

Assessing triclosan-induced ecological and trans-generational effects in natural phytoplankton communities: a trait-based field method

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Abstract We exposed replicated phytoplankton communities confined in semi-permeable membrane-based mesocosms to 0, 0.1, 1 and 10 $\mu\text{g L}^{-1}$ triclosan (TCS) and placed them back in their original environment to investigate the occurrence of trans-generational responses at individual, population and community levels. TCS diffused out of mesocosms with a half-life of less than 8 h, so that only the parental generation was directly stressed. At the beginning of the experiment and after 7 days (approximately 2 generations) we analysed responses in the phytoplankton using scanning flow-cytometry. We acquired information on several individually expressed phenotypic traits, such as size, biovolume, pigment fluorescence and packaging, for thousands of individuals per replicated population and derived population and community aggregated traits. We found significant changes in community functioning (increased productivity in terms of biovolume and total fluorescence), with maximal effects at 1 $\mu\text{g L}^{-1}$ TCS. We detected significant and dose-dependent responses on population traits, such as changes in abundance for

several populations, increased average size and fluorescence of cells, and strong changes in within-population trait mean and variance (suggesting micro-evolutionary effects). We applied the Price equation approach to partition community effects (changes in biovolume or fluorescence) in their physiological and ecological components, and quantified the residual component (including also evolutionary responses). Our results suggested that evolutionary or inheritable phenotypic plasticity responses may represent a significant component of the total observed change following exposure and over relatively small temporal scales.

Keywords Phytoplankton · Community ecology · Phenotypic evolution · Trait-based approach · Micropollutants · Mesocosms

Introduction

Ecological risk assessment (ERA) is currently facing a number of challenges, including (a) the need for methods capable to increase the ecological realism of exposure and effect assessment, (b) the unknown importance of trans-generational relative to immediate responses and effects, and (c) the understanding of how responses scale through levels of biological complexity (from individuals to ecosystems).

Ecology and evolution are tightly interconnected: ecological dynamics determine natural selection pressures and gradients (Svanbäck et al. 2009; Collins 2011; Vonlanthen et al. 2012), and evolutionary dynamics shape natural ecological settings and community dynamics (Harmon et al. 2009; Ellner et al. 2011). One common misconception is that evolutionary processes act on long temporal scales. On the contrary, rapid contemporary evolution can

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be widespread, in particular in response to anthropogenic environmental change (Seehausen et al. 1997; Olsen et al. 2004; Ellner et al. 2011; Vonlanthen et al. 2012).

Anthropogenic stressors include the chronic presence in the environment of a multitude of chemical pollutants at sub-lethal levels whose interaction with complex biological systems is largely unexplored. Exposure may induce cumulative effects that become detectable only across increasing scales of time and biological complexity as a response to combined physiological, ecological and evolutionary processes (Fischer et al. 2013). ERA practises are important aspects of the sustainable management of chemicals (ECHA 2012). However, current testing and risk assessment paradigms have provided a stepwise simplistic scheme whose lack of capability for projecting the risk to complex biological systems is becoming increasingly evident (Schmitt-Jansen et al. 2008; Dalkvist et al. 2009; Preuss et al. 2009; Artigas et al. 2012).

Traditionally, ecotoxicology follows a bottom-up approach assuming that detection of effects at the sub-organisms/organism level is diagnostic of possible impacts at the population/community level and on ecosystem stability. What is neglected in this extrapolation step is that responses propagate in this system non-linearly, since new properties emerge at each hierarchical level (Segner 2007) and influence final effects. Among others, these properties include: species interaction, acclimation, adaptation, evolution.

Evolutionary toxicology is an emergent branch of ecotoxicology whose objective is to investigate trans-generational and inheritable impacts of pollution exposure that are not necessarily predictable from the mode of toxicity of the pollutants (Bickham 2011). A number of studies observed genetic responses in populations exposed to chemical pollution (e.g. (Theodorakis and Shugart 1997; Baker et al. 2001; Matson 2006; Wirgin et al. 2011)). Similarly to more traditional effect end-points, trans-generational and evolutionary impacts can result in altered population dynamics and possibly ecosystem structure and functioning. Linking these types of toxicological responses to exposure is challenging in experimental terms. Difficulties are related to detailing the complex link between phenotypic changes to genetic structure and ecological dynamics, the time-lag between stimulus and response and the influence of multiple stressors or stochasticity.

This work introduces an alternative/complementary view to the bottom-up framework of traditional ecotoxicology. Here we describe a trait based experimental approach to simultaneously look at responses at various hierarchical levels of biological complexity while targeting temporally delayed responses. The method is based on (1) semi-permeable membrane-based enclosures for in situ exposure of natural phytoplankton communities, and (2) high-throughput phenotypic trait analysis to track effects at

the individual, population and community levels, across multiple generations. The scope is to allow elevated ecological realism and a holistic approach while preserving high levels of test manageability and statistical resolution.

The specific goals of this proof of principle study were: (1) to describe community effects; (2) to study how functional groups and their expressed trait values responded to micropollutants' exposure; (3) to assess within population trait variance changes over time as a consequence of exposure (phenotypic evolution); and (4) to scale and assess the relative importance of all these response components (ecological sorting of species, physiological change in phenotype and evolutionary adaptation of populations) on community effects.

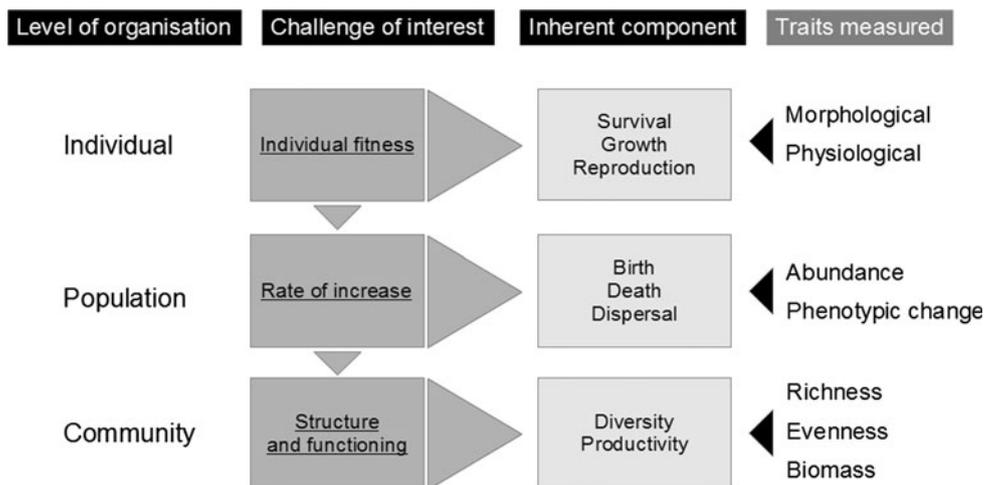
Methods

Approach

Trait-based response and effect framework

Traits are attributes of organisms or species encompassing genetic, morphological, physiological or life-history features such as size, metabolic activity or development stage. Trait-based approaches generally focus on phenotypic traits of species as the units to understand how natural communities are organised or change (McGill et al. 2006; Reiss et al. 2009). Phenotypic traits of organisms can in fact capture responses to abiotic and biotic environmental factors and their effects on community-level processes. Another advantage of functional trait-based approaches is to offer a “common currency” by which to numerically compare taxa. For example, trait-based approaches can provide a mechanistic understanding of the success of certain organisms under given environmental conditions and to relate individual- or population-level phenotypic changes to higher level changes in structure and functioning (McGill et al. 2006; Litchman et al. 2007; Suding et al. 2008; Reiss et al. 2009; Edwards et al. 2013). Trait-based approaches are also being used in ecotoxicology to improve the description of ecological responses to pollutants (such as pesticides) and to extrapolate between different species or populations using traits related to the vulnerability to stressors (Rubach et al. 2011; Ippolito et al. 2012). In this study we focused on individual phenotypic traits, associated with the morphology and physiology of organisms and therefore their individual fitness (Fig. 1). From individuals and their traits, we derived population level traits (demographic parameters) that related to population phenotypic structure and dynamics, and community level traits associated with structure (biodiversity) and functioning (productivity) (Fig. 1). We consider individual

