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Micropollutants affect the ability of phytoplankton communities to track environmental changes

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1. INTRODUCTION

"Biodiversity is the variety of life, including variation among genes, species and functional traits" (Cardinale et al, 2012)

1.1 Human-induced biodiversity loss and consequences for ecosystem functioning

Biodiversity is a largely used term in scientific literature. It is a very general concept applicable to the whole variety of life on Earth. The term has been defined in the Article 2 of the Convention on Biological Diversity as:
"the variability among living organisms from all sources, including, inter alia, terrestrial, marine and other aquatic ecosystem and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems"(Office of Technology Assessment, USA, 1987 - UN Conference on Environment and Development in Rio, 1992).
Biodiversity is strictly related to ecosystem functions. In this work the term "functions" is used as a synonymous of "processes" and it refers to all biological activities responsible for the ecosystem self-maintenance as nutrient cycling, biomass production and decomposition (Cardinale et al., 2012; Reiss et al.,2009).
Human activities and global changes are the main drivers of global environmental change (Carpenter et al., 2011). Human activities strongly influence environmental properties, and while human domination increases, biodiversity progressively declines in ecosystems worldwide; conversion of natural ecosystems for urban and agricultural use, pollution and resource over-harvesting represent the main causes of acceleration in the rate of biodiversity loss. The biodiversity decline results in a "habitat shift" from a more complex system to a less complex one; this loss of complexity has important effects on the ecosystem structure, processes and dynamics that contribute to the self-maintenance of ecosystems. The global change makes therefore crucially important to understand how the environmental properties affect biodiversity and how biodiversity loss alter ecosystem processes (Pereira et al., 2010; Sala et al., 2000; Cardinaleet al., 2012).
Changing in environmental properties due to human activities affects directly biodiversity. Genetic, species and functional diversity control ecosystem functioning and, in turn impact on services that ecosystems provide to humanity (Fig.1.1).

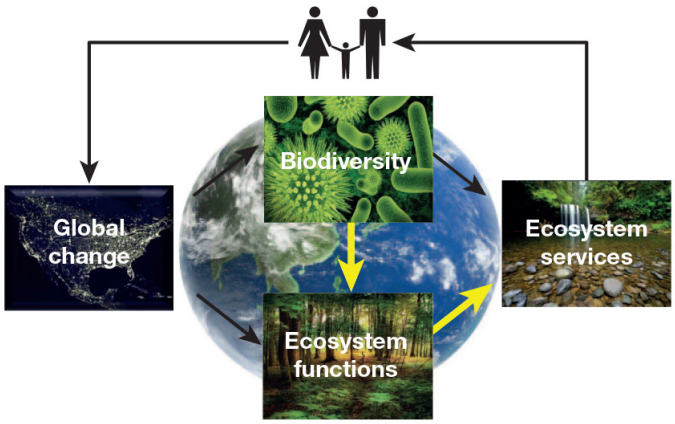

Fig. 1.1: Global changes due to human activities affect biodiversity and , in turn, ecosystem functions and services (Cardinale, 2012).

Many studies have been designed to understand how biodiversity matters for ecosystem functions (Cardinale et al., 2009). The main task of Biodiversity-Ecosystem Functioning (BEF) research is to describe how genetic, taxonomic and functional diversity control ecological processes in ecosystems. Results from twenty years of research show that biodiversity loss strongly affects the efficiency with which organisms perform processes (Cardinale et al., 2012). Cardinale in his review (Cardinale et al., 2012) shows that as the diversity (in number of genes, species, traits and in functional groups) increases, the process rate also increases until saturation (Fig.1.2). Saturation curve is reached when characteristics of organisms are redundant and they perform the same functions. This means that initially the redundancy can buffer the loss in diversity, and the impact on processes is low, but with increasing diversity loss the changement becomes important (Chapin et al., 2000). This may explain the reason why, for example, the primary productivity in large ecosystems like lakes can remain relatively constant despite a change in number and composition of species (Schindler et al., 1986).

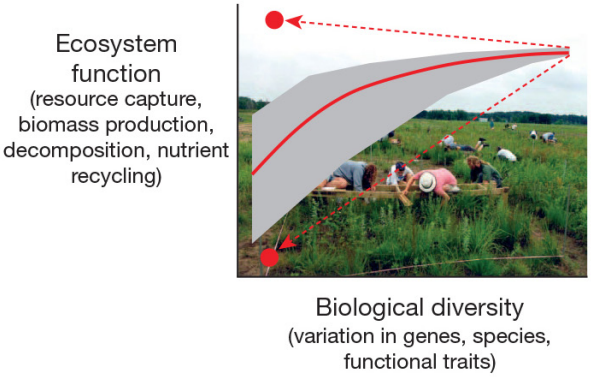

Fig.1.2: Biodiversity - functions relationship: red line show the change in ecosystem functions due to a genes, species and functional trait variation (Cardinale et al., 2012).

Many studies have shown that high genetic and phenotypic variability ensures functionality and stability of ecosystem processes through time and increases the resilience and the adaptive capacity of populations and communities (Cardinale et al., 2012). On the other hand experiments on plant polycultures have shown that in some cases higher diversity lead to a reduction in productivity (Cardinale et al., 2007). It is therefore important to consider that the scenario of possible changements in ecosystem function depend on which traits are lost and which traits are important for processes.

1.2 Aquatic ecosystems are threatened by human activities

Aquatic ecosystems are dynamic systems in which living organisms interact with non–living elements and the physical environment in a water body. They include a wide range of environments as rivers, lakes, wetlands, marine systems and underground aquifers. Among aquatic ecosystems, freshwaters are the most endangered systems on Earth.
Human-induced stressors such as chemical inputs, climate change and habitat transformation have modified physical, chemical and biological properties of water systems (Vörösmarty et al., 2004). All these transformations are affecting the structure, functioning and resilience of natural populations and communities. Freshwater are the first ecosystems to encounter human pollution and to experience local changes in climate and land use. In particular lakes have shown to be highly sensitive to climate and respond rapidly to environmental changes (Williamson et al., 2009). Plankton (especially phytoplankton) composition and abundance are generally considered as indicators of lake environmental changes (Adrian et al., 2009).
Freshwater ecosystems provide many services to human society including irrigation, fishery, drinking water, and fibre production and cultural benefits in terms of education and recreation. Freshwater are also linked to several processes that regulate ecosystems and their ability to provide services as flow regulation, recovery from fishery collapse, invasion resistance or waste removal (Carpenter et al., 2011; Palumbi et al., 2009). All ecosystem processes are mediated by the diversity of organisms. At present freshwater ecosystems are suffering severe biodiversity loss affecting both processes and services.

1.3 Chemical pollution as driver of biodiversity loss

A pollutant is defined as a substances that occurs in the environment as a result of human activities, and which represents a danger for health and life of humans, animals and plants. The issue of environmental pollution dates back to the development of human activities. During the industrial revolution people started moving from countryside to cities. The indu-

strialization and the consequent growth of the major European cities affected air and water quality. Anthropogenic industrial and urban waste products were released into rivers passing through urban areas, and in most cities sewage were washed through the streets with health and environmental consequences.

Water and air pollution from urban areas continued to increase until the 20th century, when new legislations imposed limits on the discharge of air pollutants and an improvement of sewage treatment (Cutler and Miller, 2005), but the problem of pollution is not solved yet.

The sources and behaviour of macropollutants, which are compounds occurring at µg/L to mg/L concentrations such as salts, acids, nutrients and natural organic matter, are relatively well understood. More difficult is to predict the effect of micropollutants which can be present in trace (pg/L to ng/L concentrations). Micropollutants have been found ubiquitously in all water environments. They include compounds such as heavy metals which are not degraded, persistent organic pollutants (DDT or lindane) which are degraded very slowly, and compounds such as hormones and therapeutic drugs, less persistent, but however problematic due to the toxicity for aquatic organisms and humans (Carpenter et al., 2011) and for the continuous emission in the environment (Schwarzenbach et al., 2006). Pharmaceuticals and personal care products (PPCPs) were detected decades ago in freshwater bodies, but now their presence in the environment is progressively increasing (Daughton et al., 1999). PPCPs refers to any kind of products used for personal health including therapeutic drugs, veterinary drugs, sun-screen products, fragrances and cosmetics. These compounds are mostly excreted unmetabolised by humans in urban areas or by animals in farms. Some products are lastly washed down the drain after use. At present, despite the treatment technology improvement, not all chemicals are retained in the treatment plants and so they make their way into soils and aquatic environments (Bedoux et al., 2012; Daughton et al., 1999). PPCPs have become one of the major challenge to freshwater ecosystems because many compounds are suspected to have toxic effects on aquatic organisms, particularly when present as components of complex mixtures (Schwerzenbach et al., 2006).

Nowadays chemical releases in freshwaters are the result of diffusive inputs (diffusive source pollution) from agricultural landscape and urban and industrial drains (point source pollution) (Gerecke et al., 2002). Effects of pesticides from agriculture on aquatic organisms are already known: mesocosm experiments show that pesticides can strongly reduce the diversity and productivity of aquatic communities (Relyea, 2005). Only in the past decades attention has been focused on potential environmental impacts of pharmaceuticals as a new group of contaminants because they are designed to have biological effects on humans at small doses. For this reason they can have strong effects on non-target organisms even if present in the environment at low concentration (Arnold et al., 2013). However there is a lake of knowledge with respects environmental risk associated with PPCPs use (Farré et al, 2008).

Traditionally ecotoxicology studies focus on the effects of exposure to a single compound on a model organism, but at present there is a lack of knowledge on the effect of chemical mixtures in a complex system. Even if micropollutants can be present in the environment at concentration too low to raise concerns relying on lethal dose values, it is crucial to consider that organisms in the natural environment are exposed to a complex chemical mixture and the toxicity of PPCPs on non-target organisms may occur at concentrations lower than expected due to interactions between the different toxicants (Schnell et al., 2009). Compounds in a mixture can have additive effects (sum of single compounds toxicity), synergistic effects (greater than additive effects) and antagonistic (less then additive effects) (DeLorenzo and Serrano, 2003). Chemical interactions in the mixtures can strongly amplify the effects of single compounds (Pomati et al., 2006; Pomati et al., 2008), and also sub-lethal doses have been shown to alter community structure (Pomati and Nizzetto, 2013).
PPCPs degrade in in the environment with approximately a half-life of hours to a few days, with some exceptions (Loos et al., 2009). The degradation of the parent compounds leads to the formation of by-products (due to physic and chemical reactions) and metabolites (by biotransformation processes), but at present there is a lake of information about the interactions between PPCPs taking palace in the mixture (Wilson et al., 2003), and bioactivity of these transformation products (Farré et al., 2008).

1.4 Trait diversity

Diversity is multidimensional and it can be measured and defined at different levels of biological organisation: genetic, taxonomic and trait level (Harper and Hawksworth, 1994). One of the classical approaches to define biodiversity is based on species richness, but with the development of new technologies (e.g. flow-cytometry, next generation sequencing and single cell genomics), nucleotide, allelic, chromosomal, genotypic and phenotypic variability have become important measure of biodiversity and received increased attention (Naeem et al., 2012). Traits are defined as any genetic, morphological, physiological and phenological feature measurable at the individual level such as cell size, shape, motility, nutrient uptake, behaviour, type/time of reproduction (Violle et al., 2007). Variation at genetic and genomic level can result in phenotypic individual features, so populations consist of individual characterised by different competitive ability, resistance to abiotic conditions, resource use efficiency, and in general different fitness that is the ability of individuals to survive and reproduce (Fisher et al., 1930). The selection on traits due to abiotic environmental filters changes the community assemblages (Bolnick et al., 2011). In the last years trait diversity has become increasingly important as a biodiversity measure.
A phenotypic trait that shows a response to environmental factors and determines effects

on processes (e.g. cell size on nutrient uptake) is called functional trait (Reiss et al., 2009). Traits can be considered as the link between environment, biodiversity and ecosystem functioning. The environment plays an important role in determining diversity through selection of traits. This consequently is reflected on the biological assemblage composition in terms of number and type of organisms and their ecological interactions. Characteristics of biological assemblages determine ecosystem processes (Reiss et al., 2009).

The definition of traits becomes more and more relevant in the study of the variation in organisms and species composition in space and time (community ecology) (Vellend, 2010).
The mechanisms that maintain biodiversity have been debated for a long time. Dynamics in communities are driven by deterministic (niches) including selection processes due to environmental filters and biotic interactions, and stochastic (neutral) factors including unpredictable events (random birth-death events, dispersal and drifts) (Bell, 2000). All these factors govern the composition of communities and contribute to biodiversity maintenance through time (Adler et al., 2007). Consequently, two controversial community assembly theories jointly explain biodiversity in communities. In particular, the niche of a species, that is the role and behaviour of organisms in a specific environment, include the responses and impact of organisms on the abiotic and biotic environment. Environmental filters and biotic interactions shape the community composition and dynamics over time through selection processes due to individual fitness differences and determine the trait composition in the communities (Lambers et al., 2012).
Traits are therefore becoming more and more relevant to understand how communities are assembled and how they respond to environmental change. In this study we used an individual-trait-based approach to analyse the variation of expressed individual phenotypic traits (response traits) in a changing environment.

1.5 Community dynamics and adaptive capacity in a changing environment

When the environment is in a condition of equilibrium, traits of organisms tend to reach a value that is optimal for those specific conditions. In a natural environment, conditions are never static, but it is constantly fluctuating. When the environment changes, individual traits change accordingly due to phenotypic plasticity (physiological responses), ecological interactions and evolution. Norberg's model (Norberg et al., 2001) shows that in a condition of environmental change the difference between the optimal trait of an ideal community and the average trait expressed by organisms in a real community increases. This difference will be much higher than the rate of change is higher. Norberg (2001) suggested that the capacity of a community to change in response to changes in environmental conditions is

proportional its trait diversity. This means that a community with a large variability in traits can respond more quickly to the environment and maintain functions.

