Micropollutant-induced phenotypic adaptation in cyanobacteria *Microcystis aeruginosa*

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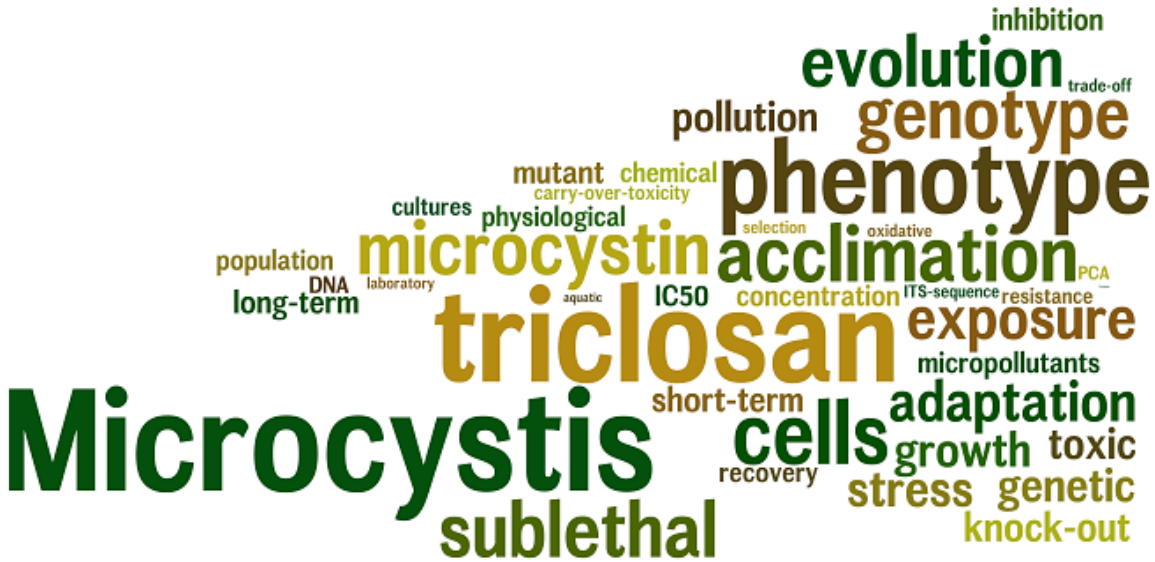

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Abbreviations

TCS	Triclosan
PCC7806	*Microcystis aeruginosa* strain out of Pasteur Culture Collection 7806
ΔmcyA	*M. aeruginosa* strain with knocked-out mcyA-gene
LT5	*M. aeruginosa* strain that underwent long-term 5.5 µg L^{-1} TCS treatment
WT	Wild type *M. aeruginosa* strain (referring to PCC 7806)
PC(A)	Principle Component (Analysis)
OD	Optical density
ELISA	Enzyme Linked Immunosorbent Assay

Central message

Realistic exposure scenarios to a common micropollutant (TCS) can induce reversible phenotypic changes (acclimation) at short timescales and stable inheritable changes (evolution) at longer timescales. Acclimation and adaptation seem to affect phenotype in opposite directions. Short-term exposure evokes a stressed phenotype (large cells, more fluorescent) as a consequence of homeostatic mechanisms or carry-over toxicity due to bioaccumulation of TCS. Long-term exposure on the contrary results in a resistant phenotype (smaller size, decreased fluorescence) but reduced growth in standard conditions compared to unexposed controls, suggesting a trade-off between resistance and growth and possibly a cost associated with the shift in phenotype

Summary

Aquatic organisms are often repeatedly exposed to low, fluctuating concentrations of environmental pollutants over a long time period. Populations exposed to such pollutants may then acclimate or adapt to survive in the changing environment.

Which durations and levels of exposure result in evolution of resistance traits and whether physiological acclimation plays a leading role in the acquirement of resistance is not well understood. We assessed the effects of different exposure scenarios using Triclosan (TCS), a water-borne micropollutant, on the growth, microcystin production and phenotype of *Microcystis aeruginosa*, a ubiquitous toxic freshwater cyanobacterium. The results revealed that 1) *M. aeruginosa* is sensitive (apparent in inhibited growth, larger cell size and changed overall phenotype) to TCS at concentrations present in the environment, 2) a second, sublethal exposure causes increased toxic effects of TCS compared to control cultures due to incomplete organism recovery over generations, and 3) pulsed sublethal exposure results in more resistant populations with changed phenotype (smaller, less fluorescent), altered microcystin metabolism and reduced growth in standard conditions compared to unexposed controls.

Our data therefore suggests realistic exposure scenarios imply different response mechanisms: a single dose causes short-term acclimation achieved by phenotypic plasticity and altered homeostasis, with the drawback of carry-over toxicity across generations. Long-term exposure on the contrary allows evolutionary adaptation to take place. Possible evolutionary mechanisms are selection on the best-fit phenotype or addition of genetic variance. Our results pose important implications in ecological risk assessment of TCS. Furthermore we advocate considering sublethal exposure scenarios more carefully, and to account for evolutionary adaptation in further ecotoxicologic experiments.

Introduction

Darwinian evolution is the driving process of innovation and adaptation across the world's biota. In order for evolution through natural selection to take place, there must be heritable genetic variation and differential reproduction associated with genetic variance (Darwin, 1853). One common misconception is that evolutionary processes act on long temporal or even geological timescales. Evolution is not only speciation but represents an important component of adaptive processes in natural populations and occurs over short times.

Examples of rapid evolution over contemporary time have become increasingly common in the last few decades (Ellner et al., 2011; Stockwell et al., 2003) and should more likely be seen as the rule rather than the exception (Kinnison et al. 2007). DDT resistance in houseflies (Busvine, 1954) and the rise of melanic forms of the peppered moth (Kettlewell, 1955) became the first two textbook examples of evolution in action. In the meantime many examples have been added. A main underlying cause is anthropogenic environmental change (Palumbi, 2001). Bacterial drug resistance, plant and insect resistance to pesticides, rapid changes in invasive species and life-history change in commercial fisheries are current examples of human induced evolution (Allendorf & Hard, 2009; Jørgensen et al., 2007; Lesch et al., 2001).

In particular, inland freshwater systems are being transformed rapidly by human activities (Vörösmarty et al., 2010). Organic pollution and eutrophication are main threats to lake ecosystems and led for example to speciation reversal in whitefish (Vonlanthen et al., 2012). But also chemical pollution through municipal wastewater has become a major challenge in aquatic systems (Schwarzenbach et al., 2006). Thousands of chemicals out of personal care

products and pharmaceuticals are being washed down the drain and enter the environment without being degraded in wastewater treatment plants.

Toxicologists and ecotoxicologists have extensively investigated the detrimental effects of chemicals after short-term exposure and documented the biological mechanisms responsible for their toxicity. But trans-generational responses including adaptation and evolution have been neglected for a long time. This lack of ecological and evolutionary theory in the environmental risk assessment (ERA) of chemicals periodically therefore recurs in the literature as a major gap to fill (Brink, 2008; Coutellec & Barata, 2011; van Straalen, 2003).

Adaptation to new conditions linked to chemical pollution can be achieved by two different kinds of mechanisms: acclimation or genetic adaptation. Acclimation is defined as increased tolerance of an elevated, usually lethal, concentration of a toxicant arising from chronic exposure to a sublethal concentration of that toxicant. It is achieved through phenotypic plasticity (the property of a given genotype to produce different phenotypes in response to particular environmental conditions) supported by altered gene expression. Phenotypic changes due to acclimation are generally reversible when stressor is released. However, in some cases acclimation can be maintained over multiple generations by epigenetic processes (Seong et al., 2011). When stress exceeds physiological operating range, survival depends only on adaptive evolution driven by selection on standing genetic variation or on *de novo* mutations that confer resistance. Genetic adaptation happens at population level and is trans-generational.

Evolutionary toxicology is an emergent branch of ecotoxicology whose objective is to investigate the trans-generational and inheritable impacts of pollution exposure that are not

necessarily predictable from the mode of toxicity of the pollutants (Bickham, 2011). It focuses mostly the effects of pollutants on the genetics of populations. Chemical toxicants have the potential to affect genetic systems in two general ways. First, they can act as genotoxicants and directly induce heritable mutations in the DNA. And second, they can influence survival and reproduction, which leads to selection of more tolerant individuals, altered gene flow between populations and genetic drift because of bottlenecks. A number of studies observed such genetic responses in populations of aquatic organisms (Matson et al., 2005; Rinner et al., 2011; Theodorakis et al., 2000; Whitehead et al., 2012; Wirgin et al., 2011).

All of those studies reported altered population structure in context with high levels and durations of pollution exposure. But, in general, aquatic organisms seldom encounter acute contamination by toxicants. Usually, they face relatively low concentration over a long duration, or pulses of exposure due to occasional releases. Most synthetic micropollutants are present in water at low to very low concentration (Brausch & Rand, 2011; T. Ohe et al., 2004), and they follow seasonal and short term dynamics in their environnemental release (Castiglioni et al., 2006). This exposure modality has been associated with detrimental effects on natural populations, including feminisation of wild fish (Harris et al., 2011) and carry-over toxicity in aquatic organisms (Adersen et al.,2006; Ashauer & Hintermeister, 2010). Carry-over toxicity is defined as increased toxic effects (compared to organisms which were not prestressed) occurring after a second pulse of exposure due to incomplete organism recovery (Ashauer & Hintermeister, 2010). Such incomplete recovered organisms may exhibit weaker molecular, biochemical, physiological, or morphological responses than acute exposed. But the consequences for survival of populations and species could be severe unless

phenotypic plasticity, acclimation or adaptation, produce individuals more tolerant in a polluted environment (Gillardin & Dorts, 2012).

Which durations and levels of exposure result in evolution of resistance traits and whether physiological acclimation plays a leading role in the acquirement of resistance in natural populations is not well understood and should be incorporated in ecotoxicological experiments (Fischer et al., 2013).

The purpose of this work is therefore to investigate the effects of sublethal exposure level of water-borne micropollutants on the growth and phenotypic changes after short and long term exposure scenarios in aquatic organisms. Previous work on the effects of sublethal doses of TCS, a biocide of emerging concern, suggested that evolutionary responses might represent a significant component of the total observed change in phytoplankton communities after single pulse exposure at environmentally relevant levels (Pomati & Nizzetto, 2013).

Here we chose cyanobacteria as a laboratory model system for a number of reasons. They are important components of aquatic phytoplankton communities where they contribute significantly to primary production and ecosystem functioning. They have been the subjects of extensive research, so that effects of pollutants on their growth and physiology are well described (Wright, 1978; Ramakrishnan et al., 2010). They have a fast generation time and are usually the most sensitive amongst aquatic microorganisms (Huertas et al., 2010). Some previous studies demonstrated very rapid adaptive evolution of cyanobacteria to lethal selective agents (Garcias et al., 2004; Dvornyk et al., 2001; Maneiro et al., 2006). Other laboratory studies pointed out that resistance of cyanobacteria and microalgae to anthropogenic pollutants at lethal exposure levels arose as a result of selection on standing

genetic diversity (mutations that occurred randomly prior to stress) (Marvá et al., 2010; González, 2012; López-Rodas, 2006; López-Rodas, 2008). Beside the great adaptive potential cyanobacteria are able to survive in adverse habitats as a result of physiological acclimation supported by generally plastic phenotypes (Fogg, 2001; Stomp et al., 2008).

Aims of this work

In this work we used laboratory experiments to study:

1) the dose-dependency of changes in population abundance and phenotypic traits in toxic and non-toxic cyanobacterial strains as a response to short term exposure to a micropollutant of emerging concern, TCS;

2) the long term exposure effects to TCS on the phenotype, toxin production, growth and genetic diversity, over a trans-generational experiment where cyanobacterial populations were chronically exposed to sublethal doses of TCS, and;

3) if micropollutant's induced phenotypic and sensitivity changes corresponded to evolutionary adaptation (i.e. were maintained across generations), or observed changes could be reverted to the original phenotype after a period of detoxification and recovery.

To address these questions we chose the cyanobacterium *Microcystis aeruginosa* and the biocide TCS as models. We carried out 96-hour growth inhibition tests, exposure-re-exposure experiments where organisms were released from stress in between exposures and a 6-month long-term experiment where cultures were exposed to chronic sub-lethal doses.

The following specific hypotheses were tested:

- Realistic levels of exposure to micropollutants induce individual responses as phenotypic plasticity in *M. aeruginosa*
- Phenotypic transitions occurring at low exposure levels are maintained over generations and not completely reversible
- Phenotypic transitions are adaptive and associated with acquired resistance to the chemical stressors
- Chronic sublethal exposure does increase resistance of *M. aeruginosa* towards TCS
- Increased resistance comes with a cost and is the consequence of selection on standing genetic variation

Materials and Methods

Test Organism

Microcystis aeruginasoa (Kützing, 1846) (Fig. 1) is a species of cyanobacteria with a worldwide distribution in temperate reservoirs and lakes. They frequently occur in dense blooms, and its toxins are considered to be the most prevalent cyanotoxins in freshwater ecosystems (Codd, 2000). Microcystins pose a challenge for wildlife conservation because they are the cause of repeated mass mortalities of water birds, fishes and even elephants (Bengis & Naicker, 2010). Moreover, microcystin-polluted drinking water is a serious threat to humans because of its hepatotoxicity (Martinez et al., 2009). Recent accumulation of toxic blooms and hypothesised increased development of blooms under the future scenario of global change, makes them management-wise important (Paerl & Huisman, 2008). Beside the environmental relevance, *M. aeruginosa* has proven its suitability as model organism in laboratory experiments in all different kinds of studies. In particular the sequenced genome (Aneko et al., 2008; Frangeul et al., 2008), the demonstrated existence of very rapid evolution (López-Rodas et al., 2006) as well as the wide range of morphological diversity that does not necessarily reflect genetic diversity (Marcos, 2006) highlights the suitability of *M. aeruginosa* as model system for trait-based evolutionary approaches.

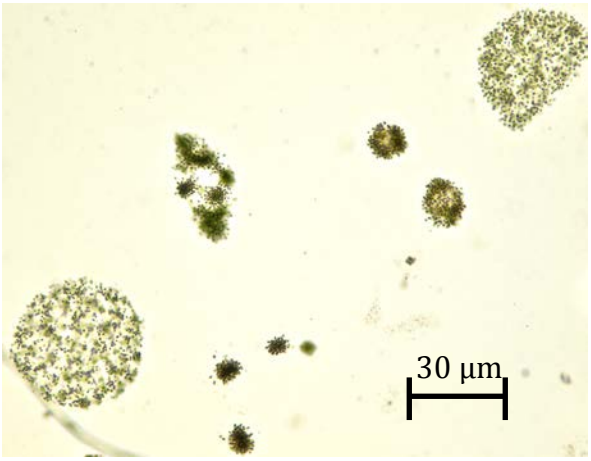

Fig. 1: ***M. aeruginosa***. Colonial form, isolated out of lake Greifensee.

Test chemical

TCS (MW=289Da) (Fig. 2) is a biocide incorporated in a wide variety of household and personal care products such as hand soap, toothpaste, and deodorants but also in textile fibres and other consumer products. Personal care products containing TCS are commonly washed down the drain and thus become part of the domestic wastewater. In sewage treatment plants, TCS is only partially eliminated and is therefore widely spread in aquatic environments. Most reports describe TCS as one of the most commonly encountered substances in solid and water environmental compartments (Bedoux et al., 2012). Actual environmental concentrations in surface waters and rivers range from 0.001 – 40 µg L^{-1} (Davison & Maillard, 2010). Beside its potential for bioaccumulation in phytoplankton (Coogan et al., 2007), TCS and its by-products adversely affect phytoplankton-growth. High sensitivity in algae and cyanobacteria is likely due to TCS antibacterial characteristics, through disruption of lipid synthesis (Mcmurry et al., 1998), membrane destabilisation (Franz et al., 2008), or uncoupling of oxidative phosphorylation (Brausch, 2011). Currently there's a need for research on the extent of the effects of chronic, low-level TCS exposure *in situ* and *in vitro* (Davison & Maillard, 2010; Novak et al., 2011; Dickson et al., 2010).

