, S., Resistance and recovery of river
682 biofilms receiving short pulses of Triclosan and Diuron. *Sci. Total Environ.* 2011, *409*, (17), 3129-
683 3137.
684 28. Proia, L., Vilches, C., Boninneau, C., Kantiani, L., Farre, M., Romani, A. M., Sabater, S.,
685 Guasch, H., Drought episode modulates the response of river biofilms to triclosan. *Aquat.*
686 *Toxicol.* 2013, *127*, 36-45.
687 29. Ricart, M., Guasch, H., Alberch, M., Barcelo, D., Bonnineau, C., Geiszinger, A., Farre, M.,
688 Ferrer, J., Ricciardi, F., Romani, A. M., Morin, S., Proia, L., Sala, L., Sureda, D., Sabater, S.,
689 Triclosan persistence through wastewater treatment plants and its potential toxic effects on river
690 biofilms. *Aquat. Toxicol.* 2010, *100*, (4), 346-353.
691 30. Backhaus, T., Porsbring, T., Arrhenius, A., Brosche, S., Johansson, P., Blanck, H., Single-
692 substance and mixture toxicity of five pharmaceuticals and personal care products to marine
693 periphyton communities. *Environ. Toxicol. Chem.* 2011, *30*, (9), 2030-2040.
694 31. Porsbring, T., Arrhenius, A., Backhaus, T., Kuylenstierna, M., Scholze, M., Blanck, H., The
695 SWIFT periphyton test for high-capacity assessments of toxicant effects on microalgal
696 community development. *J. Exp. Mar. Biol. Ecol.* 2007, *349*, (2), 299-312.
697 32. Eriksson, K. M., Clarke, A. K., Franzen, L.-G., Kuylenstierna, M., Martinez, K., Blanck, H.,
698 Community level analysis of psbA gene sequences and irgarol tolerance in marine periphyton
699 *Appl. Environ. Microbiol.* 2009, *75*, (4), 897-906.
700 33. Azim, M. E., Beveridge, M. C. M., van Dam, A. A., Verdegem, M. C. J., Periphyton and
701 Aquatic Production: an Introduction. In *Periphyton: ecology, exploitation and management.* , Azim,

M. E.; Verdegem, M. C. J.; van Dam, A. A.; Beveridge, M. C. M., Eds. CABI Publishing: Cambridge, 2005.

34. Johansson, H., Janmar L., Backhaus, T., Triclosan causes toxic effects to algae in marine biofilms, but does not inhibit the metabolic activity of marine biofilm bacteria. In PeerJ PrePrints 2:e371v1 http://dx.doi.org/10.7287/peerj.preprints.371v1, 2014.

35. Schmitt-Jansen, M., Altenburger, R., Community-level microalgal toxicity assessment by multiwavelength-excitation PAM fluorometry. *Aquat. Toxicol.* 2008, *86*, (1), 49-58.

36. Heinz Walz GmbH. 2003. Phytoplankton Analyzer PHYTO-PAM and Phyto-Win Software V 1.45. System Components and Principles of Operation. 2.130 / 01.99. Effeltrich, Germany.

37. Johansson, H., Janmar, L., Backhaus, T., Toxicity of ciprofloxacin and sulfamethoxazole to marine periphytic algae and bacteria. *PeerJ PrePrints* 2014, *2:e330v1*, (http://dx.doi.org/10.7287/peerj.preprints.330v1).

38. Scholze, M., Boedeker, W., Faust, M., Backhaus, T., Altenburger, R., Grimme, L. H., A general best-fit method for concentration-response curves and the estimation of low-effect concentrations. *Environ. Toxicol. Chem.* 2001, *20*, (2), 448-457.

39. Schmitt-Jansen, M., Altenburger, R., The use of pulse-amplitude modulated (PAM) fluorescence-based methods to evaluate effects of herbicides in microalgal systems of different complexity. *Toxicological and Environmental Chemistry* 2007, *89*, (4), 665-681.

40. DeLorenzo, M. E., Keller, J. M., Arthur, C. D., Finnegan, M. C., Harper, H. E., Winder, V. L., Zdankiewicz, D. L., Toxicity of the antimicrobial compound triclosan and formation of the metabolite methyl-triclosan in estuarine systems. *Environ. Toxicol.* 2008, *23*, (2), 224-232.