Individuals and populations are able to adapt and change the phenotypic, genetic, taxonomic and trait composition of the assemblage due to mechanisms acting over different spatial and temporal scales (Collins and Gardner, 2009). To understand the impact of chemical stressors on biological systems, physiological, evolutionary and ecological processes should be studied together (Pomati et al., 2013). Ecological changes refer to changes in the species composition and abundance in the assemblages due to multiple abiotic and biotic interactions.
Adaptation linked to new condition of temperature or chemical pollution can be achieved by two kind of mechanisms: acclimation or genetic adaptation (evolution). Acclimation allows an increasing in tolerance to changing environmental condition and it is achieved by phenotypic plasticity (the property of a given phenotype to produce different phenotype in response to environmental conditions). Phenotypic changes due to acclimation and are generally reversible when stressors are released (Fisher ae al., 2013). On the contrary genetic adaptation take place when stress exceed physiological operating range and survival depends only on adaptive evolution driven by selection of more resistant features (due to genetic variation and mutations). Evolutionary changes take place over a larger timescale and refer to changes in genetic characteristics that are handed down through generations. Stephan Gerber (2013) in his Master Thesis project on micropollutant-induced phenotypic adaptation in Mycrocystis aeruginosa found that changes in phenotype after a single exposure occur within one generation. Cells after a single triclosan (TCS) exposure showed an increasing in size and fluorescence, but they become smaller and less fluorescent after chronic sublethal exposure. Krüger and Eloff (1981) suggested that stressed cells become bigger and this parameter is a likely indicator of the physiological state of cells.

When pollutants act as selection factor, they can alter the sensitivity of individuals to other additional stressors (Fischer et al., 2013). Chemical exposure on populations and communities can result species sorting through selection of tolerant individuals and in a change of traits properties. Studies made with pesticide exposure on periphyton community (Schmitt and Alterburg, 2005) showed a changing in the community composition due to the replacement of sensitive species by less sensitive species at low level of contamination. This study also showed the importance of functional redundancy on ecosystem functions maintenance: at low chemical concentration the biomass remained constant or even increased due to the succession of species. On the contrary, at high doses the biomass production showed a drastic decrease.

1.6 Phytoplankton as a model system

The term phytoplankton refers to autotrophic microorganisms freely floating in water and characterised by a wide range of shape and dimensions.
Phytoplankton is a fundamental component of aquatic ecosystem and it provides roughly the 50% of the primary production of the planet (Watson et al., 2003). Organic material synthesised by autotrophic microorganisms supports pelagic food webs as it ensures the energy flux required by heterotrophic organisms. Phytoplankton also determines in large part the water quality and ecosystem services provided by aquatic ecosystems (Austen et al., 2007). Accordingly, any alteration that occurs at this level can change structure and function of the entire ecosystem.
Phytoplankton communities are highly diverse and dynamic and they are constituted by small microorganisms with fast generation time. Phytoplankton organisms are considered sensitive indicators of water quality, because they respond quickly to anthropogenic input of nutrients and toxic substances. Furthermore, relevant phytoplankton traits (size, shape, motility, pigments) responsible for important processes (photosynthesis and biomass production), and factors affecting them are already well known (Litchman and Klausmeier, 2008). These characteristics make them a classic model group of organisms in ecology and environmental toxicology, and allow to study responses over different spatial (individuals, populations and communities in micro- and mesocosms both in laboratory and in field) and temporal scales (hours to days and months), at the individual and community levels.

2. OBJECTIVE

The purpose of this study was to assess the responses of a phytoplankton community in a scenario of combined pollution and environmental change. Within this context we focused on community diversity and biomass production. We manipulated trait variance at the individual level directly (by selection of size classes) and indirectly (through exposure to PPCPs) and studied how reduction in trait-diversity affected populations community dynamics, production of biomass and the ability of the community to track a changing environment (increasing in temperature),

In particular the following specific questions were tested:

1. Does the reduction in traits-diversity affect community dynamics?

2. Does a direct manipulation of trait diversity (filtration of size classes) affect community dynamics and functioning differently from and indirect manipulation (PPCPs)? In other words, does the type of selection applied to community traits (mechanical and physiological) plays an important role to determine responses?

3. Does the temporal trajectory of treatments (in production of biomass, community structure, trait-diversity) follow the same trajectory of controls with a certain time lag or the trajectory is totally different?

The overall aim was to assess whether exposure to chemical pollution impairs the ability of a simple ecosystem to adapt (maintain structure and functions) in a changing environment.

3. MATERIALS AND METHODS

3.1 Test organisms

To study to what extent a phytoplankton community is able to adapt to a changing environment after manipulation of trait variability, a phytoplankton community composed by four species commonly found in European freshwater ecosystems was artificially assembled (all the four species together). Since the goal was to obtain a high diversity in terms of morphology and physiology (e.g. individual shape and size, presence or absence of colonies, amount and typology of pigments), and different processes in the community, both eukaryotic and prokaryotic, colonial and single cells organisms were selected. The list of four cultured phytoplankton species used for assembling artificial communities is shown below:

Kingdom: *Bacteria*
Phylum: *Cyanobacteria*
Order: *Synechococcales*
Family: *Synechococcaceae*
Genus: *Synechococcus*
Species: *Synechococcus leopoliensis*

Empire: *Eukaryota*
Kingdom: *Plantae*
Phylum: *Chlorophyta*
Class: *Chlorophyceae*
Order: *Sphaeropleales*
Family: *Selenastraceae*
Genus: *Pseudokirchneriella*
Species: *Pseudokirchneriella subcapitata*

Empire: *Eukaryota*
Kingdom: *Plantae*
Phylum: *Chlorophyta*
Class: *Chlorophyceae*
Order: *Chlamidomonadales*
Family: *Sphaerocystidaceae*
Genus: *Sphaerocystis*
Species: Sphaerocystis sp.

Empire: *Eukaryota*
Kingdom: *Plantae*
Phylum: *Chlorophyta*
Class: *Chlorophyceae*
Order: *Sphaeropleales*
Family: *Scenedesmaceae*
Genus: *Scenedesmus*
Species: *Scenedesmus obliquus*

3.2 Culture conditions

Cyanobacteria and Green algae were grown in 150 mL batch culture with freshwater WC Medium (Guillard & Lorenzen, 1972). WC Medium is an inorganic salt medium with a pH of 7.5. The WC Medium solution was made with six stock solutions and a TES buffer (Tab. 3.1 and Protocol 8A, Appendix).

Tab. 3.1: Chemical composition of WC Medium used for culturing Cyanobacteria and Green algae

WC Medium	
Compounds	**Nutrient Solution (mg/L)**
$CaCl_2 \cdot 2H_2O$	36,8
$MgSO_4 \cdot 7H_2O$	37
$NaH\ CO_3$	12,6
$K_2HPO_4 \cdot 3H_2O$	11,4
$NaNO_3$	85
Micronutrient solution*	

TES Buffer	0,115 g
Deionized water	994 mL

* Composition of micronutrient solution: Na_2EDTA 4,36 mg/L, $FeCl_3 \cdot 6H_2O$ 3,15 mg/L, $CuSO_4 \cdot 5H_2O$ 0,01 mg/L $ZnSO_4 \cdot 7H_2O$ 0,022 mg/L, $COCl_2 \cdot 6H_2O$ 0,01 mg/L, $MnCl_2 \cdot 4H_2O$ 0,18 mg/L, $Na_2MoO_4 . 2H_2O$ 0,006 mg/L, H_3BO_3 1,00 mg/L.

Stock solutions and nutrient solutions were autoclaved after preparation.
Stock cultures (50 mL) were grown in the laboratory at room temperature (approximately 20°C). Experiments were carried out in an incubator chamber (Multitron 2, Infors HT) at following specific conditions:
- Temperature:18°C
- Light cycle: 16h light/ 8h dark
- Light intensity: 56µE/m-1s-1

All cultures were handled under the sterile hood to avoid contaminations.

3.3 Single species analysis

Size determination
In order to define the cell size of the species chosen for the experiment, 30'000 particles for each species were analysed with the scanning flow-cytometer CytoBuoy (protocol 7D, Appendix).
Sideward scattering (SWS) and the maximum fluorescence red (FL.Red.Maximum) of raw CytoBuoy data were visually inspected in CytoClus 3 in order to define threshold values to distinguish living cells from cell debris (Fig. 3.3). SWS, that is the light reflected laterally from a particle after passing through the laser beam, gives a measure of the length of the particles. The Max.FL.Red correspond to the maximum value reached for each particle. The lowest limit for cell size was set at 1 µm on SWS length. Cut-off level for eliminating non-fluorescent green algae particles was set at Max.FL.Red≥10. Due to the lower fluorescence values of S. leopoliensis, the cut-off level eliminating for non-fluorescent particles was set at Max.FL.Red≥1.
Data were then cleaned and analysed in R (R-Development-Core-Team, 2012). Selected cell size values were used to obtain the cell size distribution for each species in order to set a cut-off level for the filtration treatment in the experiment. The community, composed by the four species, were filtered with different cut off level to reduce the cell size variability.

Growth assessment
The growth of cultures was assessed by measuring the optical density (OD_{750nm}) of the single cultures with the microplate reader (Spectra Max 190, Molecular devices corporation) set at 750 nm wavelength (protocol 7A, Appendix). The optical density is a measure of the absorbance of a suspension at specific wavelengths and it can be used as a proxy of cell concentration.
The OD_{750nm} of the single cultures was adjusted to OD_{750nm} = 0,1 by diluting them with WC

Medium. Cultures in two replicates were then grown in the incubator chamber (Multitron 2, Infors HT) at 18°C (starting experiment condition). Cell growth was monitored for 34 days, and every 2 days 250 µL sample were taken from each culture to perform the OD_{750nm} measure.

Correlation between optical density and biomass

Six serial dilutions were performed with the single cultures, and 250 µL were taken for the OD_{750} determination with the microplate reader (Spectra Max 190, Molecular devices corporation) in order to relate the OD_{750} measure with the biomass measure (dry mass). In order to have a dry mass measure, cultures were filtered with GF/F glass microfiber membranes in a vacuum filter apparatus.

Filters were placed at 480°C for 48 h and then their dry mass was measured with an analytical scale (protocol 3, Appendix).

The correlation between OD_{750} and biomass was calculated. In order to assemble the community with the same amount of biomass for each species, a value of 0,06 mg/mL was chosen, and the OD_{750} corresponding to 0,06 mg/mL was calculated for each species. The volume to be taken from each culture was then calculated accordingly.

3.4 Community assemblage

Single cultures were grown in the incubator at 18°C until they reached approximately an OD_{750}= 0,2.

The volume to be taken from each culture in order to have the same biomass level for each species in the mother culture was calculated by dividing the measured OD_{750nm} of each culture by the OD_{750nm} that corresponds to 0.06 mg/mL (protocol 4, Appendix). The starting culture was assembled accordingly.

3.5 Experimental design

Phytoplankton communities were subjected to a manipulation in trait variability at the individual level directly (by filtering out size classes) and indirectly (by using PPCPs mixture).

5 treatments were used: two size filtering treatments (F1 and F2), two PPCPs doses (D1 and D2), and a control (C), with three replicates each. After treatments, the replicates were subjected to a steady increase in temperature (1°C every day for 17 days) (Fig: 3.1).

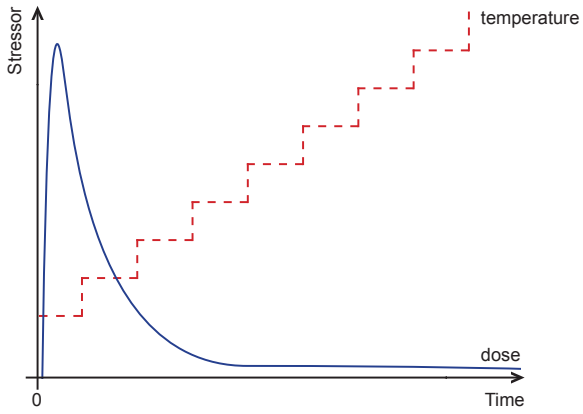

Fig. 3.1: Conceptual exposure scenario over experiment. Blue line = PPCPs mixture exposure, Red dashed line = increasing in temperature (1°C increasing per day) .

Every day 5 mL sample were taken from each replica for analysis and replaced (starting from day 1) with 5 mL fresh WC-medium.
Cells were fixed with Lugol fixative solution for microscopy (protocol 8D, Appendix) and with glutaraldehyde fixative solution for flow-cytometry (protocol 8B, Appendix). Analysis on fresh samples were performed in day. Endpoints were: cell concentration, biomass production and Chlorophyll-a (Chl-a) concentration, changes in functional traits.
The following measurements were performed:

- Optical Density
- Chl-a fluorescence intensity
- Microscopy counting
- Flow-cytometry

Micropollutant mixture

A mixture of 11 main therapeutic drugs detected in European rivers was used as physiological stressor. The median value of concentration of each compound found in rivers was taken from literature (Pomati, 2006). These values were round up at the highest order of magnitude and taken as a reference in order to calculate the experimental compounds concentration (Tab. 3.3). Micropollutants were obtained (in powder form) from Sigma-Aldrich (St. Gallen, Switzerland). The concentration of the single compound was 1g L^{-1}. The stock solution of the experimental mixture was prepared combining and diluting the single drugs in 100% ethanol to reach a dose 20,000 fold higher than the environmental level reported in Tab. 3.3, and stored at -20°C. The stock solution was then diluted in experimental cultures to reach the desired exposure concentrations. In tab. 3.4 the final compounds concentration in the treatments are shown: compounds concentration were 10 and 100 fold higher than the environmental levels (Tab. 3.3). 27,5 µL and 275 µL of mixture respectively were added in 55 mL culture.

