

1 **Triclosan causes toxic effects to algae in marine biofilms, but does not inhibit the metabolic**
2 **activity of marine biofilm bacteria**

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11
12 **Abstract**

13 Effects of the antimicrobial agent triclosan to natural periphyton communities (biofilms, com-
14 prising primarily microalgae and bacteria) were assessed in two independent experiments during
15 spring and summer. For that purpose a semi-static test system was used in which periphyton was
16 exposed to a concentration range of 5 – 9 054 nmol/L triclosan. Effects on algae were analyzed
17 as content and composition of photosynthetic pigments. The corresponding EC50 values were
18 39.25 and 302.45 nmol/L for the spring and summer experiment respectively. Effects on pe-
19 riphytic bacteria were assessed as effects on carbon utilization patterns, using Biolog Ecoplates.
20 No inhibition of either total carbon utilization or functional diversity was observed, indicating a
21 pronounced triclosan tolerance of the marine bacteria. In contrast, a small stimulation of the total
22 carbon utilization was observed at triclosan concentrations exceeding 100 nmol/L.

1. Introduction

The antimicrobial agent triclosan (for molecular structure and physico-chemical characteristics, see Table 1) is widely used in various consumer products such as toothpastes and soaps, antiseptic cosmetics and toys (Bedoux et al., 2012). Consequently, triclosan is routinely detected in STP effluents, receiving waters and sediments. Typical surface water concentrations range between <0.001-0.98, 0.012-7.94 and 0.0021-3.53 nmol/L in Europe, North America and Asia respectively (Lyndall et al. 2010; Bedoux et al. 2012). Triclosan concentrations in the marine environment have recently been reviewed by Bedoux et al., who compiled concentrations in the marine environment of up to 0.024, 0.047 and 0.1 nmol/L in European, North American and Asian marine waters respectively (Bedoux et al. 2012).

Being a broad-spectrum antimicrobial agent it is effective against both gram-negative and gram-positive bacteria (Bedoux et al., 2012). Several mechanisms of action have been suggested for triclosan toxicity. It has been demonstrated that triclosan blocks the active site of enoyl-acyl carrier protein reductase (FabI) in bacteria and hence specifically inhibits fatty acid synthesis (Levy et al., 1999; McMurry et al., 1998). Triclosan has also been shown to destabilize membranes (Lygre et al., 2003; Villalaín et al., 2001) and Franz et al. (2008) observed indications of an uncoupling mode of action which previously has also been described for rat liver mitochondria (Newton et al. 2005).

Several authors have studied the acute toxicity on the marine bacterium *Vibrio fischeri* with EC50 values ranging between 183.05 and 1795.95 nmol/L, as reviewed by Bedoux et al. (2012). Chronic studies with freshwater microbial communities revealed higher toxicity values with a

LOEC value of 10 nmol/L (Johnson et al. 2009). However, microalgae have been shown to be at least as sensitive as bacteria, with chronic EC50 values ranging from 1.8 to 15 nmol/L for green algae and cyanobacteria and between 65.97 and 1 347 nmol/L for diatoms (Bedoux et al., 2012; Yang et al., 2008).

The aim of this study was to assess the long-term toxicity of triclosan to the algae and bacteria residing in natural marine periphytic biofilms.

Periphyton are biofilm communities that cover submerged surfaces in the aquatic environment. They consist of a variety of autotrophic and heterotrophic species and are responsible for important ecological processes such as primary production and nutrient cycling (Azim et al., 2005). As periphytic organisms grow in a closely confined space in the biofilm, they compete for nutrients, space and light and any change in ecological fitness as a result of an exposure to toxic compounds is likely to not only change the overall physiological activity of the biofilm species, but also affect community biodiversity. Communities exposed to toxicants are dominated by more tolerant species (Blanck 2002).

2. Material and methods

We studied effects on microalgal and bacterial biofilms (periphyton) using the semi-static SWIFT periphyton test (Porsbring et al 2007) as described in Johansson et al. (2014). Two independent experiments were carried out between April and June 2010 at the Swedish west coast (long 11.4, lat 52.23). Biofilms were established on glass slides in the environment and then transferred to the lab where they were exposed to a concentration series (5 – 9 054 nmol/L) of triclosan. Algal and bacterial members of the periphyton were hence exposed simultaneously to

exactly identical triclosan concentrations. The exposure time was 72 and 96 hours for determining effects on periphytic bacteria, respectively algae.

