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Mariana Alves Pires. Regulation at the gene expression level of nitrogen acquisition in a nitrogen-fixing cyanobacteria: implications in ecological interactions

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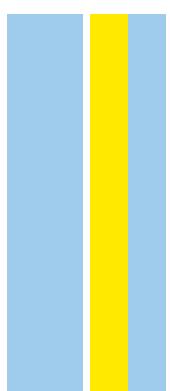
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**Regulation at the gene expression level of nitrogen acquisition
in a nitrogen fixing-cyanobacteria: implications in ecological
interactions**

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Toxicology and Environmental
Contamination submitted to the Abel
Salazar Biomedical Sciences Institute
from the Porto University.

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Abstract

A strain of the freshwater cyanobacteria *Phormidium* sp., available in our laboratory culture collection, was found to produce nitrogen-rich toxic compounds, called portoamides (Leao, et al. 2010). These compounds were shown to have an important role in planktonic community interactions.

In previous works, the production of portoamides during *Phormidium* sp. growth was shown to increase in the presence of microalgae competitors and with high consumption of inorganic nitrogen (nitrate). On the other hand, *Phormidium* sp. growing alone almost does not consume inorganic nitrogen and produces very few amounts of portoamide.

The aim of this project was to determine if the strategy of nitrogen acquisition by *Phormidium* sp. (fixation/uptake of inorganic forms), is regulated at the level of gene expression, induced by the presence/balance of competitors, and its implications for the production of portoamides. The experimental design consisted in long-term incubations of *Phormidium* in the presence/absence of a competitor and also a natural community of phytoplankton, in this later case both at low and high concentrations of nitrate. Regularly will be performed analysis of expression levels of genes selected among those related with nitrogen fixation, nitrate uptake and portoamide synthesis.

Due to difficulties in the extraction of genetic material from our strain of *Phormidium* sp., many replicates of these experiments were lost. In addition, probably due to contamination by bacterial RNA, many samples did not show expression of the house-keeping gene selected as a control. For these reasons, the results of this part of the work were not conclusive.

Another aim of the present work was to study the role of these allelochemical compounds in the structure and diversity of phytoplankton communities in interaction with the effect of predation. For this aim were incubated phytoplankton communities sampled from a freshwater pond and cultured in long-term laboratory continuous cultures. In these continuous cultures, these communities were treated as controls, other inoculated with our allelopathic species (*Phormidium* sp.), others with zooplankton predators (rotifers) and others with both the allelopathic species and the predators. The results of phytoplankton counting revealed an increased diversity and evenness of the communities with time, in which a few chlorophytes and diatoms dominated the total biomass. All measures of diversity employed indicated that the presence of the allelopathic species favoured greater diversity, and also the presence of predators. When both factors were in interaction, diversity was increased in an

additive way. This increase in diversity, however, was not due to an increase in species richness, but to an increase in evenness.

Resumo

Numa estirpe da cianobactéria *Phormidium* sp., disponível na coleção do nosso laboratório, foi detectado produção de compostos alelopáticos ricos em azoto, chamados portoamidas (Leão, et al. 2010). Estes compostos apresentaram um papel importante nas interacções das comunidades planctónicas.

Estudos anteriores mostraram que a produção de portoamidas em *Phormidium* sp., aumentava na presença de microalgas competidores e também aumentava com elevado consumo de azoto inorgânico (nitrato). Por outro lado, se *Phormidium* sp., estiver em monocultura, consome pouco azoto inorgânico e produz poucas portoamidas.

O primeiro objetivo deste trabalho é determinar se a estratégia de aquisição de azoto por *Phormidium* sp. (fixação, captação de formas inorgânicas), é mediada, ao nível de expressão genética, pela presença/ausência de competidores e em consequência, a produção de portoamidas. Outro objetivo foi estudar o efeito da alelopatia das portoamidas na diversidade de uma comunidade fitoplanctônica em presença e ausência de um competidor.

A experiência consistiu na incubação de *Phormidium* sp. em sistemas de longa duração, tanto em monocultura (apenas *Phormidium* sp.) como em policulturas (*Phormidium* sp. juntamente com competidor). Nas amostras recolhidas destas culturas, foram feitas regularmente análises da expressão de genes relacionados com a fixação de azoto, consumo de nitrato e síntese de portoamidas.

Devido a dificuldades na extração do material genético da nossa estirpe de *Phormidium* sp., muitas réplicas desta experiência foram perdidas. Além do mais, devido a uma possível contaminação bacteriana, muitas amostras não mostraram expressão do gene selecionado como controlo. Devido à ausência de expressão deste gene em muitas das amostras, não foi possível obter resultados conclusivos.

Relativamente ao outro objectivo do trabalho, foi cultivada uma comunidade de fitoplâncton em sistemas de cultura continua por um período de mais de 40 dias. Foram estabelecidos os seguintes tipos de tratamento: controlo (só a comunidade natural de fitoplâncton), presença de *Phormidium* sp., presença de (rotíferos) e um tratamento com *Phormidium* sp. e os predadores. Os resultados de contagem do fitoplâncton mostraram que houve um aumento de diversidade das comunidades com ao longo do tempo, onde clorofíceas e diatomáceas dominaram a biomassa. Todas as medidas utilizadas para estudar a diversidade, mostram que a presença da espécie

alelopática favorece a diversidade e também a presença de predadores. Quando ambos estes factores interagem, a diversidade também aumenta numa forma aditiva. Este aumento de diversidade não foi devido à riqueza das espécies, mas devido ao aumento da equidade entre elas.

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Abbreviation list

16S RNA – 16S Ribosomal RNA

Ank – *Ankistrodesmus falcatus*

Bp – Base pair

C – Control

CaCl₂.2H₂O – Calcium chloride dihydrate

cDNA – Complementary DNA

CoCl₂.6H₂O – Cobalt chloride hexahydrate

cphA – Cyanophycin synthase

CuSO₄.5H₂O – Cooper (II) sulfate pentahydrate

DMS - Non-metric multidimensional scaling

DNA – Deoxyribonucleic acid

Fe-EDTA – Ferric ethylenediaminetetraacetic acid

gDNA – Genomic DNA

H₃BO₃ - Boric Acid

K₂HPO₄ - Dipotassium hydrogen phosphate

KBr – Potassium bromide

KNO₃ - Potassium Nitrate

LEGE – Blue Biotechonology and Ecotoxicology

MgSO₄.7H₂O – Magnesium sulfate heptahydrate

MnCl₂.4H₂O – Manganese (II) chloride tetrahydrate

N – Nitrate

Na₂MoO₄.2H₂O – Sodium molybdate dihydrate

NaHCO₃ - Sodium bicarbonate

narB - Nitrate reductase

nifH – Nitrogenase reductase

ntcA – Global nitrogen regulator

P – Phosphate

PCR – Polymerase chain reaction

Ph3 – *Phormidium* sp. chemostast number 3

Ph4 – *Phormidium* sp. chemostasts number 4

Ph7+Ank – *Phormidium* sp. and *Ankistrodesmus falcatus* chemostasts number 7

Ph8+Ank - *Phormidium* sp. and *Ankistrodesmus falcatus* chemostasts number 8

Ph – *Phormidium* sp.

Ph+R – *Phormidium* sp. and rotifers

QS – Quorum Sensing

R – Rotifers

RNA – Ribonucleic Acid

rRNA – Ribosomal ribonucleic acid

RT-PCR – Reverse transcription polymerase chain reaction

secA – Preprotein translocase subunit SecA

Sol – Solution

V₂O₅ - Vanadium pentoxide

ZnSO₄.7H₂O – Zinc sulfate heptahydrate

1. Introduction

1.1. Cyanobacteria

The cyanobacteria are an abundant and diverse group of photoautotrophic prokaryotes. These microorganisms, also named blue-green algae, are divided into the following orders: Chroococcales, Chroococcidiopsidales, Gloeobacterales, Nostocales, Oscillatoriales, Pleurocapsales, Spirulinales, Synechococcales, *Incertae sedis* and Plastids. They are usually found in all types of water masses around the globe, typically fixate atmospheric nitrogen and are important contributors to global carbon fixation.

The ability to adapt to different environments, is characteristic of cyanobacteria, because they have developed adaptations to extreme temperatures, pH, radiation and drought (Hruedy, *et al.* 1999). They are important for biotechnological applications due to a rich secondary metabolism (Nagarajan, *et al.* 2013).

Cyanobacteria were probably the first oxygen producers as photoautotrophs. Its vital processes require only water, carbon dioxide, inorganic substances, and light, with photosynthesis being its main source of energy (Hruedy, *et al.* 1999).

1.2. Eutrophication

In recent decades, eutrophication has been rapidly increasing in continental and coastal waters. This phenomenon is mainly due to agricultural and livestock waster increasing nutrient loads (Pearl & Huisman 2008). One consequence associated with this phenomenon is the increase in occurrence of toxic cyanobacterial blooms (Huisman *et al.* 2018). Blooms consist in the development of abnormally high levels of biomass of a species of aquatic microorganism (or a few of them). Although these phenomena are natural in the seasonal cycle of phytoplankton communities, they can have devastating consequences depending on the species that proliferates due to the toxins that they might release. For example, neurotoxins or hepatotoxins, produced by several genus of cyanobacteria (Carmichael 1997). The increase in water temperature due to climate change is another aspect that favours cyanobacteria blooms, since the latter have a higher optimal temperature of growth than most eukaryotic algae (Whitton, *et al.* 2012).

The most common cyanobacteria to produce toxins blooms are from the genera *Dolichospermum* (previously known as *Anabaena*), *Planktothrix* and the most abundant, *Microcystis*. The latter contains gaseous vesicles, that allows for buoyancy (Walsby 1994), allowing for this genus to be dominant in almost every stall water mass.