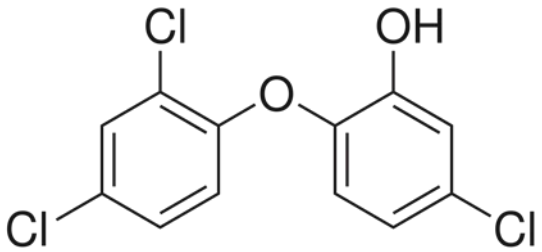

Fig. 2: Chemical structure of triclosan $C_{12}H_7Cl_3O_2$. Trade names on products are Irgasan, Irgacare, Irgacide, Cloxiphenol, Lexol, Ster-Zac, Aquasept, Sapoderm.

TCS-Powder (Irgasan, Sigma-Aldrich, St. Gallen, Switzerland, catalog No. 72779) with a purity ≥ 97% was diluted in absolute ethanol to reach a stock solution of 1 g L^{-1}. Stock solution was kept in the freezer and diluted in WC-Medium to reach concentrations of working solutions.

Cyanobacterial strains and culturing

We used two strains of *M. aeruginosa*, the toxic wild type strain PCC 7806 and the non-toxic mutant ΔmcyA in this study (obtained from Brett A. Neilan laboratory, UNSW, Sydney Australia). ΔmcyA is a transformed version of PCC 7806. It's not producing microcystin because of insertional inactivation of the peptide synthethase gene mcyA (Dittmann, Neilan 1997).

Cultures were initially grown in BG-11 Medium (Stanier, 1971) - a nutrient-rich medium for the cultivation of cyanobacteria and protozoa, prepared by diluting 20 mL cyanobacteria BG-11 freshwater solution 50x (Sigma Aldrich, St. Gallen, Switzerland) in 980 mL deionized water. pH-value was adjusted to 7.5 (protocol 1, Appendix). Only back-up cultures were grown in BG-11 because it allowed longer cultivation periods without changing medium.

Cultures that were used for experiments were acclimated to WC-medium (Guillard & Lorenzen, 1972). WC-medium is an inorganic salts medium for culturing of freshwater green algae and cyanobacteria. Its chemical composition is closer to environmental conditions, less prone to contamination and therefore more suitable for experiments compared to other similar media and BG-11. Nutrient solutions were made out of 6 stock solutions (Protocol 2, Appendix). Both, stock solutions and nutrient solutions were autoclaved after preparation.

Stock cultures and cultures kept under chronic sub-lethal exposure were grown on the lab bench at room temperature. All other experiments were carried out in a cultivation chamber under continuous light (30µE $m2^{-1}$ s^{-1}), 25°C and no shaking. Incubating cultures of a density of 2 x 10^6 cells L^{-1} in the cultivation chamber 5 days prior to the experiments allowed cells to acclimate to prevailing conditions and ensured that cells were in the mid-exponential phase of growth.

PCR-based test optimisation

To adopt DNA-extraction- and PCR-protocols to available lab-facilities and *Microcystis*-strains, a phylum- and genus-verification was conducted. We used a primer pair flanking the cyano-specific 16S rRNA gene and the microcystin-synthethase-gene cluster respectively. The most adequate protocol (for details see protocol 6, Appendix) included two cell lysis steps (mechanical disruption through freezing and shaking in cell lysis buffer), a DNA isolation step (using the Plant-DNA-Isolation-kit Nucleo Spin Plant II from Macherey-Nagel, Düren, Germany) followed by the PCR reaction. DNA quantity and quality were assessed with a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, Germany). Polymerase chain reaction amplification was performed in a volume of 25 µL, containing 12.5 µL MasterMix, 2.5 µL of each primer (1 µM), 2.5 µL nuclease-free water and 5.0 µL DNA template. The PCR thermal cycling protocol included an initial denaturation at 95°C for 10 min, followed by 35 cycles at 95°C for 60 s, at an annealing temperature of 60°C for 60 s, and elongation at 72°C for 60 s. PCR products (5µL of the reaction mix) were visualized by electrophoresis in 3% agarose in 0.5 × TBE (Tris-borate-EDTA buffer + ethidium-bromide staining) for 20 min at 100V.

Growth assessment, cell count and cell fixation

To ascertain a reasonable experimental running time and choose an appropriate starting cell density we assessed the growth of our cultures with the corresponding cell densities in each phase. Cell density was estimated by either visually counting cell numbers in a Neubauer improved-counting chamber (for exact procedure see protocol 4, Appendix), counting the cells in a Cytobuoy flow cytometer (www.cytobuoy.com) or by measuring the optical density (OD) of the cultures in a microplate reader (Tecan infinite M200) set at a wavelength of 750 nm. It was evaluated which OD_{750} corresponded to a cell concentration of $2x10^6$ cells mL^{-1}.

Exponential phase of growth was examined by determining OD_{750} of cultures. Three 150 mL Erlenmeyer culture flasks of each strain starting with 2 x 10^6 cells mL^{-1} were grown in a cultivation hood. OD_{750} of 200 mL aliquots out of each flask was measured in three-day or four-day intervals.

Experiments

To investigate the short- and long-term effects of sublethal exposure levels of TCS on *M. aeruginosa*, we designed experiments where cultures underwent different intervals and intensities of stress (Fig. 3).

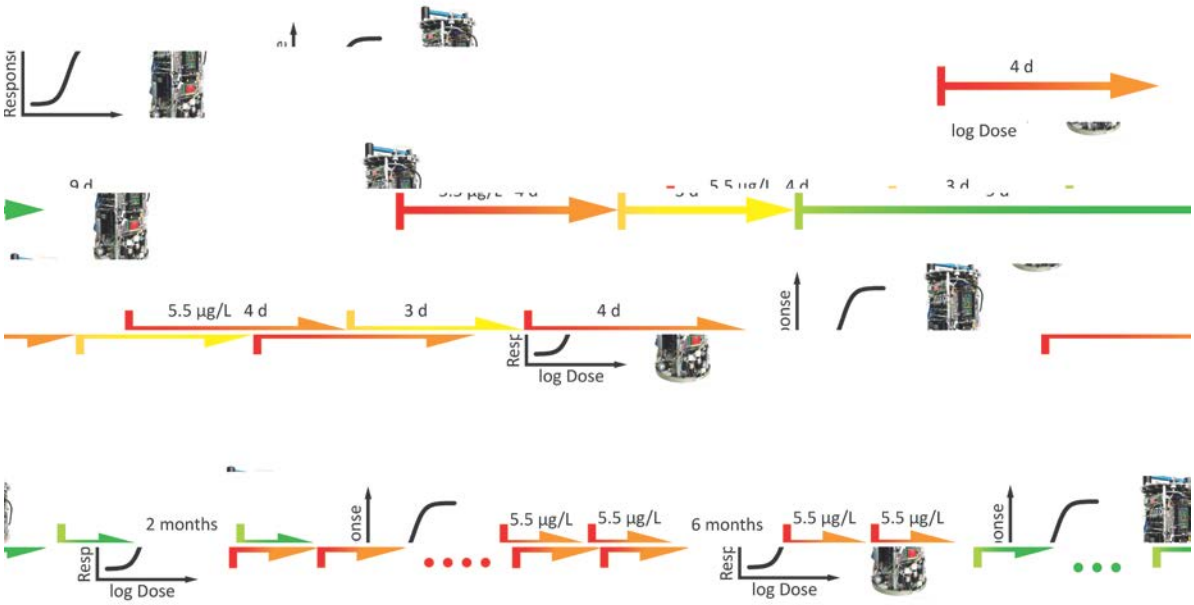

Fig. 3: Graphical overview over experiments. Red arrow = exposure, yellow arrow = detoxification, green arrow = growth in standard conditions. Short term response was assessed with I) dose-response experiments, II) stress – recovery experiments, III) stress-restress experiments and long term effects were assessed with a IVa) long-term exposure experiment and a IVb) a long-term recovery experiment.

At the end of every experiment, cells were fixed with a fixative solution for flow-cytometry (protocol 3, Appendix) and analysed. Endpoints were: abundance of cells, growth inhibition and changes in functional traits and the overall phenotype. Cells abundance and functional traits were monitored using the Cytobuoy scanning flow-cytometer (see chapter Flow-cytometry), growth inhibition was plotted against concentration (see chapter 96-h Dose-response analysis) and overall phenotype was expressed using principal component analysis (See chapter PCA)

Short-term exposure experiment

To see at which concentrations TCS has an effect on the phenotype of *M. aeruginosa*, we carried out several 96 hour growth inhibition tests with protocols based on OECD guideline 201 (OECD, 2002) (for detailed protocol see protocol 5, Appendix). Batch cultures starting with 2 x 10^6 cells mL^{-1} were treated with TCS. Test-concentrations were 50, 16, 5.5, 1.8, 0.6, 0.2, 0.06, 0.02 and 0 µg L^{-1}. These concentrations ranged across the actual environmental concentrations in surface waters and rivers. Testing at each dose was done in five replicates.

Reversibility of phenotypic change

To see if phenotypic transitions occurring at low exposure levels are maintained over generations, a reversibility experiment was conducted in two experimental replicates. Populations were exposed to a single pulse of TCS and changes in cellular phenotype were monitored over a period of time after stress was released. Two flasks containing 100 mL PCC 7806-cells starting with $2x10^6$ cells mL^{-1} were exposed to TCS (5.5 µg L^{-1}) for four days. Two flasks containing the same volume and cell density but no TCS were run in parallel as a control. After four days, cultures were filled into permeable membrane dialysis bags (Spectra/por, Spectrum Europe, Breda, Netherlands) and put into 1 L beakers filled with WC-

medium for three days (approximately one generation for uninhibited cells). Medium in beaker was replaced every 12 hours to allow TCS to diffuse out and cultures to detoxify. After three days of detoxification, cultures were reset to 2 x 10^6 cells mL^{-1} and filled back into 100 mL flasks. Five mL of culture were fixed for later analysis on the day cultures were brought back to flasks and every third consecutive day.

Stress-re-stress

To assess whether phenotypic transitions are associated with acquired resistance to the chemical stressors, we conducted a stress-re-stress-experiment. Populations of *M. aeruginosa* PCC7806 were exposed to a first dose of TCS to allow phenotypic transition and acclimation. A subsequent 96h-growth inhibition test revealed if pre-exposure made cells more resistant or phenotype more stable towards a second exposure.

Three hundred mL PCC 7806-cells starting with 2 x 10^6 cells mL^{-1} were exposed to 5.5 µg L^{-1} TCS for four days. A control treatment was run in parallel. Cells detoxified for three days in permeable membrane dialysis bags floating in 1 L beakers by replacing WC-medium every 12 hours. Test and control bags were not de-stressed in the same bucket to avoid any contact of the control with TCS. After two days of recovery, a dose response including 8 doses reaching from 0 to 150 µg L^{-1} over 96h was conducted in 12-well plates in triplicates.

Long-term exposure experiment

To test whether chronic sublethal exposure increases resistance of populations or induces a constantly altered phenotype of individuals, we executed a long-term exposure experiment. Two experimental replicates of each strain were weekly exposed to 5.5 µg L^{-1} TCS in 100 mL flasks. By replacing 50 mL of culture with fresh WC-medium every two weeks it was assured that cultures were kept in exponential growth and TCS did not exceed sublethal concentrations. Unexposed controls were run in parallel. Endpoints after 6 months, i.e.

roughly 60 generations and 24 pulse exposures, were phenotype, fitness, resistance and maintenance of resistance.

Long-term exposed PCC 7806 (called hereafter LT5) cultures were then detoxified for three days in permeable membrane dialysis bags floating in 1 L beakers by replacing medium every 12 h. One fraction of detoxified cultures was directly used for growth assessments and a 96-hour growth inhibition test. The other fraction of detoxified cultures was kept in exponential growth without new TCS being added. After 6 month of chronic sublethal exposure and two month of growth without any contact to TCS, a 96-hour growth inhibition test was repeated with the same cultures. This extended stress-re-stress experiment allowed to explore if acquired resistance remained maintained after stress had been released from long-term exposed cultures. Evaluation of possible acquired resistance of ΔmcyA remains to be explored.

Triclosan ELISA Assay

To verify the initial TCS concentration and determine the residual concentration after the long-term-EC50 test, we used a competitive ELISA-kit (Abraxis kits, PA, USA) according to the manufacturers protocol. Seven standard solutions (std 0–6), of known TCS concentrations (0, 0.05, 0.1, 0.25, 0.5, 1.0 and 2.5 µg L^{-1}) and a positive control (0.75 µg L^{-1}) were supplied with the kit.

Samples in which the residual concentration was measured were taken at the end of the 4 day incubation and filtered through a 0.2 µm filter (Whatman International, Maidstone, England). Samples to verify the initial concentration were freshly prepared by diluting the stock solution to the nominal concentrations in the same manner it was done prior to the experiment. Additional, two environmental samples out of Chriesbach, Dübendorf and one sample of a nominal concentration of 0.6 µg L^{-1} that has been exposed to UV-Light for 1h were analysed.

Standard, control and test samples were processed according to the maufacturers protocol. Reactions were stopped by adding 50 µL of stop solution and absorbance was measured at 450nm using Tecan infinite M200 microplate reader. A standard-curve was generated from the results of standard solutions. The mean absorbance value (Bx) was calculated for each standard and divided by the mean absorbance value for standard "0" (%Bx/B0). A standard curve was plotted from the %Bx/B0 values of standards 1–6 in Excel with a linear Y axis and log X axis. The equation of the line of best fit was used to interpolate the TCS concentration in ppb of the controls and test samples.

ITS-sequencing

The ITS-sequence of the rRNA operon has previously been demonstrated to be a suitable marker to monitor spatiotemporal changes in the genetic diversity of *M. aeruginosa* (Misson et al., 2012). Favourable environmental conditions led to the selection of *Microcystis* genotypes with higher fitness (Briand et al., 2009). To address if chronic sublethal TCS exposure led to the selection of resistant genotypes or if genotypic structure remained stable, we sequenced the internal transcribed spacer (ITS) of long-term exposed and unexposed control cultures. DNA-extraction and PCR were conducted according to protocol 6, Appendix. Primers were: forward 5'-TGTAAAACGACGGCCAGTCCATGGAAG(CT)-TGGTCA(CT)G-3'; backward 5'-CCTCTGTGTGCCTAGGTATCC-3'(Iteman et al., 2000). DNA was purified using PCR-Wizard Clean-Up System (Promega, Madison, USA) following the manufacturers protocol. Sequencing was performed by microsynth (Balgach, Switzerland). Sequence data were manually checked for quality including background noise, peak intensity and accuracy via the chromatograms using Chromas Lite 2.4 (http://www.Technelysium.com.au) and analysed using the BLAST tool from NCBI to confirm identity and position.

Flow-cytometry

We used the scanning flow-cytometer Cytobuoy (www.cytobuoy.com, Woerden, Netherlands) for counting and morphophysiological characterisation of *M. aeruginosa*-cells, with 20'000 particles scanned for each sample. Each particle was intercepted by a coherent solid-state Sapphire 488 nm laser beam (15 mW) at the speed of 2 m/s (Pomati et al., 2011). Digital data acquisition was triggered by the sideward scatter (SWS) signal with a trigger-level of 34 mV, which excludes particles smaller than 0.7 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 the particles. The fluorescence (FL) emitted by photosynthetic pigments in *M. aeruginosa*-cells was detected at three different wavelengths: red (FL.Red and FL.2.Red), orange (FL.Orange) and yellow (FL.Yellow) signals were collected in ranges of 668 – 734 (Chlorophyll- a), 601–668 (phycocyanin) and 536 –601 nm (phycoerythrin and decaying pigments), respectively (Dubelaar et al., 2004). A cytogram showing two important channels was displayed for instant proof reading (Fig. 4). Cell abundance was determined by dividing the amount of green events (Fig. 4) in the cytogram by the volume that has been sucked in.