Compounds	Molecular formula	Therapeutic category	Provisional initial experimental concentration [ng/L]	River concentration [ng/L]
atenolol	$C_{14}H_{22}N_2O_3$	anti-hypertensive	1000	351
bezafibrate	$C_{19}H_{20}ClNO_4$	lipid regulating	100	46
carabamazepine	$C_{15}H_{12}N_{20}$	anticonvulsant/antidepressant	1000	121
clarythromycin	$C_{38}H_{69}NO_{13}$	antibacterical	1000	143
diclofenac	$C_{14}H_{11}C_{12}NO_2$	antiinflammatory	1000	190
furosenide	$C_{12}H_{11}ClN_2O_5S$	diuretic	100	59
hydrochlorothiazide	$C_7H_8ClN_3O_4S_2$	diuretic	1000	174
ibuprofen	$C_{13}H_{18}O_2$	antiinflammatory	100	97
ranitidine	$C_{13}H_{22}N_4O_3S$	ulcer healing	10	8
sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	antibacterical	10	7
triclosan	$C_{12}H_7C_{13}O_2$	antibacterical/fungicide	100	59

Tab. 3.3: List and therapeutic category of eleven PCPPs assembled in EtOH 100%. The forecast initial experiment concentration of each compound was based on the concentration level found in European rivers (Pomati, 2006).

Compounds	Stock solution [mg/l]	Compound concentration Dose 1 (10x) [mg/L]	Compound concentration Dose 2 (100x) [mg/L]
atenolol	20,0	0,01	0,1
bezafibrate	0,2	0,0001	0,001
carabamazepine	20,0	0,01	0,1
clarythromycin	20,0	0,01	0,1
diclofenac	20,0	0,01	0,1
furosenide	0,2	0,0001	0,001
hydrochlorothiazide	20,0	0,01	0,1
ibuprofen	0,2	0,0001	0,001
ranitidine	2,0	0,001	0,01
sulfamethoxazole	2,0	0,001	0,01
triclosan	0,2	0,0001	0,001

Tab. 3.4: The table shows the concentration of PCPPs in the stock solution and the compounds concentrations in the treatments (Dose 1 = compounds concentration 10 time higher than environmental levels, Dose 2 = compounds concentration 100 time higher than environmental levels).

Filtration

According to the cell size data obtained from flow-cytometry on the single cultures, two different cut off levels were applied to the community in order to reduce the cell size variability. In order to remove the extreme lower and upper 10% of the community cell size distribution (treatment F1), replicates have been filtered through a 12 µm and 5 µm porosity Nucleopore PC filters respectively.

In order to remove of the lower 20% of the community cell size distribution (treatment F2), a 8µm Nucleopore PC filter was used to filter cultures. The 5 µm filters with particles were then washed in 55 mL WC Medium to collect cells. In order to have the same amount of nutrients in each treatment, the fresh WC Medium was diluted with medium obtained by filtering cultures with GF/F glass microfiber filter. The final 55 mL medium were composed by 41,25 mL fresh WC Medium and 13,75 mL old medium. For the exact filtration procedure see protocol 5A, Appendix.

Controls and treatments with the PPCPs mixture were diluted with fresh medium to reach 0.05 OD_{750nm} in order to start the experiment with the same density of particles for each treatment.

Control preparation

Because chemicals were diluted in ethanol, in order to have the same amount of ethanol in all treatments and replicates, the following volume of EtOH 100% was added in 55 mL culture:

- 275 µL in filtrated treatments
- 275 µL in controls
- 247,5 µL in dose 1 chemical treatments

Temperature

Treatments and controls were placed in the incubator Multitron 2 at the following specific conditions:

- Cycle light/dark: 16/8 h
- Irradiance: 56 $\mu E/m^{-1}s^{-1}$
- Revolutions per minute (RPM): 20

Cultures have been subjected to a steady increase in temperature of 1°C every day starting from 18°C to 34°C. The experiment had lasted 17 days.

3.6 Measurements

Optical density

The spectrophotometer microplate reader Spectra Max 190 was used to measure the optical density of samples. This instrument allows measuring the absorbance of a solution or a suspension at specific wavelength in order to know the concentration of particles in the sample and have an estimate of the biomass production.

250 µL fresh sample were taken every day from each treatment and placed in the microplate (Greiner, 96 well, PS, F-bottom, crystal-clear). The absorbance was measured at 750 nm wavelength.

For more information about the instrument see the manual: VersaMaxTM and SpectraMaxR user guide.

Chl-a fluorescence intensity

Fluorometry is a measure of fluorescence (FL) designed to determine quantitatively the fluorescence of a substances. Fluorescence occurs when a particles absorbs photons from the spectrum (excitation) and then rapidly emits photons (emission) when it return to its ground state. The absorbed photon is always more energetic than the emitted photon, that is the wavelength (λ) of emitted light is always greater than the λ of excitation wavelength. Fluorometry is therefore a quantitative analytical technique used to characterize the relationship between absorbed and emitted photons at specific wavelengths.

Chl-a absorbs the light in two specific wavelength range with two peaks at 465 and 665 nm. The fluorescence emission by Chl-a in the photosystem II is used as a measure of the pigment content in cells (Lorenzen et al., 1966).

The microplate reader TECAN infinite 200 with the i-Control software was used to measure the Chl-a concentration in the samples.

250 µL fresh samples were put in the microplate (Greiner, 96 well, PS, F-bottom, crystal-clear). Chl-a was excited at 435 nm wavelength, and the Chl-a FL intensity at 685 nm emission wavelength was measured.

Microscopy

Microscopy was used to determine the abundance of cells for each species. 15 µL samples were put in a Hemocytometer Neubauer chamber, and the number of cells was counted with the optical microscope Nikon Eclipse 80i set at 40x magnification. Cells were counted in three different square dimensions in the hemocytometer according to the species cell dimension and concentration of cells in samples (protocol 7F, Appendix).

Samples for microscopy were fixed with LUGOL fixative solution (protocol 8D, Appendix).

Flow-cytometry

The scanning flow cytometer CytoBuoy (www.cytobuoy.com, Woerden, Netherlands) was used for counting and morpho-physiological characterisation of the community. This instrument allows us to measure responses at the individual level in order to derive information at population and community level.

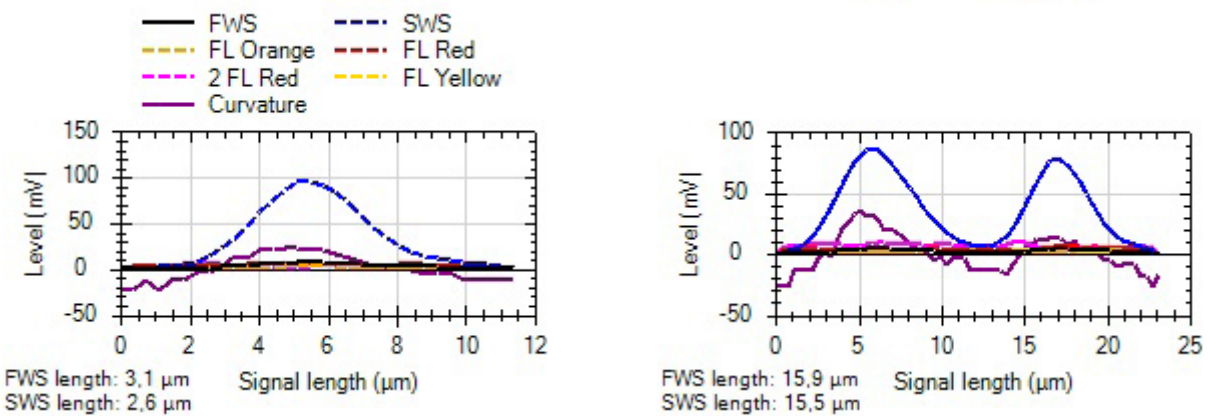

Fig. 3.2: CytoBuoy signals: Length signal on SWS scatter allowed clear distinction of single cell (left) and colonies (right).

For each sample 250 µL were processed. Each particle was intercepted by two laser beams (Coherent solid-state Sapphire, 488 nm and 635 nm, respectively, 15 mW) at the speed of 2 m/s^{-1}. More details on the instrument can be found elsewhere (Fontana et al. 2014). 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 (Fig. 3.2). The fluorescence (FL) emitted by photosynthetic pigments in 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 (Chl- a), 601–668 (Phycocyanin) and 536 – 601 nm (Phycoerythrin and decaying pigments), respectively (Dubelaar et al., 2004).

Digital data acquisition was triggered by the sideward scatter (SWS) signal with a triggerlevel of 31 mV, which largely excludes particles smaller than 1 µm, and by the FL.Red with a trigger level which exclude particles with FL.Red total < 5 mV.

Signal length of the different channels allowed distinction of different cellular morphotypes. Output files of CytoBuoy measurements contained 65 descriptors with information about structure and FL profile for each particle.

Samples for flow-cytometry were fixed with Glutaraldehyde fixative solution 25% (protocol 8B, Appendix). Fixative solution was added 1:100 to samples.

3.7 Data analysis

Data analysis and graphs were performed with CytoClus 3 (version: 3.7.15.0), SigmaPlot 10 and R statistical programming language (R-Development-Core-Team, 2012).

Cytobuoy parameter analysis

Raw CytoBuoy data from the single species culture were visually inspected in CytoClus 3 (Fig. 3.3) in order to define threshold values to select living cells from cell debris. Data were then cleaned and analysed in R. For the single culture cell size determination the lower limit for the size was set at Length.SWS>= 1 µm. Cut-off level for non-fluorescent particles was set at Max.FL.Red>=10 for green algae and at Max.FL.Red>=1 for Cyanobacteria.

Data acquired during the experiment with the scanning flow cytometer needed to be cleaned to remove measurements of dead and degraded cells. Instead of using the method explained above, an unsupervised clustering algorithm, FlowPeaks (Ge and Sealfon, 2012) was used to classify a subset of 100,000 particles into clusters. The parameter values used for the clustering algorithm were: tol=0.25, h0=0.05, h=2. These parameter values were identified as optimal based on previous tests with single species cultures. Clustering was performed using 6 important parameters: FWS.Average, FL.Red.Average, X2.FL.Red.Average, FL.Orange.Average, FL.Yellow.Average, and SWS.Length. The clusters were visually inspected and the cluster with the lowest fluorescence level (the same cluster was lowest on all axes) was selected as representing dead and degraded cells.

We then used a machine learning tool, random forests (Breiman et al., 2001), to train a classifier with this clustered dataset. This was then used to identify the low fluorescence cluster in the full dataset and eliminate particles corresponding to this cluster. This process (clustering + classification) was then repeated to remove an additional small cluster of very low fluorescence particles.

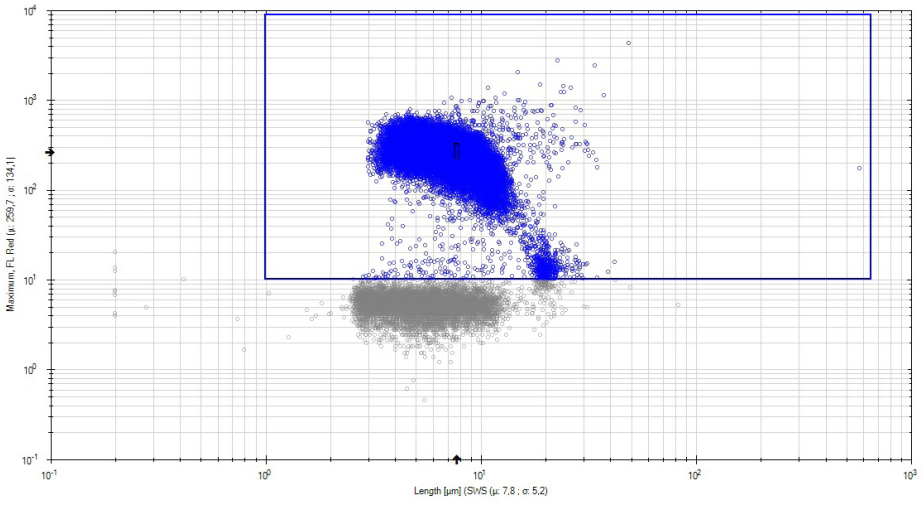

Fig. 3.3: Scatter plot (obtained in CytoClus 3) displaying lenght and maximum fluorescence. Pseudokirchneriella subcapitata cells (blu cloud) were separated from debris (gray cloud) by assessing particles >1µm and Max FL Red>10

Boxplot of the size distribution of particles for each species was performed in R. Then all size data obtained from the single culture were assembled to have the cell size value distribution in the community. Quantile function was performed in R, and the lengths of particles corresponding to the extremes lower and higher 10% values and lower 20% values of the particles size distribution were found in order to select the filters pore size for the treatments.

Communities compositional dissimilarity

Bray-Curtis (B-C) dissimilarity index used to quantify the compositional dissimilarity between treatment and control communities during the experiment, using microscopy measurements.

$$d[jk] = (sum(abs(x[ij]-x[ik])))/(sum (x[ij]+x[ik]))$$

Where d[jk] is the index of compositional dissimilarity between treatments, x[ij] is the abundance of cells in species i (Synechococcus, Pseudokirchneriella, Sphaerocystis, Scenedesmus) in treatment j, x[ik] is the abundance of cells in specie i in treatment k. The calculation is made between each pair of treatments. The B-C index is bounded between 0 and 1, where 0 means the two treatments have the same composition and 1 means the maximum dissimilarity.

4. RESULTS

During the experiment the first replica of the filtration treatment 2 showed OD_{750nm} (Fig. 4.1) and Chl-a fluorescence intensity (Fig. 4.2) around 0. Replicate F2.1 was not considered for the analysis. Graphs including replica F2.1 are shown in the Appendix.