Fig. 1 General framework for response and effect traits that were measured in this study. Pathways link the challenges of interest at different organisational levels through their related inherent components, and to measured traits. Adapted from (Violle et al. 2007). Morphological and physiological traits measured at the individual level mentioned in the Figure refer to flow-cytometry derived parameters, as reported in Table 2



and population traits as response traits, since they vary in response to changes in environmental conditions and determine the aggregated effects at community level (Violle et al. 2007; Suding et al. 2008).

Phytoplankton as a model system

Phytoplankton is a classical ecological and toxicological model system with much knowledge about ecological traits, toxicity to chemicals, and their relationship. Examples of key traits include cell size, shape and coloniality (which influence sensitivity to toxicants and nutrient uptake through surface/volume ratio), and photosynthetic pigment type and concentration (which relates to photosynthetic performance and acclimation) (Litchman and Klausmeier 2008). Recently, scanning-flow cytometry has been used to derive quantitative phytoplankton traits and group individual phytoplankton particles into morpho-functional categories, whose dynamics and trait-environment relationships reflect the structural and functional changes in natural phytoplankton communities under natural dynamics (Pomati et al. 2011, 2013).

Flow-cytometry

We used the scanning flow-cytometer Cytobuoy (Cytobuoy.com, Woerden, The Netherlands) for analysis, counting and characterisation of phytoplankton (Dubelaar et al. 2004; Malkassian et al. 2011; Pomati et al. 2011). This instrument is designed to analyse the naturally occurring size range from small (e.g. picoplankton) to large (e.g. colonial) phytoplankton species (0.5–700 μm in diameter and a few mm in length). Particles were intercepted by two laser beams (Coherent solid-state Sapphire, 488 nm and 635 nm, respectively, 15 mW) at the speed of 2 m s^{-1} . More details on the instrument can be found elsewhere (Pomati et al. 2011). Digital data acquisition was

triggered by the sideward scatter (SWS) signal with a trigger-level of 40 mV, which excludes particles smaller than circa 0.9 μm . The light scattered (908 nm) from each passing particle was measured at two angles, forward scatter (FWS) and SWS, to provide information on size and shape of particles. The fluorescence (FL) emitted by photosynthetic pigments in algal cells was detected at three different wavelengths: red (FL.Red and FL.2.Red from the 488 and 635 nm lasers, respectively), orange (FL.Orange) and yellow (FL.Yellow) signals were collected in ranges of 668–734 (chlorophyll-*a*—Chl-*a*), 601–668 (phycocyanin) and 536–601 nm (phycoerythrin and decaying pigments), respectively (Dubelaar et al. 2004). Laser alignment and calibration were done before analysis using yellow fluorescence beads of 1 and 4 μm . In this study, we scanned roughly 20,000 particles for each sample, comprising background and phytoplankton. Our Cytobuoy allows the analysis of pulse-signals providing, in the structural configuration used here, 54 descriptors of 3D structure and FL profile for each particle.

Permeable mesocosms

Membrane mesocosms used in this study were based on cellulose ester (CE) dialysis bags (Spectra/por, Spectrum Europe, Breda, The Netherlands), with molecular cut-off in the range of 100–500 Da. Bags were obtained from extruded tubes (20 mm diameter) sealed by two nylon clips. These membranes allow efficient exchange with the outer environment of compounds with molecular weight (MW) <300 Da. These in particular include mineral nutrients and gases essential for phytoplankton growth, while allowing the products of metabolism free to equilibrate with the external environment. CE membrane are highly hydrophilic, a property that strongly reduces interaction with the hydrophobic components in the water (e.g. cell membranes, dissolved organic carbon, hydrophobic

chemicals), limiting the growth of organic biofilms. Membranes were preserved in a 0.05 % sodium azide solution, at room temperature and dark conditions. Before experiments, membrane bags were rinsed with MQ water (circa 30 min each) for three times.

Test chemical

Triclosan (TCS, MW = 289 Da) has been used as antibacterial and antifungal in disinfecting and personal care products and in a number of other products including clothing, toys, and kitchen utensils. As an environmental contaminant, TCS is widely spread in waste, surface and coastal waters. Measured environmental levels generally range between a few tenths of ng L^{-1} to the low range of $\mu\text{g L}^{-1}$ (Bedoux et al. 2012). TCS is a relatively hydrophobic compounds (Log $K_{\text{OW}} = 3.5 - 4.8$) (Halden and Paul 2005). The bioconcentration factor in algae is in the order of 10^3 (Coogan et al. 2007), which means that a fraction of the compound present in water is sorbed to phytoplankton cells. The non-dissociated form of TCS has photolytic degradation half-life in surface water in the order of 4–8 days (Aranami and Readman 2007; Lindström et al. 2002). Under solar radiation conditions TCS can be partially converted to the more stable Dichlorodibenzo-p-dioxins (Aranami and Readman 2007; Mezcuca et al. 2004). TCS standards (in powder) were obtained from Sigma-Aldrich (St. Gallen, Switzerland) and diluted in ethanol to reach a stock (10 mg mL^{-1}) and the final working solutions (10 mg L^{-1}).

Experiments

Laboratory membrane tests for chemical half life

In order to assess the residence time of the test chemical in the microcosms, five sets of triplicate CE-bags were filled with tap water and spiked with 250 ng of TCS (Sigma-Aldrich, purity >97 %) dissolved in 100 μL of methanol. Membrane bags were then placed in a 60 L bath constantly flushed with tap water at 20 °C and kept in dark condition to reduce the effect of photodegradation. Samples were extracted using Oasis MCX (Waters) (3 cc, 60 mg) solid phase extraction cartridges conditioned with 6 mL methanol and 6 mL milliQ water. Before extraction 10 ng of chrysene-d12 was added to each sample as internal standard. The cartridges were eluted using 3 mL dichloromethane. Extract was concentrated under a gentle nitrogen stream to about 200 μL and reconstituted to 1 mL with cyclohexane for gas chromatography.

TCS was analysed by gas chromatography coupled to high resolution time-of-flight mass spectrometry (GC–ToF–MS; GCT, Waters) in electron impact mode (70 eV) using full scan (m/z 50–500 mD) and a source temperature

of 180 °C. Gas chromatographic separation was carried out using a DB-5MS column ($30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \mu\text{m}$) (J&W Scientific, Agilent). The temperature program was as follows 60 °C for 2 min, 10 °C min^{-1} to 200 °C, held for 10 min and 20 °C min^{-1} to 280 °C, held for 5 min. Splitless mode injection at 270 °C was used. The m/z used for TCS determination was 287.9512 Da.