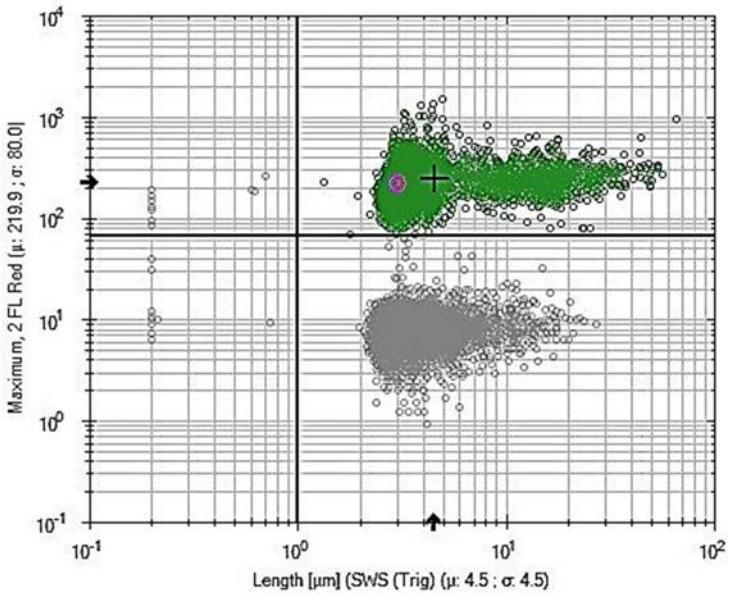

Fig. 4: Cytogram displaying length and maximum fluorescene of particles. *M. aeruginosa*-cells (green cloud) were separated from cell debris (grey cloud) by assigning particles >1µm and Max.2.FL.Red >50 (green gating lines) to the green cloud.

Signal length of the different channels allowed clear distinction of different cellular morphotypes (Fig. 5). Output-files of Cytobuoy-measurements contained 54 descriptors with information about 3D structure and FL profile for each particle.

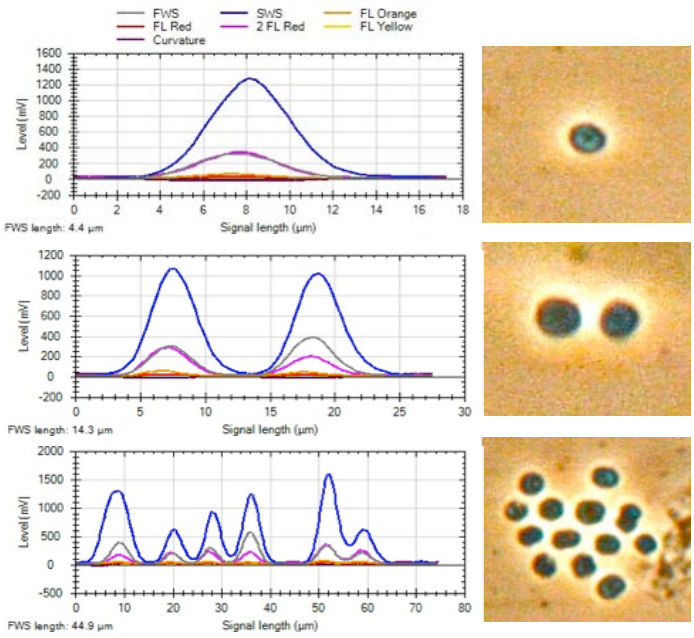

Fig. 5: Cytobuoy signals and corresponding cellular phenotypes. Signal length out of six channels allowed clear distinction of single cells (top) doubling cells (middle) and small colonies (bottom).

Microcystin analysis

Cultures of PCC 7806, ΔmcyA and LT5 and were grown in triplicates for 14 days in flasks with a starting density of 2 x 10^6 cells L^{-1}. Before microcystin analysis, triplicates were pooled together to avoid unnecessary costs. Microcystin was isolated following extraction methods as described by Fastner (1998) and determination was performed by TZW (Technologiezentrum Wasser, Dresden, Germany) according to Pietsch (2001). Sample preparation involved filtration (0.45 µm cellulose nitrate filters) of batch samples to separate intra- and extracellular microcystins. Extracellular toxins were determined by a combination of ion-pair-supported solid-phase extraction (SPE) coupled with high-performance liquid

chromatography and tandem mass spectrometry (MS-MS). Cell-bound toxins were analyzed after freezing the filters and elution of the toxins by methanol: MS-MS was used to determine microcystin variants.

Microcytin concentrations (µg L^{-1}) were expressed relative to biovolume (g L^{-1}). Biovolume was estimated by summing up the volumes of every single particle (Pomati &Nizzetto, 2013):

$$Biovolume = \sum 0.1630\ \sqrt{0.0017\ Total.FWS} - 0.0133)/volume.ml$$

Data analysis

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

Growth curves

Growth rate (μ_{0-n}) was estimated in exponentially growing cultures as:

$$\mu_{0-n} = \frac{ln(OD_n) - ln(OD_0)}{T_n - T_0}$$

where OD_n and OD_0 are optical densities at time t=5 and 0 days, respectively. For this purpose, the values of T_0 and T_n were determined at 3 and 7 days after the transference of cells to fresh medium. Modelling of growth-curves was performed by nonlinear regression analysis. A four-parametric logistic model with the lower limit fixed at starting OD_{750} 0.06 was fitted to the data using the drm function of R-package drc (Ritz, 2012).

96-h Dose-Response analysis

The average specific growth rate (μ_{0-4}) of each replicate within the 96-h growth inhibition tests was calculated as the logarithmic increase in cell abundance from the equation:

$$\mu_{0-4} = \frac{ln(OD_4) - ln(OD_0)}{(4-0)}$$

where OD_4 and OD_0 are optical densities at the end (after 4 days) and at the beginning of the test, respectively. The percent inhibition of growth rate for each replicate was calculated with the equation:

$$\% \, Inhibiton = \frac{\mu_C - \mu_T}{\mu_T} 100$$

where μ_c is the mean value across all replicates in the control and μ_T is the value for growth rate in each single treatments. In treatments where inhibition exceeded 100% i.e. mortality occurred, inhibition was set to 100%. Percentage of inhibition for each concentration was plotted against the logarithm of TCS concentration. Modelling of dose-response curves and calculation of IC50 was performed by nonlinear regression analysis. A four-parametric log-logistic model was fitted to the data using the drm function of R-package drc (Ritz, 2012). IC50 and confidence interval were calculated using the ED function of the same package.

Cytobuoy parameter analysis using PCA

The raw Cytobuoy data giving information about 54 scattering and fluorescence derived traits for each particle was visually inspected in R in order to set threshold levels to extract living cells from cell debris. Cut-off level for non-fluorescent particles was set at Max.FL.2.Red = 50 (Fig. 6). Particles < 1µm (fluorescent background) were also cut off.

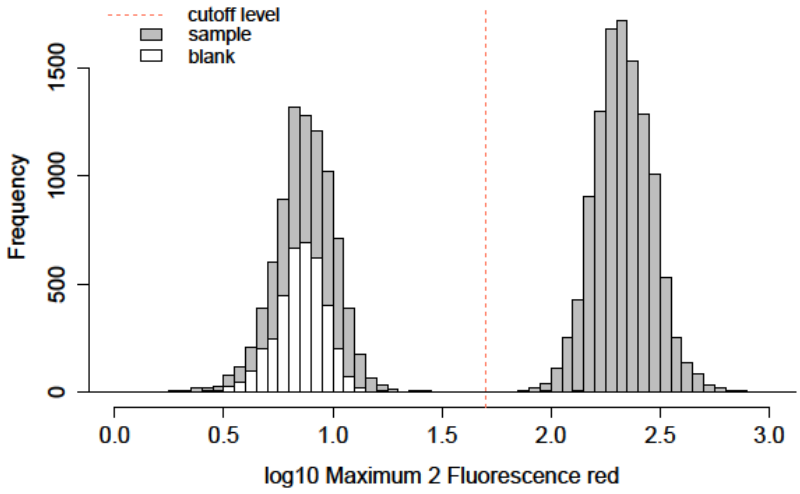

Fig. 6. Histogram of particle-fluorescence. Cut-off-level (red) was set visually to separate *M. aeruginosa* cells from cell debris and dust.

Cytobuoy parameters of remaining cells were used to derive aggregated measures of *M. aeruginosa* individual phenotypes using principal component analysis (PCA) (Pomati et al., 2011). Before PCA, data were standardised by centring and scaling. PCA was not applied on every single experiment itself but included all experiments of this study. Displaying the loadings of each trait onto each principle component showed the relative contribution of parameters. Overall population phenotype in a replicate was expressed by the mean of each principle component across the whole population.

Results

PCR-based test optimisation

PCR profiles of PCC 7806 and ΔmcyA strains revealed that both strains produced amplicons for the cyano-specific 16S rRNA gene amplification and the microcystin-synthethase-gene amplification (Fig. 7). This confirmed that the strains were indeed cyanobacteria containing the microcystin-synthethase-gene-cluster. Previously tried protocols failed because cell lysis and extraction was not efficient enough and (low DNA yield and quality determined with NanoDrop). Therefore the suggested DNA extraction protocol was used in further experiments.

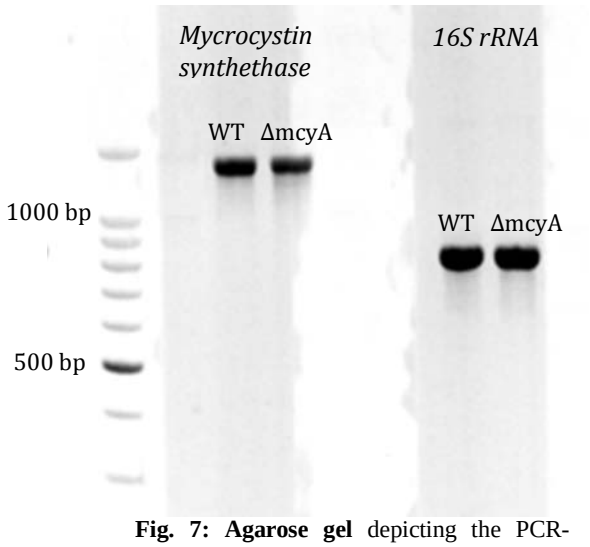

Fig. 7: Agarose gel depicting the PCR-profiles of aplified microcystin synthethase gene (left) and 16 S rRNA gene (right)

Growth assessment

OD_{750} was linearly correlated with both, optical cell counts and cell counts from Cytobuoy measurements (Fig. 8). At higher cell densities, optical cell counts were accurate and effective whereas at lower cell densities, Cytobuoy counts were more reliable. Regression functions of the two counting methods revealed that an OD_{750} between 0.06 and 0.07 corresponded to 2000 cells μL^{-1} i.e. 2 x 10^6 cells mL^{-1}. Adjusting OD_{750} was therefore regarded as a valid tool to dilute pre-cultures to actual starting cell densities for experimental use.

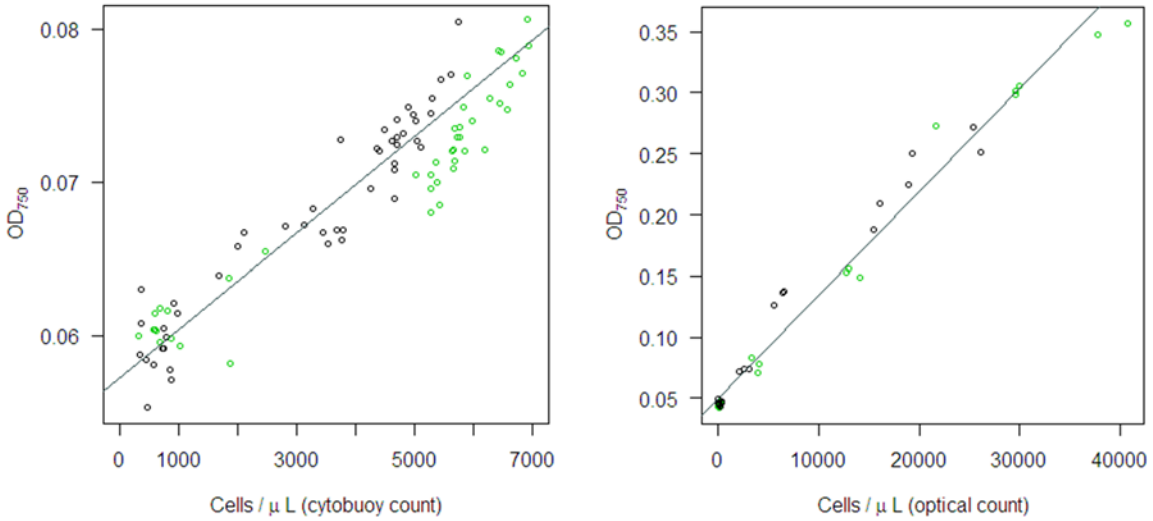

Fig. 8: Correlation of optical density and cell count. Optical density correlated with cytobuoy and opical cell counts of PCC7806 (green dots) and ΔmcyA (black dots). Linear relationship expressed as function was $y = 3.14e^{-6} x + 0.057$ for cytobuoy counts ($R^2 = 0.85$, $p < 0.001$) and $y = 8.47e^{-6} x + 0.049$ for optical count ($R^2 = 0.98$, $p < 0.001$) (darkgreen lines).

Specific growth rate in the exponential growth phase was 0.31 ± 0.01 d^{-1} (mean ± standard error) for PCC7806 and 0.189 ± 0.01 d^{-1} for ΔmcyA. With a generation time of 3 days, PCC7806 grew faster under prevailing conditions in the hood than ΔmcyA with a generation time of 7 days. Maximal OD_{750} approached 0.35 for WT and 0.25 for knock-out cultures (Fig. 9). Hence, cell densities at stationary phase were almost twice as high for WT cultures as cell densities for knock-out cultures (approximately 4 x 10^7 cells mL^{-1} for PCC7806 and 2 x 10^7 $cells^{-1}$ for ΔmcyA).

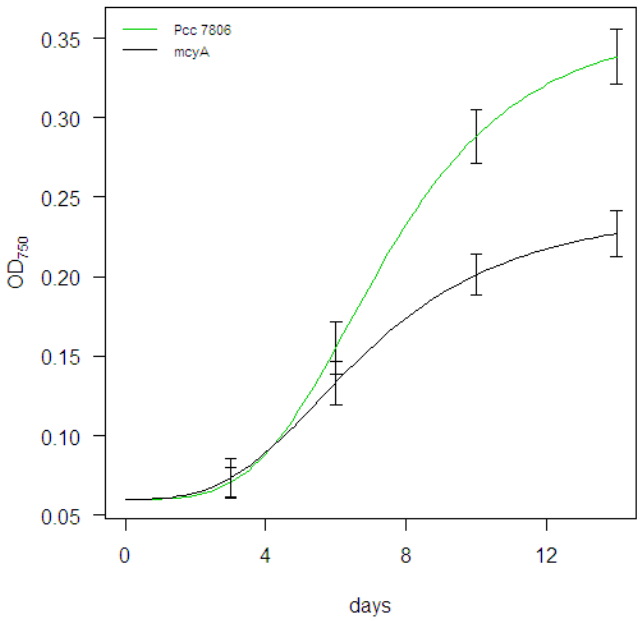

Fig. 9: Growth curves of PCC7806 (green) and ΔmcyA (black) with model based errors. The knock-out mutant grew slower and reached lower densities than WT.

PCA

The dimensionality of cytobuoy derived particle descriptors was reduced to 32 orthogonal vectors covering 99% of the total variance in the data by PCA. The first four principle component axes described 75% of the variation in the 54 traits (Tab. 1). The first PC explained 37% of the variance. Traits were equally loaded on PC1 (maximal contribution 3%). However, size-related traits seemed to be among the most important ones. The second PC explained 23% of variance and was associated with fluorescence related traits. The third PC explained 10% of variance and was associated with traits defining cellular shape. And PC 4 explained 5% of variance and was associated with plasticity. For contribution of further PC's and more detailed loadings see Tab.2 in appendix.