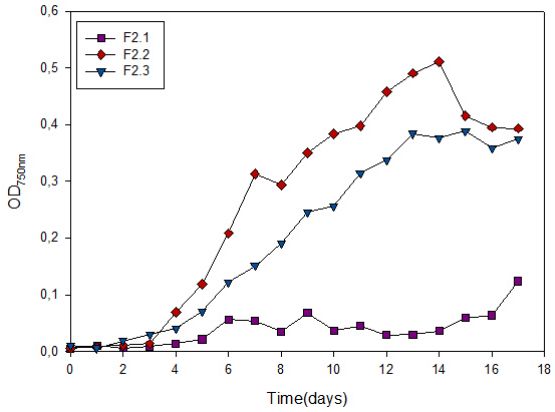

Fig. 4.1: The graph shows the OD_{750nm} trend in the replicates of filtrated treatment (lower 20% community cell size cut off). The replica 1 shows an OD_{750nm} near 0.

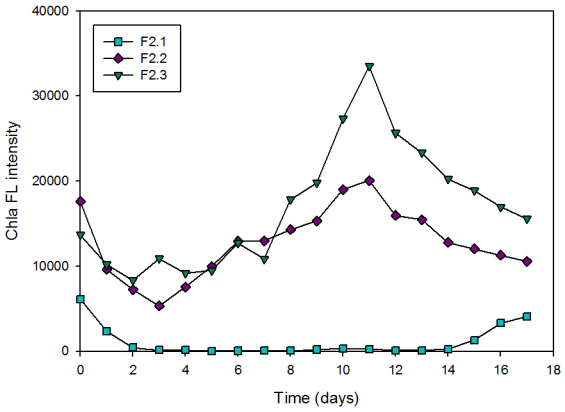

Fig. 4.2: The graph shows the Chl-a fluorescence intensity trend in the replicates of filtrated treatments (lower 20% community cell size cut off). The replica 1 shows a Chl-a fluorescence intensity near 0.

4.1 Single cultures cell size

The size distribution of particles for community cultures are reported in graphs (Fig 4.3). The filtration treatment was applied to the community. Filter pore size dimensions used for filtration are represented in graphs with blue lines: 8 µm pore size filter was used to remove the lower 20% of the community cell size distribution (Fig. 4.3.b), 5 µm and 12 µm pore size filter were used to remove the lower and upper 10% of the community cell size distribution respectively (Fig. 4.3.a).

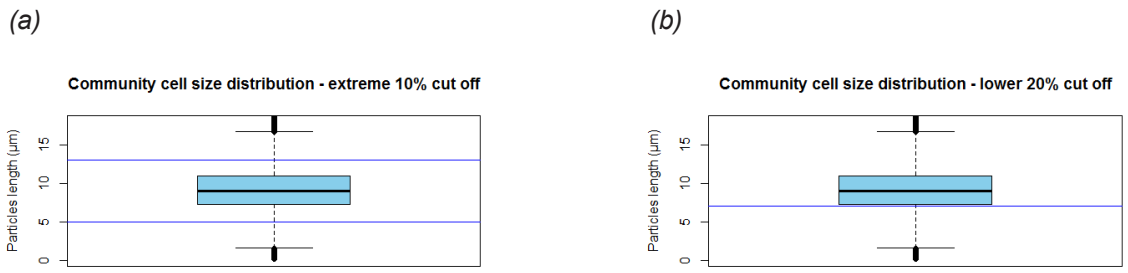

Fig. 4.3: Cell size distribution in the community. (a) The blue lines set at 5 µm and 12 µm represent the filters pore size used to remove the extreme lower and upper 10% of the cell size distribution. (b) The blue line set at 8 µm represent the filter pore size used to remove the lower 20% of the cell size distribution.

4.2 Cultures growth assessment

S. leopoliensis culture reached a maximum OD_{750nm} = 0,392 and then declined, while green algae cultures continued to grow until the end of the measuring period. All cultures started the exponential growth phase between 0,1 and 0,15 OD_{750nm} (Fig. 4.4).

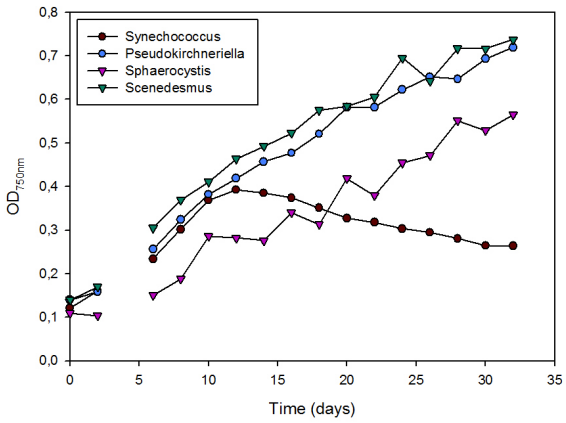

Fig.4.4: Cultures (S. leoponliensis, P. subcapitata, Sphaerocystis sp. and S. obliquus) were grown at 18°C for 34 days. OD_{750nm} was measured every 2 days interval. Data from day 4 is missing.

4.3 Single species correlation between optical density and biomass

OD_{750nm} was linearly correlated with dry mass measures for each specie (Fig. 4.5). Linear relationship expressed as function was y = 1,77x + 0.02 for *S. leopoliensis* (P-value < 0,0001 and R2 = 0,9986), y = 2,04x + 0,02 for *P. subcapitata* (P-value <0,001 and R2 = 0,9987), y

= 0,57x + 0,04 for *Sphaerocystis sp.* (P-value < 0,001 and R2 = 1) and y = 1,26x + 0,03 for *S. obliquus* (P-value < 0,0001 and R2 = 0,9918).

For *S. leopoliensis*, *P. subcapitata* and *S. obliquus* cultures the value of 0,06 mg/mL corresponded to a value between 0,1 and 0,15 OD_{750nm}.

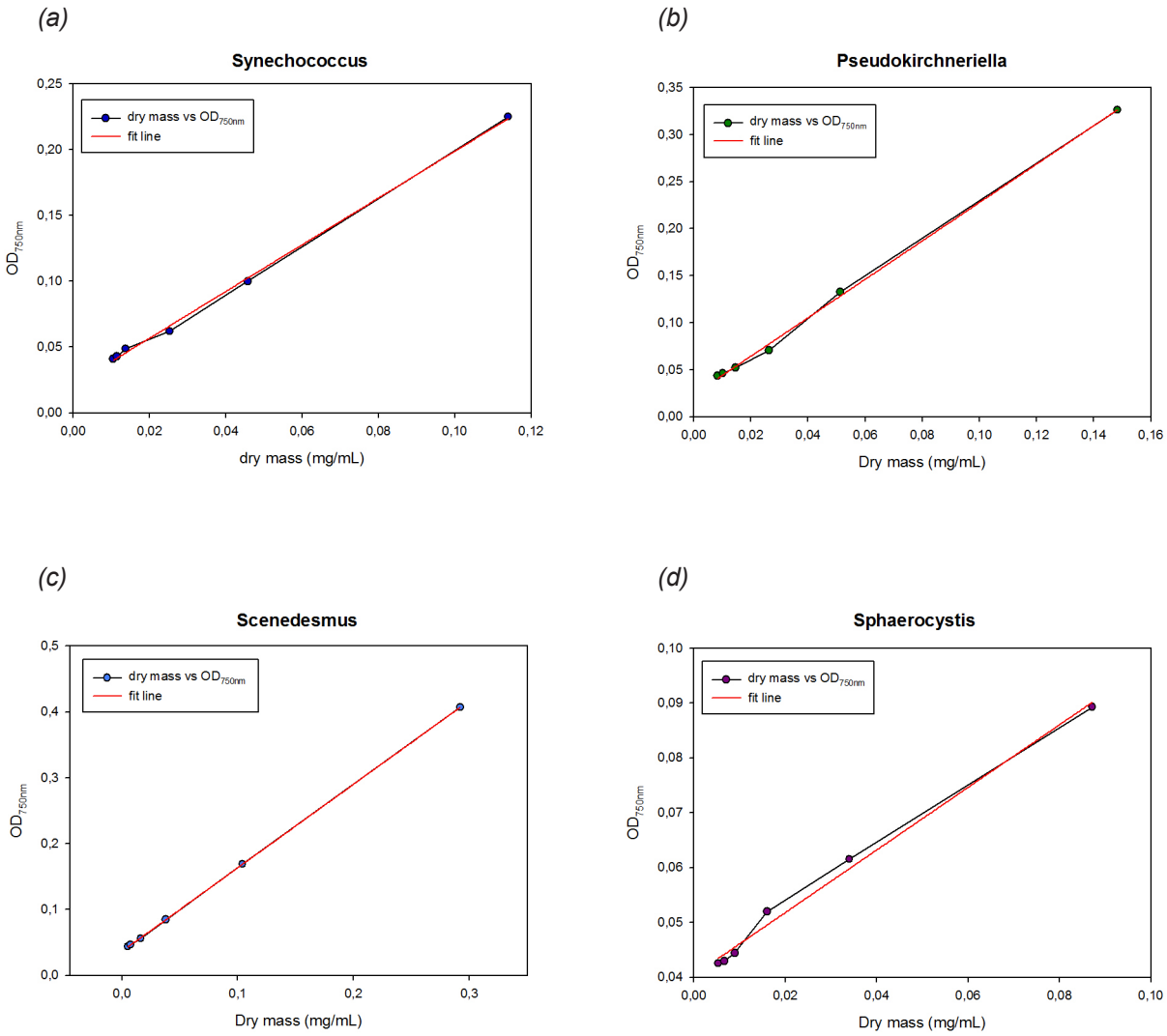

Fig. 4.5: Correlation of OD_{750nm} and dry mass calculated for the four species used in the experiment: S. leopoliensis (a), P. subcapitata (b), S. obliquus (c), Sphaerocystis sp. (d). OD_{750nm} and dry mass were measured on six serial dilutions. Linear relationship is shown in the graphs.

4.4 biomass – OD_{750nm} correlation for all species

OD_{750nm} values and dry mass values obtained after serial dilution of single specie cultures were combined in the graph (Fig. 4.6) in order to obtain the correlation between optical density and biomass of the community. Linear relationship expressed as a function was y = 1,40x + 0,03 (P-value < 0,0065 and R^2 = 0,92). Trend line equation was used to calculate the biomass of communities during the experiment based on OD_{750nm} values. Graphs representing OD_{750nm} trend during the experiment are shown in the appendix (Fig. 8.2).

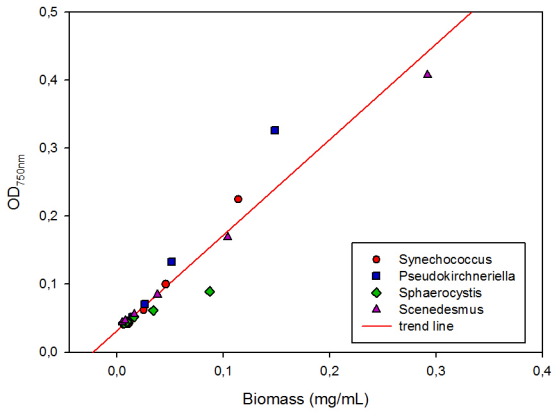

Fig. 4.6: Correlation between biomass and OD_{750nm} for the four species together. Red line represent the trend line equation.

4.5 Functional endpoint

Biomass production

The biomass average value of replicates for each treatment is represented in the graphics (Fig.4.7.a). The graphic shows a consistent trend in biomass production between Control, filtered treatments and lower PPCPs dose treatment. The community in D2 deviates from controls and other treatments in the biomass production. Biomass production in D2 is lower than the control and other treatments starting from day 6.

Treatment D2 shows a deviation in biomass production from control (Fig. 4.7.b) Differences in biomass production between D2 and C increase rapidly starting by day 2 until day 8, and then D2 tend to recover until day 13, but it never reaches control level.

In the treatment with lower PPCPs dose (D1), biomass production decreases and deviates from C starting from day 4 to day 6, and then the values increase again until it exceed the biomass of control.

Biomass in F1 and F2 tends to increase through time reducing the difference from control.

(a) (b)

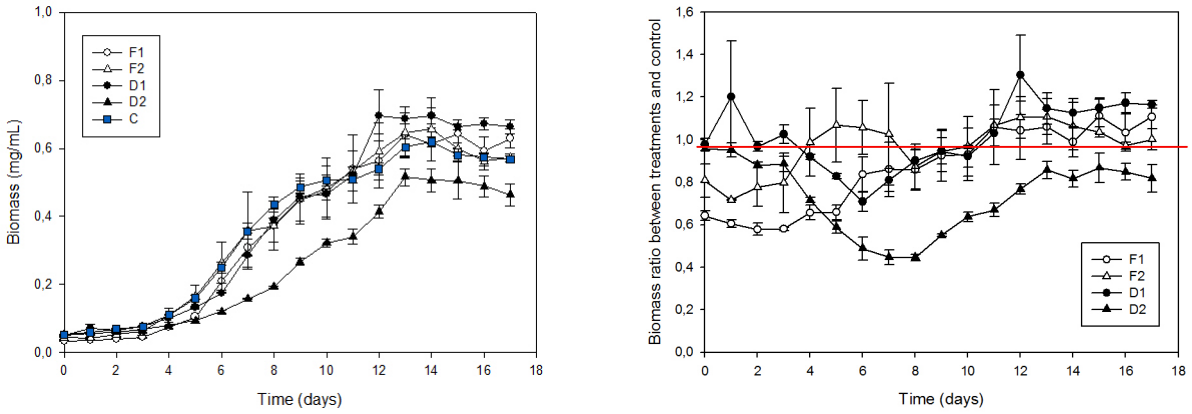

Fig. 4.7: (a) Biomass accumulation with time: biomass average value of 3 replicates for each treatment is reported. (b) Deviation in biomass production between treatments and control through time obtained dividing the mean values of replicates for each treatment by the mean values of replicates in control. Values lower than 1 mean lower treatments values compared to control. Values equal to 1 (red line) mean no differences between treatment and control. Values higher than one mean higher treatments values than controls. F1 = filtration with 5 and 12µm porosity filters, F2 = filtration with 8 µm pore filter, D1 = exposure to lower PPCPs dose (10x), D2 = exposure to higher PPCPs dose (100x).