1.3. Nitrogen metabolism of cyanobacteria

The ability to perform nitrogen fixation is an important trait of cyanobacteria. They are the most abundant microorganisms that can perform nitrogen fixation (Zehr 2011) they have an important role in the nitrogen cycle in the ocean-atmosphere fluxes. The expression of the genes needed for nitrogen fixation is part of a complex genomic expression stimulus (Ohashi, et al. 2011). An alternative to the nitrogen fixation is the uptake of inorganic forms, like nitrate. This way of nitrogen acquisition is more efficient than fixation. The regulation of metabolism at genomic level will promote one or the other way, as a result of environmental conditions.

Since nitrate concentrations are usually low in the environment, photosynthesis organisms are required to have a system that will uptake rapidly the nitrate before the reduction takes place. It is believed that the system that cyanobacteria use, is linked to the photosynthesis itself. (Flores, et al. 2005).

When the nitrogen becomes a limiting resource, there's an accumulation of 2-oxoglutarate (2-OG). This accumulation will lead to the activation of nitrate assimilatory genes (Ohashi, et al. 2011)

It was shown that concentrations of ammonia above 1×10^{-5} M inhibit the uptake of nitrogen in *Anabaena cylindrica* (Ohmori, et al. 1977) however this study didn't show the mechanism behind. Other studies (Flores, et al. 2005) suggested that this could be achieved by inactivation of glutamine synthetase.

1.4. Allelopathy in cyanobacteria

The term allelopathy was first labelled by Molisch (1937) and later redefined (Rice 1984) to chemical interactions between microorganisms or plants mediated by chemical compounds, that can favour or inhibit the growth. This phenomenon was first addressed in natural aquatic systems by Keating 1977. Nowadays many cyanobacteria have been identified to produce allelopathic compounds. Fig.1 shows pictures of some

of these species:



Figure 1: Figure taken from Sliwinska et al 2021. A) *Microcystis* sp; B) *Nostoc* sp; C) *Synechococcus* sp. Scale bar = 10 µm.

The allelopathic compounds could provide significant advantage to their producer over other species in a competitive scenario. This means that allelopathy can make an impact in the structure and diversity of the phytoplankton community (Legrand, et al. 2003), (Felpeto, et al. 2018). It has been hypothesized that allelopathic properties are a factor contributing to development of cyanobacteria blooms (Legrand, et al. 2003).

1.5. Ecological implications of allelopathy in phytoplankton communities

The principle of ecological exclusion states that if two species compete for the same limiting resource, the best adapted of the two will competitively exclude the other one (Hardin 1960). This principle was extended to a theoretical model by Tilman (1981) through the R* theory, employing competition for nutrients in phytoplankton as a model mechanism. According to this R* theory, only n species could coexist in equilibrium in n limiting resources (Tilman 1981). Because phytoplankton communities share a few resources (a few forms of nitrogen, phosphate and light) their biodiversity is paradoxically high. Trying to find explanations for this paradox, ecologists came across with several potential mechanisms that could enhance biodiversity: differential predation, complex life cycles, chaotic dynamics allelopathy (summarized in (Roy & Chattopadhyay 2007)). Some of these mechanisms were demonstrated in theory but not in experimental systems.

The effect of allelopathy promoting diversity at intermediate strength was recently demonstrated in a two-species system (Felpeto, et al. 2018) and a natural planktonic community driven to equilibrium in the laboratory (Barreiro Felpeto et al. unpublished) employing a strain of the cyanobacterium *Phormidium* sp. as a model allelopathic species. With weak allelopathic effect, the best competitors will win competition and dominate the community. With strong allelopathic effect, the allelopathic species dominates the community, and at intermediate allelopathic effect, there is coexistence and hence, higher diversity (Felpeto, et al. 2018).

The strength of allelopathy was determined by the initial abundance of the species. However, this species was shown to modulate the allelopathic effect depending on the environmental conditions and the mechanism by which this modulation happens is unknown. Also, the interaction of this mechanism with other important mechanisms enhancing phytoplankton biodiversity (differential preparation) is unknown.

Phormidium sp from the order Oscillatoriales is a filamentous cyanobacteria that can be found primarily in shallow and calm freshwater water masses, in benthic or tychoplanktonic (accidentally in the water column) habitats.

In a strain of *Phormidium* sp. from our laboratory culture collection (<https://lege.ciimar.up.pt>), were isolated and identified, using bioassay-guided fractionation, a group of allelochemicals compounds called portoamides (Leão, et al. 2010) (Fig. 2). These compounds are small cyclic peptides and were shown to inhibit growth of several microalgae, bacteria and cyanobacteria (Leão, et al. 2010; Antunes et al. 2019; Monteiro, et al. 2021). Their mechanism of action seems to be interfering with the respiratory chain (Ribeiro, et al. 2017) (Sousa, et al. 2019) thereby affecting energy production.

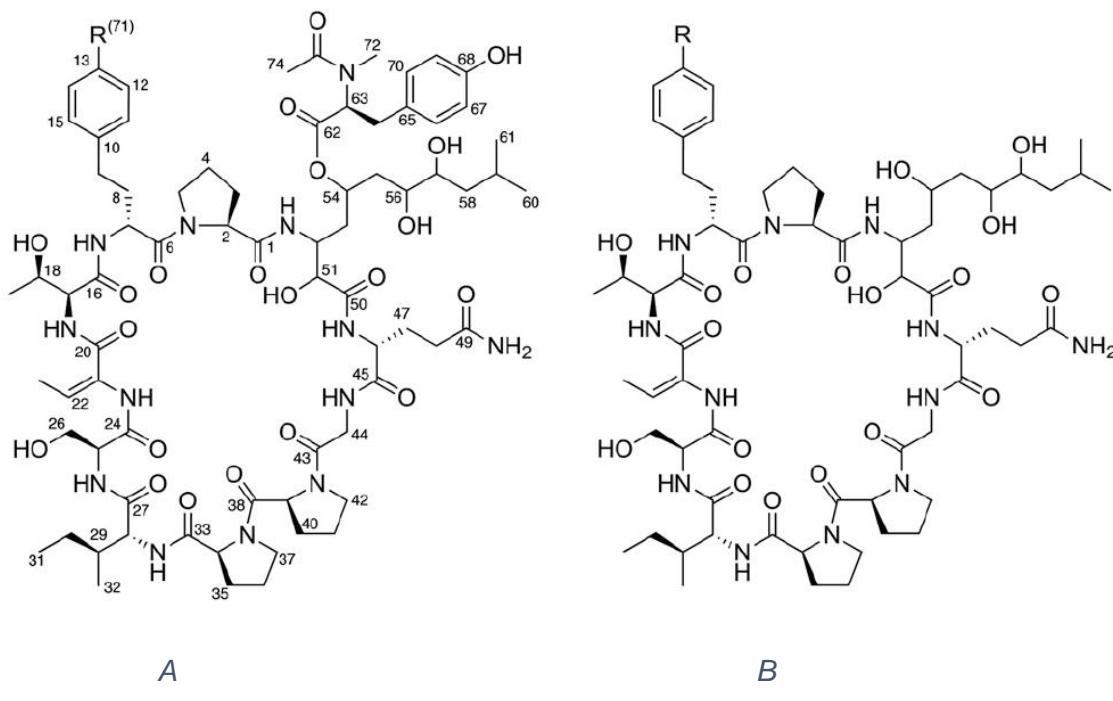


Figure 2: Portoamides A and B (taken from Leão et al. 2010).

Experimental work performed in our research group (Barreiro et al. unpublished) suggest that this strain switches its nitrogen acquisition strategy from fixation in monoculture to uptake of inorganic forms (nitrate) in co-culture with a competitor (the

chlorophyte *Ankistrodesmus falcatus*). This can be mediated by a Quorum Sensing (QS) mechanism (Zhou, et al. 2016). QS is a language of chemical signals of particular importance in microorganisms. Wyss (2013) reported that a nitrogen fixing bacteria, by a QS mechanism, detects the presence of a microalgal competitor (*Chrorella* spp.) and switches from nitrogen fixation to nitrate uptake, depriving its competitor from available nitrogen. The first part of the present work aims to study whether if this mechanism could exist in the interaction between our strain of *Phormidium* sp with *Ankistrodesmus falcatus*.

The genome of this *Phormidium* sp. strain has been sequenced. Hence, the effect of culture conditions (presence/absence of the competitor *Ankistrodesmus falcatus*) in the expression levels of the genes responsible for nitrogen fixation and the nitrate uptake could be studied with common molecular techniques.

1.6. Aims

The aims of this work were I) to determine if the physiological strategy of nitrogen acquisition in *Phormidium* sp. is conditioned by the presence of competitors (in this case *Ankistrodesmus falcatus*), by looking at the expression patterns of selected genes and II) Determine the effect of *Phormidium* sp. allelopathy in the diversity of phytoplankton community with and without the interaction with zooplanktonic grazers (rotifers).

2. Methodologies and materials

2.1. Phytoplankton cultures and growth medium

The *Phormidium* sp. strain used (strain LEGE 05292), formerly classified as *Oscillatoria* sp. (Barreiro & Vasconcelos 2014; Felpeto, et al. 2018; Leão, et al. 2010) is available in our laboratory collection (<http://lege.ciimar.up.pt>). The chlorophyte *Ankistrodesmus falcatus* strain employed was ACOI 252.

The medium composition employed to maintain the cultures and in all the experimental work (except modifications of the nitrate concentration that will be detailed when due) is shown in table 1, as is the same as in Barreiro & Vasconcelos (2014):

Table 1: Medium composition.