Tab.1: Principle components (PC1 – PC4), explained variance and their loadings

	PC1	PC2	PC3	PC4
% variance	37	23	10	5
Improtant Loadings	Center.of gravity. Total.fluo. Chl-a Number.of.cells	Inertia Average fluorescence Maximum fluorescence	Asymmetry Fill factor Average.FL.orange	Scattering Asymmetry Fill factor

Short-term exposure

TCS inhibited 50% of population abundance (IC50) in *M. aeruginosa* at concentrations of 1.53 ± 0.16 µg L^{-1} for WT, and 0.72 ± 0.1 µg L^{-1} for ΔmcyA (Fig. 10).

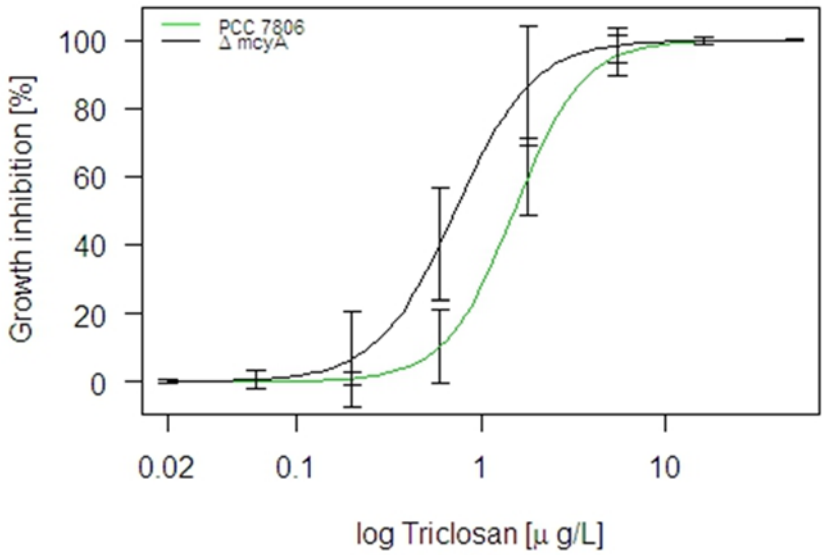

Fig. 10: Dose-reponse curves showing the extent of how a given concentration of Triclosan inhibits the growth of the toxic M. aeruginosa strain PCC 7806 (green) and the nontoxic strain ΔmcyA (black).

The non-toxic mutant therefore appeared to be more sensitive to the chemical and showed an overall different phenotype. Median cell size in controls was 3.6 µm for WT and 5.1 µm for ΔmcyA. A significant observable transition in cellular phenotype occurred for WT at IC50 levels and above (Fig. 11). ΔmcyA showed a weaker phenotypic transition than PCC 7806 and the shift in phenotype occurred at doses higher than IC50 (Fig. 12). Cells exposed to increasing concentrations of TCS were bigger, scattered more light, were more fluorescent,

more asymmetric, had a lower fill factor and were sticking more together forming cell-clusters (Fig. 4-8, Appendix). However cell clusters were on average up to 3 cells sticking together and thus could not be considered as a colony yet. Big clusters were rare and looked different from real colonies (Fig. 9, Appendix).

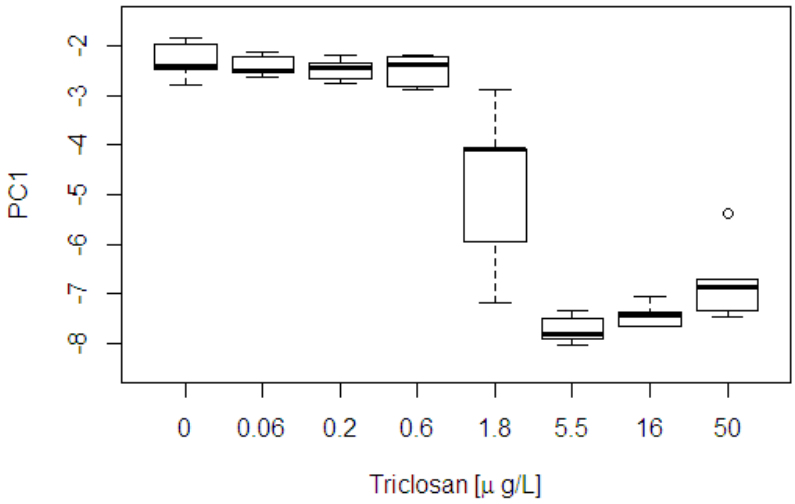

Fig. 11: Cellular phenotype (PC1) of PCC 7806 at different TCS concentrations. TCS concentrations above 0.6 µg L^{-1} induced changes in cellular phenotype after 4 days of exposure. Boxes consist of population means of 5 replicates per concentration.

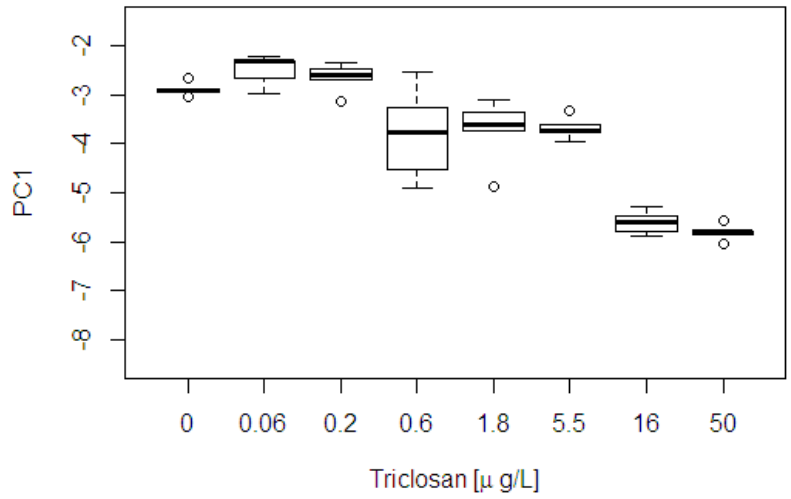

Fig. 12: Cellular phenotype (PC1) of ΔmcyA at different TCS concentrations. TCS concentrations above 0.2 µg L^{-1} induced changes in cellular phenotype after 4 days of exposure. Boxes consist of population means of 5 replicates per concentration.

Reversibility of phenotypic change

Changes in the WT phenotype, acquired after exposure to TCS, were maintained only over a short period of time after cultures were released from chemical stress. In experimental replicates, the overall phenotype of stressed cells reverted gradually back to unstressed phenotype and converted completely after 6-9 days of growth (circa 3 generations) in TCS-free medium (Fig. 13. Directly after TCS exposure cells were bigger and more fluorescent than unexposed cells. But simultaneously with PC1 also size and fluorescence reverted back (Fig. 2 and 3, Appendix)

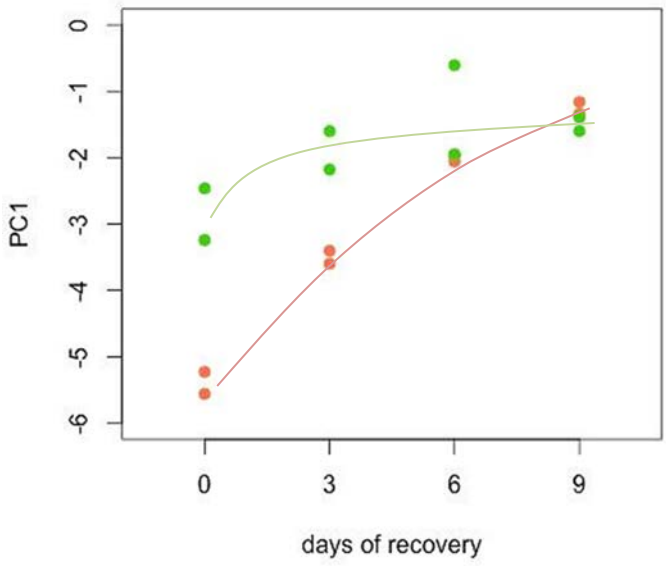

Fig 13: Recovery of cellular phenotypes. Phenotypic transition of TCS exposed cultures (red) reverted back to unstressed phenotype (green) after 9 days (i.e. 3 generations) when stress has been released. Every point represents the mean PC1 of thousands of cells within a replicate.

Exposure-re-exposure

Pre-exposure to 5.5 µg L^{-1} TCS for four days reduced cellular growth of *M. aeruginosa* in a subsequent 96-h growth inhibition test. Growth rate of 0-treatments (no TCS dosing) in pre-exposed cultures was 0.23 ± 0.01 d^{-1} whilst control 0-treatments showed a growth rate of 0.4

± 0.01 d^{-1}. Resistance was impaired too. Pre-exposed cultures were not able to grow when they were exposed to a second dose of 5.5 µg L^{-1} TCS. Mortality following the second exposure was greater than what was observed for the same dose with cultures that were not stressed previously (Fig. 14)

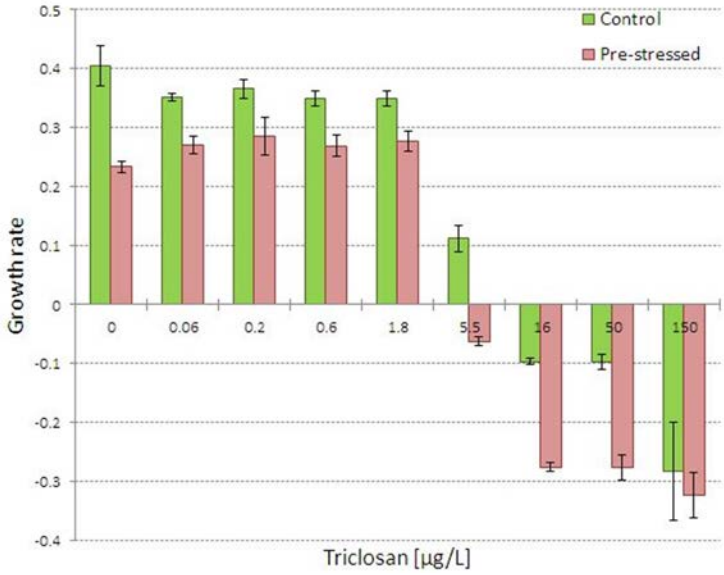

Fig. 14: Growth rate of pre-stressed and control cultures at different TCS concentrations. Growth following the second exposure was reduced, mortality occurred at lower concentrations and was higher than in controls.

In previously untreated cultures of PCC 7806, low-stressed cells (<IC50) were phenotypically clearly separated from high-stressed cells (>IC50) after a 96-h test (Fig. 11, Appendix, see also Fig. 18, main text). In pre-stressed cells (5.5 µg L^{-1} for four days), on the contrary, separation of phenotypes was not that obvious after the same 96-h test (Fig. 11, Appendix). Pre-stressed cells that received a low dose in the second exposure maintained a stressed phenotype. Surprisingly, pre-stressed cells that received another dose of 5.5 µg L^{-1} were phenotypically the closest to cells that have never been exposed (fig. 11, Appendix). A first exposure made cells bigger and a second exposure to the same level of toxin made cells small again (Fig. 15)

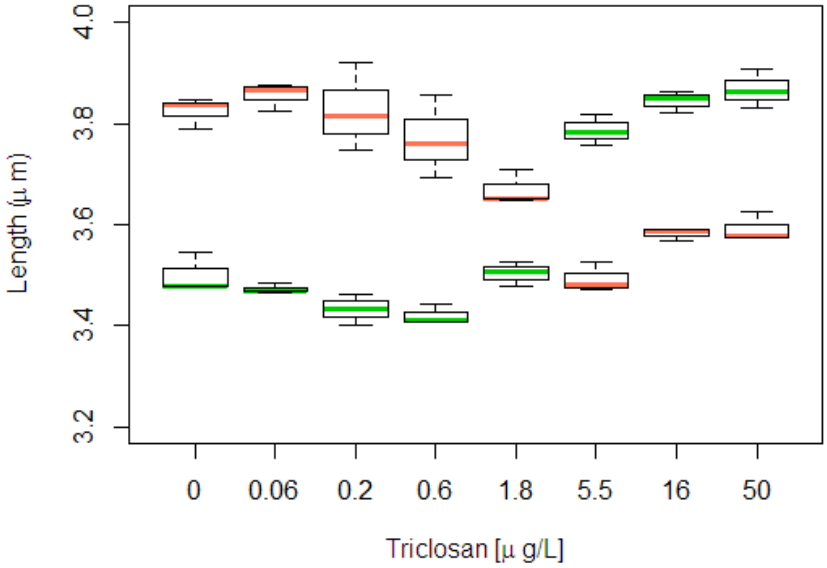

Fig. 15: Median cell size of *M. aeruginosa* populations with increasing TCS dosage (n=3 per dose per treatment). Controls (green) enlarged cell size and prestressed populations (orange) became smaller with increasing dose.

Long-term exposure experiment

Long-term exposure of *M. aeruginosa* to sublethal doses of TCS led to more resistant populations, able to maintain the acquired resistance across several generations. After 6 month of growth under chronic 5.5 µg L^{-1} TCS exposure, cultures survived concentrations up to 16 µg/L at which growth of control PCC 7806 was completely inhibited (Fig. 10, Appendix). This resistant "strain", is referred to as LT5 (for long-term 5.5 µg L^{-1} TCS). LT5 cultures had an EC50 of 16.89 ± 1.01 µg L^{-1} and were over 15 times more resistant than their ancestor cultures (Fig. 16). But also control PCC7806 cultures (LTC), that underwent the same experimental procedure as LT5 but without TCS added, changed sensitivity slightly. IC50 of LTC was 4.9 ± 0.31 µg L^{-1} (Fig. 12, Appendix).

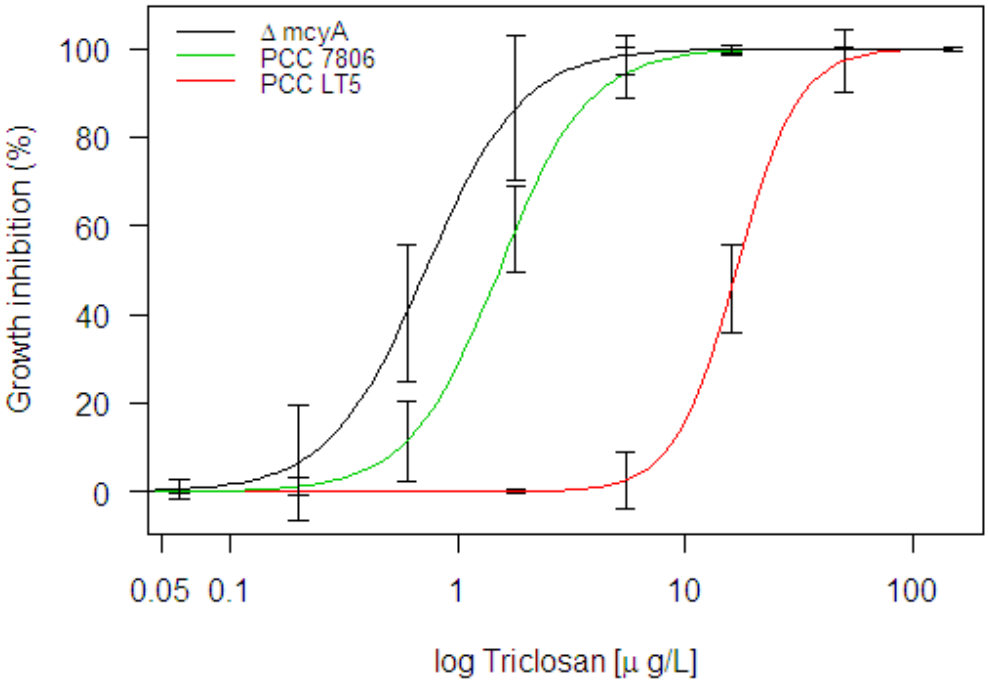

Fig. 16: Dose-reponse curves of three *M. aeruginosa* strains. Chronic sublethal exposed cultures (LT5, red) were significantly more resistant than ancestor control cultures (PCC 7806, green) and ΔmcyA (black).