Laboratory membrane tests on phytoplankton

Synchococcus sp. and *Scenedesmus* sp. were obtained from the Eawag culture collection and maintained in WC medium (Guillard and Lorenzen 1972). Laboratory tests on phytoplankton community diversity and productivity were carried out by collecting a water sample (1L) from outdoors mesocosms in early summer (June). The sample was filtered through a 90 μm mesh to exclude zooplankton and then split into aliquots for laboratory tests. These included natural community controls in flasks without the addition of culture medium, the natural community aliquoted in CE-bags suspended in WC medium, and community controls in flasks with the addition of WC medium in the same volumetric proportion as in CE-bags suspended experiments (Fig. S1 A, B, Supplementary online material). All experiments were performed in triplicate inside a controlled culture room at 20 °C and 12 h light/dark cycles. At the end of experiments, all bags were sacrificed and utilised for flow-cytometry analysis.

Mesocosm experiments

A two litre sample of lake phytoplankton community, acclimated for several weeks in an outdoor experimental mesocosm (total volume = 1 m^3) was collected and filtered through a 90 μm nylon mesh to exclude zooplankton. This sample was split into four aliquots of 0.5 L volume in 1 L borosilicate glass bottles. Three bottles were spiked with 1 mL solution of ethanol dissolved TCS at 0.05, 0.5 and 5 mg L^{-1} to obtain the final concentrations of 0.1, 1 and 10 $\mu\text{g L}^{-1}$, respectively. These concentrations reflected the environmental range of TCS exposure at the lower end of waste water treatment effluent levels and at the higher end of surface water levels (Singer et al. 2002; Fernandes et al. 2011; Bedoux et al. 2012), and fall within the range of sensitivity for phytoplankton species and communities (DeLorenzo et al. 2008; Backhaus et al. 2011). The fourth bottle was spiked with 1 mL of ethanol (vector control). Immediately after spiking, bottles were shaken and 20 mL samples were taken from each of the four bottles for flow-cytometry (time 0 samples used for calculation of haldane rates and for application of the Price equation partitioning, see below). For each treatment and the vector control, we prepared three CE-mesocosms of 40 cm length (12 in total, circa 80 mL of volume each), closed them

with dialysis-bag clips leaving circa 2 mL of air inside to let them float, and placed them into the outdoor mesocosm from which the phytoplankton was initially sampled (Fig. S1 C, D). After 7 days of incubation (corresponding to a minimum of two generations for most phytoplankton), we collected 20 mL from each CE-bag for flow-cytometry analyses. All water samples collected for flow-cytometry were fixed using a filter-sterilised solution of paraformaldehyde and glutaraldehyde (0.01 and 0.1 % final concentration, pH 7) and stored at 4 °C in the dark prior to analyses.

Data analysis

Data processing, analysis and graphics were performed with the R statistical programming language (R-Development-Core-Team 2012).

Data preparation and clustering of phytoplankton particles

Raw Cytobuoy data were visually inspected for the distribution of FL signals in order to set threshold levels to extract FL particles (phytoplankton) with a size larger than 1 μm . Phytoplankton concentrations were calculated by inferring the number of cells from the number of humps in the SWS signal of each particle to account for colonial species (Dubelaar et al. 2004). Phytoplankton biovolume was estimated for each particle assuming an ellipsoid shape and based on Total FWS signal using the formula $\text{Biovolume}^2 = 0.0017 \text{ Total FWS} - 0.013$ (Foladori et al. 2008). Cytobuoy particle descriptors were standardised and, by principal component analysis (PCA), reduced to 30 orthogonal vectors covering 99 % of the total variance in the data. Principal components (PCs) were utilised for grouping particles into functional categories based on Cytobuoy-derived morphology (Pomati et al. 2011, 2013). In this study we used unsupervised model-based clustering to group phytoplankton, applying maximum likelihood estimation and Bayes criteria to identify the most likely model and number of clusters. Specifically, we used the R package *mclust* in which the optimal model is selected according to Bayesian Information Criterion initialised by hierarchical clustering for parameterised Gaussian mixture models (Fraley and Raftery 2002). To accommodate for computational issues, we limited the final clustering dataset to 20 % of the totally acquired phytoplankton particles (6,000 out of a total of 30,000), randomly extracted from the database. The optimal model in our study corresponded to 12 ellipsoidal, equally shaped clusters.

Dose–response relationships

Dose–response relationships in community effects and population responses were assessed using regression

models based on the ordinary least squares method for the estimation of linear and quadratic model parameters (Legendre and Legendre 1998). The significance of regression coefficients was tested by analysis of variance (ANOVA). Control treatments (no dose) were not included in dose–response analyses.

Following TCS exposure, we characterised the average phenotype of Cytobuoy-derived functional groups in terms of size (length by SWS), pigment fluorescence (total signals) and their intracellular distribution (fill factor), coloniality (number of cells per colony by SWS), cells scattering (total FWS, total SWS and their ratio) and cells biovolume (Supplementary Table S1). Average functional group trait levels were logged (ln) before analysis. Using the two-step fourth corner method (Dray and Legendre 2008) we tested for statistical links between this trait-by-group table (matrix Q), a group abundance table (matrix L, abundance for phytoplankton functional groups in the different replicated treatments), and a table (matrix R) consisting of TCS concentrations for each treatment. This technique allows the correlation between Cytobuoy-derived traits and dose to be tested while weighing by the abundances of phytoplankton clusters, in order to identify traits that relate to population abundance under stress conditions. The link between quantitative phytoplankton traits and initial TCS concentration was measured by a Pearson correlation coefficient and the significance was tested by comparing results of two permutation models (9999 iterations): model A (permutes values for experimental replicates, i.e. rows of L) and model B (permutes abundance values of populations, i.e. columns of L) (Dray and Legendre 2008). Model A tests H_0 (no link between R and Q) against H_1 (L and R are linked), and model B tests H_0 against H_2 (L and Q are linked). Combining results allows a test of H_0 against H_3 (matrices R, L, and Q are linked): the significance of the H_3 test (final p value) was extracted as the maximum of individual p values for H_1 and H_2 tests (ter Braak et al. 2012).

Rates of phenotypic change

To study changes over time of within-population trait variance, and how these were affected by TCS treatment, we calculated “haldane” rates of phenotypic evolution (Gingerich 2009). Being broadly defined as a change in standard deviations per generation, the haldane rate takes into account variations in population structure for a trait of interest at the appropriate scale of at which evolutionary processes operate. As originally reported by J.B.S. Haldane, “the variation within a population at any time constitutes, so to say, the raw material available for evolution” (Haldane 1949). We calculated haldane rates for each control and treated experimental replicate by taking the

difference between two sample means at the at the end and the beginning of the 7 days test, and dividing this difference by the pooled within functional group variance and then by the number of elapsed generations using the equation proposed by Gingerich (2009) (traits were naturally logged before analysis). For this study, we assumed a conservative generation time for phytoplankton of circa 3 days (Reynolds 2006), corresponding to a total of two generations spanning across our 7 days test. In order to survey the overall TCS dependency in the observed rates of phenotypic evolution, we fit linear models to the relationship between haldane rates and log TCS dose, and extracted regression coefficients and relative p values. Results of this meta-analysis were visualised in coloured-coded image maps using CIMminer (Weinstein et al. 1997).