Compounds	Final µM	Final mg/L
Major salts		
CaCl ₂ .2H ₂ O	250	36.760
MgSO ₄ .7H ₂ O	166	36.970
Trace metals		
MnCl ₂ .4H ₂ O	2.100	0.5400
ZnSO ₄ .7H ₂ O	0.073	0.0287
CoCl ₂ .6H ₂ O	0.091	0.0300
Na ₂ MoO ₄ .2H ₂ O	0.074	0.0230
CuSO ₄ .5H ₂ O	0.038	0.0125
KBr	0.100	0.0120
V ₂ O ₅	0.007	0.0009
Vitamins		
Thiamine-HCl	0.2965	0.10000
d-Biotin	0.0020	0.00050
Cianocobalamin (B ₁₂)	0.0004	0.00055

Iron source		
Fe-EDTA*	-	-
Boric acid		
H ₃ BO ₃	74.397	4.560
Sodium bicarbonate		
NaHCO ₃	150.000	12.600
Macronutrients		
KNO ₃	3200	323.520
K ₂ HPO ₄	200	34.800

*Sol1: 28g FeCl₃.6H₂O + 1L HCl 0.1 N;

Sol2: 39g Na-EDTA + 1L NaOH 0.1 N;

Fe-EDTA = 10 ml Sol1 + 9.5 ml Sol 2 in 1L

This culture medium was prepared with ultrapure water and autoclaved. Vitamins were added after cooling down of the medium, by filtering them through 0.2 µM filters (Fisherbrand Syringe Filter)

2.2. Chemostat experiments with monocultures of *Phormidium* sp. and co-culture with *Ankistrodesmus falcatus*.

With the aim of testing the expression levels of genes related with nitrogen acquisition, were performed long-term competition experiments in continuous cultures (hereafter chemostats). The experiments were performed as *Phormidium* sp. monocultures and co-cultures of this species with the chlorophyte *Ankistrodesmus falcatus*, as explained in the introduction, the aim of these experiments was to test whether the strategy of nitrogen acquisition in *Phormidium* changes between these two conditions.

A picture of the chemostat system is shown in figure 3. It consists of three 1 L Erlenmeyer. Culture medium circulates throughout them. Fresh culture medium is pumped by a peristaltic pump at constant rate from the first flask (inflow flask) to the next flask, containing the culture of the phytoplankton species. This one is kept at constant volume by a fixed overflow tube thorough which culture goes to waste flask. The fresh medium is dropped into 60 mL syringe, where it converges with an air flow,

pushing it into the culture flask. The purpose of this syringe is to work as a “microorganism trap”, by constituting a space with air that breaks the continuous flow of liquid medium, preventing the growth of microorganisms in the tubes, that otherwise would reach the inflow flask. The air enters through an air inlet connected with a 0.2 µm filter (Fisherbrand Syringe Filter). The air flow, together with the medium, enters the culture flask through a tube reaching the bottom of the flasks. The air flow helps to homogenise the culture flask and increase the surface of exchange of gases.

The chemostats were set in a culture room, with 12:12h day-night cycle and incident light intensity of 60 µmols m⁻² s⁻¹ and at a temperature of 20 °C.

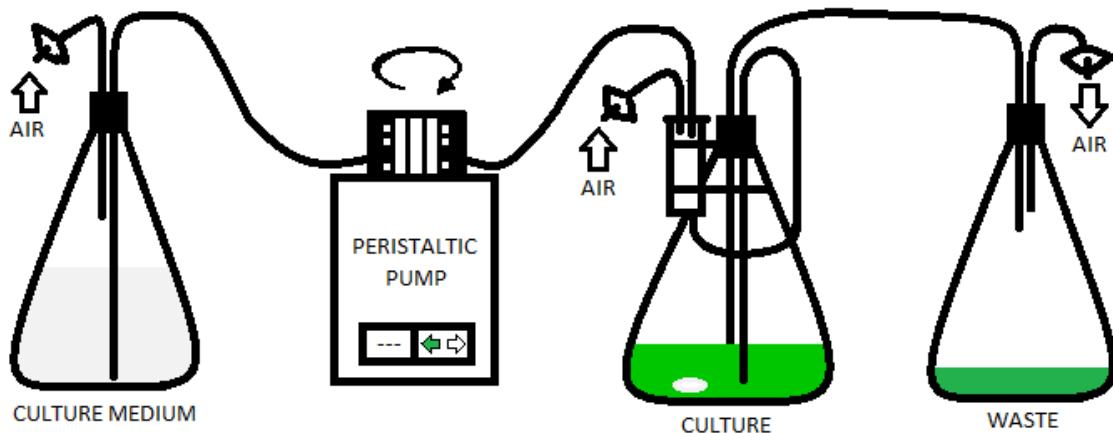


Figure 3: Chemostat representation

The walls of the culture flask were periodically cleaned with a magnet placed inside the flask, to prevent growth of the microorganisms that would shade light and make the culture medium non-homogeneous.

In these chemostats was employed the medium composition shown in table 1, with nitrate concentration reduced to 320 µM. This constitutes a N:P ratio of 1.6:1, thereby ensuring that nitrate was the limiting resource (The optimal ratio is 16:1 according to Redfield 1934).

1.5 mL samples for cell counting were taken per chemostat during the whole length of the experiment (40 days). These samples were preserved with Lugol for further cell counts under the microscope. Both *Phormidium* sp. and *Ankistrodesmus falcatus* were counted in a Neubauer chamber, under a Leica Microscope.

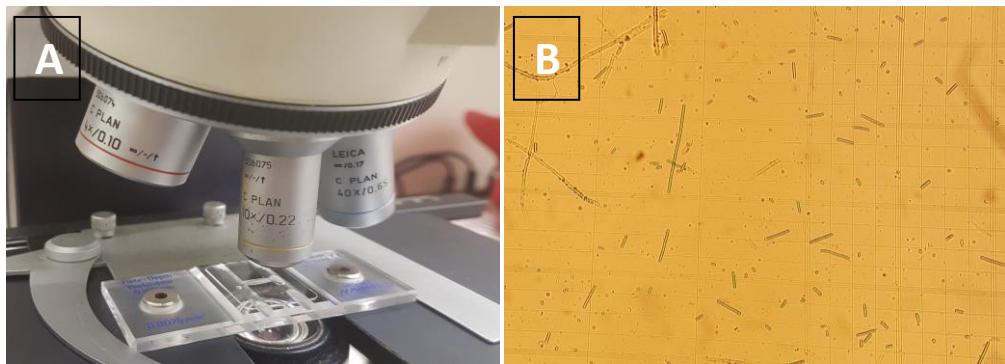


Figure 4: A) Microscope Leica with Neubauer chamber; B) Example of cell count through microscope Leica, mostly showing *Phormidium* sp.

Additionally, samples were taken for nitrate analysis, portoamide analysis (for these two later analyses, results are not shown in this thesis) and RNA extraction will be described below.

2.3. Gene Expression in *Phormidium* LEGE 05292 strain.

2.3.1. Sampling and RNA preservation

The sampling for RNA extraction for the chemostat competition experiment occurred in separate days: I) samples from continuous growth culture with only *Phormidium* sp were taken in days 0,1,5,9,18 and 40; II) samples from continuous growth culture with *Phormidium* sp and *Ankistrodesmus falcatus* were taken in days 0,1,7,15 and 37. Day 0 was the day of the inoculation. The sample days were determined by the quantity of biomass available.

Biomass was collected by centrifugation from the culture flasks on the respective sampling day. RNA stabilization agent (Qiagen) was added to protect the RNA from degradation, accordingly to the manufacturer recommendations. Later, all samples were stored in -80°C until further use.

Prior to the RNA extraction, the laboratory surfaces and all working materials were cleaned with RNase-away (Thermo Scientific) reagent, to remove any RNase and DNA contamination. The samples were then defrosted and grinded in a mortar with liquid nitrogen to break the cells and release the RNA.

2.3.2. RNA Extraction and treatment

For the RNA extraction the PureLink™ RNA Mini Kit (Invitrogen) was used, accordingly to the manufacturer's protocol. Briefly, it was added to the *Phormidium* sp. suspension 0.6 mL of Lysis Buffer and 0.6 µL of 2-mercaptoethanol. One volume of 70% ethanol was added and all samples were then processed in a spin cartridge with a collection tube. All the samples were centrifuged at 12.000 x g for about 1-2 minutes throughout the protocol. Two elution's were carried out using ultra-pure water, in a total volume of 60 µL. The extracted RNA was treated with DNase I to rapidly remove genomic DNA from total RNA using the RapidOut DNA removal Kit (ThermoFisher Scientific) following the manufacturer's instructions. Briefly, after gently vortex, DNase I was added to the samples, followed by incubation at 37°C for 30 min. After, the DNase Removal reagent, was added and after gently mixing and centrifugation at ≥ 800 x g, the supernatant, containing DNA-free and DNasefree RNA was transferred into a new tube.

The samples were quantified on NanoDrop nA-1000 spectrophotometer (DS-11 FX from DeNovix). If the RNA sample was too diluted, it was used the speedvac equipment to evaporate the solvents and concentrate the sample (program V-AQ at

45°C for 5 to 10 minutes). For immediate use, the sample was stored on ice and for longer storage it was kept at -80°C.

To verify the quality of the RNA treatment, a 4% agarose gel electrophoresis (2g of agarose in 50 mL of TAE 1X buffer with 1.8 µL of Syber Safe). In each well it was loaded 2.5 µL of ladder VI (Nzytech) and 2 µL of sample (treated and non-treated RNA) + 0.5 µL of loading dye and the gel was run for 45 minutes at 120V.

2.3.3. cDNA synthesis

For the conversion of RNA to cDNA, it was used the NZY First-Strand cDNA Synthesis Kit (NZY Tech), according to the manufacturer's recommendations. The resulting single-stranded cDNA is then suitable for use in the further quantitative Reverse Transcription PCR (RT-PCR). Briefly, NZYRT 2x Master Mix and NZYRT Enzyme Mix, and the target RNA sample (final concentration of 0.5 µg for all samples) were mixed and incubated at 25 °C for 10 min, followed by incubation at 50 °C for 30 min, and heating inactivation of the reaction at 85 °C for 5 min. Finally, the-NZY RNase H reagent was added to the suspension and incubated at 37 °C for 20 min to remove RNA bond to cDNA. All samples were then stored at -20°C.

2.3.4. Gene expression by RT-PCR

The primers for the RT-PCR were designed with the software Primer3Plusand and its quality was confirmed in Oligocalc online tool. The primers were designed to have approximately 20 bp of length and to yield amplicon sizes of ~100 bp (Table 2) The primers annealing temperature (T^o) was calculated using the NEB Tm calculator (NEB tools).