Growth rate of LT5 cultures was reduced compared to PCC 7806 cultures but still higher than growth rate of ΔmcyA (Fig. 17). Specific growth rate in exponential phase of PCC7806 was 0.25±0.01 d^{-1}, 0.164±0.01 d^{-1} for ΔmcyA and 0.22±0.01 d^{-1} for LT5.

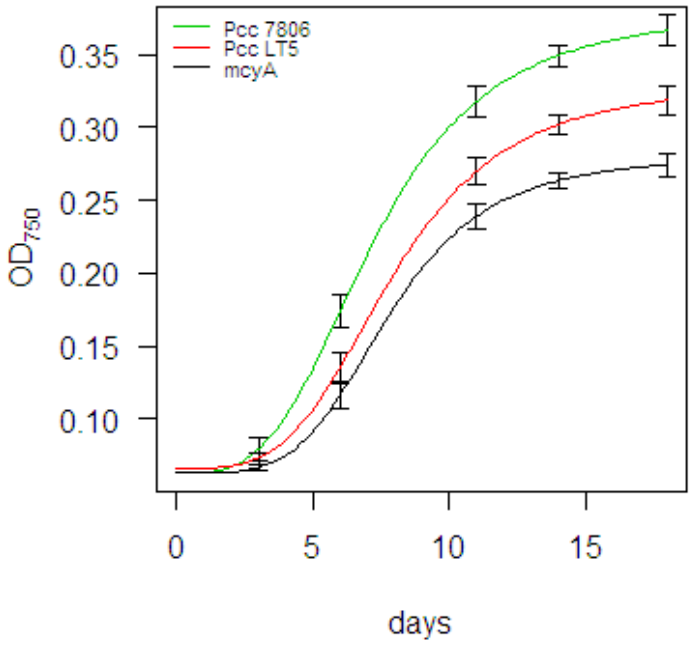

Fig. 17: Growth curves of LTC (green) LT5 (red) and ΔmcyA with model based errors. Both, resistance and the loss of a gene comes with the cost of growing slower.

Overall, across all the experiments, the phenotypes of LT5, PCC 7806 and ΔmcyA were clearly different. Not only between strains but also within strains we observed a phenotypic separation between stressed and unstressed cells (Fig. 18). The range of mean population phenotype under identical experimental conditions was smaller for LT5 than for the other two strains. *M. aeruginosa* strains, across all treatments, aligned along a trade-off phenotypic axis between PC1 and PC2 (Fig. 18). Along this axis, one major denominator that separated strains was cell size. Median cell size measured by forward scattering in unexposed cultures was 4.3 µm for ΔmcyA, 3.4 µm for PCC 7806 and 2.9 µm for LT5. Besides being the smallest, LT5 cells were more symmetric, scattered less light, had a higher fill factor and a higher inertia. Further, fluorescence (absolute and relative to size) measured by all FL-channels was reduced in long-term exposed cells suggesting a decreased chlorophyll-a and phycocyanin content. Stressed LT5 cells were phenotypically more similar to unstressed PCC 7806 cells and stressed PCC 7806 were phenotypically more similar to unstressed ΔmcyA cells. Short term exposure shifted phenotypes more to the top left of the axis whereas long term exposure shifted phenotypes to the bottom right. Hence, different exposure scenarios seem to affect phenotype in 2 opposing ways. As depicted in Fig. 18, along the PC1/PC2 axis our analysed cultures sorted not only based on their phenotypic characteristics, but also on their sensitivity to TCS (high for ΔmcyA, medium for WT, low for LT5).

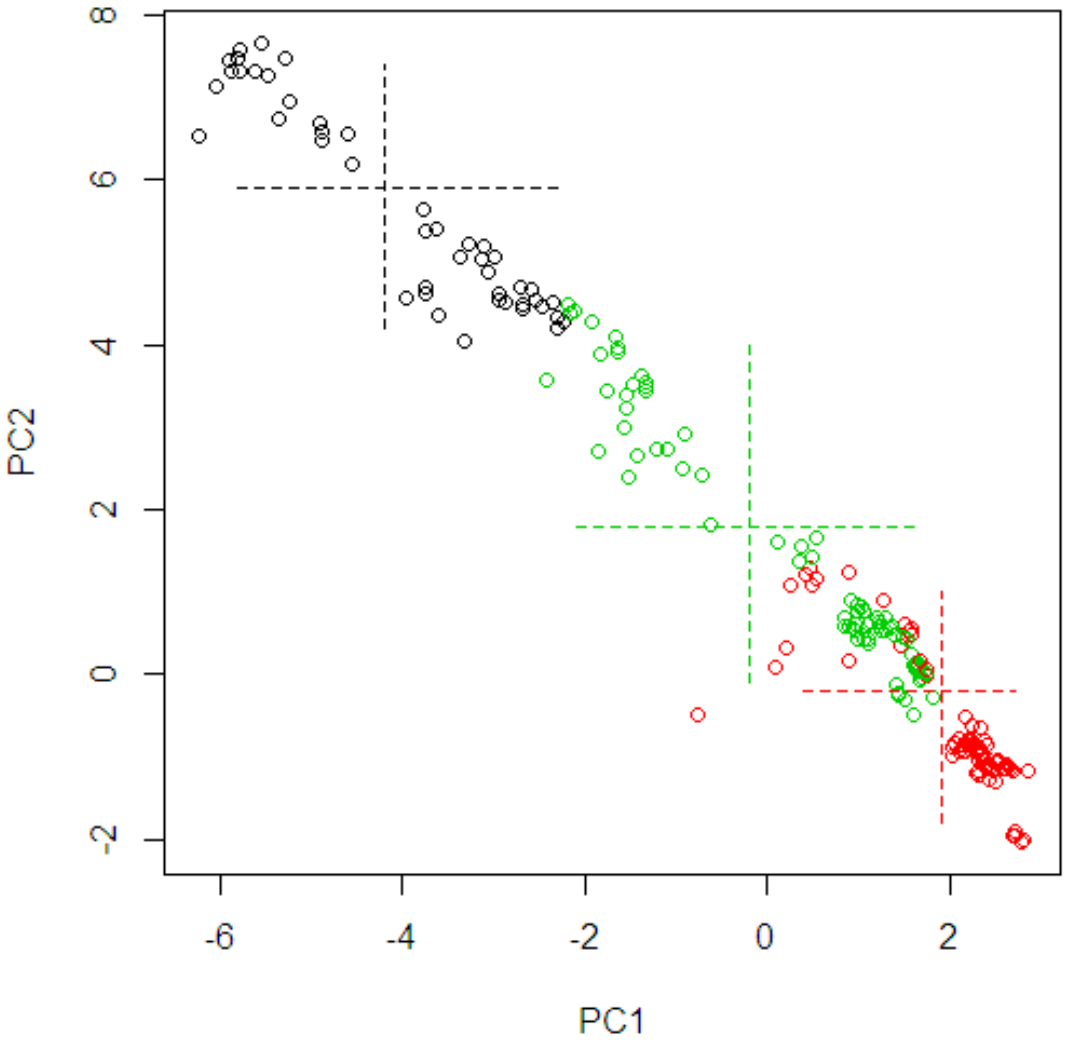

Fig. 18: Mean population phenotype of three different *M.aeruginosa* strains expressed by PC1 and PC2. Within strains (separated by colour), phenotype differed in stressed (>IC50) and unstressed (<IC50) state (separated by dotted lines). Points at the top left reflect populations with large, fluorescent cells that grow slow and are TCS-sensitive whereas points at the bottom right reflect small, denser packed - although less fluorescent cells that are TCS-resistant. Length of dotted lines represents the range where 90% of mean expressed phenotypes are in. Position of dotted lines represents the mean phenotype at IC50

Increased resistance of LT5 cultures was maintained even after two month of growth without any contact to TCS, although not at the same level (Fig. 19).

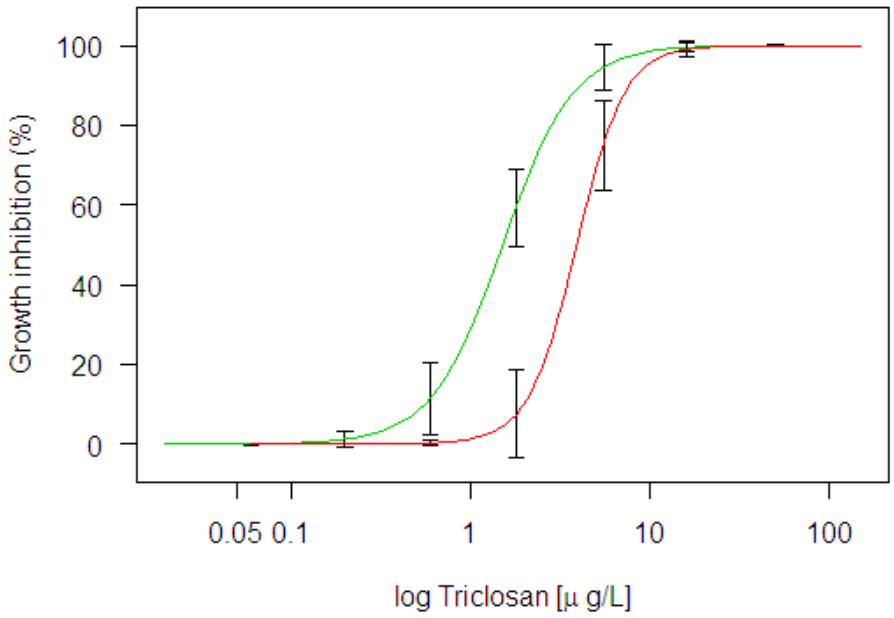

Fig. 19: Dose-reponse curves after long-term recovery. Resistance of LT5 (red) was partially maintained after 2 month of recuperation and significantly more resistant than PCC 7806 (green).

IC50 dropped down to 3.9 ± 0.3 µg L^{-1} but was still significantly higher than in experimental controls (Fig. 13, Appendix). Maintained resistance over 20 generations let us assume that changes were not only of physiological nature but that there was also a partition of genetic contribution. That's the reason why we looked at neutral genetic diversity by sequencing the ITS-region of 16S rDNA.

ITS sequencing

The 488 bp long ITS-sequence of the 16 rRNA was sequenced from strain PCC 7806 and resistant strain LT5. Sequences were identical except one undetermined nucleotide in the LT5 sequence on position 475 (whole sequence see fig. 1, Appendix). BLAST-search showed 100% congruency of sequences to GeneBank accession no. AM778951.1 and uncovered the

undetermined nucleotide in the LT5 sequence as a known polymorphism. Chromatograms pointed out that PCC 7806 populations also harboured both nucleotides on position 475 (A & G) but G was not detected because of a higher partition of A (Fig. 20).

No neutral genetic variance could therefore be found in original and long term exposed populations.

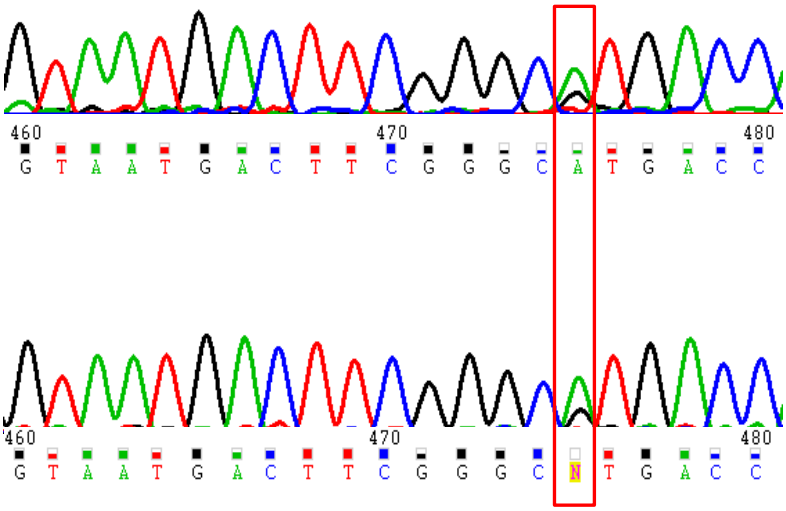

Fig. 20: Section out of the ITS-DNA-sequence chro-matograms from *M. aeruginosa* PCC 7806 (top) and resistant LT5 strain (bottom) with a shared polymorphism.

Triclosan immuno assay

To verify the nominal TCS concentration and measure residues after the experiments, we used a competitive ELISA. Standard curve was plotted on a semi-log graph using the calculated %Bx/B0 representing the ratio between the mean absorbance value for standards and the mean absorbance of standard „0“ (Fig.14, Appendix). From the standard curve, the resultant TCS concentrations were interpolated for test samples and internal positive control (Tab. 2).

Tab. 2: Triclosan concentrations in experimental and environmental Samples. Nominal TCS concentrations are theoretically derived values. Actual TCS concentrations were derived out of the mean values of experimental replicates (n = 2).

Sample	Nominal Triclosan [µg/L]	Actual Triclosan [µg/L]
Internal standard	0.75	**0.66**
LT5 flask	5.5	**11.26**
PCC 7806 flask	0	**0.10**
start control	0	**0.11**
start 0.06	0.06	**0.07**
start 0.6	0.6	**2.06**
start 5.5	5.5	**6.50**
start 16	16	**16.88**
start 150	150	**183.00**
end PCC7806 0	0	**0.08**
end PCC7806 0.06	0.06 - x[a]	**0.10**
end PCC7806 0.6	0.6 - x	**0.12**
end PCC7806 5.5	5.5 - x	**2.90**
end PCC7806 16	16 - x	**9.25**
end PCC 7806 150	150 - x	**125.18**
end LT5 0	0	**0.06**
end LT5 0.06	0.06 - x	**0.06**
endLT5 0.6	0.6 - x	**0.17**
end LT5 5.5	5.5 - x	**2.56**
end LT5 16	16 - x	**5.46**
end 150	150 - x	**106.89**
Chriesbach I	?[b]	**28.03**
Chriesbach II	?	**30.44**
Freezing 1 h	0.6	**0.52**
1 UV-Light	0.6	**0.31**

[a] x corresponds to the unknown amount of degraded TCS during the experiment.

[b] unknown concentration

TCS concentration in the positive control was slightly lower (0.65 µg L^{-1}) than expected (0.75 µg L^{-1}). In experimental flasks on contrary, concentrations (0.1 µg/L in PCC7806 and 11.26 µg L^{-1} in LT5) were higher than nominal concentrations.

Experimental TCS concentrations used in the 96-growth inhibition assays were verified to be accurate in the higher rage of exposure levels and too high in the lower range, questioning the accuracy of the kit at low concentration levels. Over the experimental duration TCS-concentration was not maintained throughout. After 4 days around 50% of initial TCS

concentration remained. Degradation of toxin was not faster in LT5 cultures than in PCC 7806 cultures. Freezing of a sample for 1 h, reduced TCS-concentration only slightly whereas UV-radiation was fatal for 50 % of the compound within 1h.

The two environmental samples out of Chriesbach showed TCS concentrations above the kit's upper detection limit (2.5 µg L^{-1}). Extrapolation of the standard curve hypothesised concentrations around 30 µg L^{-1}.