Application of the Price equation approach

The Price equation is a general mathematical theorem that describes how the average value of any trait changes in a population from one generation to the next (Gardner 2008). In this study, we applied the Price equation approach to partition a community aggregated effect into physiological, ecological and residual (evolutionary) components as proposed by Collins and Gardner (2009). Briefly, this approach proposes that the change in the average value of a community trait of interest (total change) during a time interval is given by the sum of three components: (1) the physiological change, e.g. the change in the trait value exhibited by a species averaged over all species in the community (tendency of species to change their trait value); (2) the ecological change, e.g. the covariance between a species' new trait value and its growth factor (tendency for species with certain trait values to increase in relative abundance within the community); and (3) the evolutionary change, which should be calculated as the covariance between a lineage's new character value and its relative growth factor, averaged over all species in the community (tendency for lineages with certain trait values to increase in relative abundance within their species). Similarly to Collins and Gardner, in this study we could not discriminate between different genetic lineages within our identified functional groups. The third component of change in the Price equation (evolutionary) was therefore estimated as residual after subtracting the sum of physiological and ecological terms from the total change (for more details, see (Collins and Gardner 2009; Collins 2011)). The Price equation partitioning approach was applied to each experimental replicate, including controls and treatments, using trait values at the beginning and at the end of the experiment.

Results

Testing CE-membrane mesocosms

Residence time of TCS in the CE-bags and exposure modality

After spiking, the concentration of TCS in the CE-bags declined relatively rapidly with a half-life in the order of few hours (Fig. S2, Supplementary online material). After 24 h only less than 10 % of the initially added TCS mass was still present in solution inside membrane mesocosms (Fig. S2). The observed disappearance curve did not match a first order decay. Half-life visually estimated from the chart (Fig. S2) for the early phase of the kinetic experiment (namely $\tau \approx 5$ h, between $t = 0$ and $t = 5$ –10 h) exceeded that estimated for the second phase (namely $\tau \approx 10$ –15 h, between $t = 0$ and $t > 10$ h).

Phytoplankton growth and diversity

With the aim of assessing the effectiveness of CE dialysis bags as a suitable environment for enclosed phytoplankton, we initially explored the effects of growth inside membrane bags for two species maintained in the laboratory (*Synchococcus* sp. and *Scenedesmus* sp). Experimental CE-bags allowed phytoplankton cultures confined within them to proliferate exponentially with no significant differences (Wilcoxon test) in abundance of cells compared to control cultures in standard flasks after 9 days of incubation (data not shown). CE-membranes did also not shade light in the PAR range (data not shown). We then tested the suitability of CE-membrane mesocosms as a growth environment for phytoplankton natural communities (Fig. 2). Compared to natural samples maintained in the laboratory, and natural samples supplemented with culture media in the same proportion of suspended CE-bags (Fig. S1 A, B), communities in membrane mesocosms showed no statistically significant difference (Wilcoxon test) in diversity metrics such as richness of Cytobuoy-derived clusters or Shannon index (Fig. 2a, b), as well as in their average productivity (Fig. 2c, d), 7 days after inoculum. In addition, 7 days after incubation no evident presence of biofilms was observed on the CE membrane surfaces.

Effects of TCS on natural phytoplankton communities within CE-mesocosms

Effects on community traits

Phytoplankton assemblages spiked with a pulse of TCS at 0.1, 1 and 10 $\mu\text{g L}^{-1}$ in the field did not show changes in community richness compared to untreated controls. By

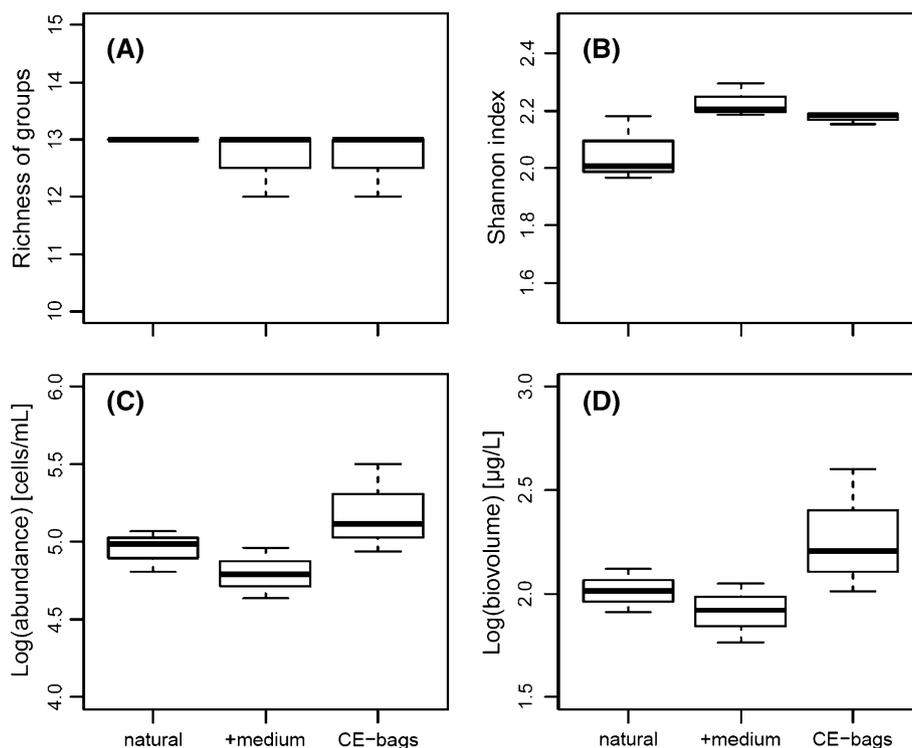


Fig. 2 Growth of natural phytoplankton communities in membrane mesocosms (CE-bags) compared to control samples in flasks without the addition of enriched culture media (natural), and community controls in flasks with the addition of WC medium in the same volumetric proportion as in CE-bags suspended experiments (+medium). Richness (a) and Shannon diversity (b) of phytoplankton groups were derived by clustering of Cytobuoy data. Total abundance of

phytoplankton cells (c) and total biovolume (d) were similarly obtained by Cytobuoy analysis. In plots, whiskers represent the range between the lowest and the highest values, boxes represent the 75th and 25th percentiles, and thick lines represent the median of data distributions ($n = 3$). X-axis tick labels in (a) and (b) are the same as in (c) and (d)

cluster analysis, we identified 12 Cytobuoy-derived functional groups in all controls and treated CE-bags, with no group disappearing as a consequence of TCS stress (data not shown). No statistically significant differences were also observed among controls and treatments with regards to Shannon diversity index (Fig. 3a). Although a slight trend is visible in Fig. 3a, no significant dose–response was detected both for linear and quadratic terms in the relationship between Shannon diversity and TCS (Table 1).

On the other hand, pulsed TCS treatments appeared to affect the productivity of natural phytoplankton communities exposed in CE-bags in their environment. Both total community biovolume and FL of active Chl-*a* showed a statistically significant increase after exposure to TCS at $1 \mu\text{g L}^{-1}$ ($p < 0.05$, *t* test) compared to non-spiked mesocosms (Fig. 3b, c). Dose–response relationships were detected in these two aggregated community traits, with both linear and quadratic terms being highly significant (Table 1). Linear terms in dose–response relationships seemed to be larger than quadratic terms, which in turn were negative suggesting a decrease in total biovolume and Chl-*a* FL with increasing doses of pulsed TCS, above the

pulsed exposure concentration of $1 \mu\text{g L}^{-1}$ (Table 1). Also the total FL of phycocyanin, a cyanobacterial specific pigment, increased in spiked mesocosm communities compared to untreated CE-bags (data not shown), suggesting that cyanobacteria became more abundant in TCS stressed phytoplankton communities.