Effects to bacteria were assessed using Biolog Ecoplates™ (purchased from Dorte Egelund ApS, Roskilde, Denmark). These 96-well plates, pre-loaded with 31 different carbon-sources and a tetrazolium dye, provide information on functional diversity and total metabolic activity of the bacteria growing in them. Optical densities were measured over 96 hours at 595 nm (absorbance of the oxidized tetrazolium dye) and 700 nm (in order to correct for turbidity). Total content and relative fractions of photosynthetic pigments were used as a measure of algal biomass and community structure (Porsbring et al 2007). For further details see Johansson et al. (2014).

Triclosan was bought at highest available purity ($\geq 97\%$, Sigma-Aldrich Sweden AB, Stockholm Sweden) and dissolved in methanol (Lichrosolv purity, VWR international AB, Göteborg, Sweden) to a final concentration of 9 mmol/L. This stock solution was then diluted in methanol, a dilution factor of 2.3, resulting in one stock solution for each final test solution. To prepare test solutions, 200 μ L of each stock solution was pipetted into a 250mL pyrex flask and the methanol was let to evaporate before 200mL nutrient amended GF/F filtered sea water (0.7 mmol/L PO_4^{2-} and 8 mmol/L NO_3^-), which had been collected the day before the start of the experiment at the periphyton sampling site, was added. New test solutions were prepared daily and shaking vigorously at 4 °C in the dark for at least 12 hours prior to use.

A concentration range of 5 to 9054 nmol/L final concentration was used in the present experiment, which was based on previous rangefinding experiments and was tailored towards describ-

ing the full concentration-response curve for effects on algae, the more sensitive organism group, see below. 10 concentrations were tested in total and every second concentration up to 1 000 nmol/L was tested in triplicates, while higher concentrations were only tested once.

2.1 Data analysis – Biolog Ecoplates

The optical densities measured for each Biolog Ecoplate were analyzed in accordance with Johansson et al. (2014). Here we only report on the background corrected average carbon utilization (AWC), as no significantly toxic effects on bacteria were observed (see below).

2.2 Data analysis – Pigment composition

Effects on pigment content were expressed as percent inhibition compared to the arithmetic mean of the untreated controls.

Additional to investigating changes on total pigment content, we performed nonlinear nonmetric multidimensional scaling (nMDS) with all the individual pigments detected in each experiment. nMDS is an ordination method that condenses a multidimensional data structure into a 2-dimensional plot. The distance between two points in an nMDS plot reflects the multivariate dissimilarity between those samples (Clarke 1999). This was performed using Manhattan Distance for describing the dissimilarity between pairs of samples.

3. Results and discussion

Triclosan effects on the heterotrophic and the phototrophic part of biofilm communities were investigated in two independent experiments during spring and summer 2010. Effects on the algal

part of the periphyton communities will be discussed first, followed by an analysis of the effects on bacteria.

The tested concentration range (5 – 9 054 nmol/L) describes the full concentration response curve for periphytic algae in both experiments (Fig. 1 and Table 2). The algae were more sensitive towards triclosan in the spring experiment (EC₅₀ of 39.25 nmol/L) compared to the summer experiment (EC₅₀ of 302.45 nmol/L). Due to the well-known shortcomings of classic NOEC determinations (e.g. Warne and van Dam, 2008), we instead used the lower 95% confidence belt of the EC₁₀ as an estimate for the first toxic effects, which were observed at 10.81 respectively 32.74 nmol/L (table 2). The difference in sensitivity between spring and summer periphyton is most likely caused by different thicknesses of the biofilms of both experiments, indicated by the 60% higher chl a content of the summer periphyton, as well as a 20% higher catabolic activity of the bacteria isolated from the summer periphyton. A thicker biofilm would lead to a decreased exposure of the individual algae that are embedded in the biofilms.

Additionally, the relative pigment composition also indicates that the species composition of the spring and summer periphyton was slightly different. Fig. 2 shows that the summer communities contained slightly elevated relative amounts of fucoxanthin, diadinoxanthin and diatoxanthin, which are abundant pigments in diatoms, the more tolerant algal species towards triclosan, but are absent from e.g. green algae that have a higher triclosan sensitivity. The pigment pattern hence shows a greater presence of diatoms in the summer periphyton, leading to a higher tolerance of the periphyton communities.

The pigment data was further analyzed using nonmetric multivariate scaling (nMDS) and the resulting plots are shown in Fig. 3A and 3B. With increasing concentrations there is a clear trajectory in both experiments, from left to right in the nMDS plots. The steady movement towards the right indicates that the change in pigment composition is uniform for all pigments and that triclosan affects the total biomass rather than specific pigments. This is further supported by an analysis of the relative content of the individual pigments, which shows that no major changes occur until the highest test concentrations (Fig. 4A-D).