Firstly, in order to check for DNA contamination, a 16S PCR, using Supreme NZYTaqII 2x Green Master Mix (NZYtech), was executed following the manufacturer's recommendations. The cyanobacterial primers CYA359F (forward) and CYA781R (reverse) were used to amplify the 16S rRNA gene (Table 2) The PCR conditions were as following: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 53 °C for 30 sec, extension at 72 °C for 30 sec and a final extension also a 72 °C for 10 min. Positive control (genomic DNA from LEGE 05292) and negative control (water) were also included in the PCR. The PCR products were analysed on a 1% agarose gel electrophoresis (422 bp size band). If a 422 bp band was amplified for the cDNA samples but not for the RNA samples, it means that the samples were free from DNA contamination and further RT-PCR to investigate gene expression was conducted.

Two housekeeping-genes were used in the RT-PCR: 16S ribosomal RNA and *secA* (Szekeres, et al. 2014) and (Pinto et al. 2012). The expression of nitrogen fixation and storage related genes (the nitrogenase-*nifH*; the ferrodoxin-dependent nitrate reductase *narB*; the global nitrogen regulator *ntcA*; the cyanophycins *cphA* and *cphB*) and the genes with allelopathic functions, (portoamide a and b) (Table 2) was evaluated using RT-PCR in the same conditions as mentioned above, with exception of the annealing temperature that was specific for each gene and calculated using the Tm calculator from www.thermofisher.com. The gene sequence of the selected genes can be viewed in the attachments section (Annex 1).

To test the efficacy of the primers, it was conducted a PCR with all the designed primers with the gDNA sample of *Phormidium* sp. After the PCR to evaluate if the designed primers worked, it was also examined the expression of *nifH*, *narB*, *ntcA*, *cphA*, *cphB*, portoamidas a and portoamidas b. Two control genes were used in this experiment: 16S ribosomal RNA and *secA*. The latter it's a house keeping gene and is a marker that was used (successfully) on previous studies (Szekeres, et al. 2014) and (Pinto et al. 2012). Their genetic code can be viewed in the annex section (Annex 1).

Table 2: Primer sequences

Primer Name	Primer Sequence	Primer size	Primer TM	Product Size (bp)
05292_16S-F	CTACAATGCGACGGACAA	18	61.5°C	82
05292_16S-R	AGCCTACAATCTGAAGTGTGAG	20	60.8°C	
05292_SecA-F	TAACCTTCACTCTTATACTCA	21	56.9°C	94
05292_SecB-R	AACACTTACAAGCAATGG	18	56.9°C	
05292_NifH-F	CCAGTTCGTCCATAGAGAT	19	59.8°C	85
05292_NifH-R	AATGAAGAATACAAGCAATTAG C	23	59.7°C	
05292_NarB-F	ATACTGCTGAGGACATACA	19	59.0°C	142
05292_NarB-R	CAAGGCGAAGATGCTATT	18	58.9°C	
05292_NtcA-F	ACCTCGCAGCATTAACATT	19	61.7°C	79
05292_NtcA-R	ATTACTCTCAGCACCAATCG	20	61.6°C	
05292_CphA-F	TGATAAACAGAGGCAGAGA	18	58.3°C	140
05292_CphA-R	TGACAGTCGCACTATCTA	18	58.4°C	
05292_CphB-F	TACCAATCAACCATACATC	19	55.8°C	133
05292_CphB-R	TACACATCCTCACCTAA	18	55.7°C	

05292_Portoamidas 1-F	CGAGCAAGTAAGTCAAGT	18	58.4°C	98
05292_Portoamidas 1-R	ATTCTACAGCCGTCAATG	18	58.5°C	
05292_Portoamidas 2-F	TCGGATTGGTGAATACTAT	19	56.9°C	103
05292_Portoamidas 2-R	CTGTTGTTGTTCTTCTTGA	19	57.3°C	

Finally, the PCR products were analysed on a 4% agarose gel electrophoresis as previously described.

2.4. Effect of *Phormidium* sp. allelopathy and microzooplankton grazing on the diversity of a natural phytoplankton community.

A community was sampled in a freshwater pond from Parque da Cidade (Porto) by taking a 5L of water from the sub-surface. This sample was immediately taken to the laboratory and filtered through a 40 µM mesh to remove zooplankton and large particles. Then, this field community was allowed to acclimate to the culture room (same as above) for 24h.

Then, 250 mL of this community were inoculated in 500 mL chemostats, with the same medium composition as in table 1, but with 120 µM of nitrate. Previous works in the research team (Barreiro, et al. 2019) showed that intermediate levels of allelopathic effect by *Phormidium* sp. increased phytoplankton diversity in a similar field community. As we suspect from our previous studies, the level of allelopathic effect in *Phormidium* sp. can be modulated with nitrate. A concentration of nitrate of 160 µM showed to increase diversity compared to concentrations of 40 and 640 µM. In our case, we employed a slightly lower concentration (120 µM) to avoid an excessive growth of photoautotrophs that might increase the risk of anoxia of the microzooplankton grazers.

A factorial experiment was performed, consisting in the following treatments: I) only with the field community (labelled Control (C) in the samples); II) the field community + microzooplankton grazers (the rotifer *Brachionus calicyflorus*)(labelled rotifers (R) in the samples); III) the field community + *Phormidium* (labelled *Phormidium* (Ph) in the samples) (5 mL of culture in steady-state phase, with medium composition from table 1

and 320 µM of nitrate) and IV) the field community + *Phormidium* + rotifers (labelled Ph+R). With two replicates each.

The chemostats were running 24 hours with the medium alone, and the natural community and *Phormidium* was inoculated the day after.

Three different variables were sampled every week:

- Nitrate concentrations: filtration through 0.2 µM filters 7 (Fisherbrand Syringe Filter) mL samples placed in 10 mL tubes, 2 replicates. Sampled day 0 (pond water) and days 6, 14, 21, 28, 35 and 40. These data are not shown in this thesis.
- Portoamide concentration: filtration through 0.2 µM filters, 15 mL samples (30 mL on day 0) in 15 Falcon tubes, 1 replicate. Sampled days 0 (pond water) 6, 21 and 40. These data are not shown in this thesis.
- Prokaryote community composition: 30 mL centrifuged at 4500 x g and the pellet was frozen at -20°C in 2 mL tubes. Sampled days 0 (pond water) 6, 21 and 40. This samples were later treated for sequencing of the amplicon of the 16S ribosomal subunit, to identify major taxonomic groups.
- Eukaryote phytoplankton community: One 15 mL sample was taken and fixed with Lugol solution from each chemostat for diversity estimation by microscope counts. 10 mL of this solution were placed in a Ütermöhl chamber, allowed to sediment, and counted in an inverted microscope (Leitz Labovert). Sampled days 0 (pond water) and 6, 14, 21, 28, 35 and 40.

Rotifer strain was obtained from the culture collection of aquatic organisms from ECIMAT (Universidade de Vigo).

The inoculation of rotifers was done on day 21 of the experiment. About 1000 rotifers were picked the day prior to the inoculation and acclimated 24 h with a Petri dish with sterile medium. Then, after transferring all the rotifers to a flask, the same volume of these was added to each of the chemostats. After removing rotifers by filtration through 40 µM mesh, the same volume of this was added to the chemostats corresponding treatments without rotifers. This was done because this medium, where the rotifers were grown, might contain some cells of *Ankistrodesmus falcatus* and *Selenastrum capricornutum*, which are used as food to maintain the rotifer cultures and might change community compositions.

2.5. Diversity Analysis

All the diversity indexes and analysis were calculated and performed with functions from the *vegan* R package that calculate indexes and measures of alpha diversity (diversity of a single isolated community). The function *diversity* for the Shannon, Simpson and inverted Simpson. The function *renyi*, for the degrees of Renyi diversity. The function *specnumber* for species richness, and the Pielou evenness index was calculated as the quotient between Shannon index and natural logarithm of species richness.

A non-metric multidimensional scaling with square root transformation of the abundances and Bray-Curtis distance as a measure of similarity was performed with *metaMDS* function. This analysis calculates the similarity between communities as a function of the species composition and also the similarities between species according to their appearance and abundance in the communities. Communities and species are then geometrically projected in a chosen number of dimensions (2 in our case).

3. Results and Discussion

3.1. Determine gene expression

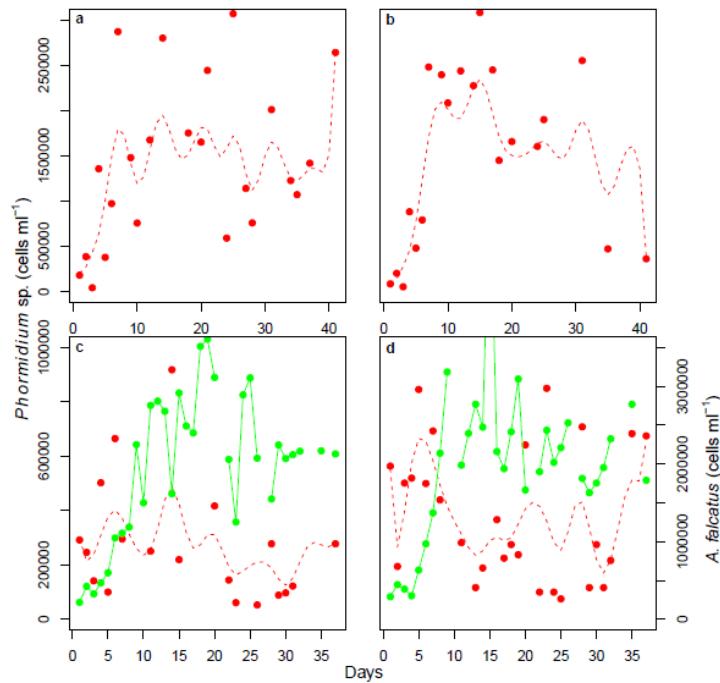


Figure 5: a and b chemostats with *Phormidium* sp. monoculture growth; c and d) chemostats with co-culture of *Phormidium* sp (red) and *A. falcatus* (green). The hyphenated lines represent a smooth non-parametric fit to *Phormidium* abundances, in order to show more clearly its general trend.