Microcystin content of *M. aeruginosa* strains

Microcystin knockout strain ΔmcyA did not produce any microcystin. Both toxic strains produced microcystin-LR (MC-LR), and the two demethylated variants D-MC-LR 967 and D-MC-LR 981 (Tab. 3). Content of D-MC-LR 967 was extremely low in both strains. Absolute amount of total (intra- and extracellular) microcystin was 311.9 µg L^{-1} and 291.7 µg L^{-1} for PCC 7806 and LT5 respectively (data not shown). These values exceeded guideline value of >100 µg L^{-1} above which public water bodies are closed for swimmers (Bundesgesundheitsblatt, 2003). Long-term exposed cultures produced less total microcystin relative to biovolume (102.8 µg g^{-1}wet weight vs. 128 µg g^{-1} wet weight in PCC 7806). Proportion of demethylated microcystin 981 appeared to be slightly higher in LT5. Interestingly there was no extracellular microcystin in LT5 wherease 10% of total microcystin in PCC7806 was extracellular.

Tab. 3: Microcystin content of different *Microcystis aeruginosa* strains. Toxin per biovolume (µg g^{-1}) n.d. = not detectable)

Strain	**PCC 7806**			**ΔmcyA**			**LT5**		
Microcystin	*LR*	*M 967*	*M 981*	*LR*	*M 967*	*M 981*	*LR*	*M 967*	*M 981*
Extracellular	6.58	n.d. [a]	6.17	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Intracellular	62.96	0.37	52.26	n.d.	n.d.	n.d.	49.65	0.32	52.82
Total	69.54	0.37	58.43	n.d.	n.d.	n.d.	49.65	0.32	52.82

[a] n.d. = not detectable

Discussion

Human use of pharmaceuticals and personal care products is resulting in chronic or pulse sublethal exposure to micropollutants for aquatic organisms in natural water bodies. Organisms may adapt to those chemicals by acclimation or evolution. This topic has been approached in the present study. We evaluated the adaptive response of a cyanobacterium, the toxic bloom-forming species *M. aeruginosa*, to the widespread aquatic contaminant TCS.

The growth curves of *M. aeruginosa* with no TCS added indicated that all algal cells were in exponential phase of growth between day 3 and 7 after inoculation of fresh medium with 2 x 10^6 cells L^{-1}. Yet, growth of the non-toxic mutant was significantly slower than growth of WT. Even though the biological role of microcystin is poorly understood, it has been suggested that microcystins increases the fitness of *M. aeruginosa* under oxidative stress conditions (Zilliges et al., 2011). Considering 40 µMol of photons m^{-2} s^{-1} as oxidative stress (light irradiance when non-toxic mutants became chlorotic (Phelan, 2009)) it's possible that the 30 µMol of photons m^{-2} s^{-1} used in the present study already posed a certain oxidative stress to the cells and that ΔmcyA could cope worse with the present light conditions and therefore grew slower.

Dose dependency of growth

TCS inhibited growth of both our strains at concentrations that can be present in aquatic environments (see Tab. 2 with levels in the Chriesbach river).

In previous reports, IC50 of TCS ranged from 0.7 to 66 µg L^{-1} (96 h growth inhibition test) in microalgae and MIC (minimal inhibitory concentration) in bacteria ranged from 10 and 3000 µg L^{-1} (Orvos et al., 2002). Our 96h growth inhibition test with TCS on the cyanobacteria *M. aeruginosa* yielded IC50 values within the range of 0.7–1.5 µg L^{-1}. Thus, TCS was found to

be toxic for *M. aeruginosa* at observed environmental concentrations ranging from 0.001 – 40 µg/L in surface waters (Davison & Maillard, 2010). Moreover this confirms that TCS may be even more effective against cyanobacteria than against green algae (Lyndall et al., 2010) or the specifically affected bacteria. Further, our study discovered that responses of toxic and nontoxic *M. aeruginosa* to TCS stress were different. EC50 value of toxic strain PCC7806 was higher (1.5 µg L^{-1}) than that of the nontoxic strain ΔmcyA (0.7 µg L^{-1}). By contrast, another study found that nontoxic *M. aeruginosa* were more resistant to nonylphenol than toxic strains (Wang et al., 2007). It is true that we also expected the nontoxic strain to be more resistant in our growth inhibition tests because of its slower growth rate (less deviation from control cultures per unit of time is expected compared to a fast growing strain). Slow growth means also reduced metabolic activity and therefore effects of inhibition should occur later. The fact that nontoxic mutants were less resistant could be explained by the speculation that cells were slightly pre-stressed because of prevailing light regime and therefore showed a higher susceptibility towards toxic stress.

Changes in phenotype after single exposure

Cytobuoy analysis allowed us to measure multiple individually expressed morphological and pigment related traits in phytoplankton with high precision. Principal components, representing aggregated measurements of Cytobuoy derived traits, expressed overall cellular phenotype and allowed separation of stressed and unstressed cell populations. Changes in phenotype after a single exposure occurred fast (within one generation) and are therefore likely to be the result of acclimation through phenotypic plasticity. Cell size of both toxic and nontoxic strains tended to become bigger with increasing concentration. Krüger & Eloff (1981) suggested that cell size is a likely indicator of the physiological state of a cell with stressed cells being larger. Instead of investing energy in division, cells probably invested

more in growth to achieve a better surface / volume ratio. Minimizing relative exposed surface would also explain the observed increased number of cells sticking together and the occasional colony formation. Colony formation by cyanobacteria is a known stress response to abiotic factors such as temperature, salinity, light intensity and nutrients (Jang et al., 2003). Observed cell clusters and colonies occurred at higher exposure levels. However they were not of the same type as colonies occurring in the wild (Fig. 8, Appendix). Mucilaginous envelope was missing. Increased size and cells sticking together also explain why stressed cells scattered more light and were more asymmetric. Beside changes in size and related traits we also observed increased fluorescence in stressed cells indicating enhanced pigment production as an answer to a growing energy demand necessary for toxic response. By contrary fill factor was reduced, suggesting that cell internal membranes weren't as dense packed as before because of the cell enlargement.

Recovery after exposure

Phenotypic changes observed after short-term exposure (5.5 µg L^{-1} TCS) were partially maintained for 2 generations but reverted completely after nine days of recovery. Cell counts of recovering cultures indicated that cells must have gone through 3 generations within that time (Fig. 12). Thus the recovery of cell division must have occurred earlier than the recovery of phenotype. Possible effects of TCS residues in the medium were excluded by prior detoxification in dialysis membranes. Residence time of TCS in membranes was shown to be rather short (Tab. 2). A previous study demonstrated that after 24 hours only less than 10% of the initially added TCS mass was still present in solution inside membrane mesocosms (Pomati & Nizzetto, 2013). Our finding of a partially maintained phenotypic acclimation over 2 generations even under complete absence of toxin in the medium might suggest a form of phenotypic inheritance such as maternal or epigenetic effects. 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, 2009). To our knowledge there is no study so far that demonstrates with what mechanism phenotypic inheritance is achieved in cyanobacteria, including epigenetic effects.

Alternatively, bioaccumulation and carry-over toxicity (incomplete eleimination and slow reversibility of inhibitory mechanisms) of TCS could be responsible for delayed recovery. Algal bioaccumulation factor (BAF) of TCS and degradation products has been shown to be in the range of approximately three orders of magnitude (Coogan, 2007).

Delayed recovery has also been reported in algae when exposed to S-metolachlor (disrupts lipid biosynthesis, as TCS) (Vallotton et al., 2008), but not after exposure to atrazine and isoproturon (inhibitors of photosystem II) (Rain et al., 2012; Laviale et al., 2011). It could be hypothesized the recovery time necessary for the restoration of the fatty acid balance is longer than for the restoration of photosystem II.

Characterisation of exposure scenario

ELISA tests for LT exposed flask (11.26 µg L^{-1}) indicated that TCS concentrations periodically could have exceeded nominal value (5.5 µg L^{-1}). However, the mentioned concentration represents the upper possible limit concentrations could have reached because sample was taken directly after the second spike (one week before culture was diluted again). Exposure pattern of LT5 cultures in experimental flasks therefore more likely simulated the dynamics of pulsed exposure with an average of 5.5 µg L^{-1} rather than a constant exposure of 5.5 µg L^{-1}. *M. aeruginosa* is anyway likely to encounter pulsed TCS exposure in the wild because it's a typical exposure mode for many emerging contaminants including

pharmaceutical, drug of abuse, personal and house care products originated from human use (Reid et al., 2011).

Surprisingly TCS-concentrations in Chriesbach (the creek in front of Eawag) were over the ELISA-kit upper detection limit (2.5 µg L^{-1}). Extrapolation from standard curve gave values for sample concentrations around 30 µg L^{-1}. Even if extrapolation overestimated the actual concentration, values are rather high. One reason could be that that samples were taken in winter when sorption, biodegradation and photolytic degradation are decreased and TCS availability increased.

Response to multiple sublethal exposures – Acquirement of resistance

When *M. aeruginosa* cells were stressed two times sequentially, response to the second pulse was stronger despite the fact that they were exposed to the same doses (fig. 14). Together with the delayed recovery this finding provides evidence for carry-over toxicity of TCS. It implies that prestressed cells had not yet recovered from the impact of the first pulse at the onset of the second pulse.

Phenotypes of cells exposed twice to TCS showed an interesting pattern. While a first exposure induced the usual stressed phenotype (bigger, more fluorescent), a second exposure made cells smaller again (Fig. 15) and phenotypically more similar to unstressed cells (Fig 9, Appendix). It could be, that getting smaller is an important step in reaching a morphophysiological state that enables cell division under prevailing conditions.

During the long-term experiment *M. aeruginosa* retrieved the ability to divide under multiple consecutive stress events. Cells became tolerant to an elevated, usually lethal, concentration of TCS arising from chronic exposure to a sublethal concentration (5.5 µg L^{-1}). Resistance increased 15-fold. The induction of resistance mechanisms following exposure to a low

concentration of TCS has been reported in a number of studies for bacteria (Mcmurry et al.,1998; Pycke et al., 2010; Braoudaki & Hilton, 2004). In rare cases, association between TCS resistance and resistance to other antimicrobials have been demonstrated (Christensen et al., 2011). McBain (2004) however, failed to demonstrate a biologically significant induction of drug resistance in a number of bacterial species exposed to sublethal concentrations of TCS, suggesting that TCS-induced drug resistance is not generally readily inducible. The present study seems to be the first report of induced TCS resistance in cyanobacteria as non-target species occurring in the environment.

Not only treatment cultures became more resistant during the long-term experiment but also experimental control cultures acquired more tolerance to TCS. Reasons for this can be various. Considering that they have been kept in exponential growth for 6 month, it could be that there occurred selection for fast growing cells. Another explanation is that also control cells came into contact with TCS due to cross-contamination when transferred into new flasks washed together with TCS contaminated flasks, or washed with TCS-containing rinsing agent in the washing facility. This hypothesis is supported by the finding that also control samples contained low concentrations of TCS according to the ELISA-assay. Finally, the different experimental containers (glass vs. microplates) used in the 96 h growth inhibition tests may also explain some result discrepancies. There is a chance that lipophilic TCS adhered to the plastic wall of microplates and more light was passing through the cover-lid, leading to a faster degradation. However, increased resistance of stressed cultures was so significant that parallel increased resistance of controls did not invalidate the observed pattern.

Costs of resistance

Commonly, microalgal resistant mutants show lower fitness compared to those of the sensitive wild type (Garcias et al., 2004). According to our results, TCS resistance seemed indeed to involve an associated cost. TCS-resistant mutants exhibited a diminished growth rate in the absence of TCS. This cost in fitness would represent a disadvantage for surviving in uncontaminated water bodies.

While a single exposure made cells bigger and more fluorescent, chronic sublethal exposure made cells smaller and less fluorescent. In our experimental context, the enlargement of cells at a single inhibiting concentration represents probably the consequence of homeostatic mechanisms: altered osmotic regulation and homeostasis comes with metabolic costs and higher energy requirement. Hence increased pigment content, becoming larger and inhibited cell division are consequences of stress response and homeostasis maintenance. The reduced cell size of long-term stressed cultures on contrary is probably the outcome of selection for the best-fit phenotype under prevailing conditions. TCS can inhibit lipid synthesis and destabilises cell membranes (Dann & Hontela, 2011; Mcmurry et al., 1998). Therefore small cells with reduced lipid content and low absolute membrane surface are able to survive and reproduce better in a TCS-contaminated environment than alternative phenotypes.

Thus, getting bigger seemed to be just a physiological response of cells at short term but at long term only small cells were able to get resistant and continue growing.

After all, it can't be excluded that the smaller cell diameter in resistant cells may simply be related to the resistance related costs. This explanation would be consistent with the finding of another study that observed decreased cell size in copper (no interaction with lipid-pathway) resistant *M. aeruginosa*, with lowered fitness in standard growth conditions (Garcias et al., 2004).

Mechanisms of resistance and resistance trait adaptation

Persistence of TCS in cultures of LT5 and PCC7806 showed similar patterns (Tab. 2). This finding suggested that faster biodegradation of TCS in resistant strains was not a possible mechanism mediating resistance. Possible remaining mechanisms are the upregulation of the expression of efflux pumps and/or down regulation membrane permeability as well as the mutagenesis of some TCS targeted proteins in *M. aeruginosa*.

So far, there has been scarce information available on the maintenance of resistance after TCS exposure has been relieved. This lack of knowledge occurred in the literature as an important scientific gap to fill (Davison & Maillard, 2010). In our study resistance was maintained (although at lower levels compared to continuously stressed cultures) two months after stress has been released. It is unlikely that persistent physiological effects can explain our result because the longer the period after stress is relieved, the less probable are any lingering physiological effects. It has previously been shown that mild environmental stress elicits mutations affecting fitness (Goho & Bell, 2000). Here we also assume that increased resistance had a genetic basis. Our data suggest that evolutionary adaptation could have happened during the course of the long-term stress experiment. The distinctive phenotypes of our three *M. aeruginosa* strains, aligned along a sensitivity axis to TCS, also supported this idea (fig. 18). When considering that the pre- or absence of a whole gene-cluster is responsible for the phenotypic separation of PCC 7806 and ΔmcyA, it is very likely that a few mutations underlie the separation of phenotypes of PCC 7806 and LT5.

As our data also show, adaptation to stress comes with a cost and a lower fitness in standard conditions. Therefore is unlikely that altered phenotype and increased resistance persist, as the recovery period is extended. Assuming that our LT5 population is mixed and dominated by a resistant phenotype, this resistant phenotype will decrease in frequency compared to the other

variants after extended periods of recovery. Because resistant cells showed a lowered fitness, selection will act to restore the prior level of fitness, removing the mutational variance in the process. Twenty generations of recovery seemed to be long enough so that any physiological acclimation would have disappeared, but not too long that the reversed selection gradient would have entirely effaced any effects on the fitness.

ITS-sequencing revealed that there was no neutral genetic variation in the original populations, as well as in the long-term exposed populations, suggesting no variance in functional genes. This is expected as the cultures were originally monoclonal and may bring important implications for mechanisms of induced resistance: instead of selection on standing genetic variation such as proposed by different authors (Fischer et al., 2013; Gingerich, 2009; López-Rodas et al., 2006), our data suggest that resistance may have been acquired either as 1) a quantitative trait (see Klerks, 2011), by changes in the constitutive expression of a set of genes involved in the response (selection then would not have acted on specific genotypes but on the ability of individuals within one genotype to express a certain phenotype) or 2) appeared *de novo* as a consequence of beneficial mutations, recombination or duplication of important functional genes. Mobile DNA fragments (insertion sequences) are able to rearrange chromosomes and are responsible for the instability of phenotypic markers and for the acquisition of resistance and accessory functions in bacteria (Mahillon, 1998). Furthermore they are able rearrange chromosomes and involved in the instability of phenotypic markers (Mahillon, 1998). Insertion sequences have also been described for diverse genera of cyanobacteria including M. aeruginosa PCC 7806 (Mlouka et al.,2004). Therfore it is not unlikely that additive genetic variance induced by insertion sequences also occurred in our strains.