Backhaus et al. previously reported a slightly higher EC50 value of 1 166 nmol/L for total pigment composition of marine periphytic algae Backhaus et al. (2011). As the different sensitivities that were observed for the spring and summer periphyton in the present study indicate (Fig. 1), such differences are most likely due to the natural variability of the species composition of the test material. The toxicity of triclosan observed in the present study is quite similar to previously published data from single species experiments. The range of EC50 values (65.97-1 347 nmol/L) described for diatoms by (Bedoux et al. 2012) corresponds well with the EC50 values of the present study that was using a diatom-dominated biofilm community (39.25, resp. 302.45 nmol/L). Similarly, the EC50 values of 383 and 3 100 nmol/L from studies with limnic communities Ricart et al. (2010), Franz et al. (2008) indicate a comparable triclosan-sensitivity of limnic communities. As mentioned earlier, green algae seem to be noticeably more sensitive, an EC50 of 12 nmol/L was for example observed for the marine chlorophyceae *Dunaliella tertiolecta* by DeLorenzo et al (2008).

No inhibition of the carbon source metabolism was observed for the periphytic bacteria up to the highest test concentration of 9 000 nmol/L in neither experiment. Instead, a small stimulation of the AWC was observed at higher concentrations (Fig. 5), a pattern that was similar in both experiments. The stimulatory effects were visible at concentrations >140 nmol/L at which point the algae from the exposed biofilms were already strongly inhibited (30 and 80% inhibition of the total pigment content in the spring and summer experiment, respectively). The stimulation might be caused by indirect effects as competition for space with the algae is relieved and/or exposed algae release carbohydrates and other biomolecules due to the membrane-damaging effects of triclosan.

However, the complete lack of inhibition on marine periphytic bacteria that was observed in the present study does not completely agree with previously published results. Acute toxicity EC50 values for *Vibrio fischeri* ranges between 183 and 1 796 nmol/L Bedoux et al. (2012) which is within the tested concentration range of this study.

Triclosan has also been shown to be toxic to limnic bacterial communities. Ricart et al. (2010) measured an EC50 value for the ratio of live/dead bacteria of 151 nmol/L, which is the concentration range in which first signs of stimulation occur in the present study.

In an experiment carried out by Nietch et al. (2013) effects on stream periphyton communities were assessed on a wide range of endpoints and organisms. All tested concentrations (0.34 – 34.54 nmol/L) resulted in a significant toxicity, even though the effects were not uniform over the tested concentration range. For example, bacterial cell numbers were elevated when the periphyton was exposed to low triclosan concentrations (up to 3.45 nmol/L) while concentrations

above 17.27 nmol/L lead to an inhibition of bacterial cell numbers. Inhibitory effects on periphytic bacteria were also observed in a study by Lawrence et al (2009), who exposed river biofilms to 34.5 nmol/L triclosan.

Two reasons might be responsible for these sensitivity differences between marine and freshwater periphyton. First of all, triclosan has a pK_A of 8.1, which very close to the pH of the marine water used for our tests (7.9 – 8.1). This means that roughly half of the compound exists in its phenolate form under test conditions, which is known to be significantly less toxic than its non-ionized counterpart (Orvos et al. 2002). At neutral pH, i.e. under typical conditions in a freshwater system, 82% of the molecule exist in its non-ionized form (calculated with JChem, ChemAxon, Vers. 6.2.1), which has a significantly higher lipophilicity, leading to an elevated internal concentration (bioaccumulation).

Secondly, sensitivity differences might also be simply caused by different species composition of limnic and marine periphyton and the known differences in bacterial sensitivity towards triclosan. While several bacterial species are obviously susceptible to the compound, others are known to be resistant and can even utilize triclosan as a carbon source Meade et al. (2001), Nietch et al. (2013).

However, the principal pattern that was observed in the present study corresponds to the earlier discussed study by Lawrence et al., (2009), who observed a general shift towards a more heterotrophic community. This is consistent with our observations (i.e. strong toxic effects on periphytic algae, slight stimulations of periphytic bacteria), confirming the generally higher toxicity of

triclosan to algae than to bacteria – despite the fact that the compound is used in consumer products for its bactericidal purposes.