Population responses

Within CE-mesocosms, phytoplankton populations responded differently to a pulse of increasing TCS doses. Most functional groups showed a positive trend with regards to abundance, few groups were affected negatively by exposure to TCS (see linear coefficients in Table 1), and between-populations variability resulted in most dose–response relationships being statistically not significant (Table 1). The abundance of three functional groups, however, appeared to be significantly and positively affected by pulsed TCS exposure, with both linear and quadratic terms being significant (Table 1). The negative coefficient of quadratic terms, as mentioned above, suggested a decrease in population abundance for increasing pulsed TCS levels exceeding $1 \mu\text{g L}^{-1}$ (Table 1).

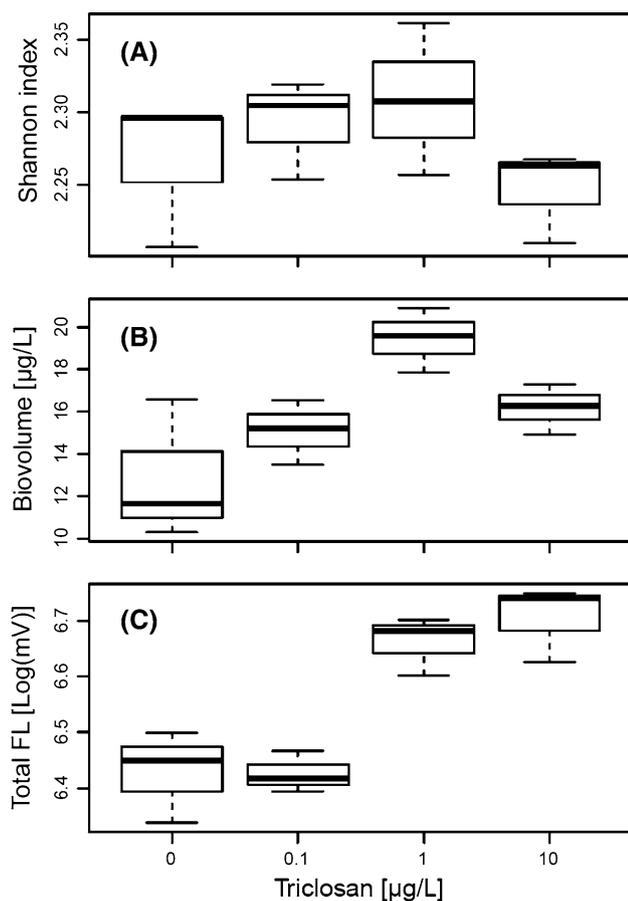


Fig. 3 Effects of TCS on the diversity (a), total biovolume (b) and total Chl-*a* fluorescence (D) of natural phytoplankton communities confined within CE-bags and maintained in an outdoor mesocosm for 7 days. *Boxplots* are as in Fig. 2, zero exposure level of TCS represents untreated control communities ($n = 3$). X-axis tick labels in (a) and (b) are the same as in (c)

Identification of response traits

Each Cytobuoy-derived functional group was characterised by specific trait values after pulsed TCS exposure, defining the group's "average response phenotype" (Table S1). We used the fourth-corner method to detect which traits were statistically linked to TCS stress. We consider these as response traits, potentially affecting individual fitness and population growth in phytoplankton groups after exposure. Cells fluorescence, scattering and biovolume showed the strongest links with TCS pulse exposure (Table 2). All selected traits displayed a positive correlation with dosing of pulsed TCS with the exception of fill factors, which relate to within-cells pigment distribution (Table 2). These results suggest that, after TCS spiking of communities, phytoplankton groups became on average larger and more fluorescent, with FL signals more concentrated in certain areas of cells and colonies.

Table 1 Triclosan dose–response relationships for community and Cytobuoy derived phytoplankton group effects: regression coefficients and their statistical significance are reported for both linear and quadratic model parameters

Variable	Coefficient		<i>p</i> Value	
	Dose	Dose ²	Dose	Dose ²
Shannon diversity	0.021	−0.003	0.632	0.545
Total biovolume	5.437	−0.527	0.009	0.009
Total FL Chl-<i>a</i>	0.290	−0.026	0.002	0.003
Abundance of GR1	266.733	−24.033	0.152	0.172
Abundance of GR2	−48.430	1.580	0.651	0.876
Abundance of GR3	10.809	−1.067	0.924	0.921
Abundance of GR4	90.436	−9.930	0.270	0.211
Abundance of GR5	47.655	−5.363	0.599	0.536
Abundance of GR6	88.248	−8.558	0.680	0.675
Abundance of GR7	−16.946	−0.184	0.940	0.993
Abundance of GR8	40.971	−6.087	0.817	0.719
Abundance of GR9	69.323	−6.558	0.303	0.306
Abundance of GR10	330.720	−31.621	0.024	0.024
Abundance of GR11	528.435	−50.722	0.022	0.021
Abundance of GR12	518.445	−48.078	0.051	0.056

The most significant relationships are highlighted in bold

Table 2 Relationships between phytoplankton traits and triclosan exposure, as obtained from Cytobuoy analysis and the two-step fourth corner method

Trait	<i>p</i> Value	Linkage ^a
Fill factor FL phycocyanin	0.007	−0.087
Total FL phycocyanin	0.008	0.085
Total SWS	0.010	0.082
Total FWS	0.010	0.082
Total FL yellow	0.011	0.070
Length by SWS	0.013	0.070
Biovolume	0.015	0.081
Fill factor FL Chl- <i>a</i>	0.016	−0.080
Total FL Chl- <i>a</i>	0.021	0.090

Length by SWS represents the most accurate measure of particle size; Total FL represents the integrated fluorescence signal, while the fill factor, which ranges between 0 and 1, gives information on how the signal resembles a square signal, i.e. very low values indicate that the signal is concentrated in a very narrow peak, indicative of an uneven distribution of pigments within cell/colony (pigment packaging)

^a Pearson correlation coefficient: low values are expected for reduced number of species or if data are affected by the presence of background random noise

Rates of phenotypic evolution

Out of the identified response traits, we selected phytoplankton size, biovolume, total FL and packaging of the two pigments phycocyanin and Chl-*a* to assess possible within-population evolutionary consequences of pulsed