Chl-a concentration

Treatment D1 shows a higher and steady Chl-a FL intensity compared to all other treatments until day 11, and then the intensity decreases until it reaches the control. Filtered treatments show a trend similar to control but at lower values. Chl-a FL intensity in F1 and F2 converges to control after day 11. At day 11 Chl-a FL intensity reach a peak in C, F1 and F2 and fluorescence decrease until the end of the experiment (Fig. 4.8.a).

Differences in Chla FL intensity between filtered treatments and control decrease progressively through time (Fig. 4.8.b). F1 and F2 tend to reach the fluorescence intensity of control progressively. D1 initially differs from control until day 5, and then it tends to converge to control level. D2 shows an increase in difference from control starting from day 10.

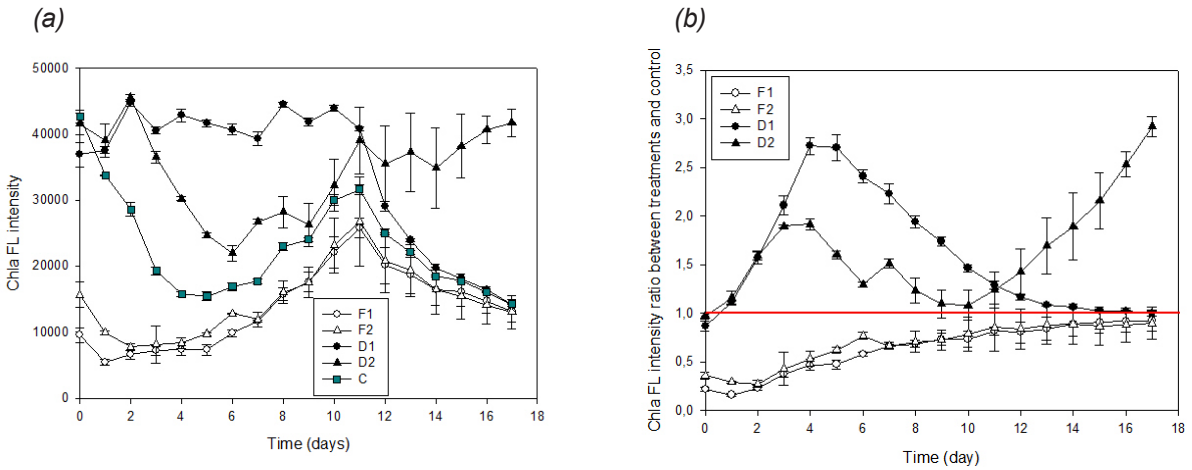

Fig. 4.8: (a) Chl-a fluorescence variation per 200 µm during time. Excitation wevelength was set at 435 nm and the emission wavelength was measured at 685 nm. (b) Deviation of treatments from control based on Chl-a fluorescence intensity values. F1 = filtration with 5 and 12µm porosity filters, F2 = filtration with 8 µm pore filter, D1 = exposure to lower PPCPs dose (10x), D2 = exposure to higher PPCPs dose (100x).

Total community cell concentration

In Fig. 4.9.a the cell concentration of in the different treatments based on cells counting with the optical microscope are represented. D2 shows a lower total cell concentration compared to control until day 11 when treatment reach control. After day 11 the cell concentration in control starts to decline, while cell concentration in higher dose treatment reaches the control and stays constant at higher level compared to control. D1 follows the same trend of the control, but at a higher cell concentration.

No differences in cell concentration are present between filtered treatments and control.

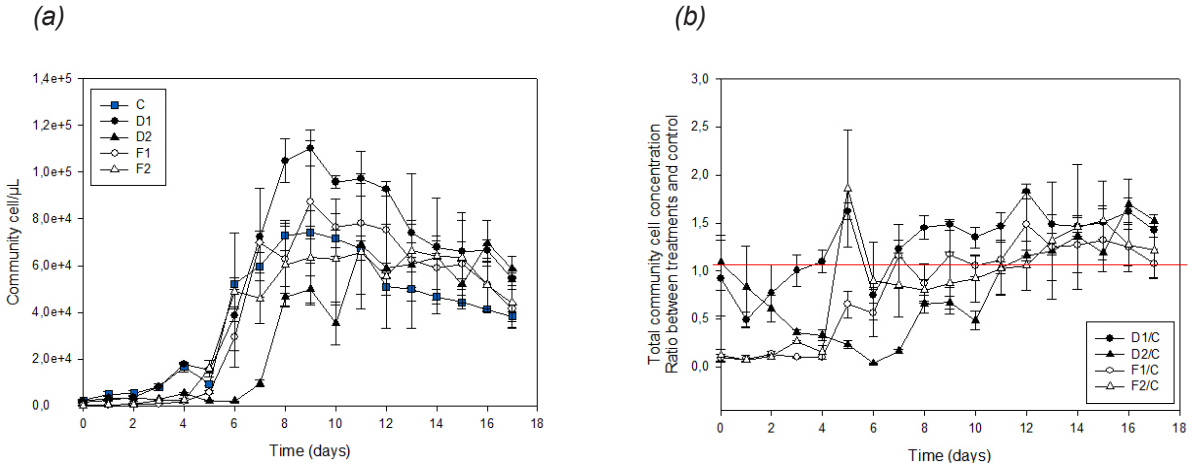

Fig. 4.9: (a) Total community cell concentration. Cells were counted with hemocytometer and optical microscope set at 40x magnification. (b) Ratio between treatments and control calculated on the total community cell concentration (cell/µL) values. F1 = filtration with 5 and 12µm porosity filters, F2 = filtration with 8 µm pore filter, D1 = exposure to lower PPCPs dose (10x), D2 = exposure to higher PPCPs dose (100x).

4.6 Structural endpoints

Bray-Curtis dissimilarity between treatments

The Bray-Curtis dissimilarity index (Fig. 4.10) between filtered treatments and control is high during the first 4 day of the experiment, and then the index decrease rapidly and reach the D1 treatment.
D1 fluctuates around the same values for all the experimental period. D2 shows a peak in dissimilarity at day 6. Communities structures converges among treatments at day 8 and fluctuate in the same range until the end of the experiment.

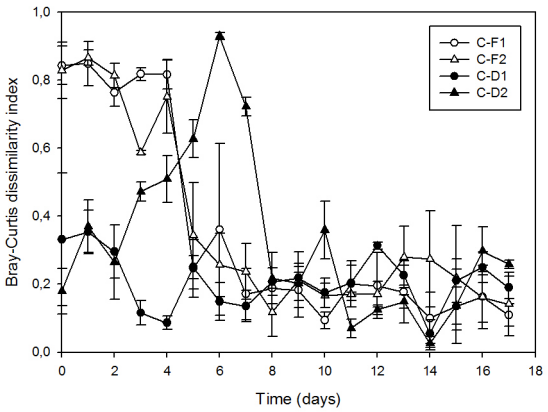

Fig. 4.10: Bray-Curtis dissimilarity index between treatments and control obtained with microscopy data.

Single species cell concentration
Filtration treatments acted mainly on *S. leopoliensis* and *P. subcapitata* that presents cell concentration of approximately one order of magnitude lower than control at the beginning of the experiment (Fig. 4.11). During the experiment cell concentration in filtered communities increase and tend to reach the control.
In D2 (Fig. 4.11.c) *P. subcapitata* and *S. leoponliensis* cell concentration reflect the control trend (Fig.4.11.a). *S. leopoliensis* and *Sphaerocystis sp.* appear not to grow until day 6, and then start increasing the cell number until day 8. After day 8 the cell concentration in all treatments remains approximately constant.

(a)

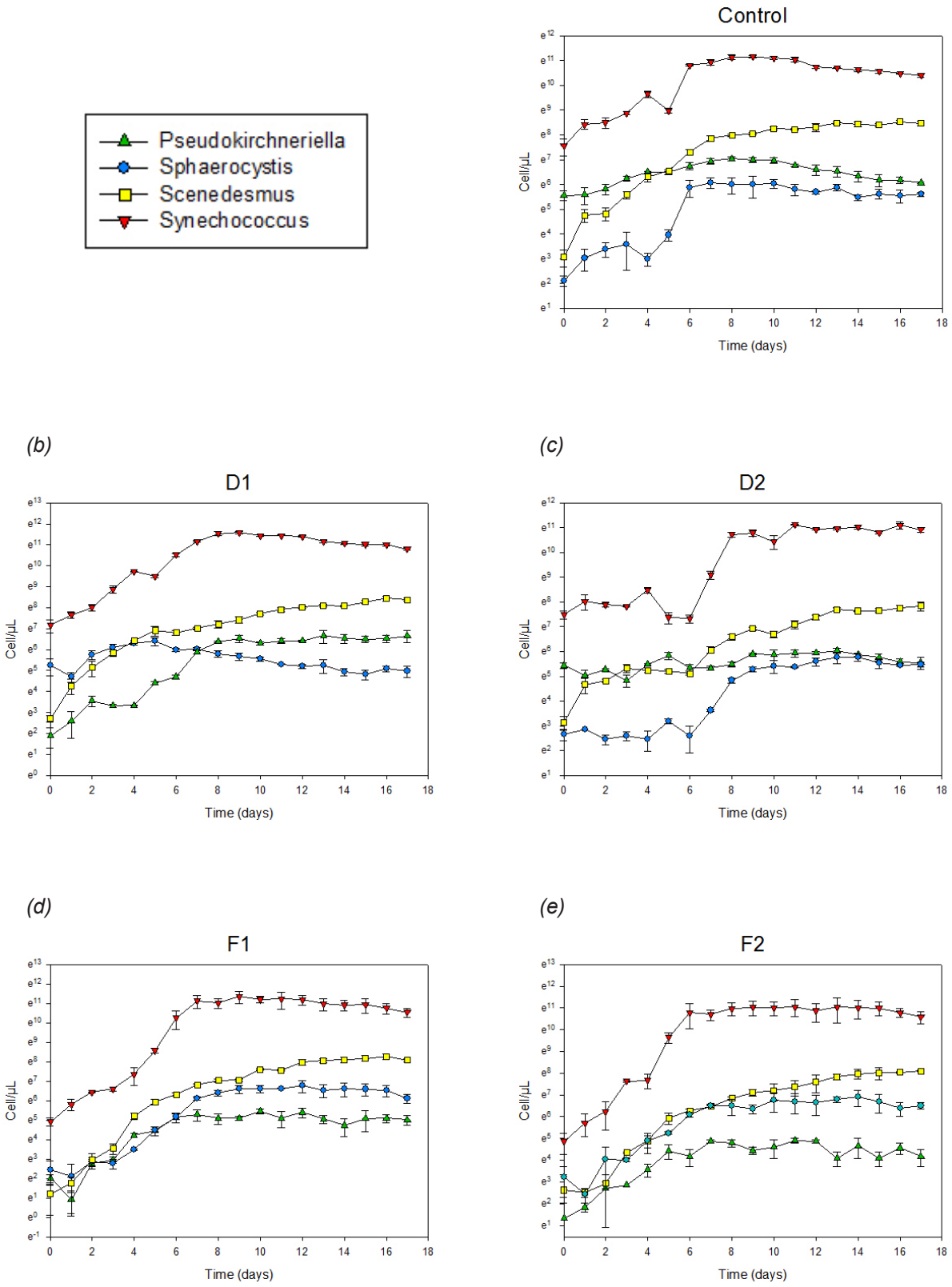

Fig. 4.11: Single species cell concentration (Cell/µL) in C (a), D1 (b), D2 (c), F1 (d) and F2 (e). Cells were counted with Hemocytometer and optical microscope set at 40x magnification.

4.7 Trait changes

In Fig. 4.12.a Is represented the changing in cell size based on SWS of the overall communities derived from single particles values. F1 shows a starting mean value of length similar to the F2. Mean length values of both filtered treatments are higher than control. Cell size increase through time for each treatment until day 8, then tend to stabilise until the end of the experiment. Treatments with PPCPs show an increase in size faster than control in the first four days of experiment.

Differences between filtered cultures and control decrease through time (Fig. 4.12.b) due to the increasing in particles dimension of control. PPCPs treatments tend to diverge from control in the first 4 days (Fig. 4.12.b) that correspond with an increasing in particles dimension in treated cultures. Starting from day 5 the particles dimension in the control increase and tend to exceed treatments.

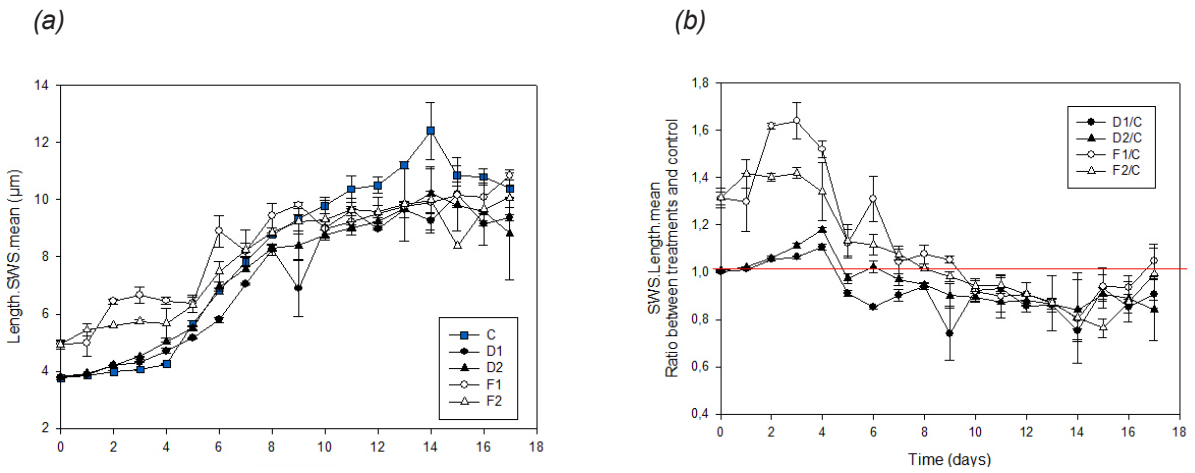

Fig. 4.12: (a) Communities cell size (SWS scattering) change through time. Values were calculated for each particles. (b) Ratio between treatments and control related to particles length (SWS). F1 = filtration with 5 and 12µm porosity filters, F2 = filtration with 8 µm pore filter, D1 = exposure to lower PPCPs dose (10x), D2 = exposure to higher PPCPs dose (100x).