4. Hazard and risk of triclosan towards microbial communities

The freshwater PNEC for triclosan has been previously determined at 0.17 nmol/L, based on a NOEC-value from freshwater green algae (*Scenedesmus*), using an assessment factor of 10 (Samsøe-petersen et al., 2003; Dye et al., 2007). The same ecotoxicological data are used to calculate the same PNEC of 0.17 nmol/L also for the marine environment in a report by the Australian Government (2009), which, however, is not entirely in line with the strategy used within REACH. Here, an additional assessment factor of 10 is generally required, if freshwater data are used for estimating hazards to marine life (ECHA, 2008). This strategy would then result in a PNEC for marine life of 0.017 nmol/L. It should, however, be pointed out that this additional assessment factor is supposed to account for the uncertainty that results from missing data for specifically marine taxonomic groups, such as echinoderms. Under the assumption that algae are the most sensitive taxonomic group also in the marine environment, freshwater and marine PNECs can be assumed identical, see discussion in Lyndall et al. (2010).

Capdevielle et al. (2008) established a Triclosan-SSD (species-sensitivity distribution) for freshwater species and estimated a PNEC of 5.36 nmol/L for freshwater based on the HC5 (hazardous concentration for 5% of the species) (Capdevielle et al. 2008). This comparatively high PNEC is a result of (a) the fact that no assessment factor was applied to the HC5, and (b) the misfit of the used log-logistic SSD-model, which clearly underestimates the actual data in the critical low-effect region, near the HC5. Using a non-parametric approach, Lyndall et al (2010) estimated a

SSD-based HC5 of 1.73 nmol/L, which would, using the REACH-recommended assessment factors of 1-5 for SSD-based NOECs, result in a PNEC of 0.35 – 1.73 nmol/L for the freshwater environment. This would then result in a PNEC of 0.035 – 0.17 nmol/L for the marine environment, almost identical to the PNEC values discussed at the beginning of this section.

Risk assessments of triclosan for the limnic aquatic environment have been performed by several authors. Brausch and Rand (2011) as well as Tamura et al. (2013) calculated risk quotients for algae in the freshwater environment that exceed 10, based on maximum environmental concentrations of up to 7.9 nmol/L. Similarly, Samsøe-petersen et al. (2003) calculated risk quotients of 3-25 for low-technology STP plants, based on the freshwater PNEC of 0.17 nmol/L and effluent concentrations of up to 4.36 nmol/L. Taking into account the distribution of monitored triclosan concentrations, Lyndall et al. (2010) concluded that the 95th percentile of modeled and measured triclosan concentrations in surface water, sediment and biota is below the HC5 for triclosan. Still, several autotrophs are among the most sensitive species and triclosan might hence directly affect primary production at environmental hotspots, such as wastewater effluent dominated waters.

We observed first toxic effects on periphytic algae at 10.81 and 32.74 nmol/L (lower 95% confidence intervals of the EC10 values for total pigment composition) for the spring and summer periphyton, respectively. A PNEC of 0.17 nmol/L (Samsøe-Petersen, 2003; Dye, 2007), respectively 0.35 nmol/L Lyndall et al (2010) would hence be sufficiently protective. A PNEC of 5.36 nmol/L, as estimated by Capdevielle et al (2007), however, might not provide an adequate level of protection.

The highest detected triclosan concentration in limnic surface waters (6.9 nmol/L), (Lyndall et al. 2010) is just below a concentration that would cause toxic effect to the biofilms in the present study. As only few analytical surveys on triclosan occurrence in marine waters have been performed, overall conclusions on its risk for the marine environment are currently not possible. The reported triclosan occurrences of up to 0.1 nmol/L in the marine environment (Bedoux et al., 2012) result in a factor of at minimum 108.1 between environmental concentrations and clear toxic effects on marine algae. However, concentrations of up to 0.55 nmol/L triclosan have been reported in a monitoring report (Remberger et al., 2002) for a heavily used part of the marine environment near Gothenburg. This would reduce the margin of safety to a factor of 20. Conclusions on environmental risks hence seem to strongly depend on the actual exposure scenario, as well as the applied assessment factor.

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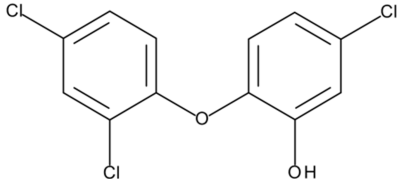
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Table 1 Physico-chemical characteristics of triclosan

Substance	Cas	M.W (g/mol)	Molecular structure	pKa	logKow (pH=8)
Triclosan	3380-34-5	289.54		pKa ₁ ^a : 7.8	Kow ^b : 4.76

^aYoung et al., 2008, ^bPhysprop Database: <http://esc.syrres.com/fatepointer/search.asp>, acquired 2013-11-20

Table 2 Inhibition of the total pigment content in periphytic algae exposed to triclosan. The estimated parameters of the Weibull fits ($\hat{\theta}_1$, $\hat{\theta}_2$, $\hat{\theta}_3$) that were used for estimating EC10, EC50 and EC90 values are given.