Figure 5 showcases the growth of both species in the span of 40 days. Graphics a) and b) represent the chemostats with a monoculture of *Phormidium* sp. The monocultures show a gradual growth of *Phormidium* sp. over the course of the experiment. Although in b there is a decline of population on the last two days. This decline only for two days is not reliable. It is very complicated to obtain reliable counts from this species because of the large aggregates that are formed in the cultures with time. These aggregates are responsible for the large variation in the estimates between adjacent days.

In the co-cultures, graphics c and d, we can see an equilibrium of coexistence between the two species. This equilibrium was possible because, although *A. falcatus* is a better competitor for nitrate, the limiting nutrient (Barreiro & Vasconcelos 2014; Felpeto, et al. 2018) the allelopathic effect of *Phormidium* sp. prevents its exclusion by killing some *A. falcatus* cells (Felpeto, et al. 2018).

Alongside with the cell counting, RNA extraction and treatment with DNase was carried out to the collected samples *Phormidium* cultures. After performing the RNA extraction and treatment, all samples were quantified in NanoDrop nA-1000 spectrophotometer (DS-11 FX from DeNovix), as previously described. Most of the samples showed to be pure since the value of the A260/280 ratio in the spectrophotometer was approximately 2. The following table (Table 3) shows the concentration of all the non-treated and treated RNA samples obtained:

Table 3: Concentration of non-treated and treated RNA samples. For the samples name codes, "D" represents the day of the experiment, "ph" the presence of *Phormidium* sp., and "Ank" the presence of *A. facetus*. The numbers are just replicate numbers.

RNA extraction and treatments concentrations		
Sample	RNA pre-treatment (ng/µL)	RNA treated (ng/µL)
D0 ph7 + Ank	65.434	86.799
D0 ph8 + Ank	125.279	91.759
D5 ph3	30.000	10.232
D7 ph7 + Ank	75.600	60.392
D7 ph8 + Ank	49.509	41.511
D9 ph3	87.087	53.888
D9 ph4	49.963	29.428
D15 ph7 + Ank	40.169	25.537
D15 ph8 + Ank	55.935	45.299
D18 ph3	47.286	56.727
D18 ph4	45.424	36.481

Prior to conversion to cDNA, an electrophoresis gel was performed to check non-treated and treated RNA samples.

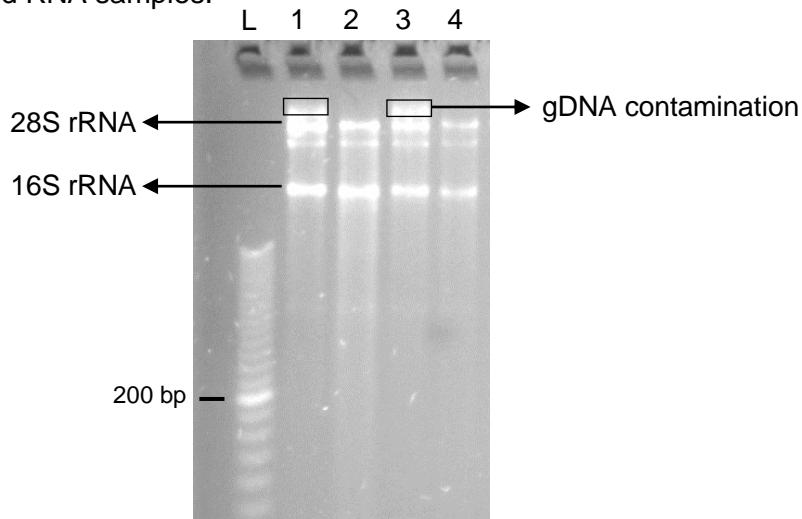
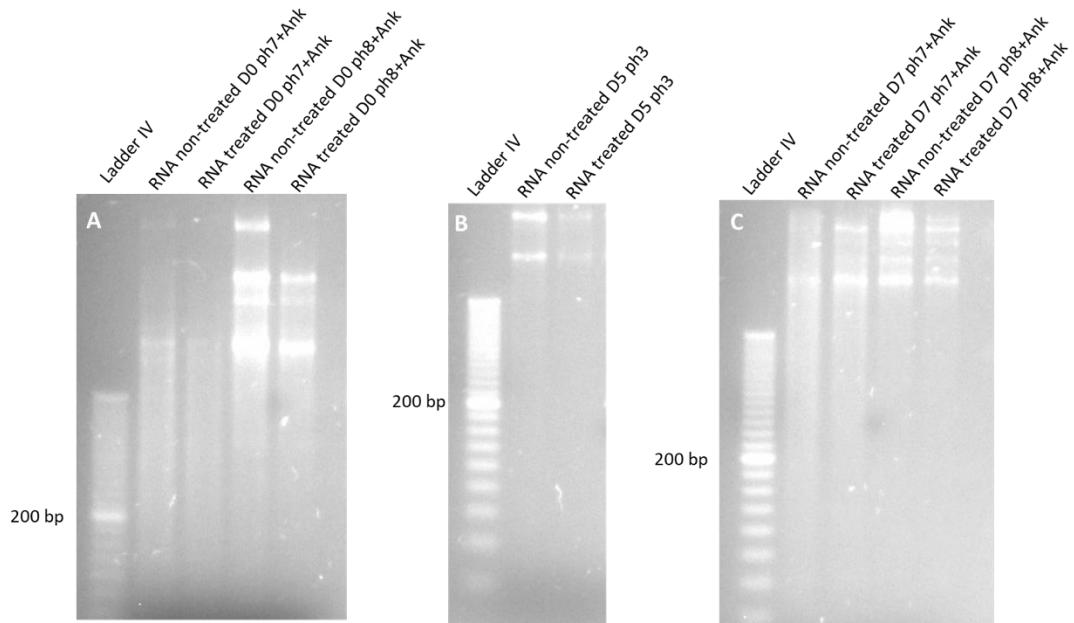


Figure 6: Electrophoresis 4% gel with non-treated and treated RNA samples. L: ladder IV; 1- RNA sample ph3 non treated day 18; 2-RNA sample ph3 treated day 18; 3- RNA sample ph4 non-treated day 18; 4- RNA sample ph4 treated day 18.

Figure 6 shows a successful treatment of the RNA samples with DNase I, since the gDNA contamination seen in the non-treated samples (sample 1 and 3) was not seen after treatment with DNase I (samples 2 and 4). In fig.7 showed below, is represented the electrophoresis gels for all the RNA samples collected, showing that both RNA extraction and treatment with DNase I was successfully, although there were some samples more concentrated than others.



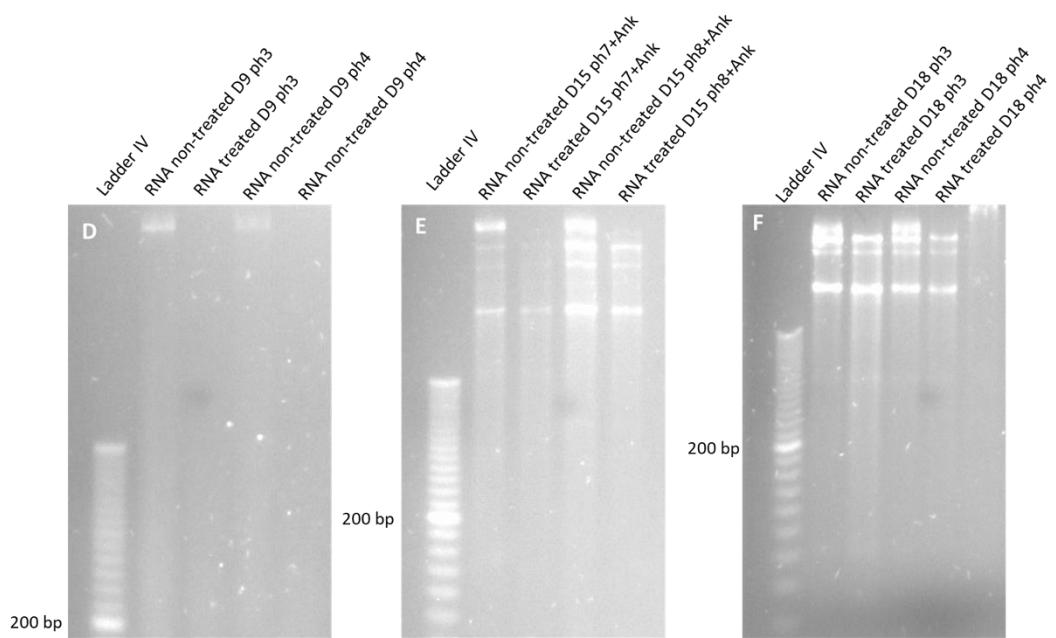


Figure 7: Electrophoresis gel with non-treated and treated RNA samples; A) Day 0 of chemostasts ph7+Ank and ph8+Ank; B) Day 5 of chemostast ph3; C) Day 7 of chesmostasts ph7+Ank and ph8+Ank; D) Day 9 of chemostasts ph3 and ph4; E) Day 15 of chemostasts ph7+Ank and ph8+Ank; F) Day 18 of chemostasts ph3 and ph4; Sample codes are the same as in previous figure.

Electrophoresis gel with day 9 RNA sample shows band aver faint band, however, checking the NanoDrop concentration (Table 3) it was confirmed that there was quantity of RNA to move forward with the conversion to cDNA.

Since all the treated RNA samples were shown to have good quality and enough concentration, ones more than others, all samples were converted to 1 µg of cDNA for further RT-PCR.

The cDNA samples were also quantified in the NanoDrop to assure that all samples had the same concentration. Table 4 shows the concentration for all cDNA samples.