41. Tatarazako, N., Ishibashi, H., Teshima, K., Kishi, K., Arizono, K., Effects of triclosan on various aquatic organisms. *Environmental sciences : an international journal of environmental physiology and toxicology* 2004, *11*, (2), 133-140.

726 42. Eriksson, K. M., Antonelli, A., Nilsson, R. H., Clarke, A. K., Blanck, H., A phylogenetic
727 approach to detect selection on the target site of the antifouling compound irgarol in tolerant
728 periphyton communities. *Environmental Microbiology* 2009, *11*, (8), 2065-2077.
729 43. Dahl, B., Blanck, H., Pollution-induced community tolerance (PICT) in periphyton
730 communities established under tri-n-butyltin (TBT) stress in marine microcosms. *Aquat. Toxicol.*
731 1996, *34*, (4), 305-325.
732 44. Dayan, F. E., Ferreira, D., Wang, Y. H., Khan, I. A., McInroy, J. A., Pan, Z. Q., A pathogenic
733 fungi diphenyl ether phytotoxin targets plant enoyl (acyl carrier protein) reductase. *Plant*
734 *Physiology* 2008, *147*, (3), 1062-1071.
735 45. Escher, B. I., Snozzi, M., Schwarzenbach, R. P., Uptake, Speciation, and Uncoupling Activity
736 of Substituted Phenols in Energy Transducing Membranes. *Environ. Sci. Technol.* 1996, *30*, (10),
737 3071-3079.
738 46. Escher, B. I., Schwarzenbach, R. P., Mechanistic studies on baseline toxicity and uncoupling
739 of organic compounds as a basis for modeling effective membrane concentrations in aquatic
740 organisms. *Aquat. Sci.* 2002, *64*, (1), 20-35.
741 47. Ruban, A. V., Lavaud, J., Rousseau, B., Guglielmi, G., Horton, P., Etienne, A. L., The super-
742 excess energy dissipation in diatom algae: comparative analysis with higher plants. *Photosynth.*
743 *Res.* 2004, *82*, (2), 165-175.
744 48. Sahinkaya, E., Dilek, F. B., The growth behavior of Chlorella vulgaris in the presence of 4-
745 chlorophenol and 2,4-dichlorophenol. *Ecotoxicology and Environmental Safety* 2009, *72*, (3), 781-
746 786.
747 49. Lima, S. A. C., Raposo, M. F. J., Castro, P. M. L., Morais, R. M., Biodegradation of p-
748 chlorophenol by a microalgae consortium. *Water Res.* 2004, *38*, (1), 97-102.

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749

750 Table 1. Nominal and analyzed TCS concentrations in the flow-through microcosms.

Number of microcosms	Number of samples analyzed	Nominal concentrations (nM)	Median analyzed concentrations (nM)	Range of analyzed concentrations (nM)
7	5	0	0.71	0 – 2.7
1	4	0.316	0.28	0.23 – 3.4
1	4	1	1.5	0.66 – 2.8
3	4	3.16	3.23	2.1 – 17
1	4	10	7.0	5.2 – 10
3	4	31.6	31	20 – 45
4	7	100	88	69 – 160
3	4	316	260	190 – 470
1	4	1000	790	500 – 1200

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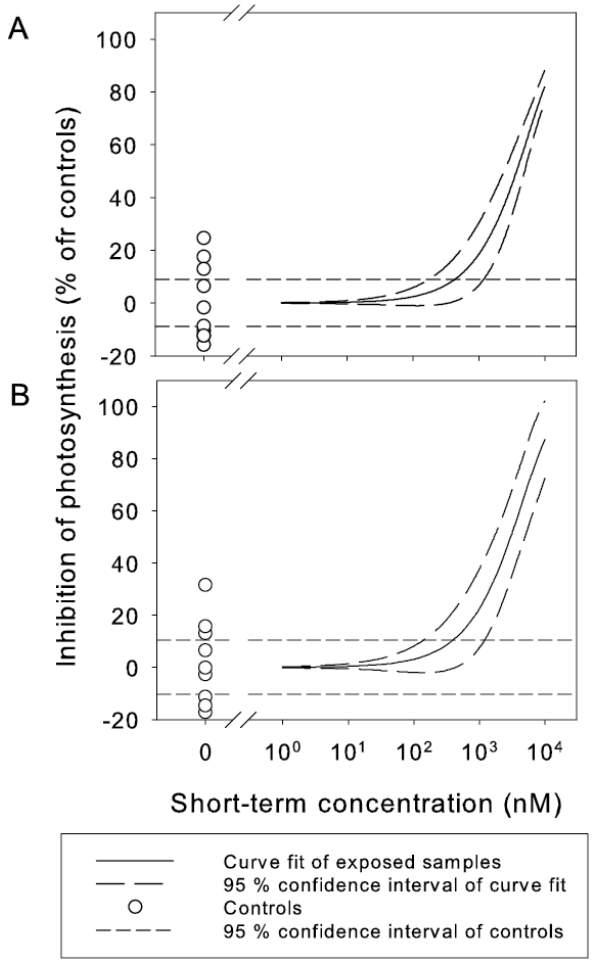