The standard error of size calculated on all particles for each samples is used as a measure of diversity (Fig. 4.13), showing the variability of size in communities. F1 and F2 show higher values than control. D2 show a reduction in diversity from day 6 to day 13 compared to control and other treatments.

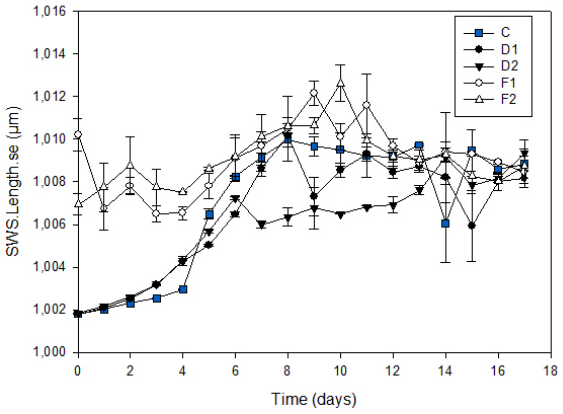

Fig. 4.13: Standard error of particles length calculated on all particles in each sample. Cells length is based on SWS. F1 = filtration with 5 and 12µm porosity filters, F2 = filtration with 8 µm pore filter, D1 = exposure to lower PPCPs dose (10x), D2 = exposure to higher PPCPs dose (100x).

5. DISCUSSION

The aim of this study was to investigate whether exposure to PPCPs impairs the ability of a phytoplankton community to maintain structure and functions in a changing environment. In particular we were interested in understand if the reduction in trait-diversity affects community dynamics and functioning and if the type of selection applied to community traits (mechanical through filtration of size class and physiological through exposure to PPCPs mixture) plays an important role to determine response. We evaluated the response (dynamics, diversity and productivity) of an artificially assembled phytoplankton community in a scenario of combined pollution and temperature change.

5.1 Filtration effects on community cell size

The filtration with 8 μm pore size filter (Fig. 4.3.b) shifted the mean of cell size toward bigger values as expected (Fig. 4.12.a). The filtration with 5 and 12 μm pore size filters showed to have a different effect on the community then expected. As filters were supposed to exclude the extreme lower and upper 10% of the community particles size distribution, the overall expected effects should have been no changes in mean, but reducing in variance. On the contrary, the mean length based on SWS suggests that the treatment F1 shifted the mean of particles size toward bigger values (Fig. 4.12.a). This was probably due to the filtration procedure: cells trapped on the filters (5 μm and 8 μm pore size) must have formed clusters, and probably resuspending in the medium did not fully work. The increasing in clusters number in communities must have shift the mean toward high values.
The increasing in size diversity in F1 and F2 after filtration was not expected as well (Fig. 4.13). Probably the presence of both small and larger particles due to the cluster formation increased the size variance.

5.2 Functional endpoint

Biomass production
High PPCPs dose exposure seems to reduce growth only in the first 6 days of experiment, after which the number of cells in treatment D2 reached and exceed the control (Fig. 4.9.a). Despite a higher cell concentration in D2 respect to control, a lower biomass production was observed (Fig. 4.7.a). The lower biomass accumulation can be explained by the lower particles dimension in the community exposed to high dose (Fig. 4.12.a).

On the contrary the addition of lower PPCPs dose seemed to stimulate biomass production although with a delay in the response compared to control (Fig. 4.7.a). A dose-dependent effect on algae after exposure to chemicals was also found by Schmit (2005). Even if Schmit (2005) used a different assemblage composition (periphyton) and different chemical (pesticide isoproturon) compared to ours, his results showed an increase in algal growth at low dose of chemical exposure and a reduction in biomass production with higher chemical concentration. PPCPs exposure seems to cause in both cases a dose response phenomenon (hormesis) characterised by a low dose stimulation and high dose inhibition of productivity (Calabrese and Baldwin, 2003). This explanation could be consistent with the finding of another study investigating the toxicity of five PPCPs (fluoxetine, propranolol, triclosan, zinc-pyrithione, and clotrimazole) on marine periphyton communities (Backhaus et all., 2011). PPCPs exposure seems to affect the ability of the community to produce biomass. A stimulatory effect on *P. subcapitata* was also found for some antibiotics individually tested (Clarithromycin and Sulfamethoxazole) at very low concentrations (Yang et al.,2008).

Starting from the beginning of the experiment one replicate of the treatment F2 showed a Chl-a fluorescence and OD_{750nm} around 0. Maybe filtration with 8 µm porosity filter had a more severe effect than expected. We did not observe a dramatic change in response patterns with the inclusion of replica F2.1 in the results, and for a better visualization and interpretation of data, replicate F2.1 (considered unrepresentative) was omitted from further analysis.
The mechanical reduction in trait diversity with filtration seemed to have no effects on the temporal trend in biomass production. Efficiency in biomass production seemed therefore to be affected more by exposure to chemicals that determine a physiological response on phytoplankton than to a change in trait diversity.

Total community cell concentration
Exposure to different PPCPs doses showed opposite effects on the community cells concentration. Low dose exposure seemed to determine an increase in the total phytoplankton abundance (sum of all four species counts), probably stimulating growth (Fig. 4.9.a). In contrast to what observed by Bishop (1973) exposing *Euglena gracilis* to two antibiotics (chloramphenicol and cycloheximide), cell division in communities exposed to the highest dose seemed to be inhibited during the first 6 days post-initiation. Considering the degradation time of PPCPs (half life of hours to a few days, with the exception of atenolol and clarythromicin that are more persistent) (Loos et al., 2009), it is possible that the chemical stress was released after the day 6 corresponding to the increasing in biomass production and cell concentration in D2. A previous study on the effects of a mixture of antibiotics (tetracycline hydrochloride, oxytetracycline hydrochloride, doxycycline hydrochloride and chlortetracycli-

ne) on a phytoplankton community showed similar results: cell abundance was significantly reduced during the exposure period, but, after releasing stress, abundance did not significantly differ from control (Wilson et al., 2004). Even though all communities are subjected to a steady increase in temperature that acts as a stress factor, we consider the control as the reference system, and the return of treatments values to a condition similar to control after the removal of chemical stressor is considered as recovery (Giddings et al, 2002). In both studies communities were able to recover after the hypothesised release of chemicals (Wilson et al., 2004).

Chl-a concentration

Chl-a concentration is a physiological property of cells that can show a very quick (within hours) plastic response due to environmental conditions (Gratani, 2014). The Chl-a concentration in cells was significantly lower in filtered treatments even if the communities started the experiment with the same biomass level.

While Chl-a is typical of phytoplankton and all photosynthetic organisms, other accessory pigments differs between taxonomic groups (Gregor et al., 2005). While accessory pigments (excited at 430nm) absorb blue-light at between 530 and 680nm, Cyanobacteria that contains phycocyanin as accessory pigment, absorb blue light weakly, and maximum emission occur at an excitation λ between 550 and 630nm. Moreover, Chl-a in Cyanobacteria is located in the photosystem I that is less fluorescent, so the emission of Chl-a in Cyanobacteria excited at 430nm is weaker than for green-algae (Gregor et al., 2005). So the contribution in FL by cyanobacteria to the total FL is lower than that of green algae, and it should not be responsible for the lower FL intensity of filtrated treatments, in which the smallest cells were removed. Pigment FL is tight to physiologic responses and it can change as a consequence of stress (Gratani, 2014). Therefore the lower FL intensity of filtered cultures can be due to the stress of filtration: stressed cells slow down metabolism. The higher FL intensity in communities exposed to the lowest PPCPs treatment (Fig. 4.8.a) is not related to a higher values in biomass accumulation (based on biomass level) compared to controls (Fig. 4.7.a). This means that PPCPs exposure has physiological effects on community Chl-a production or FL and it is in agreement with the results obtained by Gerber (2013) with Mycrocystis aeruginosa. Gerber observed an increased FL after a single TCS exposure indicating enhanced pigment production as a response to stress conditions. Treatment with higher PPCPs dose (100x) shows Chl-a FL levels trend consistent with control but at higher values until day 11, and then values remains constant while Chl-a FL in all other treatments decrease and converge with the control. An experiment conducted on *Selenastrum capricornutus* shows a reduction in photosynthetic efficiency after stressing cultures with three antibiotics (erythromycin, ciprofloxacin and sulfamethoxazole) added individually (Liu et al., 2011). Antibiotics have been shown to interfere with protein synthesis and DNA replication in

Cyanobacteria, and to affect the photosynthetic metabolism and inhibit chlorophyll synthesis (Bishop, 1973). In the experiment conducted by Bishop (1973) on *E. gracilis* the antibiotics (chloramphenicol and cycloheximide) had a concentration of 10 mg/ml while our compounds concentration were between 0,001 and 0,1 mg/mL. In our experiment we used as antibiotic chlarithromycin that is highly toxic for phytoplankton organisms (Isidori et al., 2005; Yang et al., 2008). Despite these differences in species and chemicals used, the effects seems to be similar in our experiment. It is possible that low PPCPs dose can stimulate the Chl-a production while an higher dose inhibit the Chl-a production (hormesis) (Calabrese and Baldwin, 2003). However Chl-a FL in both PPCPs treatments is higher than control. If PPCPs act on organisms inhibiting the photosystem II and reducing the photosynthetic efficiency (Liu et al., 2011), maybe cells can respond by increasing the Chl-a concentration.

5.2 Structural endpoints

During the experiment the four species utilised coexisted without any of them going to extinction (Fig. 4.11). Communities started the experiment with a different structure: filtered cultures show an initially high difference in composition compared to control and communities exposed to PPCPs (Fig. 4.10). D2 showed a peak of dissimilarity at day 6 (Fig. 4.10) that corresponds to the lower total community cell abundance (Fig. 4.11). *S. leoponliensis* and *Sphaerocystis sp.* were the species more affected by the PPCPs mixture and they started growing only after 6 days (Fig. 4.11 a, c). Among all PPCPs, antibiotics (clarithromycin in particular) and β-blockers (atenolol) have the strongest effects on phytoplankton organisms (Isidori et al., 2005; Yang et al., 2008). Atenolol and Clarytromycin are slowly degraded in water (Loos et al., 2009) and are expected to persist in our cultures for all the experimental period. Clarithromycin has been considered a significant environmental risk for aquatic organisms (Zheng et al. 2012). In particular clarithromycin (found in the environment at a concentration between 0,2-2 ng/L^{-1}) showed to have chronic effects on *P. subcapitata* and acute toxicity on *S. leoponliensis* (Zheng et al., 2012). This difference in toxicity can explain the differences in cells concentration between species in high PPCPs dose and control at day 6. A low dose on the contrary seemed not to have affected so strongly the cell growth for any of the species considered (Fig. 4.12).

5.3 Change in size structure

Cell size is a likely indicator of the physiological state of a cell with stressed cells being larger then unstressed (Krüger & Eloff, 1981). Cell size changes (Krüger & Eloff, 1981) and colony formation (Jang et al., 2003) can respond to abiotic factors such as temperature, nutrient and pollution. The increase in length (SWS) of particles exposed to PPCPs mixture (Fig. 4.12.a) during the first days of the experiment was expected in our experiment and is in agreement with results found exposing *Mycrocystis aeruginosa* to TCS (Gerber, 2013). Not expected was the increasing in cells dimension in controls and filtered treatment that is in contrast on what found by Peter (2013), according to which an increase in temperature should make particles smaller. In this experiment all communities showed an enlargement in size through time.

Communities exposed to PPCPs showed, during the first 4 days, a faster response in changing (with particles becoming bigger) than control, maybe due to the fast plastic phenotypic change that occur within hours. In the first 4 days also the diversity in size increase for PPCPs treatment compared to control supporting the hypothesis of a chemical-induced increase in size.

A previous experiment conducted on *Synechococcus* showed microcolonies formation after 3-5 days of exposure to different light intensity and UVR radiation (Callieri et.al, 2011). Considering that phytoplankton generation time is around 3 days we can hypothesize that the increase in size during the first 4 days in PPCPs treatments could be due to an enlargement in cells dimension, while the increase in size after day 4 could be also due to colony or cluster formation. Starting from day 5, control showed an increasing in particles size as well. Because the reduction in cell dimension is the response of cells to an increase in temperature (Peter and Sommer, 2013), the increase in particles size we observed in control was probably due to colony formation that is a response to stress (Jang et al., 2003) such as temperature increase. The size of particles in control and PPCPs treatments in fact reached the values found for filtered communities that are supposed to have formed clusters.

The increase in the number of clusters without an increase in cell size can lead to an increasing in size variance as we observed in the filtered treatments. The increase in diversity in control communities could be due to an increasing in the number of colonies.

6. CONCLUSIONS

This experiment was designed to assess the response of phytoplankton communities to a reduction in trait diversity (through mechanical filtration and PPCPs exposure), in a scenario of environmental change.

We found that the reduction in trait diversity through mechanical filtration did not affect community dynamics (recovery of the same structure and function of control). On the contrary, exposure to high doses PPCPs determined an inhibition in cell growth altering community structure, with an increase in community dissimilarity compared to controls. However the community structures converged among treatments at day 8 and fluctuated in the same range (Fig. 4.10). From an ecological point of view the communities reached a structure similar to control (recovery), but they were functionally different.