	$\hat{\theta}_1$	$\hat{\theta}_2$	$\hat{\theta}_3$	EC10	EC50	EC90
Spring	-22.2318	16.54862	-0.69853	14.22 [10.81 – 17.81]	39.25 [34.55 – 46.51]	182.21 [114.83 – 294.43]
Summer	-6.1564	2.334018	–	47.15 [32.74 – 69.79]	302.45 [258.60 – 347.86]	988.60 [842.04 – 1154.73]

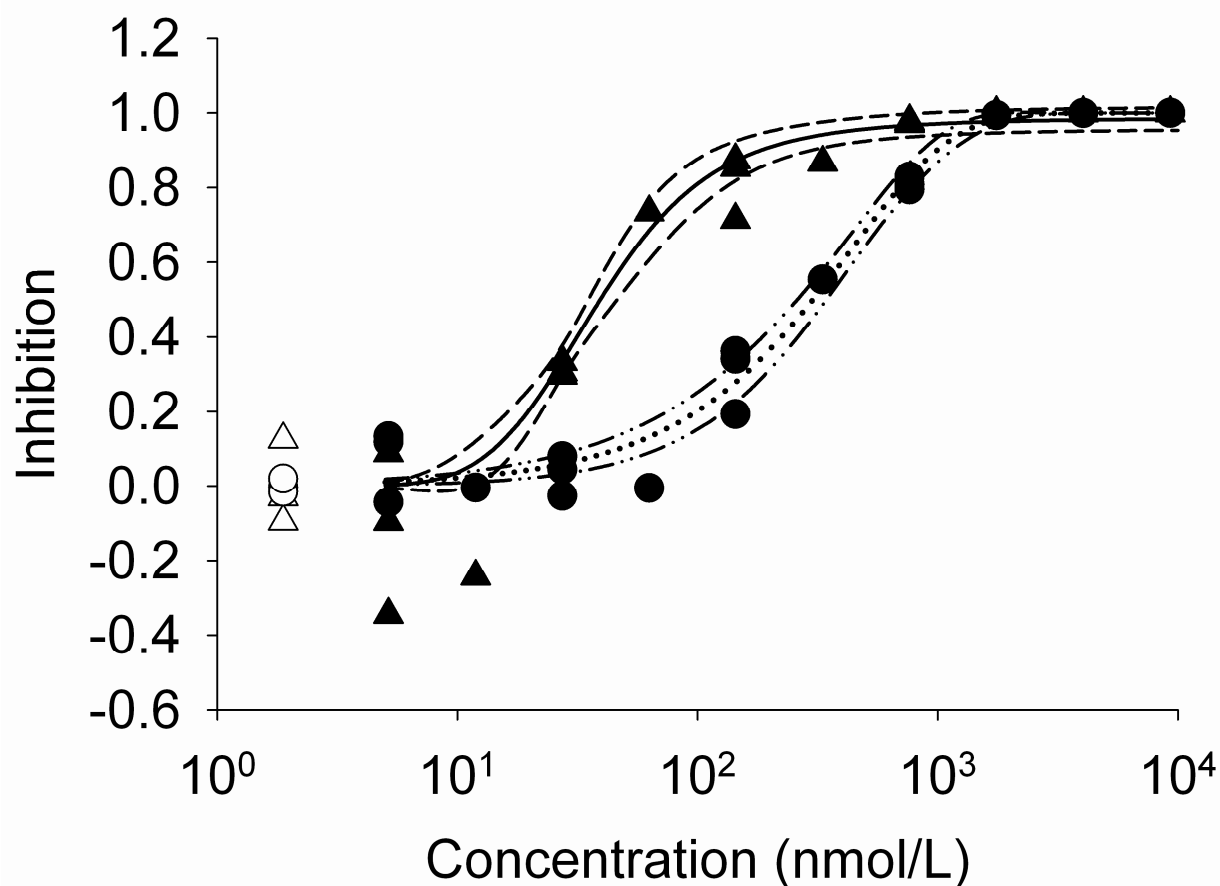


Fig. 1 - Total pigment content after 96 hours exposure to triclosan in spring (Δ) and summer (\circ), Filled symbols represent triclosan treatments, open symbols the controls. Lines give the corresponding Weibull fits.