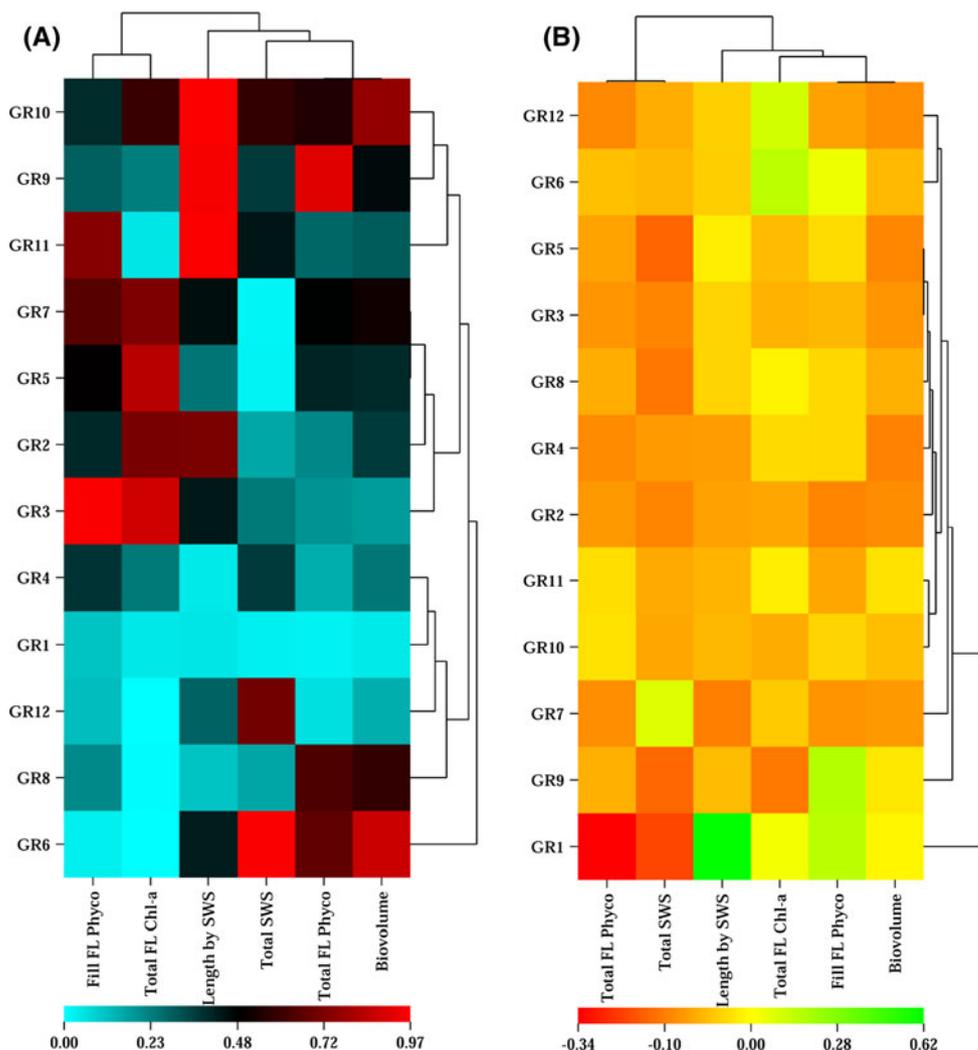
TCS exposure. We analysed the rates of change in trait variance (haldane units) within each replicated population of each functional group, across controls and treatments. In absolute terms, rates of phenotypic evolution averaged between 0 and 0.3 haldanes among unexposed populations, reaching levels of 0.7–1.5 haldanes on average in populations spiked with TCS. To test the influence of increasing TCS doses on changes in the rate of phenotypic evolution we analysed dose–response relationships using linear models. Results of this meta-analysis are visually reported in Fig. 4.

Approximately one-third of phytoplankton groups showed significant changes in the rates of evolution for more than one trait as a consequence to pulse TCS dosing (Fig. 4a, bottom cluster). Among selected traits, phycocyanin fill factor and total Chl-*a* FL appeared to be those that were more significantly affected by TCS exposure in their rate of evolution across all phytoplankton groups (left cluster in Fig. 4a). Phycocyanin fill factor (together

with total SWS) showed a general trend towards decreasing rates of change as a consequence of increasing TCS doses across all phytoplankton groups, as depicted in Fig. 4b (left cluster). The largest cluster Fig. 4b (right side) comprised traits, like total Chl-*a* FL or size, whose rates of evolution were generally increased as a consequence to TCS spiking.

Overall, most phytoplankton groups showed weak or statistically insignificant changes in their rates of phenotypic evolution as a consequence to pulses of TCS, with the exception of groups 6, 12 and, in particular, group 1. Functional group 1 was the most affected of all in terms of both statistical significance (Fig. 4a) and strength of TCS effects (Fig. 4b) on the rate of trait evolution. For example, pulse exposure to TCS in group 1 determined a dose–response increase in cell size, and a decrease in phycocyanin total FL, with peak rates of change in the order of 2 and 1 standard deviation per generation, respectively (data not shown).

Fig. 4 Visual colour-coded summary of dose-dependent changes in rates of phenotypic evolution, with 12 rows (functional groups) and 5 columns (selected traits) ordered and clustered by average linkage using Euclidean distances. The colour in each image reflects the estimated *p* values (a) and regression coefficients (b) on rates of phenotypic evolution for each trait with respect to TCS dose in each group. Colour scales are shown with aqua high significance, red no significance in (a), and red reduction, green increase in (b), respectively. Tables with data are in Supplementary online material, Tables S2–S3 (effects and *p* values) (Color figure online)



Comparing physiological, ecological and residual components of change

We partitioned temporal changes (7th day compared to start of the experiment) of community effects into the component of within-group change in trait values (e.g. physiological change) and the component due to variation in the relative abundance of different functional groups carrying different trait values (e.g. ecological change). We applied this partition approach (Price equation) across all controls and treatments and, for each sample, subtracted physiological and ecological contribution from total community change to obtain a measure of the residual change. Figure 5 reports the Price equation partitioning of total community changes in biovolume after pulse exposure to $1 \mu\text{g L}^{-1}$ of TCS.

In both control and treatment replicates, total community biovolume increased during the course of the experiment (Fig. 5). The physiological contribution (within-group trait change) to total community gain in biovolume was slightly negative (approaching zero) for the control, while was significantly positive for TCS treatments (Fig. 5). This suggested that phytoplankton acclimated to a pulse of TCS stress by increasing their average size and biovolume. The ecological contribution to community change was negative for both controls and treatments, suggesting a general transition in community composition towards smaller phytoplankton, which appeared to be stronger for TCS stressed samples (Fig. 5). The residual contribution to change was strongly positive (the largest in absolute terms) for both control and treated communities, pointing towards additional processes that favoured larger

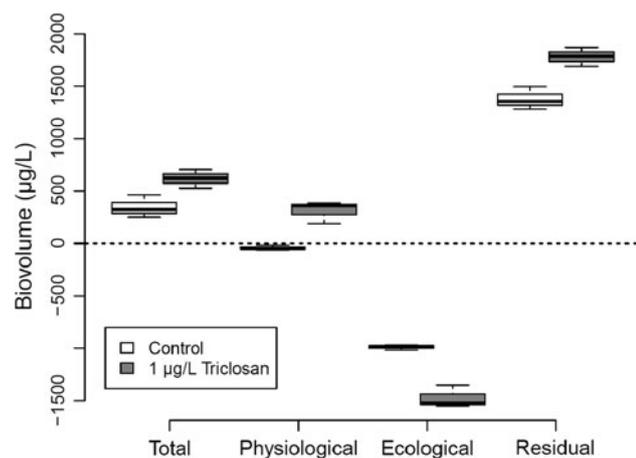


Fig. 5 Price equation breakdown of terms used in partitioning phytoplankton community effects of TCS at $1 \mu\text{g L}^{-1}$ into total, physiological, ecological and residual (including evolutionary) change, compared to changes in untreated controls. The figure depicts changes in community biovolume ($n = 3$), while changes in total Chl-*a* FL are reported in Supplementary online material (Fig. S3)

organisms during our experiment, and in particular under pulse TCS exposure (Fig. 5). General patterns of community partition into physiological, ecological and residual change were very similar across all treatments and selected traits (see, for example, Supplementary Fig. S3).