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754 Figure 1. Short-term (75 minutes) effects of TCS on maximum quantum yield (A) and effective
755 quantum efficiency (B) of PS II of field-sampled periphyton.

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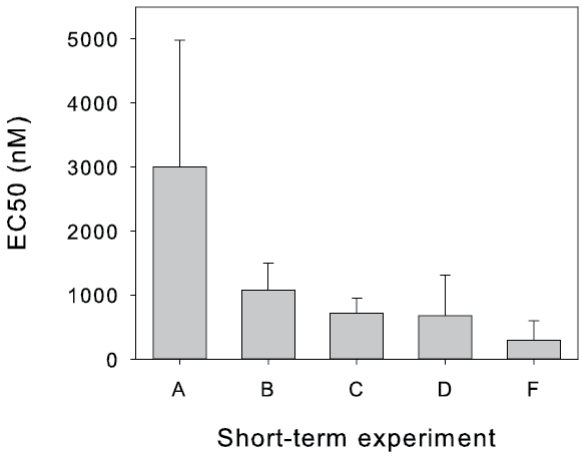

Figure 2. EC50 values from short-term toxicity of TCS to photosynthesis of periphyton communities. Toxicity detected with effective quantum efficiency in periphyton sampled from the field (A), detected with ^{14}C-incorporation in periphyton sampled from the field (B), detected with ^{14}C-incorporation in periphyton sampled from the unexposed control microcosms (C-E). The incubation times were 75 minutes for test A and 60 minutes for test B – F. The error bars indicate 95% confidence belt limits around the EC50 in each short-term test.

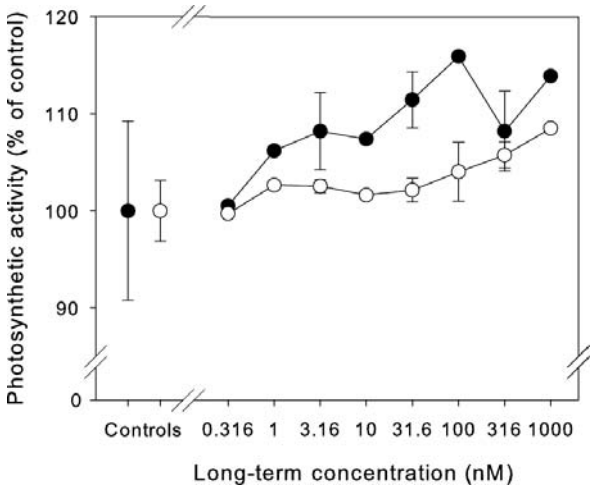

Figure 3. Long-term effects of TCS on periphyton photosynthesis detected as maximum quantum yield (white circles) and effective quantum efficiency (black circles). The error bars indicate 95% confidence intervals.

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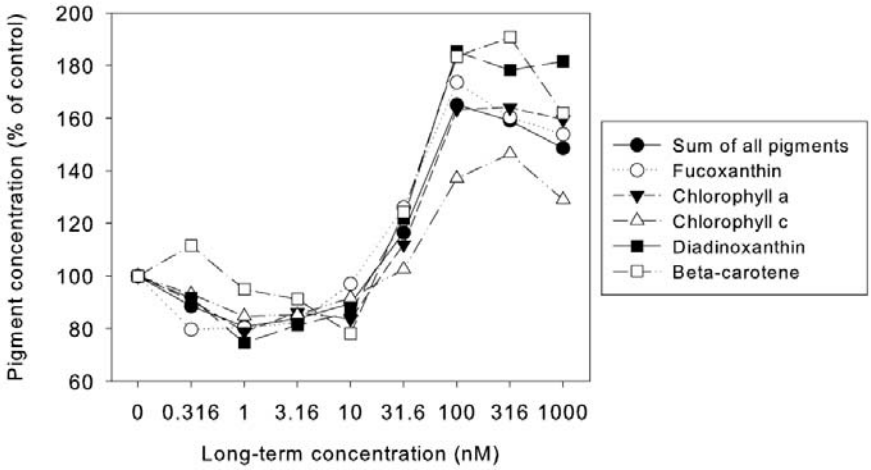