Table 4: cDNA nanodrop concentrations. Sample codes are the same as in previous figures.

cDNA nanodrop quantifications		
Sample	Concentration (μg)	Volume used for RT-PCR (μL)
D0 ph7 + Ank	0.0869	1.20
D0 ph8 + Ank	0.0918	0.90
D5 ph3	0.010	0.90
D7 ph7 + Ank	0.060	0.86
D7 ph8 + Ank	0.041	1.30
D9 ph3	0.05388	0.90
D9 ph4	0.02948	1.00
D15 ph7 + Ank	0.02537	1.00
D15 ph8 + Ank	0.045299	0.83
D18 ph3	0.056727	0.90
D18 ph4	0.036481	0.77

Prior to RT-PCR, all the primers were tested by PCR to check for the best PCR conditions and in order to see if they amplify the expected size band. The figure 8 below shows the PCR results:

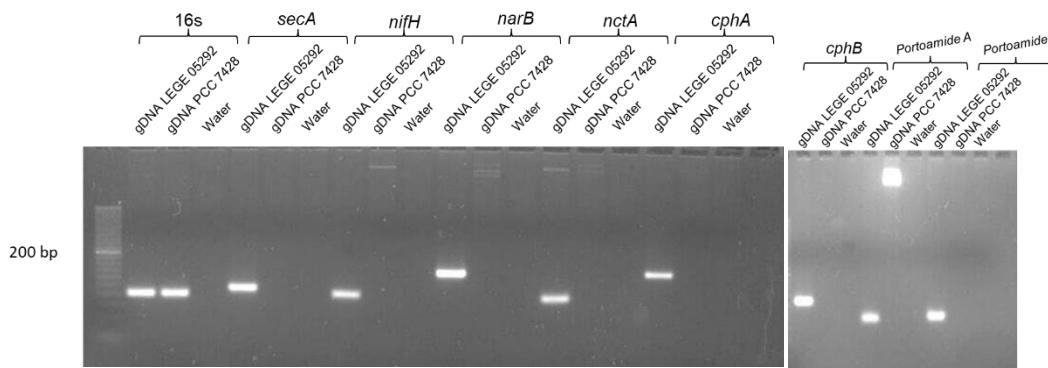


Figure 8: PCR primer results tested in our *Phormidium* strain (LEGE 05292), a different cyanobacteria gDNA strain (PCC 7428) and with water.

In the gDNA samples of *Phormidium* sp. there was amplification of a band with the expected size with all the primers. In the negative controls used, another cyanobacterial strain PCC 7428 and water there was no band amplification, except for the 16S in PCC 7428, as expected. So, the primers are efficient and can be used to test gene expression in LEGE 05292.

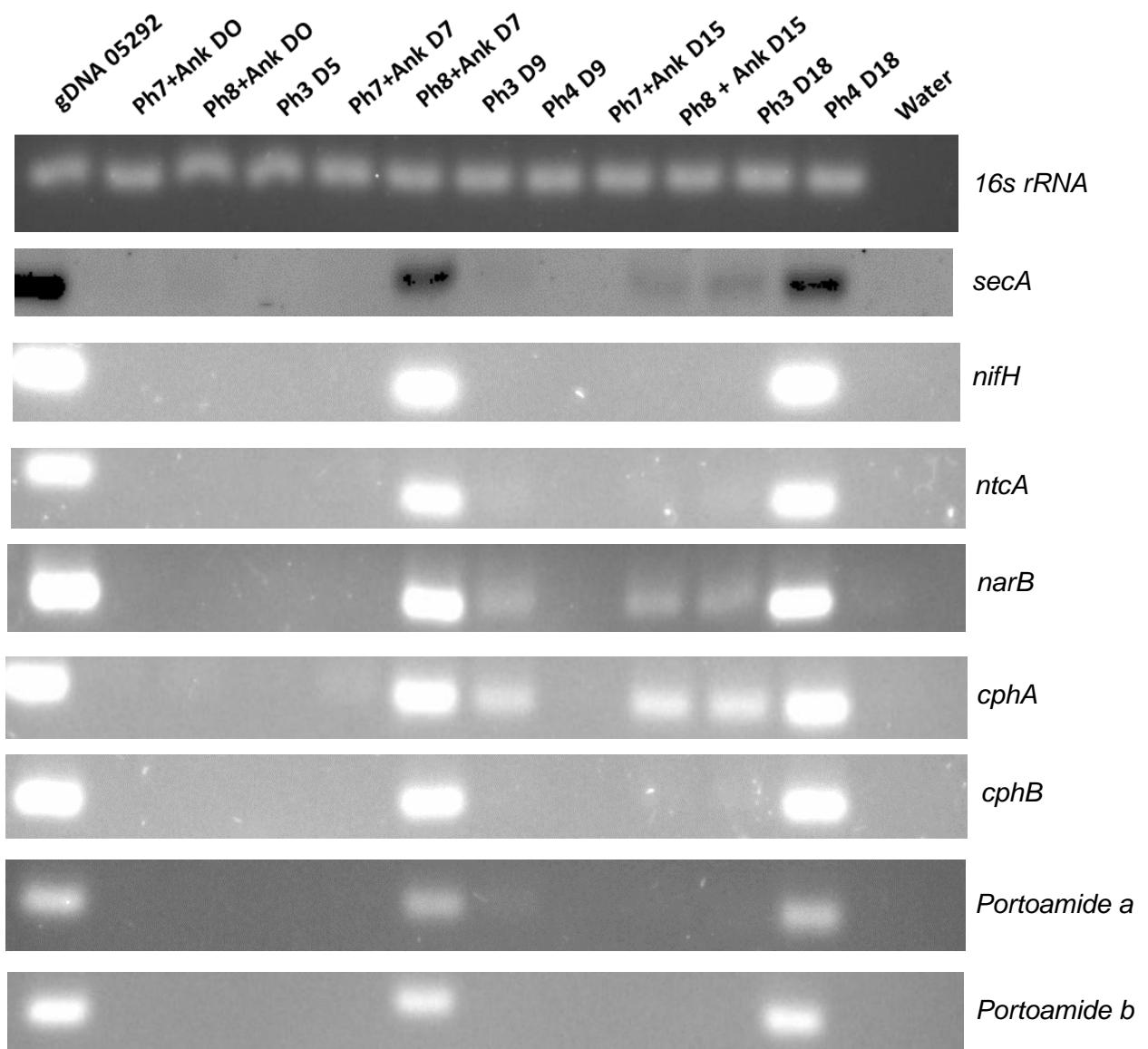


Figure 9: Electrophoresis result of the genes expression for all the primers in all samples.
Sample codes as in previous figures

Even though we observe similar expression of 16s rRNA in all samples, the same does not happen with *secA*. The *secA* gene was considered one of the best housekeeping genes for cyanobacteria gene expression and therefore a suitable control for gene expression (Szekeres, et al. 2014), (Pinto, et al. 2012). So, it was expected a 94 bp band amplification in all samples with the same density for *secA* gene. However, in this case only the control gDNA sample, *ph8+Ank D7*, *ph7+Ank D15*, *ph8+Ank D15*, *ph3 D18* show *secA* expression. Due to this, it isn't possible to have a conclusive discussion regarding the following results for the rest of the genes. While 16s rRNA

does express in all samples, it's not a good control primer for this experiment on its own.

As seen in Fig.9, we observe expression of all the tested genes in the samples ph8+Ank D7 and ph4 D18. In addition, it is possible to observe a faint band for expression of *narB* and *cphA* genes in the samples ph3 D9, ph8+Ank D15 and ph3 D18. Since the expression pattern in all the samples is the same between *secA* and the remaining test genes, it's not possible to make a coherent conclusion for the initial hypothesis.

Some explanations been thought out to explain the RT-PCR results, including: I) the biomass taken from the chemostasts was not the same across all sample days resulting in a different pellet for each sample; II) *Phormidium* sp LEGE 05292 is notorious for its hard extraction of DNA and RNA (past experiments on our lab have shown this), due to this, there is a possibility that the RNA extraction was not optimal while using the liquid nitrogen to grind without guidelines, compromising the bacteria decontamination of the sample. III) It could be that on the polyculture chemostasts, it was inoculated too much *Ankistrodesmus falcatus*, however, if this would have been the case, the monoculture chemostasts wouldn't have shown the same problem.

3.2. Allelopathy and the diversity of phytoplankton community.

3.2.1. Community composition

The absolute numerical values can be found in the appendix (Table 6). The pond from Parque da Cidade, showed in the day of the initial sampling, a relatively poor community, in which most of the biomass belonged to the large single-celled chlorophyte *Pyramimonas*, followed by the diatom *Diatoma*. All the other species were found in very small amounts. Several of the species that appeared in the experimental cultures were not found in this 15 mL sample from the pond.

The experimental cultures increased a lot their total biomass and species evenness relative to the pond, due to the enrichment in macronutrients. Day after day, the total biomass and number of species detected in the cultures, increased until reaching a maximum at equilibrium around day 21. In this equilibrium, diatoms and chlorophytes dominated the communities. Among diatoms, prominent taxons were *Diatoma*, *Melorisa*, *Synedra*, *Navicula*, *Asterionellopsis* and *Fragillaria*. Among chlorophytes were *Chlorella*, a small flagellate similar to *Chlamydomonas*, *Scenedesmus* spp., *Desmodesmus*, *Selenastrum*, *Kirchneriella*, *Monoraphidium*, *Ankistrodesmus*, *Pediastrum*, *Coelastrum*, *Oocystis*, *Volvox*. It is known that several species of

flagellates and dinoflagellates can't grow in bubbles cultures. Some other species found in the natural samples do not grow at all in laboratory cultures. In this sense, we can say that the species composition of the experimental communities is just an approximation to what can be found in the field. An example of this could be the *Cryptophyte* (a labile flagellate that normally does not resist strong bubbling) that appeared in some samples during the first days but disappeared completely afterwards.

The curves of species accumulation for each sample count are shown in the appendix (Fig. 27-34). Most of them showed a clear plateau after 10 counts, particularly the most diverse ones, in the last days of the experiment.