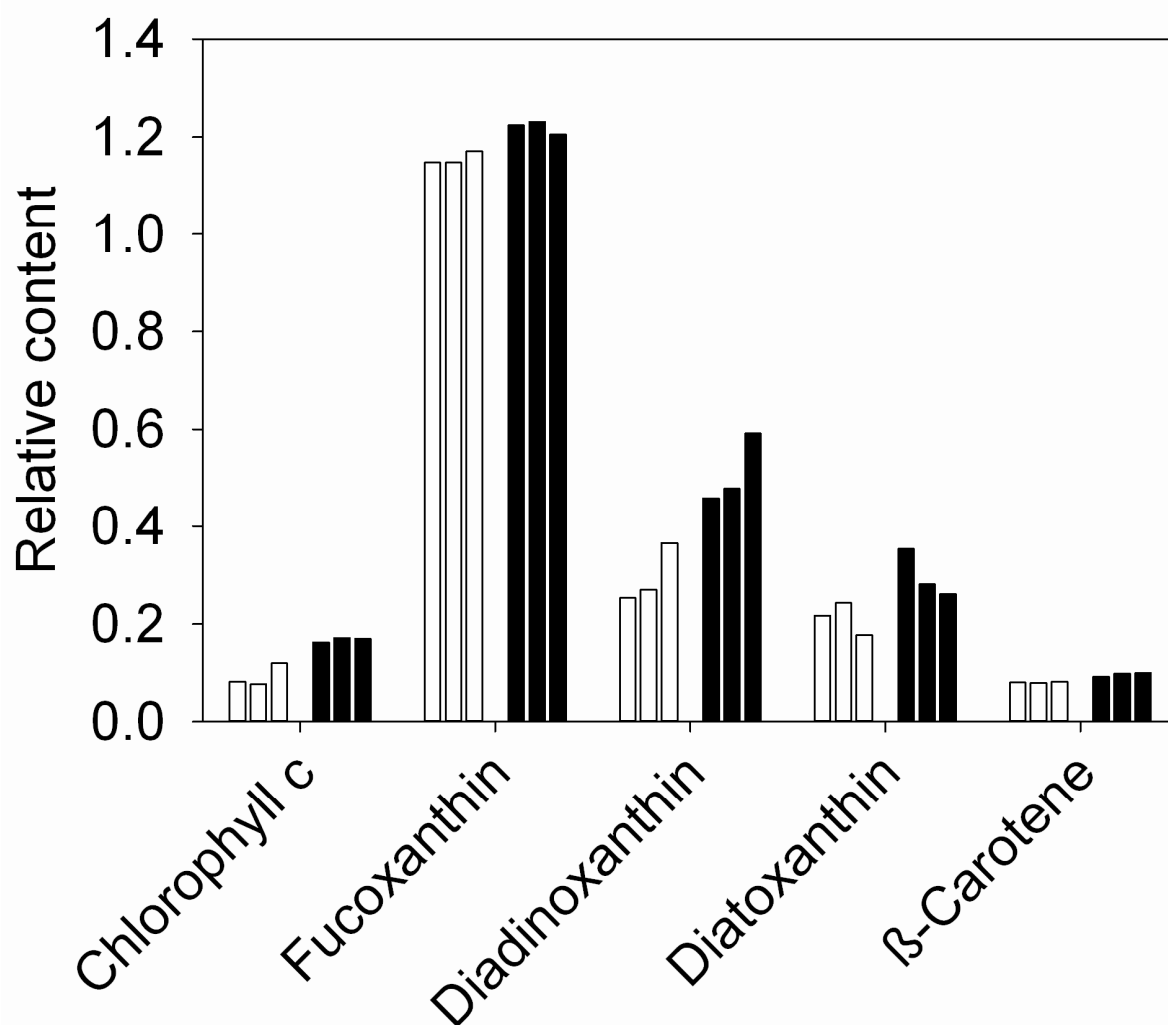


Fig. 2 Pigment content relative to chl a. Open and solid bars represent the triplicate controls, from the spring and summer experiment respectively.