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769 Figure 4. Long-term effects of TCS on pigment content in periphyton communities.

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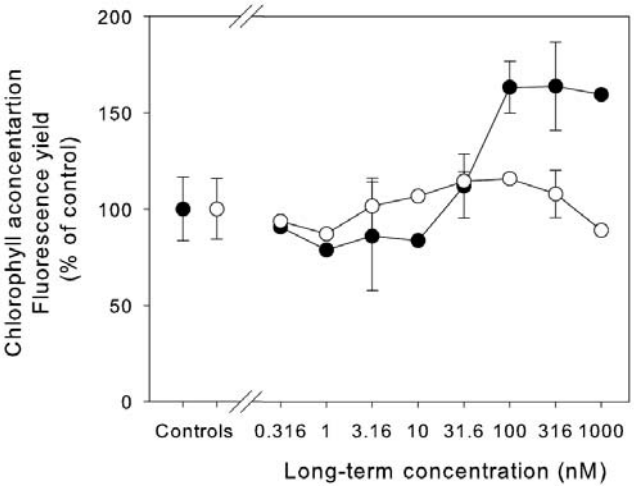

771

772 Figure 5. Long-term effects of TCS on chlorophyll *a* concentration and maximum fluorescence
773 yield. The chlorophyll *a* concentration (black circles) was determined with HPLC and the
774 fluorescence yield (white circles) was determined with PAM in dark-adapted periphyton. The
775 error bars indicate 95% confidence intervals.

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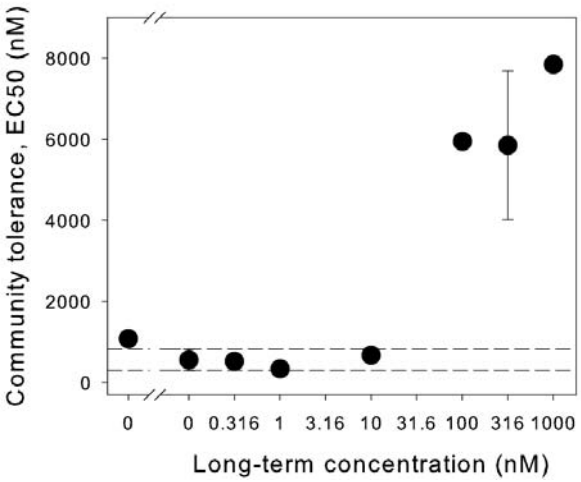

Figure 6. Community tolerance in periphytic algae and cyanobacteria over long-term exposure to TCS. Levels of community tolerance are given as EC50 values for the inhibition of photosynthesis after short-term exposure to TCS, using the incorporation of radiolabeled carbon dioxide as the endpoint. The community tolerance of periphyton sampled in the field next to the water intake of the microcosm system is shown to the left of the break on the x-axis. The community tolerance of periphyton in the microcosms is shown to the right of the break on the x-axis. The long-dashed lines represent the 95% confidence interval of three control microcosms, and the error bars indicate 95% confidence intervals of three replicated 316 nM exposure microcosms.

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798 Supplementary table 1. Description of curve fitting model, EC50 values and the 95 % confidence limits
799 around the EC50 values from short-term concentration-response testing on field- and microcosm-sampled
800 periphyton, using Pulse Amplitude Modulation (PAM) and ^{14}C-incorporation, to describe TCS effects on
801 photosynthesis.