Before the rotifer inoculation, the cultures differed only between those containing *Phormidium* sp. and those not containing it. The cultures with *Phormidium* sp. showed, in the last day before rotifer addition (day 21) less species richness and much higher biomass of diatoms relative to chlorophytes. This suggests that diatoms could be more resistant to *Phormidium* sp. allelopathy than chlorophytes in general.

The rotifers were added to four of the cultures on day 27. Their abundances are shown in Fig. 19. The abundances were not tracked every day, so the real dynamics of their populations are unknown. Normally, their abundances would oscillate together with those of their prey (Fussmann, *et al.* 2000) and (Yoshida, *et al.* 2003).

The rotifers seem to have several effects on the cultures. First, when *Phormidium* sp. was present, the dominance of diatoms was a bit reduced in benefit of chlorophytes (seen in Ph+R treatment in days 35 and 40 relative to day 21). Second, if *Phormidium* sp. was not present, there were no major changes between diatoms and chlorophytes, but, inside chlorophytes, the abundances became more even. This aspect will be further reflected in the diversity indexes. Rotifers are filter feeders, so they are mainly non-selective, ingesting all food particles that go through the flow maintained by the rotation of their cilia. Only larger particles that can't be handled would be rejected. There is no apparent reason why the direct action of feeding itself would benefit some of the species here and not others, because, although cell sizes varied a lot, all of them fit in the range that rotifers can handle, up to approx. 50 µm in the maximum length (Starkweather & Gilbert 1977). This effect of rotifers in increasing in general the evenness of the communities (both by re-balancing between diatoms and chlorophytes and equilibrating inside chlorophytes) could be simply due to the effect of reducing limitation by nitrogen by either of these two ways: I) faster remineralization or release of small nitrogen compounds due excretion, or II) simply a reduction in the total biomass

of phytoplankton by grazing, which would leave more nitrogen available (keep in mind that nitrogen is continuously being renewed in these cultures).

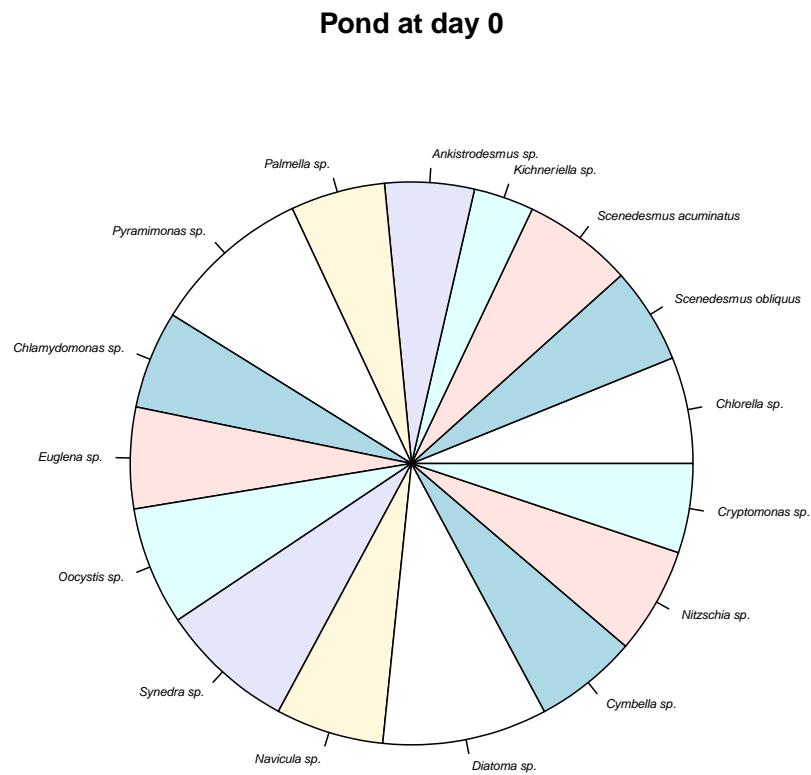
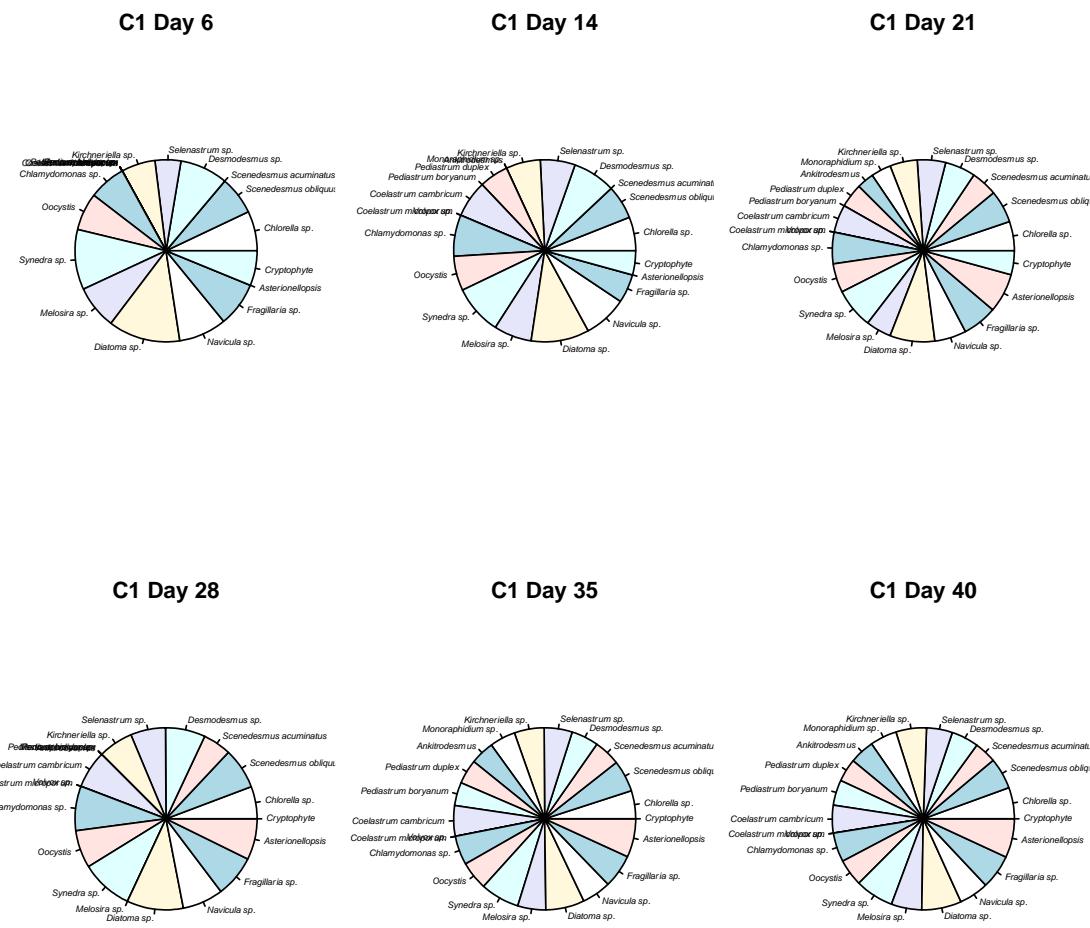


Figure 10: Pie chart of the initial community composition (as the logarithm of the abundances in biovolume) from the pond sampled in Parque da Cidade pond.

Regulation at the gene expression level of nitrogen acquisition in a nitrogen fixing-cyanobacteria: implications in ecological interactions

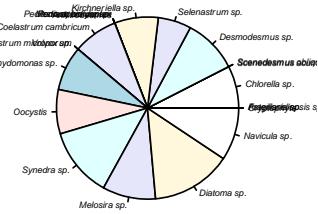


2

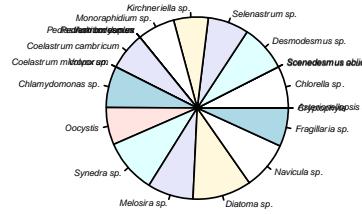
Figure 11: Pie charts (as the logarithm of the abundances in biovolume) for sample C1 (Control replicate number 1). since day 6 to day 40.

Regulation at the gene expression level of nitrogen acquisition in a nitrogen fixing-cyanobacteria: implications in ecological interactions

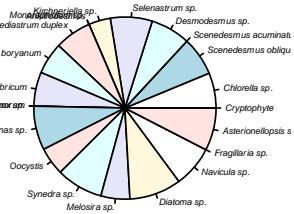
C2 Day 6



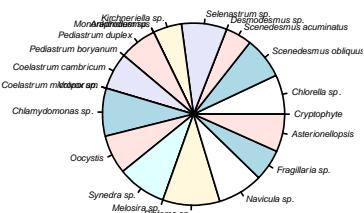
C2 Day 14



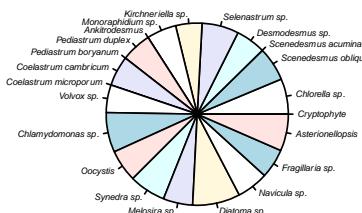
C2 Day 21



C2 Day 28



C2 Day 35



C2 Day 40

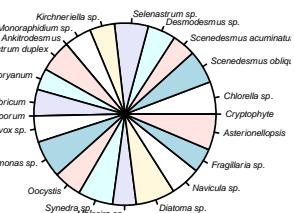


Figure 12: Pie charts (as the logarithm of the abundances in biovolume) for sample C2 (Control replicate number 2). since day 6 to day 40.