Direct and indirect manipulation of traits affect functions in a different way: efficiency in biomass production seemed to be affected more by exposure to chemicals, that determine physiological responses on phytoplankton, than to a change in trait diversity by direct manipulation. Exposure to PPCPs slowed down the adaptive capacity of communities to track an environmental change with a dose-dependent effect. These findings highlight the importance of physiological responses for ecosystem processes.
Filtration did not affect the ability to track the environmental changes compared to control. The temporal trajectory of these treatments (in production of biomass, community structure and trait diversity) followed the same trajectory of control. Exposure to low PPCPs on the contrary showed a deviation on the structure and biomass production compared to control with a dose-dependent effect. Exposure to PPCPs slow down the ability of communities to track an environmental change.

We have to consider that adaptive responses of organisms and communities in laboratory conditions can only partially predict responses in the wild due to complexity of natural ecosystem. The challenge in the future is therefore to assess responses of natural communities to realistic environmental levels of contaminants in the natural environment. Furthermore, being phytoplankton at the basis of the trophic chain, an alteration on the phytoplankton biomass production can strongly alter, by bottom-up processes, all the other trophic levels in aquatic ecosystems and have consequences on ecosystem services as O_2 production (Wilson, 2004) and fisheries.

7. REFERENCE

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8. APPENDIX

8.1 Experiment protocol

Protocol 1: Single species growth curve

Stock culture of the following species: Synechococcus leoponliensis, Pseudokirchneriella subcapitata, Sphaerocystis sp, Scenedesmus obliquus

- Optical Density (OD750nm) was adjusted by diluting the cultures with WC-Medium to reach OD750nm=0,1
  (see the appendix: protocol 7A for OD measure)

- Grow the species (OD750nm= 0,1) in the incubator Multitron in specific conditions:

- 18°C
- Light cycle: 16h light/ 8h dark
- Light intensity: 56µE/m-1s-1
- Shaking: 20 RPM

- Take a sample every 2 days and measure OD750nm until OD reach the maximum (stationary phase of the grow curve)

Protocol 2: Measuring the cell size with scanning flow cytometry (Cytobuoy)

Materials:
- single species culture
- milliQ water
- 5 mL sterile pipette
- 4 vials (20mL)
- Flow cytometer Cytobuoy
- glutaraldehyde (MilliQ solution 1%)
- EtOH 70%

Procedure:
- take a culture
- dilute culture 1:20 for a total volume of 10 mL in a 20 mL vial

- measure (manually) the culture with the Cytobuoy with following settings:

- number of particles analysed: 30000
- trigger signal SWS: 28mV
- flow rate (µL/sec): 1,99

Analyse the raw data using the program CytoClus 3.

Protocol 3: Measuring the dry mass of the cultures

Materials:

- single species culture
- 96 -well microplate, PS, F-bottom, crystal-clear
- sterile 1 mL pipette tips
- micro pure water (Milli-Q)
- GF/F, 47 mm Ø glass microfiber filters (WHATMANTM)
- vacuum filter apparatus
- aluminium foil
- oven for 48°C
- oven for 480°C
- analytical balance (1mg accuracy)
- desiccator with silica sand
- EtOH 70%
- microplate reader (Spectra Max 190)

Procedure:

- Perform a serial dilution of each culture (1, 1:3, 1:9, 1:27, 1:81, 1:243) diluting the cultures with MilliQ water. Final volume: 60 mL.
- Take a 250 uL sample of each culture on a 96-well microplate and measure the OD750nm with the microplate reader (spectra Max 190)
-
- Wrap the GF/F filters into the aluminium foil and sterilize them in an oven for 2 hours at 480°C.
- Place the filters in the desiccator and weight the filters with the analytical balance (tare)
-
- Prepare and rinse the vacuum filter apparatus first with EtOH 70% and then with MilliQ water
- Put a filter into the filter apparatus
- Filter a culture

- Clean the cylinder funnel of the vacuum filter apparatus with milliQ water before removing the filter
- Remove the filter when it is not dripping anymore and wrap it into the aluminium foil
- Rinse the filter apparatus first with EtOH 70% and then with MilliQ water between the samples and dry the cylinder funnel with clean paper

- Dry the filters in the oven at 48°C for 48 h
- After 48h put filters into the desiccator
- Weight the filters with the analytical balance (wet weight)

Calculate the wet weight by subtracting the weight of the empty filter (tare) to the weight of the filter with cells, then divide the value of the wet weigh for 60 to obtain the weight for 1mL of culture.

- Put filters in the oven at 480°C for 48 h
- Put filters in the desiccator
- Measure the weight with the analytical balance (dry weight)

Calculate the dry weight by subtracting the weight of the empty filter (tare) to the weight of the filter with cells, and then divide the obtained number by 60 to have the value of the dry weight for 1mL of culture.

Protocol 4: Culture assemblage

Materials:
- 96-well microplates, , PS, F-bottom, crystal-clear
- sterile 1mL,5 mL, and 50 mL pipette tips
- sterile 300 mL graduated tubes
- 2 L erlenmayer flask
- single species cultures (Synechococcus leoponliensis, Pseudokirchneriella subcapitata, Sphaerocystis sp, Scenedesmus obliquus)
- Glutaraldehyde fixative solition 1%
- LUGOL fixative solution
- One 20mL vial
- One Eppendorf tube (1 mL)
- microplate reader (Spectra Max 190)
- microplate reader (TECAN)

Procedure:

- Prepare 2 replicates of each species and place them into the incubator at 18°C. Grow the cultures until they reach optical density, OD750nm, 0,2 (approximately)
- measure OD750nm and calculate how much volume take from each species to prepare the mother culture:

- obtain the dilution factor by dividing the measured OD750nm for the OD750nm that correspond to 0.06mg/mL.
- divide the volume of final culture (1000 mL) for the dilution factor to obtain the volume of culture to take from each species.

- take the calculated volume from each culture and assemble them in a 2 L erlenmeyer flask (mother culture)
- add WC-medium to reach total volume of 1000 mL if necessary
- take 250 uL of the mother culture on a 96-well plate and measure:

- OD750nm with with microplate reader (spectra Max 190)
- Chla fluorescence intensity with the microplate reader (TECAN)

- sample 5 mL of the mother culture and add it into a 20mL vial. Then fix it with 50 µL Glutaraldehyde fixative solution for flow cytometry
- sample 400 µL of the mother culture and place it in a 1mL eppendorf. Then fix it with 10 µL LUGOL for microscopy

Protocol 5A: Treatment with filtration

Materials:

- autoclaved filter apparatus
- 5 µm, 47 mm Ø Nucleopore PC filter
- 8 µm, 47 mm Ø Nucleopore PC filter
- 12 µm, 47 mm Ø Nucleopore PC filter
- GF/F, 47 mm Ø glass microfiber filters
- 15 Erlenmayer 100mL autoclaved flasks
- 18 Erlenmayer 300 mL autoclaved flasks
- 6 autoclaved 100 ml beakers
- 18 Sterile 50 mL syringe (with eccentric tip)
- 2 L WC- growth medium
- micro pure water (milliQ)
- vacuum filter apparatus

Procedure for filtering 1 (F1): remove the extreme upper and lower 10% of the cell size di-

stribution
sample 390 mL of culture and split it in 3 autoclaved Erlenmayer flasks (= 130mL each replicate)

- place 12 µm Nucleopore PC filter into the autoclaved filter apparatus
- take the culture with a sterile 50 mL Luer eccentric tip syringe and filtrate the culture (keep this filtered solution)*
- pass air through the filter apparatus after all the culture have been filtered with the syringe in order to dry the filter

- put another flask under the filter apparatus. Take another sterile syringe and fill the syringe with clean WC –growth medium and pass through the filter.
- pass again air through the filter apparatus to dry the filter
- (throw this filtrated solution away)
- clean the filter apparatus with milliQ water and Bleach for 5 min rinse with milliQ water and dry it with a clean tissue

- place a clean 5 µm Nucleopore PC filter into the clean filter apparatus
- take a new sterile syringe and filter the already filtered solution*
- pass air throw filter apparatus after filtering

- filter the WC-medium out: filter again the filtrated solution with GF/F glass microfiber filter (with the vacuum filter apparatus) in order to get rid of the cells. Keep only the old medium (keep this filtered solution)
- put 13,75 mL old medium (from the filtration with GF/F filter) into a autoclaved beaker and add 41,25 mL of fresh WC -growth medium to reach final volume of 55 mL *
- in order to collect cells for the new culture after filtration: flush the cells from the filter (5 µm Nucleopore PC filter including the cells) in a beaker with the WC –growth medium*
- put the new culture into a 100 mL autoclaved Erlenmayer flask

- clean the filter apparatus in a beaker with bleach (50%) for 5 min rinse with milliQ water and dry it with a clean tissue
- Repeat the procedure for the other 2 replicates

Procedure for filtering 2 (F2): to get rid of the lower 20% of the cell size distribution
sample 390 mL of mother culture and split it into 3 autoclaved Erlenmayer flasks (=130mL each replicate)

- Place a 8 µm Nucleopore PC filter into the filter apparatus

- take culture with sterile 50 mL eccentric tip syringe and pass the culture through the filter apparatus
- pass air throw the filter apparatus with syringe to dry the filter
- (keep this filtered solution)

- filter the WC-medium out: filter again the filtrated solution with GF/F glass microfiber filter (with the vacuum filter apparatus) in order to get rid of the cells. Keep only the old medium (keep this filtered solution)
- put 13,75 mL old medium (from the filtration with GF/F filter) into a autoclaved beaker and add 41,25 mL of fresh WC -growth medium to reach final volume of 55 mL *

- in order to collect cells to have a new culture after filtration: flush the cells from the filter (8 µm Nucleopore PC filter including the cells) in a beaker with the WC –growth medium*
- put the new culture into a 100 mL autoclaved Erlenmayer flask

- clean the filter apparatus with bleach and milliQ water and dry it with a clean tissue
- repeat the procedure for the other 2 replicates

Protocol 5B: Treatment with chemical mixture

Materials:
- sterile 100 µL and 1 mL pipette tips
- chemical stock mixture in EtOH 100%: Chemical mixture was 20000 times higher than the background levels in the main European rivers (Pomati, 2006). See protocol 8E for mixture stock solution preparation.
- 3 erlenmeyer 100mL flasks
- a culture

Procedure Dose 1:
- sample 150 mL of mother culture and split it into 3 autoclaved Erlenmeyer flasks (3 replicates)
- Add into each replicates 27,5 µL of the chemical mixture

Procedure Dose 2:
- sample 150 mL of mother culture and split it in 3 autoclaved Erlenmeyer flasks (3 replicates)
- Add to each of the 3 replicas 275 µL chemicals mixture

Protocol 5C: prepare Controls

Materials:
- sterile 1mL pipette tips
- EtOH 100%

Procedure:
- to have the controls: sample 165 mL of the culture and split it into 3 autoclaved Erlenmeyer flasks (3 replicates)

- in order to have the same amount of EtOH in each replicates add the following volume of EtOH 100% :

- control: add 275 µL of EtOH 100% in each replicate
- treatment F1: add 275 µL EtOH 100% in each replicate
- treatment F2: add 275 µL EtOH 100% in each replicate
- treatment Dose 1: add 247,5 µL EtOH 100% in each replicate
- treatment Dose 2: do not add EtOH 100%

Protocol 5D: Increasing the temperature

- Place cultures into an incubator (Multitron 2). Select following conditions:

- 18°C
- Light cycle: 16h light/ 8h dark
- Light intensity: 56µE/m-1s-1
- Shaking: 20 RPM

- increase the temperature 1°C every day starting from 18°C to 34°C (17 days)

Take 5 mL sample every day from each replicates for analysis and replace it with 5mL WC-growth medium (see protocol 6)

Protocol 6: samples for analysis

Materials:
- 96 -well microplate, PS, F-bottom, crystal-clear
- sterile 100 µL and 1 mL pipette tips5 mL plastic sterile pipette
- 20 mL vials
- 39 eppendorf vials
- fixative LUGOL for microscopy samples

- fixative Glutaraldehyde (1%) for flow cytometry samples (work in the fume hood!!!)
- 75 mL WC –growth medium

Procedure:
- sample 5 mL of each culture every day and put it into the 20 mL vials
- add 5 mL WC- growth Medium to each replicates (from day 1 onwards)
- place the cultures back in the incubator increasing the temperature of 1°C

- from the 5mL samples take:
  - 250 mL of sample from the 5mL sample. Put this sampe into the microplate. Measure the OD750nm (see protocol 7A)and Chlorophyll a (Chla) fluorescence intensity(see protocol 7B) Do these analysis immediately with fresh sample
  - 500 µL of sample. Place this sample into a 1mL Eppendorf tube and measure Orthophosphate (see protocol 7C). Do the measure with fresh samples
  - 500 µL of sample (only for treatments Dose 1 , Dose 2 and Control). Place it in a 1mL eppendorf tube for High-performance-liquid-chromatography (HPLC) and put them into a freezer (-20°C)
  - 400 µL of sample. Place it in 1mL eppendorf tube for microscopy (see protocol 7F). Add LUGOL (10 µL) and store the samples at 4°C (for LUGOL fixative solution see protocol 8C)
  - add Gluteraldehyde fixative solution (1:100) in each 20mL vial with the remaining volume of culture for the flowcytometry : 40µL for the filtered treatment, 35 µL for other treatment and control. Place samples at 4°C.
  - (For Flowcytometry see protocol 7E. For Glutaraldehyde fixative solution see protocol 8B)

Protocol 7A: optical density analysis

Take 250 µL of fresh sample and place it in a microplate (Microplate, 96 well, PS, F-bottom, crystal-clear).
Set the microplate reader (Spectra Max 190) at 750 nm wavelength and measure the absorbance.