Experimental system	Long-term exposure (nM)	Endpoint	Curve fitting model	EC50 (nM)	95 % confidence limits around the EC50 (nM)
Field-sampled periphyton	0	PAM, maximum quantum yield	Weibull	3757	2380 - 4982
Field-sampled periphyton	0	PAM, effective quantum efficiency	Weibull	3002	1684 – 4984
Field-sampled periphyton (close to microcosm system water intake)	0	^{14}C-incorporation	Weibull	1079	701 - 1501
Microcosm-sampled periphyton	0	^{14}C-incorporation	Weibull	296	96 – 597
Microcosm-sampled periphyton	0	^{14}C-incorporation	Weibull	713	484 – 947
Microcosm-sampled periphyton	0	^{14}C-incorporation	Weibull	674	248 – 1307
Microcosm-sampled periphyton	0.316	^{14}C-incorporation	Weibull	522	272 – 844

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Microcosm-sampled periphyton	1	^{14}C-incorporation	Weibull	337	164 – 583
Microcosm-sampled periphyton	10	^{14}C-incorporation	Weibull	674	376 – 1063
Microcosm-sampled periphyton	100	^{14}C-incorporation	Weibull	5946	4648 – 7217
Microcosm-sampled periphyton	316	^{14}C-incorporation	Weibull	4243	2930 - 5471
Microcosm-sampled periphyton	316	^{14}C-incorporation	Weibull	7477	5097 – 10917
Microcosm-sampled periphyton	316	^{14}C-incorporation	Weibull	5829	3444 – 8671
Microcosm-sampled periphyton	1000	^{14}C-incorporation	Weibull	7841	5096 -10187

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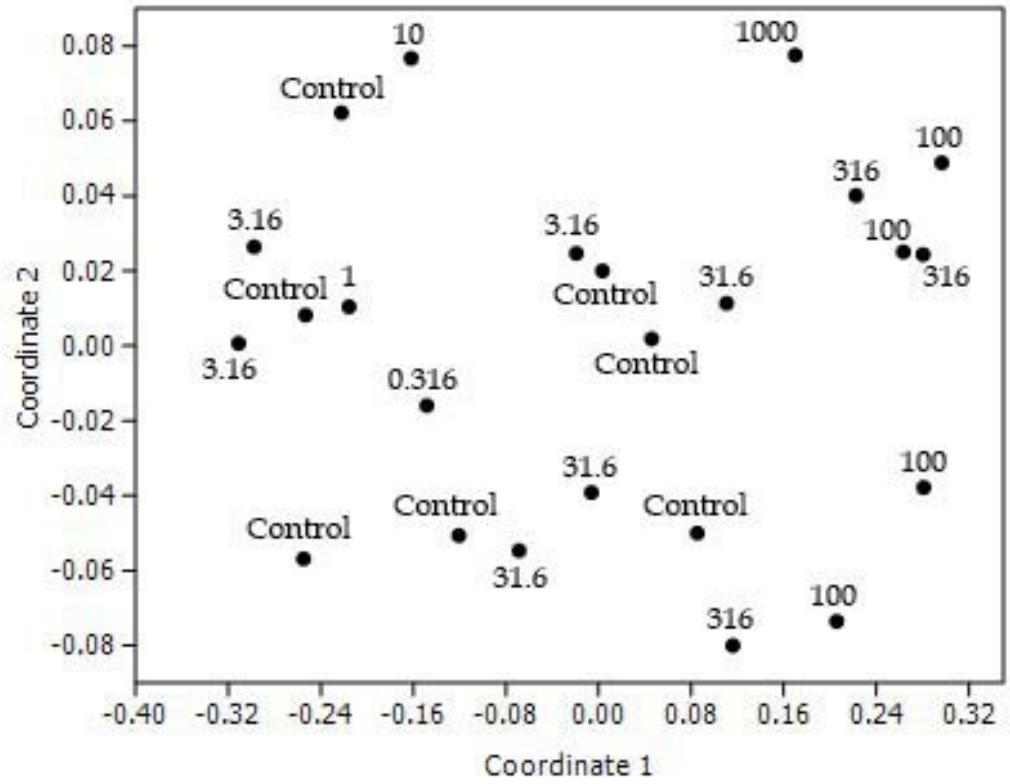

804

805 Supplementary figure 1. Multi-dimensional scaling ordination, based on the Bray-Curtis dissimilarity
806 index, of pigments in periphyton communities at different exposure levels of TCS. A label of the
807 corresponding exposure level is given next to each point. The stress value for the plot is 0.03.

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