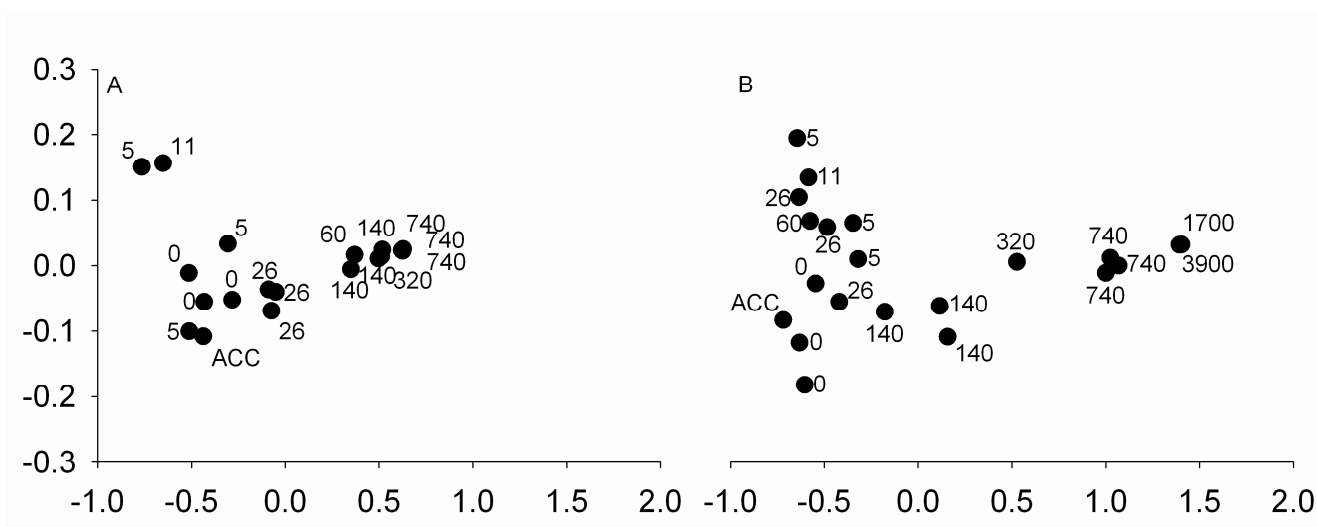


Fig. 3 Nonmetric multidimensional scaling (nMDS) showing effects on total pigment composition. Fig. 3A and 3B show data from the spring and summer experiment respectively. Concentrations >740 and >3 900 nmol/L for Fig. 3A and 3B respectively are not plotted as no pigments could be retrieved from the biofilms (100% toxicity).