Discussion

Membrane based mesocosms and trait monitoring as a tool to study phytoplankton in the field

Toxicity and community effects in laboratory cultures or microcosms can be very different from what happens in natural habitats, where population and community dynamics are influenced by multiple stressors and fluctuating environmental factors (Fischer et al. 2013). The ideal mesocosm would maintain test populations in natural conditions with their complex and fluctuating environment (de la Broise and Palenik 2007; Hassler et al. 2008), and dialysis bags have been used in the past to culture various microorganisms including phytoplankton and to assay the effects of pollutants on phytoplankton in nature (see Powers et al. 1976).

Similarly to early investigations (Powers et al. 1976), we found that phytoplankton cells in dialysis bags had a tendency to be slightly more productive than controls, although this difference was not statistically significant (Fig. 2c, d). These results suggest that nutrients were as readily available to cultures in dialysis bags as to cultures growing in glass flasks with enriched media, confirming full exchange of conditions between internal and external environment. Contrary to previous reports (Powers et al. 1976), our membrane-based mesocosms were not affected by fouling and biofilm growth both during the preliminary lab test (7–9 days incubation) and during the field test (7 days incubation). This was likely related to the different environments of deployment (salt- vs fresh-water) and possibly different properties of membrane materials compared to earlier work.

The relatively rapid decline of TCS concentration observed in the CE bags during laboratory tests simulated the dynamics of pulsed exposure. Pulsed exposure is likely a typical exposure mode for many emerging contaminants including pharmaceutical, drug of abuse, personal and house care products originated from human use. These pollutants typically have (a) a periodicity of emission peaks often concentrated in specific hours during the day or specific days during the week; and (b) a relatively short environmental half live, which results in environmental concentrations highly dependent on emission rates and fluctuations (Reid et al. 2011; Thomas et al. 2012).

Although our experimental set up allows to carry out toxicological tests preserving elevated ecological realism,

it has some limitations. Disadvantages of the use of CE-based mesocosms include the impossibility of performing tests under controlled exposure conditions (maintenance of steady state concentration of test chemical and manipulation of environmental variables), and the possible occurrence of chemical binding or adsorption to membrane surfaces. The observed two-kinetic behaviour of TCS clearance could be interpreted by hypothesising a first phase of rapid partitioning of the compound between the water phase and the bag inner surface, while the second slower phase is essentially due to TCS diffusing out from the bags. During the field experiment with phytoplankton, a significant fraction of the chemical may also have partitioned on the organisms (Coogan et al. 2007). The rapid decline in dissolved phase concentrations, however, is expected to drive the release of the chemical back to the water phase and ultimately outside microcosms. Future evaluation of the mesocosm method performance will encompass: (1) a more comprehensive assessment of chemical fate in the microcosms, including concentration dynamics of degradation by-products; (2) a better characterisation of diffusivity for a range of chemicals of ecotoxicological relevance in realistic conditions (e.g. presence of particulate and dissolved organic matter); and (3) the investigation of the use of passive donors to replenish chemicals inside the bags during the incubation and simulate chronic exposure conditions.

Flow-cytometry has been used before to test responses of individual cells to common pollutants under natural environmental conditions (Czechowska and van der Meer 2011). Scanning flow-cytometry offers the extra opportunity of measuring multiple individually expressed morphological and pigment related traits in phytoplankton with high precision (Collier 2000) and over a large number of individuals per population in natural communities (Pomati et al. 2011, 2013). Data obtained by scanning-flow cytometry allow to measure and group individuals based on their expressed phenotype, which is the results of acclimation and selection processes, and to derive population and community quantitative variables that link directly to individual responses (Fig. 1). This approach allows targeting responses to chemical stress and effects at multiple levels of biological complexity, with the possibility of studying how responses scale or partition through population and community interactions (Fig. 5). Another opportunity provided by our trait-based response and effect framework is the possibility of shifting focus from a “categorical view” of biodiversity responses (e.g. ‘How many species are there and why?’) to a “quantitative view” (‘How much variation in traits is there and why?’) (McGill et al. 2006; Reiss et al. 2009). In this last view, an approach like ours would be more effective in detecting possible early warning signals of stress or population collapse in

complex natural assemblages, since changes involving categories (e.g. presence or absence of a species) are likely to occur when the stress has already overtaken population resilience boundaries (Scheffer et al. 2012).

Community effects of TCS

In this study, we found no statistically significant effects of TCS on phytoplankton richness and evenness, and statistically significant stimulation in two community metrics associated with productivity (Fig. 3). Previous reports on TCS effects on aggregated phytoplankton community features are controversial. Pulse exposure (48 h duration) to TCS at $60 \mu\text{g L}^{-1}$ has shown to cause higher Chl-*a* content after 7 days and increased mortality of diatoms and bacteria in biofilms, consequently reducing biodiversity (Proia et al. 2011). At lower doses (similar to those used in our study), TCS treatment has resulted in stimulation of pigment content at community level (hormetic effects) in one study (Backhaus et al. 2011), and non-significant differences in biomass yields with marked shifts in the community structure (reduced species richness) at higher concentrations (Wilson et al. 2003). The lack in detection of biodiversity effects in our study may be the result of insufficient resolution of the statistical clustering method on the actual diversity of the phytoplankton, or of low statistical replication of experimental treatments ($n = 3$). We consider however more likely that the low exposure level and the very short time characterising our exposure design were not sufficient to induce disappearance of an entire functional group over the course of 2–3 generations. This idea is supported by previous studies: treatment of phytoplankton communities in outdoor mesocosms with herbicides, for example, resulted in limited effects on biodiversity after short periods of time, with many treatments increasing Shannon diversity for longer exposure time (Knauert et al. 2009). A positive control, with a known effect on community biodiversity, would have allowed to directly test the importance of methodological constraints in detecting extinction of one or more functional groups and, although challenging, should be included in future similar studies, also to assess the impact of extinction on inferred community effects.

The increase in aggregated community traits (such as biovolume and FL) could have different components: (1) individuals are becoming larger and more fluorescent, (2) large and highly FL groups become more abundant (or small and less FL become less abundant), (3) large and highly fluorescent individuals are positively selected (or small and low fluorescent individuals are negatively selected). For example, phycocyanin FL was positively linked to TCS exposure, suggesting the tendency of this chemical to favour cyanobacteria over other groups. In our

study functional groups 1, 10 and possibly 11 appear to be cyanobacteria due to the higher levels of phycocyanin FL compared to the other groups (Supplementary Table S1), and show positive growth response to TCS dosing (Table 1). We used the Price equation approach, however, to scale and summarise community components of change, and we took advantage of the independent measures of physiological and ecological components to test the validity of this approach.

Assessing the importance of physiological, ecological and evolutionary responses

Phytoplankton are able to survive in adverse habitats as a result of physiological acclimation, which is supported by generally plastic phenotypes (Stomp et al. 2008; Huertas et al. 2010). Here we identified a series of response traits (Table 2) whose average levels per population at the end of the experiment were significantly linked to TCS exposure. This response, which we call here physiological, represented for most of the groups an increase in the average cell volume (and scattering, Table 2), as well as FL (Figs. 5, S3).

The mode of toxic action of TCS in algae is not fully understood (Franz et al. 2008; Dann and Hontela 2011). TCS can inhibit lipid synthesis destabilising cell membranes, but significant differences in sensitivity can be found among phytoplankton species (Franz et al. 2008; Dann and Hontela 2011). This involvement of membrane homeostasis in toxicity points towards sensitivity being driven by size related traits, and surface/volume ratio. Lipid content is another important trait explaining sensitivity, by determining bioaccumulation and bioconcentration (Kent and Currie 1995). Total lipid content in phytoplankton can be linked to the amount of cell membranes and internal structures, which influence cells scattering (Dubelaar et al. 1987).