Protocol 7B: Chla fluorescence intensity

Material:
- microplate, 96 well, PS, F-bottom, crystal-clear

- sterile 1mL pipette tips
- microplate reader TECAN infinite 200

Procedure:
Place 250 mL of fresh sample in the 95 well-plate (PS,F-bottom,crystal-clear) and measure the Chla fluorescence intensity with the instrument TECAN infinite 200.
Instrument setting:
- excitation wavelength: 435 nm
- emission wavelength: 685 nm
- mode: top
- gain: optimal

Protocol 7D: Flow cytometry manual measurements

Materials:
- 10 mL samples
- 10 mL EtOH 70% in a 20 mL vial
- glutaraldehyde sheat fluid 1% in a 20 mL vial
- flow cytometer CytoBuoy

Procedure:
- turn on the CytoBuoy
- open the CytoBuoy inlet and outlet tubes
- place the outlet tube in a glass empty beaker (waste)
- place the inlet tube in the EtOH 70% vial
- turn on sample pump for 1 min (max speed)
- turn on the sheat pump for 2 min (max speed)
- place the inlet tube in the Glutaraldehyde sheat fluid
- turn on the sample pump for 1 min (max speed)
- turn on the sheat pump for 2 min (max spead)
- set the instrument
- place the inlet tubes in the first sample
- run the measurement

Protocol 7E: Flow cytometry measurements with autosamples

Materials:

- 1,5 mL short thread Vials 32x11,6 mm (clear), wide opening
- ND9 short thread Screw yellow Caps (without septa)
- 10mL EtOH 70% in a 20mL vial
- glutaraldehyde sheat fluid in 20 mL vial
- Gynkotek GINA 50 autosampler
- 50 mL flask with Glutaraldehyde sheath fluid 25% connected to the autosampler with a tube
- 1mL pipette tips
- flow cytometer CytoBuoy

Procedures:

- check the glutaraldehyde level in the 50mL flask: the tube should always be immersed in the liquid
- plug the autosampler trigger
- turn on the autosampler and the CytoBuoy
- open the CytoBuoy inlet and outlet tubes
- place the outlet tube in a glass empty beaker (waste)
- place the inlet tube in the EtOH 70% vials
- turn on sample pump for 1 min (max speed)
- turn on the sheat pump for 2 min (max speed)
- place the inlet tube in the Glutaraldehyde sheat fluid
- turn on the sample pump for 1 min (max speed)
- turn on the sheat pump for 2 min (max spead)
- connect the inletCytobuoy tube to the autosampler inlet tube
- turn on sample pump for 1 min (max speed)
- turn on the sheat pump for 2 min (max speed)
- prepare Blank: put 650 µL Glutaraldehyde sheat fluid in 2 Vials
- prepare samples: take 650 µL sample with the pipette and put it in the glass Vial
- put the yellow cap without membrane
- put samples in the autosampler (put blanks as the first and last measurements)
- turn on Lasers and let them warm up for 3 min
- set the Cytobuoy:

- SWS trigger: 31 mV
- Smart trigger: FL.Red total >5
- Flow rate (µL/sec): 1,99

- set the autosampler:

- start probenort: 1
- eispritzvolumen: 250µL
- analysenzeit: 6,4 min
- wieder holungen: 0
- standard interval: 9999
- standard volumen: /
- standard analysenzeit: /
- racktyp: 1

- press Start in the autosampler to run measures

- clean the instrument after use:

- turn on samplepump for 1 min
- turn on sheat pump for 2 min
- disjoin autosampler and CytoBuoy inlet tubes
- place the CytoBuoy inlet tube in the container with EtOH 75%
- turn on sample pump for 1 min
- turn on the sheat pump for 2 min
- close the inlet and outlet CytoBuoy tubes

Protocol 7F: Determination and counting cells visually

Materials:

- hemocytometer Neubauer
- cover glass
- pipette tips and 100µL pipette
- 95% EtOH
- optical microscope Nikon Eclipse 80i

Procedure:

- clean the hemocytometer with EtOH 95% and let it dry
- put cover glass on top
- take 15 µL sample and put it onto the two outer glass-bars
- let the culture be sucked under the glass until square is filled
- let cells settle down for 30s
- count the number of cell per species at the optical microscope

For high cell densities: count cells in 4 mini-squares (in 4 squares in the central big square)
Cells/µL= #cells / (0.00025µm2 x0.1mm)

For high cell densities: count cells in 4 middle-squares (in the 4 external squares)
Cells/µL= #cells / (0.00625µm2 x0.1mm)

For low cell densities: count all cells in big-square (in the 4 external squares)
Cells/µL= #cells / (0.1µm2 x0.1mm)

Protocol 8A: WC–Medium preparation

Composition of Freshwater Medium WC for cultivation of Cyanobacteria and Green algae (Tab.1)

Tab. 1: Chemical composition of WC Medium used for culturing Cyanobacteria and Green algae

WC Medium		
Compounds	**Stock Solution (g/l)**	**Nutrient Solution**
$CaCl_2 \cdot 2H_2O$	36,8	1 ml
$MgSO_4 \cdot 7H_2O$	37	1 ml
$NaH\ CO_3$	12,6	1 ml
$K_2HPO_4 \cdot 3H_2O$	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 mg/l, $FeCl_3 \cdot 6H_2O$ 3,15 mg/l, $CuSO_4 \cdot 5H_2O$ 0,01 mg/l, $ZnSO_4 \cdot 7H_2O$ 0,022 mg/l, $COCl_2 \cdot 6\ H_2O$ 0,01 mg/l, $MnCl_2 \cdot 4H_2O$ 0,18 mg/l, $Na_2MoO_4 \cdot 2H_2O$ 0,006 mg/l, H_3BO_3 1,00 mg/l.

Preparation for 1L in glass bottle:
- dilute 0.115g buffer (TES; No. 53) in 500ml „Semidest" (deionised) water
- stirr it well

- add 1mL stock solutions (Tab.1)
- fill up bottle with „Semidest" water
- autoclave for 40 min (120°C)

Protocol 8B: Fixative solution for cyanobacteria and green algae flowcytometrie

Preparation for 100 ml, work under the fume hood!!!

- dissolve 1 gram paraformaldehyde in 50 ml H2O at 65°C
- add during heating 1-3 droplets 1 M NaOH to make solution clear
- add 40 ml 25% glutaraldehyde
- adjust pH to 7.0 and bring to 100 ml
- filter solution with Nucleopore filter (0.2 µm pore size)
- store at 4-7°C in the dark

End concentration is 0.01% PF and 0.1 % GA.
Use: Add 1:100 to samples.

Protocol 8C: Sheath fluid for CytoBuoy preparation

dilute 1:100 the fixative solution for flow-cytometry (0.01% PF and 0.1% GA) with millipure water (MilliQ).

Protocol 8D: LUGOL fixative solution for microscopy (C2H3NaO2)

For LUGOL preparation add in order and stir well:
- KI 60g
- H_2O 120mL
- I 30g
- H2O 300mL
- Na-acetat 30g

Use: Add 1:100 to samples.

Protocol 8E: chemical mixture preparation

Dilute in a dark vial the solution (1g/L) of each compound with EtOH 100% to achieve the concentration shown in the following table and stir well:

WC Medium	
Compounds	**Target Concentration (mg/l)**
carbamazepine	20,0
clarythromycin	20,0
diclofenac	20,0
hydrochlorothiazide	20,0
atenolol	20,0

ranititine	2,0
sulfametholxazole	2,0

bezafibrate	0,2
furosemide	0,2
ibuprofen	0,2
triclosan	0,2

Place the vial at -4°C in the dark.

8.2 OD750nm Measures during the experiment period

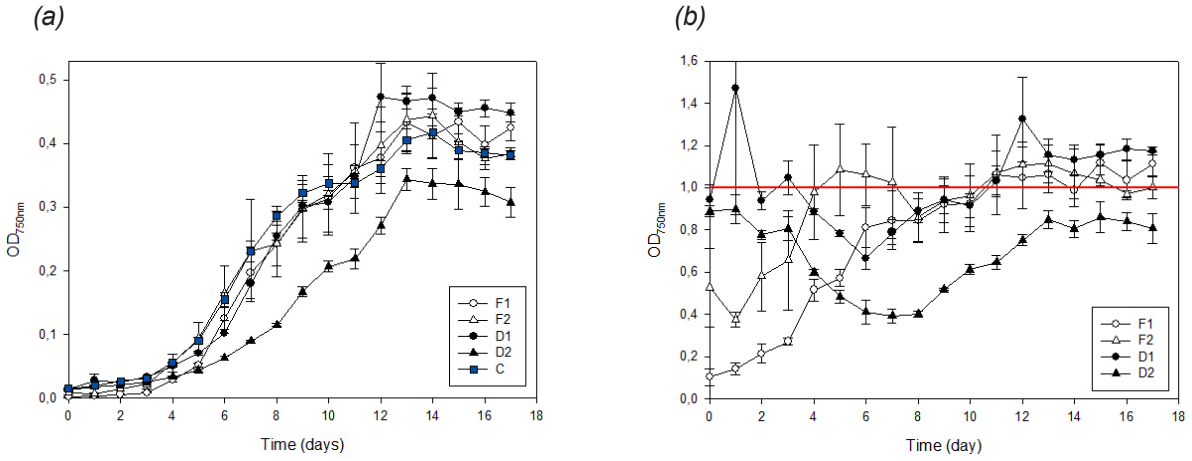

Fig. 8.2: (a) OD_{750nm} during experiment (without replica F2.1). (b) OD_{750nm} rate between treatments and control (without replica F2.1).

8.3 Results with replica F2.1

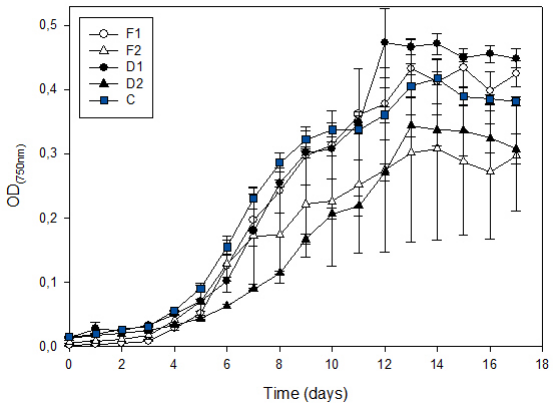

Fig. 8.3.1: OD_{750nm} measured during the experimental period (Including replica F2.1)

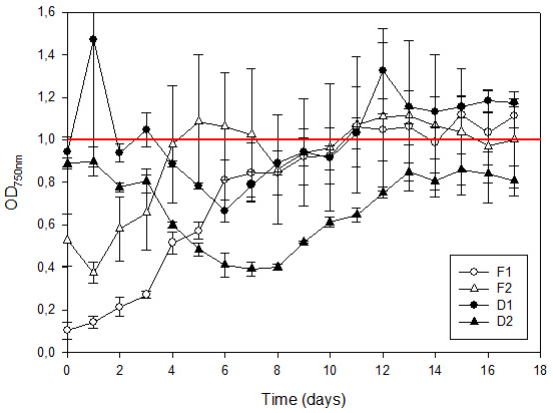

Fig. 8.3.2: OD_{750nm} ratio between tratments and control (Including replica F2.1)

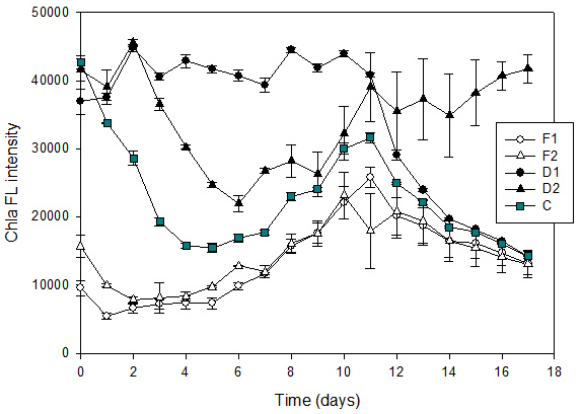

Fig. 8.3.3: Chl-a FL intensity through time (Including replica F2.1)

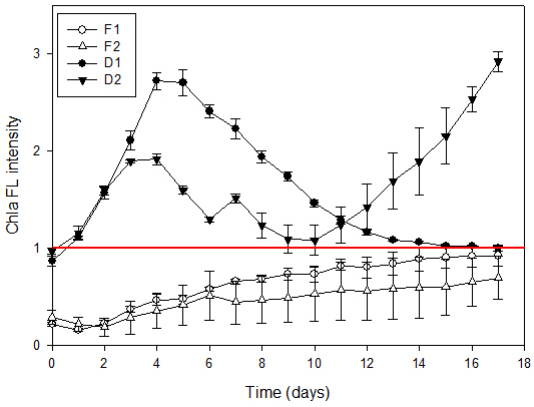

Fig. 8.3.4: Chl-a FL intensity ratio between treatments and control (Including replica F2.1)

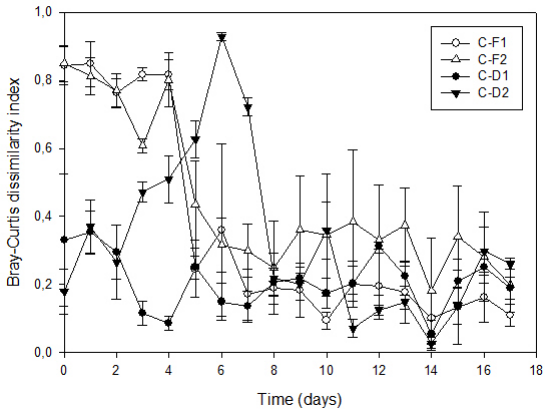

Fig. 8.3.5: Bray-Curtis dissimilarity index between treatments and control (Including replica F2.1)

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