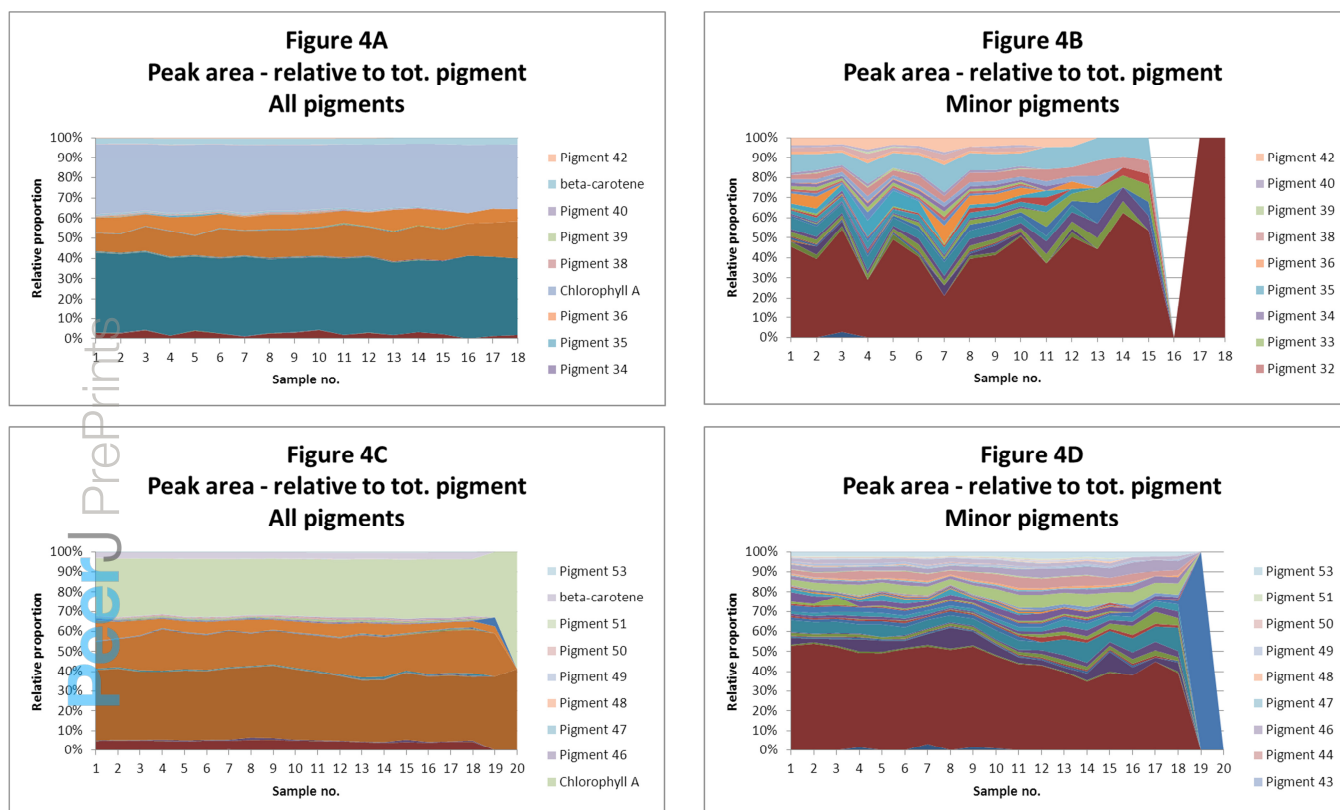


Fig. 4 Relative proportion of individual peak areas compared to the total pigment content. A and C represent all pigments in the spring and the summer experiment respectively while B and D only show minor pigments.

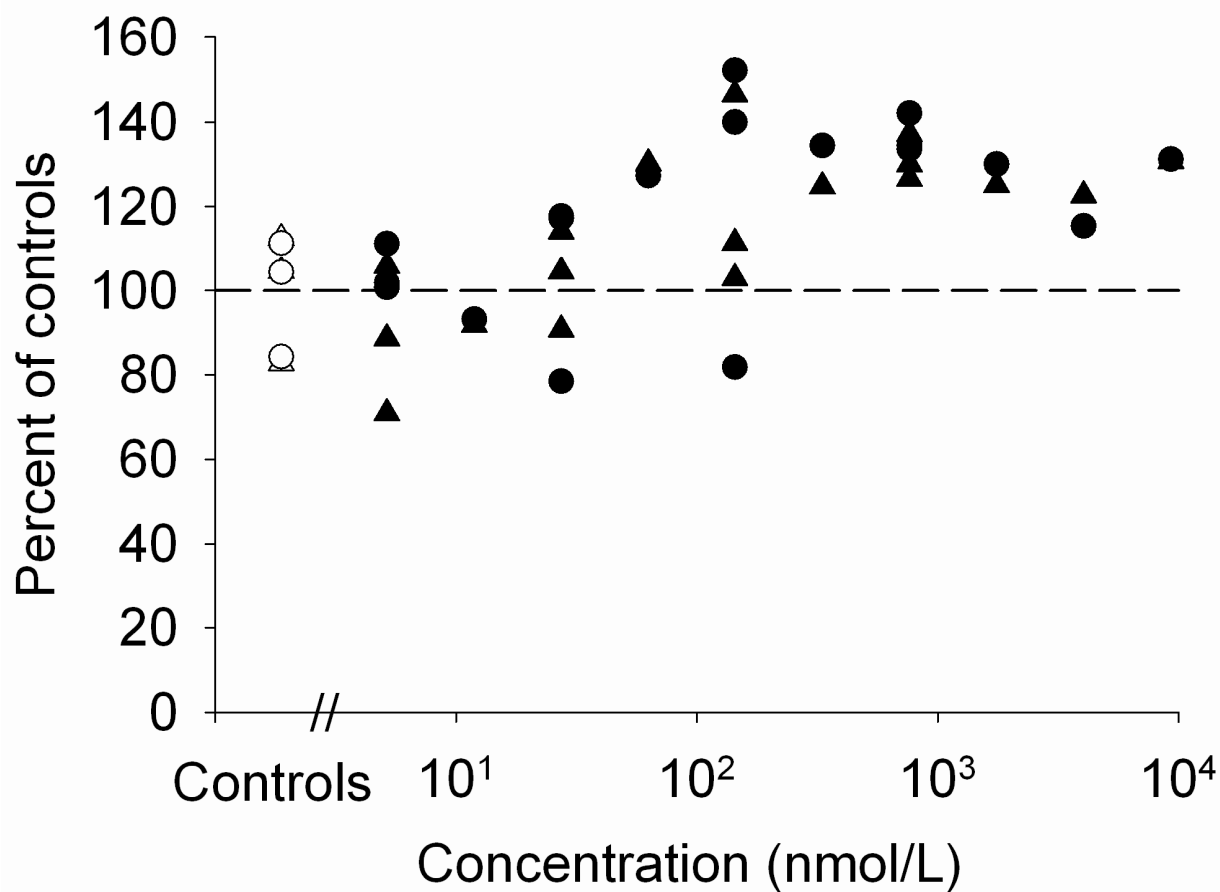


Fig. 5 Average Well Color after 72 hours of incubation in the Biolog Ecoplates or the experiment performed in spring (▲) and summer (○) respectively. Filled symbols represent triclosan treatments.