Phytoplankton cell size has been historically considered as one of the most significant factor in algal–pollutant interaction and subsequent sensitivity (Kent and Currie 1995), even in mixed assemblages (Echeveste et al. 2010, 2011). A previous study highlighted the inverse relationship between sensitiveness to TCS exposure and phytoplankton dimension (Ciniglia et al. 2005), suggesting purely surface/volume ratio related drivers (Kent and Currie 1995; Franz et al. 2008). At the individual level response, there is little information on the effects of TCS on phytoplankton FL patterns, with rare exceptions. Opposite to our results, Ricart et al. (2012) reported a decrease in algal FL for TCS exposure. Our data also indicated an increase in pigment packaging as a consequence of exposure (negative relationship between fill factors and TCS dose, Table 2).

The ecological response (change in the relative abundance of groups within the community) observed here suggested that groups characterised by average small dimensions and low FL became more dominant after TCS exposure (Figs. 5, S3) as a consequence of competitive interactions (Collins and Gardner 2009; Collins 2011). This component of community change may highlight the fact that small organisms (although more sensitive from a physiological point of view) are favoured in a stressed environment by their high surface/volume ratio, which allows more efficient harvesting of nutrients (Litchman and Klausmeier 2008). Physiology and ecology therefore acted in opposing forces, with ecological component being stronger and suggesting limited support to the hypothesis that community change is attributable to the elimination of phytoplankton groups more sensitive to TCS.

In our study, differential growth among functional groups appeared to be related to differences in traits. Previous studies investigating responses of a range of unicellular algae populations to TCS highlighted a wide range of response sensitivity (EC50 values range between 0.7 and $19 \mu\text{g L}^{-1}$ with lowest observed effect concentrations (LOEC) in the range of 0.1 and $1.2 \mu\text{g L}^{-1}$) and no relationship with any major structural or physiological trait (Bedoux et al. 2012). Here, groups with more significant responses to TCS appeared to be cyanobacteria (groups 10–11) or green algae (group 12, Table 1) growing in single cells or small colonies of medium size (between 20 and $50 \mu\text{m}$), with high pigment FL and cells scattering (Table S1). These groups showed only weak signs of TCS dependent phenotypic trait evolution (Fig. 4), suggesting that they may have been already fit for the applied stress. Additionally, our data show that some populations had a significantly higher growth for low doses (below $10 \mu\text{g L}^{-1}$) with the trend decreasing at higher levels (Table 1). This is consistent with previous reports of TCS showing hormetic responses at sub-lethal doses in periphyton (when present in mixture with other pharmaceutical and personal care products) (Backhaus et al. 2011; Brown et al. 2012). It may be argued that if smaller cells are more sensitive to exposure (as hypothesised here), they may also be more sensitive to low dose hormetic effects.

The residual component of change that we analysed in this study (calculated as difference between the total change and the sum of physiological and ecological change in the Price equation) encompassed evolutionary and stochastic population responses, and was the largest in absolute terms [as previously reported (Fox 2006; Collins and Gardner 2009)]. The positive value of this component in Figs. 5 and S3 suggested that the relative fitness of larger and more fluorescent organisms increases under pulse TCS exposure (Collins and Gardner 2009), pointing towards the

importance of micro-evolutionary processes in explaining community change.

Haldane rates of phenotypic evolution found in this study for unexposed phytoplankton populations fall within the range expected for many natural and mildly stressed populations (Hendry et al. 2008; Gingerich 2009). Rates of phenotypic change in populations spiked with TCS were however extremely high. Even assuming a faster generation time of 24 h, the most significant TCS responses would still result in changes higher than 0.5 standard deviations in biovolume or FL per generation, when most literature data report contemporary rates of phenotypic evolution between 0.1 and 0.3 haldanes (Gingerich 2009). If the modality and level of exposure simulated here can increase the rate of phenotypic change in natural phytoplankton populations by a factor of 2–3 compared to natural conditions, then background chronic chemical pollution should be cause for serious concern.

Phenotypic changes can be maintained across generations through epigenetic effects, allowing population survival in highly fluctuating environments where it is an advantage to be able to revert to original phenotype (Gingerich 2009; Svanbäck et al. 2009). Although in recent studies only doses that drastically reduced growth also induced adaptation in phytoplankton species (Huertas et al. 2010), it is possible that selection on standing phenotypic variance can drive adaptive evolution in acclimated populations (Gingerich 2009; Fischer et al. 2013). In the context of a natural community, it is possible that weak selection synergises with species interactions to influence adaptive evolutionary processes (Hendry et al. 2008; Svanbäck et al. 2009; Collins 2011; Fischer et al. 2013). A closer analysis of responses highlights that some group showing significant dose-dependent change in phenotypic evolution (e.g. Group 1, Fig. 4) did not significantly change its abundance in the community (Table 1): namely, did not contribute to the ecological component of change.

The phenotypic changes observed in this study appear to be fast, and happen over a few generations. Given the short time of the experiment and the low doses, we suggest that the greater or reduced phenotypic changes in treatments compared to controls may be mainly the result of phenotypic plasticity. Strong plastic responses to human-induced environmental change have been noted before, and can either increase or decrease the likelihood and strength of selection, and the rate of genetic change (Hendry et al. 2008). In our framework, standing genetic variation, rather than novel mutational input, accounts for most of the response to selection during the short experimental time interval. Trait changes, however, were evident after two generations, and the question is whether they reflected contemporary evolution or simply epigenetic phenotypic inheritance.

Conclusions

Contrary to recent data suggesting that, under most scenarios, adverse effects due to TCS are unlikely to occur in the real environment based on current exposure levels (Lyndall et al. 2010), our results point towards possible effects on community functioning in natural scenarios. In particular, we noted that responses to chemicals can be fast and occur at different scales of biological complexity. Although evolutionary responses have been commonly ignored in ERA and ecosystem science in general, there is no a priori reason to assume that effects at any scale are unimportant, and our study supports this view. Our data show that, similarly to what observed in other disciplines (Ozgul et al. 2009; Svanbäck et al. 2009; Collins 2011; Ellner et al. 2011), in ecotoxicology the contribution of physiological, evolutionary and ecological processes should be studied together to understand impacts of environmental stressors on complex biological systems. Overall, rapid changes in some traits and their variance within populations were stimulated by TCS dosing, others were inhibited. These chemical-induced higher or lower rates of phenotypic change may affect the adaptive potential of natural populations that have to cope with multiple stressors (Fischer et al. 2013).

We presented an approach that represents a promising tool for increasing the ecological realism in ERA and scale/partition responses simultaneously from individual to population and community level. Although the use of natural communities in the natural environment increases the ecological relevance of observed responses and effects, it implies a principally lower reproducibility of the experimental data, because the physiological state and the species composition will change between experiments. This may actually represent an opportunity to factor out ecological contingency (biodiversity and water chemistry) from the effects of chemical stressors on natural communities. More experimental studies should address in the future the relative importance and interaction of physiological acclimation, evolutionary processes and ecological dynamics in explaining community effects of micropollutants like TCS, in order to scale the effects of stressors through community level dynamics and link between response-and-effect traits (the Price equation is only one of the possible ways forward in this direction). The study of resilience and recovery time in complex natural systems also appears critical, especially after exposure to mixtures that may affect the structure and functioning of communities more deeply and unpredictably (Knauer et al. 2009).

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