Microcystin analysis

Environmental stimuli such as nutrients, pH, growth, light, temperature, predation or trace metals could change levels of toxin expression in *Microcystis* strains (Neilan, 2012; Pearson et al., 2010; Jang et al., 2003). Our study showed that also chemical exposure influenced microcystin-production and -metabolism in *M.aeruginosa*. Toxin production in LT5 cultures was reduced, proportion of demethylated variants was increased and extracellular microcystin was absent (table 3).

There are various hypotheses about the role of microcystins in cyanobacteria. It has been assumed that microcystins are involved in basic metabolism, protection against predation, competition with other organisms, alleophatic interactions and oxidative protection (Zilliges et al., 2011). However, biological functions of microcystin remain controversial. MC-LR, the variant produced by our strain, is considerably the most toxic variant. The toxicity of demethyl MC-LR is unknown. It has been reported that the majority of *Microcystis* populations produce primarily MC-LR and demethylated microcystins as major variants are rarely found in bloom samples (Via-Ordorika, 2004). Since our sublethal exposed strain produced less and majorly demethylated microcystin, this possibly implies that chemical exposure to TCS reduces toxicity and bloom-forming potential of *M. aeruginosa*. On contrary another study found increased microcystin production in response to organic pollution but also decreased extracellular microcystin (Wang et al., 2007).

Microcystin excretion is also a much-debated subject. Proportion of dissolved microcystin can vary between 0% and 70% (Wiedner et al., 2003) whereas the latter occurs only when microcystin is released into the water due to cell lysis. It is also assumed that microcystin excretion increases at high growth rates and as the cell number in the culture reaches a maximum (Lyck, 2004). Absent extracellular microcystin in LT5 therefore may be

representative for its diminished fitness. Further it indicated that no cells dyed or lysed and no microcystin was pumped out. The fact that microcystin was kept inside in exposed cells possibly highlights its importance in preventing oxidative damage (as TCS has been suggested to induce oxidative stress at sublethal concentrations (Tamura et al., 2012). Additionally it gives us further information about possible resistance mechanisms. Since microcystin is putatively pumped out through ABC-transporters (Pearson et al., 2004) which at the same time are known to mediate TCS-resistance in bacteria (Poole, 2005; Betts et al., 2003), it could well be that the pumping out of microcystin is shut down in parallel with an increased efflux of TCS through the same or substituted transporters so that the increased expense of energy (pumping out TCS in ABC transporters costs ATP) is compensated.

Conclusions

We found that realistic environmental levels of exposure to TCS induced reversible phenotypic change in *M. aeruginosa* after short term exposure and that under long term sublethal selective pressure, *M. aeruginosa* evolved a more resistant population with a diminished growth rate in standard conditions, altered microcystin metabolism and no change in the standing neutral genetic variation.

It is true that the adaptive response of organisms to chemical exposure in the laboratory can only partially predict responses in the wild (Klerks, 2011). Differences in exposure scenarios and other conditions also mean that the trait that is being selected for in the laboratory may differ from the trait that would be selected for at a contaminated site. Previous data suggested 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). More recent studies however, raised concern about possible effects in natural environments (Pomati & Nizzetto,

2013) and suggested to include TCS into routine monitoring programmes and to consider it as an important candidate for prioritisation at the European scale (von der Ohe et al., 2012).

Based on our results we additionally advocate for a more careful consideration of sublethal doses and environmentally relevant exposure scenarios in the ecological risk assessment of TCS and also other chemicals. In this study we used TCS as model chemical. But since physiological and evolutionary processes can interact and occur at low levels of exposure to any anthropogenic chemical, it is possible that adaptation may happen for other microorganisms and pollutants.

Outlook

Long-term exposure protocol has also been applied on two other mcy-knock-out mutants and with an even lower concentration of TCS (1.5 µg L^{-1}). In the meantime cultures are exposed for a year and assessment is being performed after this work. Comparing resistances of laboratory cultures with isolates from the wild out of contaminated and uncontaminated sites would reveal whether resistance already arouse in wild populations. Possible cross-resistance with other prevalent micropollutants such as antibiotics should be checked (Davison & Maillard, 2010).

To describe mechanism that mediates resistance, gene expression pattern should be displayed by microarray or mRNA sequencing. If the underlying molecular mechanism involves DNA sequence variations, a full DNA genome sequence would be required. Fortunately there is already a reference genome of PCC 7806 for alignment existing (Frangeul et al., 2008) so we would not have to generate a *de novo* sequence.

Keeping in mind that contemporary evolution should more likely be seen as a common process, rather than the exception, it is logical to believe that the thousands of chemicals out

there have an evolutionary effect on naturally exposed populations. The challenge in the future is to demonstrate this for natural populations and disentangle the relative contribution of pollutants from other possible evolutionary and ecological forces shaping biodiversity changes.

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Appendix

Protocol 1: BG-11

BG11-Medium for the cultivation of cyanobacteria

Preparation for 1L in glass bottle:

- Pipette 20ml BG-11 Concentrate into 1L glass bottle (fridge snail lab)
- Add 980 ml H_2O
- Adjust pH to 7.5 by adding few droplets 1M NaOH ; stir well
- Autoclave for 40 min (120°C)

Protocol 2: WC-Medium

WC-Medium for the cultivation of cyanobacteria (Tab.1)

Tab. 1: Composition of WC-Medium

	Stock solution (g/l)	Nutrient solution
$CaCl_2 . 2H_2O$	36.8	1 ml
$MgSO_4 . 7H_2O$	37	1 ml
$NaH\ CO_3$	12.6	1 ml
$K_2HPO_4 . 3H_2O$	11.4	1 ml
$NaNO_3$	85	1 ml
Micronutrient solution*		1 ml
TES Buffer		0.115 g
Deionized Water		994 ml

* Composition of Micronutrient solution: (Na_2EDTA 4.36 g/L, $FeCl_3\ 6H_2O$ 3.15 g/L, $CuSO_4 \cdot 5H_2O$ 0.01 g/L, $ZnSO_4 \cdot 7H_2O$ 0.022 g/L, $COCl_2 \cdot 6H_2O$ 0.01 g/L, $MnCl_2 \cdot 4H_2O$ 0.18 g/L, $Na_2MoO_4 \cdot 2H_2O$ 0.006 g/L, H_3BO_3 1.00 g/L)

Preparation for 1L in glass bottle:

- Dilute 0.115g buffer (TES; No. 53) in 500ml „Semidest" (deionised) water
- stir it well
- Add 1ml stock solutions**:** 1-5 and 7 (fridge Snail lab)
- Fill up bottle with „Semidest" water
- Autoclave for 40 min (120°C)

Protocol 3: Fix-solution

Fixative solution for cyanobacteria flowcytometrie

Preparation for 100 ml:

- dissolve 1 gram paraformaldehyde in 50 ml H_2O at 65°C (G74 big box in fridge)
- add during heating 1-3 droplets 1 M NaOH to make solution clear (hood)
- add 40 ml 25% glutaraldehyde (In fridge where WC-components are)
- adjust pH to 7.0 and bring to 100 ml (hood)
- filter solution with 0.2 =µm Nucleopore filter (use syringe)
- store at 4-7°C, dark

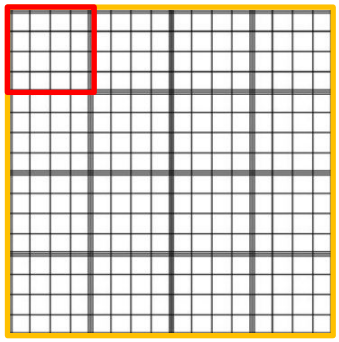
Use: Add 1:100 to samples.

End concentration is 0.01% PF and 0.1 % GA.

Protocol 4: Counting Cells visually

- Take Thoma neu counting chamber = Neubauer improved counting chamber
- Put a drop of water onto the two outer glass-bars
- Put cover glass on top
- Let a drop of sample be sucked under the glass until square is filled

- Let cells settle down for 30s
- For high cell densities: count cells in all 16 mini-squares,
- For low cell densities: count all cells in big square



Thoma neu counting chamber scheme

Cells/µL = #cells /(0.0025mm^2 x 0.1mm) counted

Cells/µL = #cells /(0.04mm^2 x 0.1mm)

To adjust cell density to 10x10^6 cells/ml, make sure you have 8 cells per big square.

Protocol 5: 96-h growth inhibition test

1 run with 5 replicates

Dilute 0.001g TCS (Powder) in 1 ml EtOH absolut to reach stock solution of 1 g L^{-1}

Dilute 100 µl stock solution in 9.9ml WC-Medium to reach start solution (10'000 µg L^{-1})

Dilute 0.5 ml of start solution in 16.6 ml WC Medium to reach work solution I (300 µg L^{-1})

Perform dilution series with work solution I (1:3) 7 steps to reach work solutions II - VIII

Bring Cyano-cultures to a densit of 4 x 10^{-6} cells ml^{-1}

Mix 2ml cyanoculture with 2ml work solution in 12-well plate to reach starting cell concentration of 2 x 10^{-6} and TCS concentrations of 150, 50, 16, 5.5, 1.8, 0.6, 0.2, 0.06 µg L^{-1}

Run also 0-treatment (only WC-medium 2ml + 2ml culture)

Cover sides of 12-well plates with parafilm to avoid transpiration of medium

Incubate plates in cultivation hood for four days (25°C, continuous light 30 µE m^{-2} s^{-1})

Mix plate once a day and put plate back randomly

After 4 days, spike every replicate with 40 µl fix-solution

Protocol 6: Lysis & PCR protocol

Centrifuge 10ml Microcystis cultures down for 30s 3000rpm

Fill 1.5 ml of darkgreen centrifugate into Eppendorf tube

Centrifuge at 3000 rpm for 5 min, reject most medium, save pellet

Add 2 beads (cleaned in Jawel, and washed in MQ-H_2O before using)

3 x (Heat for 10 min 65°C, vortex 10 s, Freeze tube in liquid nitrogen)

add 400 µl PL1 Buffer (lysis buffer, Nucleo Spin Plant II, Macherey-Nagel, Düren, Germany)

shake tubes in lyser, 10min 30'000

centrifuge 3000 rpm for 1 min to get rid of foam

add 10 µl RNAse A, incubate 20 min at 65°C

Follow NucleoSpin Plant II manual step 3 to end

MasterMix - 2.5 µl

Primer - 2.5 µl each

Nuclease-free water - 2.5 µl

DNA-template - 5.0 µl DNA

95°C - 10 min

95°C - 60 s

60°C - 60 s

72°C - 60 s

72 - 10 min

4°C - ∞

Figures and Tables

Fig. 1 ITS-Sequences of PCC7806 and LT5

PCC 7806 ITS-16S-rRNA-sequence

5‘_TGCGGTCTTCTTTTTTGGCTTTTTCACCTAGCTCTATGCAGTTTTCAAGGTTCCTCTGGACTCTTTACTCAGAGCCAGCATTCTCT
CTTACTATATAAGTAAGATAAGGTGCTGATTCCTCGCTTCTCTTGTTTTTGCCACCTGTGCAGGTGGGCCATCCTGGACTCGAACC
AGGGACCTCACCCTTATCAGGGGTGCGCTCTAACCACCTGAGCTAATAGCCCTTGTTCCTGATTCAGGTCTTGTTTGAACCCAGAA
CCTAGTTTGAAAGCCAATTTCGCCTCGCTCCGACCTTTTGGGATTGATTCTTGGTAGCGACTACTTTTTTTTCGTCTCCTACCTGAA
TTAGGTCTCCCTTTAAGGAGGTGATCCAGCCACACCTTCCGGTACGGCTACCTTGTTACGACTTCACCCCAGTCACTAGCCCTGCC
TTAGGCATCCCCCTCCTTGCGGTTGAGGTAATGACTTCGGGCATGACCAACTTCCATGGACTGGCCGTCGTTTTACA_3`

LT 5 ITS-16S-rRNA-sequence

5`_TGCGGTCTTCTTTTTTGGCTTTTTCACCTAGCTCTATGCAGTTTTCAAGGTTCCTCTGGACTCTTTACTCAGAGCCAGCATTCTCT
CTTACTATATAAGTAAGATAAGGTGCTGATTCCTCGCTTCTCTTGTTTTTGCCACCTGTGCAGGTGGGCCATCCTGGACTCGAACC
AGGGACCTCACCCTTATCAGGGGTGCGCTCTAACCACCTGAGCTAATAGCCCTTGTTCCTGATTCAGGTCTTGTTTGAACCCAGAA
CCTAGTTTGAAAGCCAATTTCGCCTCGCTCCGACCTTTTGGGATTGATTCTTGGTAGCGACTACTTTTTTTTCGTCTCCTACCTGAA
TTAGGTCTCCCTTTAAGGAGGTGATCCAGCCACACCTTCCGGTACGGCTACCTTGTTACGACTTCACCCCAGTCACTAGCCCTGCC
TTAGGCATCCCCCTCCTTGCGGTTGAGGTAATGACTTCGGGCNTGACCAACTTCCATGGACTGGCCGTCGTTTTACA_3`

Tab. 2: Principle components (PC1 - PC6), explained variance and loadings of 19 most important traits

	PC1		PC2		PC3		PC4		PC5		PC6	
% of variance	37		23		10		5		4		2	
loading 1	Center.of.gravity.FL.Yellow	0.036	Inertia.FL.Red	0.153	Asymmetry.FWS	-0.153	Average.FWS	0.166	Asymmetry.FL.Red	0.155	Total.FL.Orange	0.656
loading 2	Center.of.gravity.FL.Orange	0.036	Inertia.FL.Orange	0.145	Fill.factor.FWS	0.152	Maximum.FWS	0.146	Asymmetry.FWS	0.123	Total.FL.Yellow	0.535
loading 3	Center.of.gravity.FL.Red	0.035	Inertia.FL.Yellow	0.138	Asymmetry.SWS	-0.149	Asymmetry.2.FL.Red	0.145	Average.2.FL.Red	0.117	Total.FWS	0.204
loading 4	Center.of.gravity.2.FL.Red	0.035	Inertia.SWS	0.134	Fill.factor.2.FL.Red	0.142	Fill.factor.FL.Yellow	-0.136	Asymmetry.SWS	0.110	Fill.factor.FL.Red	0.164
loading 5	Total.FL.Red	0.034	Inertia.FWS	0.134	Asymmetry.FL.Orange	-0.135	Asymmetry.FL.Orange	0.128	Asymmetry.FL.Yellow	0.105	Inertia.2.FL.Red	0.130
loading 6	Center.of.gravity.SWS	0.034	Inertia.2.FL.Red	0.133	Fill.factor.SWS	0.133	Fill.factor.FL.Orange	0.118	Maximum.2.FL.Red	0.105	Inertia.FWS	0.123
loading 7	Center.of.gravity.FWS	0.034	Average.SWS	-0.125	Asymmetry.2.FL.Red	-0.131	Total.FWS	0.117	Inertia.SWS	0.098	Maximum.SWS	0.116
loading 8	Number.of.cells.SWS	0.034	Average.FL.Red	-0.124	Asymmetry.FL.Red	-0.130	Inertia.FL.Yellow	0.086	Total.2.FL.Red	0.093	Average.FL.Red	0.109
loading 9	Number.of.cells.FWS	0.033	Maximum.FL.Orange	-0.123	Asymmetry.FL.Yellow	-0.121	Asymmetry.FL.Yellow	0.079	Asymmetry.FL.Orange	0.092	Maximum.FL.Red	0.109
loading 10	Number.of.cells.2.FL.Red	0.032	Average.FL.Orange	-0.122	Average.2.FL.Red	0.115	Maximum.FL.Orange	0.077	Inertia.FWS	0.085	Average.FWS	0.105
loading 11	Total.SWS	0.031	Number.of.cells.FL.Red	-0.121	Average.FL.Orange	0.110	Maximum.2.FL.Red	0.077	Fill.factor.SWS	0.076	Inertia.SWS	0.102
loading 12	Number.of.cells.FL.Orange	0.030	Maximum.FL.Red	-0.120	Fill.factor.FL.Orange	0.107	Average.FL.Red	0.072	Maximum.FWS	0.069	Average.SWS	0.099
loading 13	Total.FWS	0.029	Average.FL.Yellow	-0.119	Average.FL.Yellow	0.099	Inertia.FL.Orange	0.071	Inertia.FL.Red	0.067	Fill.factor.SWS	0.096
loading 14	Total.2.FL.Red	0.029	Maximum.2.FL.Red	-0.119	Length.2.FL.Red	0.089	Fill.factor.2.FL.Red	0.069	Inertia.FL.Orange	0.067	Inertia.FL.Orange	0.095
loading 15	Length.SWS	0.028	Number.of.cells.FL.Yellow	-0.117	Inertia.FWS	0.086	Number.of.cells.FL.Oran	-0.068	Average.FL.Orange	0.065	Maximum.FWS	0.092
loading 16	Length.FL.Orange	0.027	Maximum.SWS	-0.117	Length.FWS	0.084	Number.of.cells.FL.Red	-0.068	Center.of.gravity.2.FL	-0.065	Center.of.gravity.FWS	0.090
loading 17	Maximum.FL.Red	0.027	Average.2.FL.Red	-0.113	Total.2.FL.Red	0.084	Average.2.FL.Red	0.059	Asymmetry.2.FL.Red	0.065	Center.of.gravity.SWS	0.085
loading 18	Length.FWS	0.027	Length.FL.Red	0.108	Length.FL.Orange	0.082	Number.of.cells.FWS	-0.054	Fill.factor.FWS	0.063	Length.FL.Orange	-0.085
loading 19	Total.FL.Yellow	0.026	Length.FL.Yellow	0.108	Average.FL.Red	0.079	Number.of.cells.2.FL.Re	-0.054	Center.of.gravity.FL	-0.060	Inertia.FL.Red	0.084

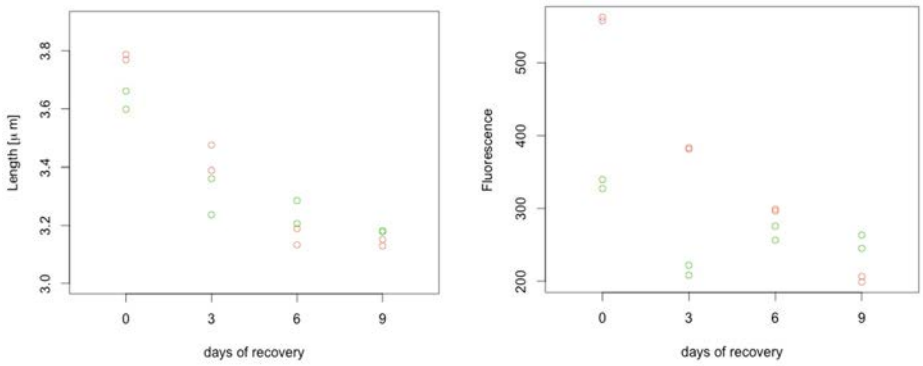


Fig. 2 and 3: Phenotypes during recovery. Increased length (left) and fluorescence (right) of TCS exposed cells (red) reverted back to unstressed phenotype (green) after 9 days when stress has been released.

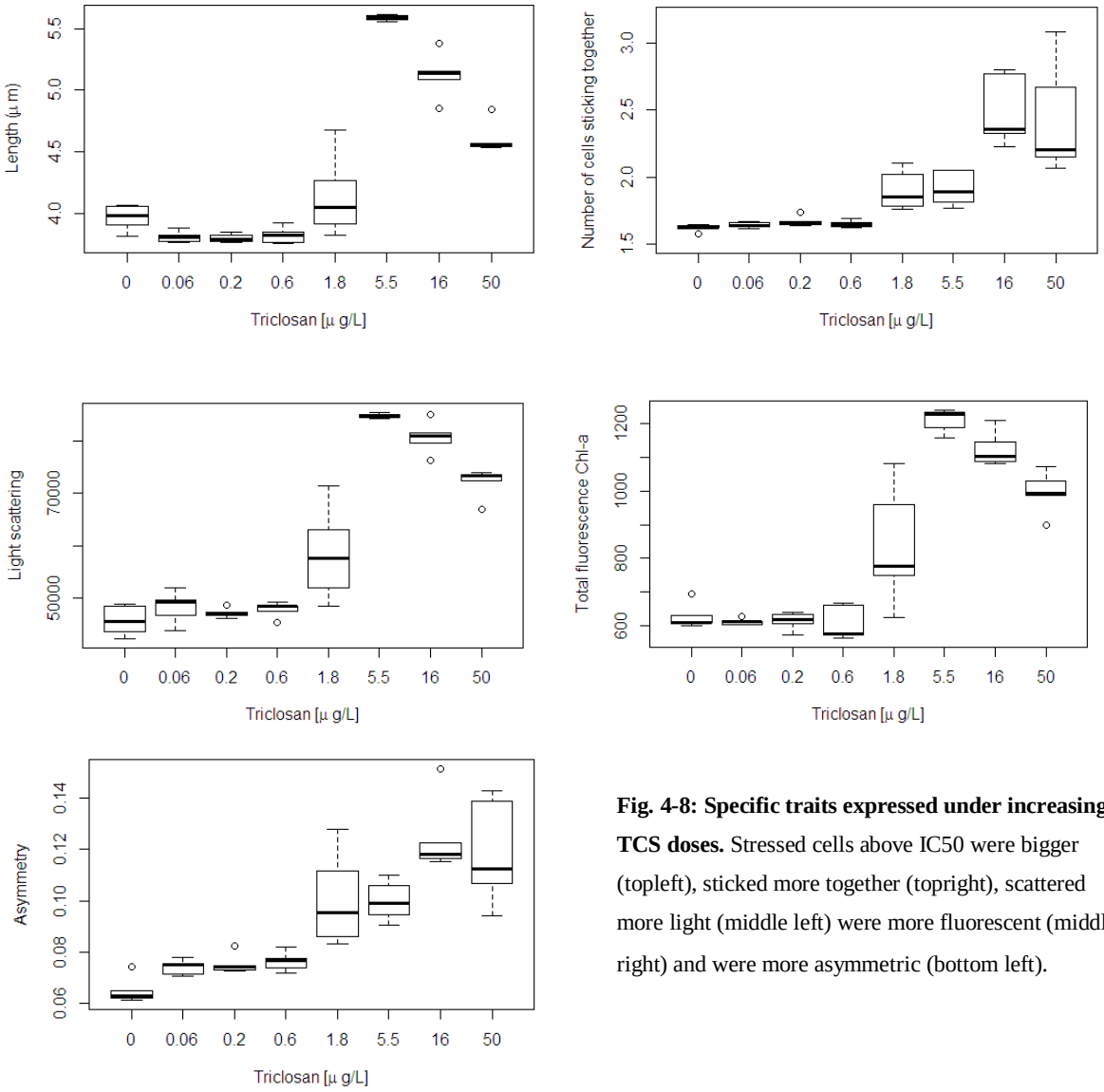


Fig. 4-8: Specific traits expressed under increasing TCS doses. Stressed cells above IC50 were bigger (topleft), sticked more together (topright), scattered more light (middle left) were more fluorescent (middle right) and were more asymmetric (bottom left).

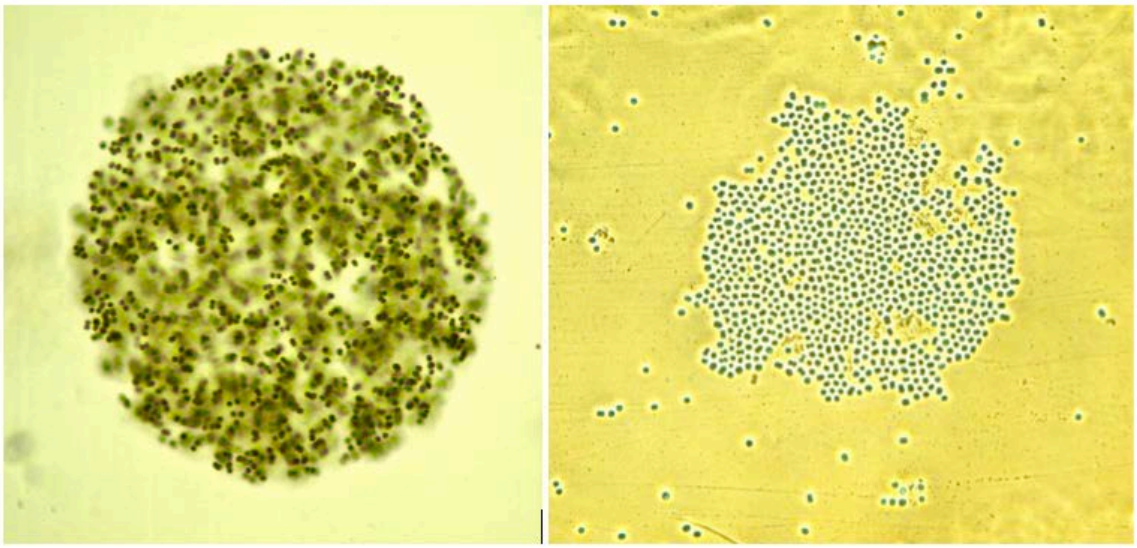


Fig. 9. Phenotypes of *M.aeruginosa,* Real colonial phenotype (left) out of lake Greifensee looked different than TCS-induced aggregated phenotype (right) out of experiments.

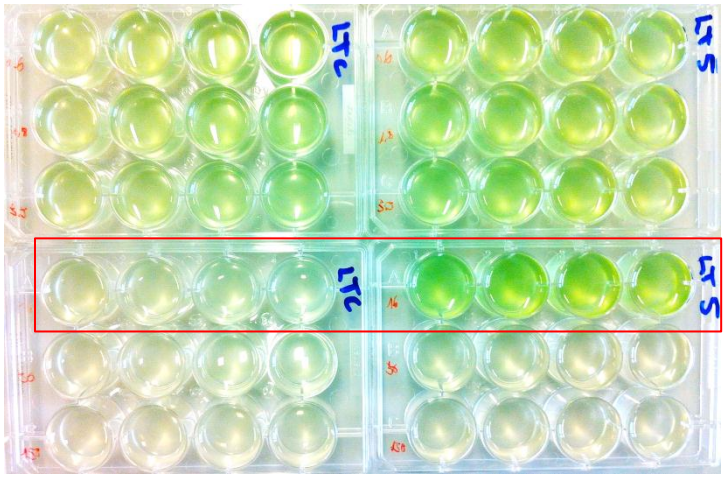


Fig. 10: Cultures after 96-h Growth inhibition test. LT5 (right) grew at 16 µg/L TCS (red box) where controls (left) did not grow.

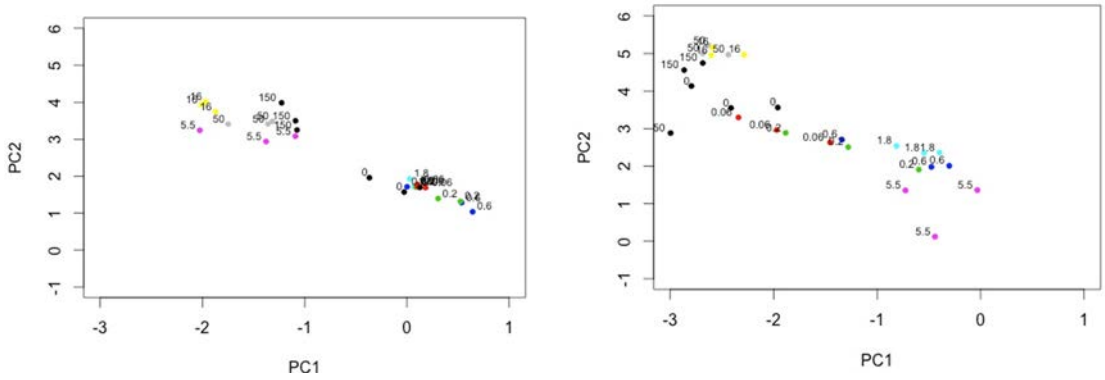


Fig. 11: Cellular phenotype (principal component 1 & 2 of measured physio-morphological traits – PC1 & PC2) of control and pre-exposed cells after a 96-h growth inhibition test

left: After a 96 h growth inhibition test, cellular phenotype of NON-pre-exposed (control) populations differed the most between populations under IC50 (0 – 1.8 µg L^{-1}) and populations above IC50 (5.5 – 150 µg L^{-1}).

right: After a 96 h growth inhibition test, cellular phenotype of pre-exposed cells was the closest to control unstressed cells at acclimated concentration of 5.5 µg L^{-1}.

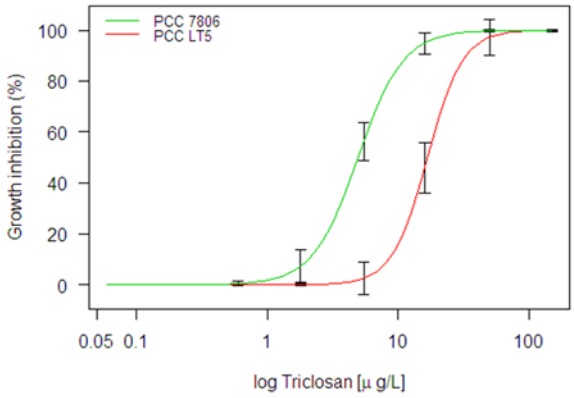


Fig. 12: Dose response curve after long term experiment. Chronic sublethal exposed cultures (LT5, red) were significantely more resistant than experimental control cultures (LTC, green)

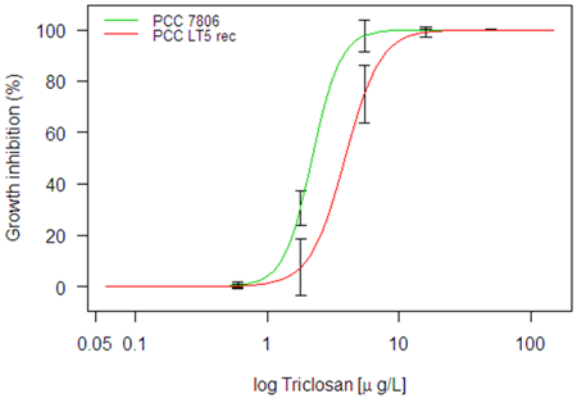


Fig. 13: Dose response curves after long term recovery. Resistance of LT5 (red) was partially maintained after 2 month of recuperation and still significantly more resistant than experimental controls (green).

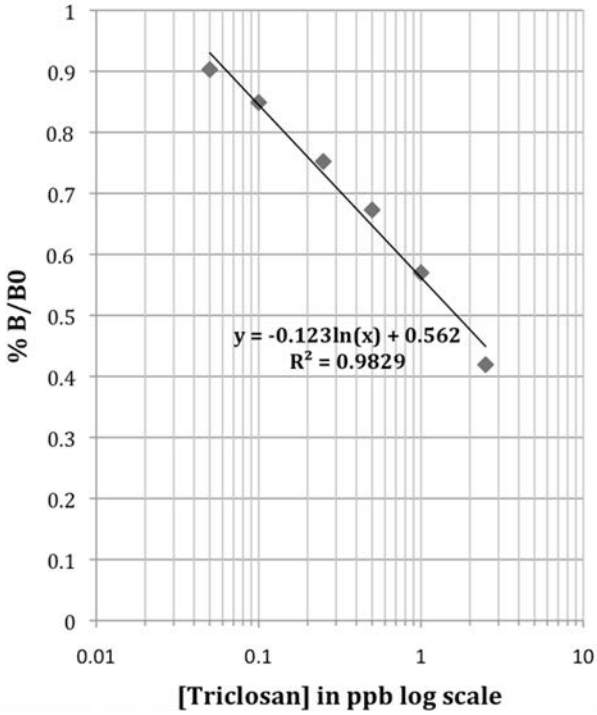


Fig. 14: Standard curve plotted using %Br/B0 values calculated for six known triclosan concentrations (standards std 1–6)