Regulation at the gene expression level of nitrogen acquisition in a nitrogen fixing-cyanobacteria: implications in ecological interactions

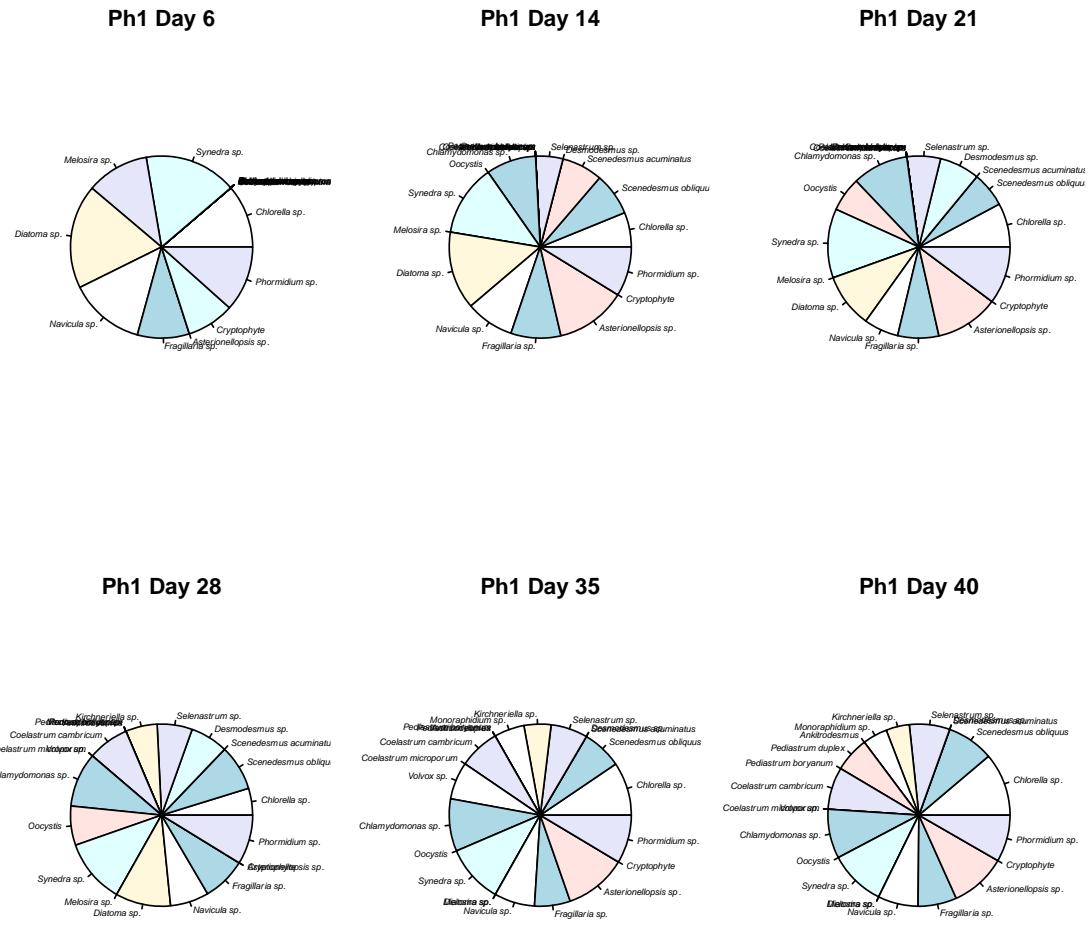


Figure 13: Pie charts (as the logarithm of the abundances in biovolume) for sample Ph1 (Phormidium replicate number 1). since day 6 to day 40.

Regulation at the gene expression level of nitrogen acquisition in a nitrogen fixing-cyanobacteria: implications in ecological interactions

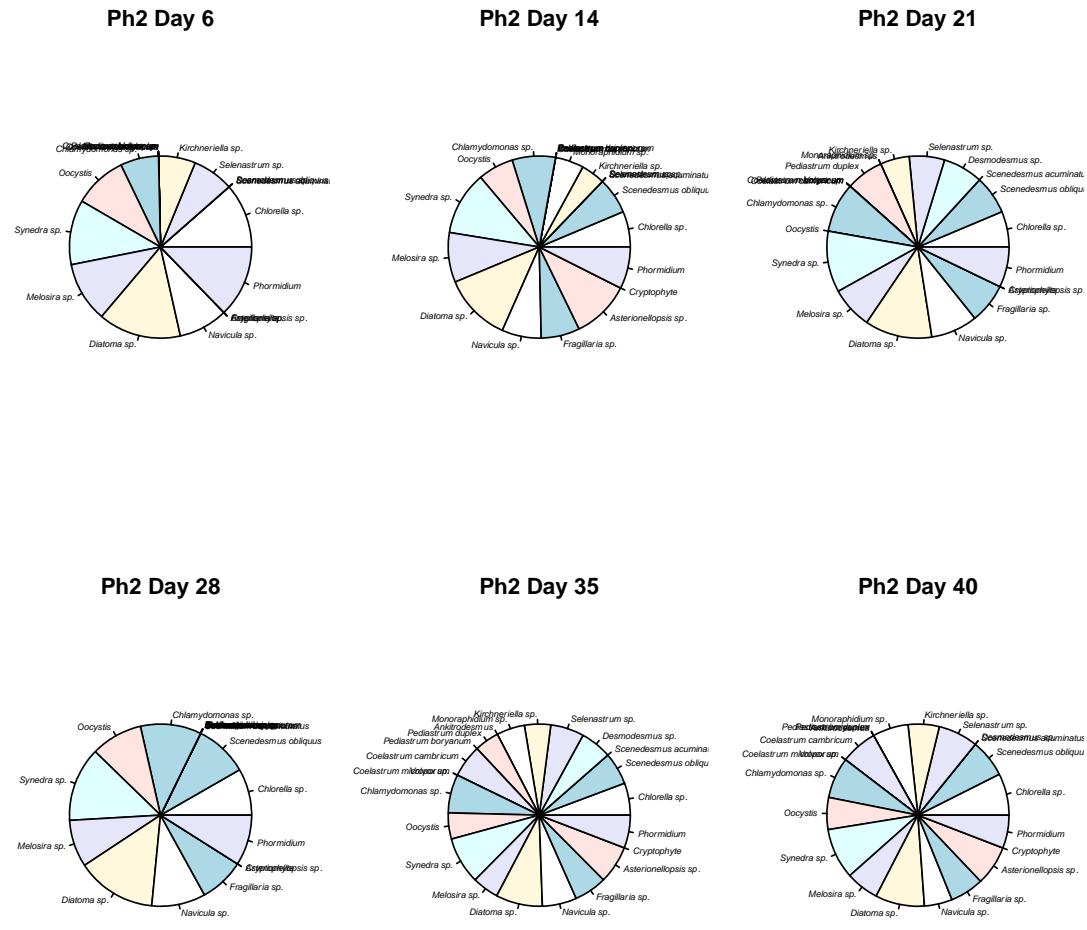


Figure 14: Pie charts (as the logarithm of the abundances in biovolume) for sample Ph2 (Phormidium replicate number 2). since day 6 to day 40.

Regulation at the gene expression level of nitrogen acquisition in a nitrogen fixing-cyanobacteria: implications in ecological interactions

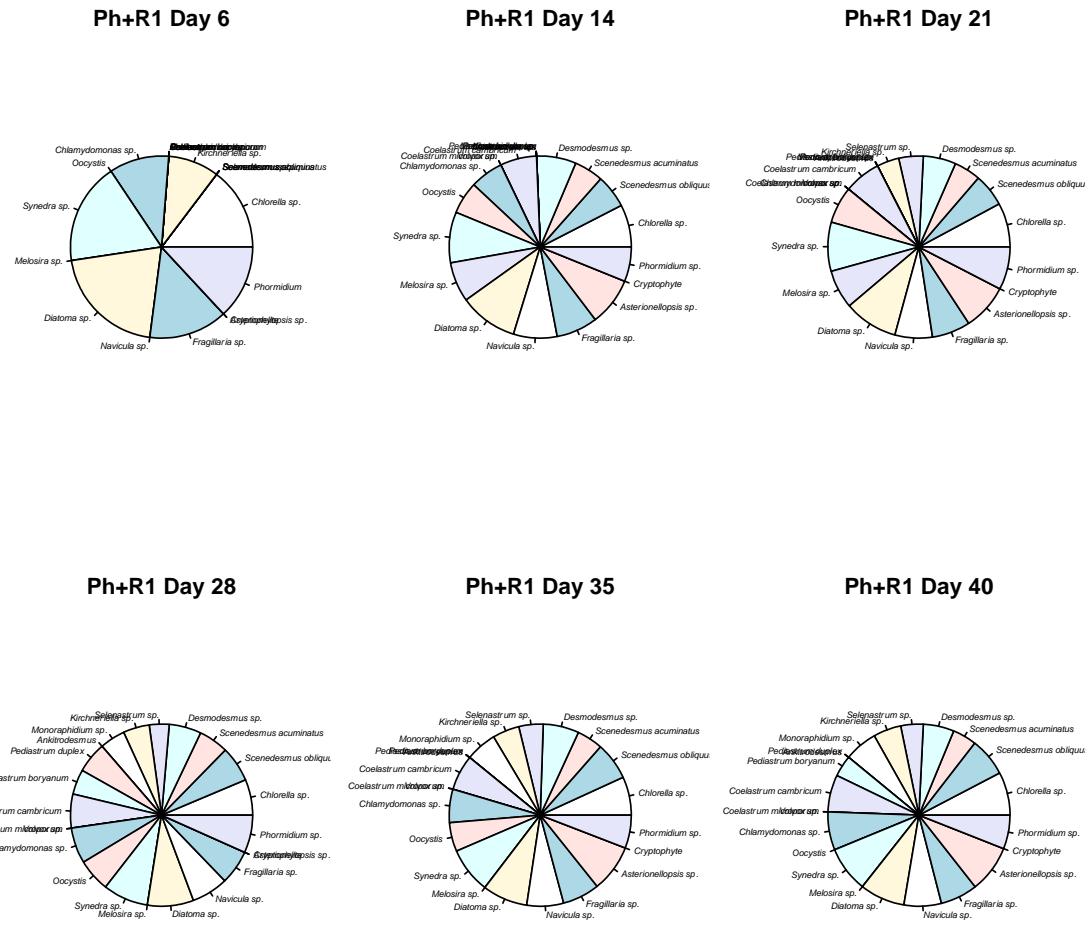


Figure 15: Pie charts (as the logarithm of the abundances in biovolume) for sample Ph+R1 (Phormidium + Rotifers replicate number 1). since day 6 to day 40.

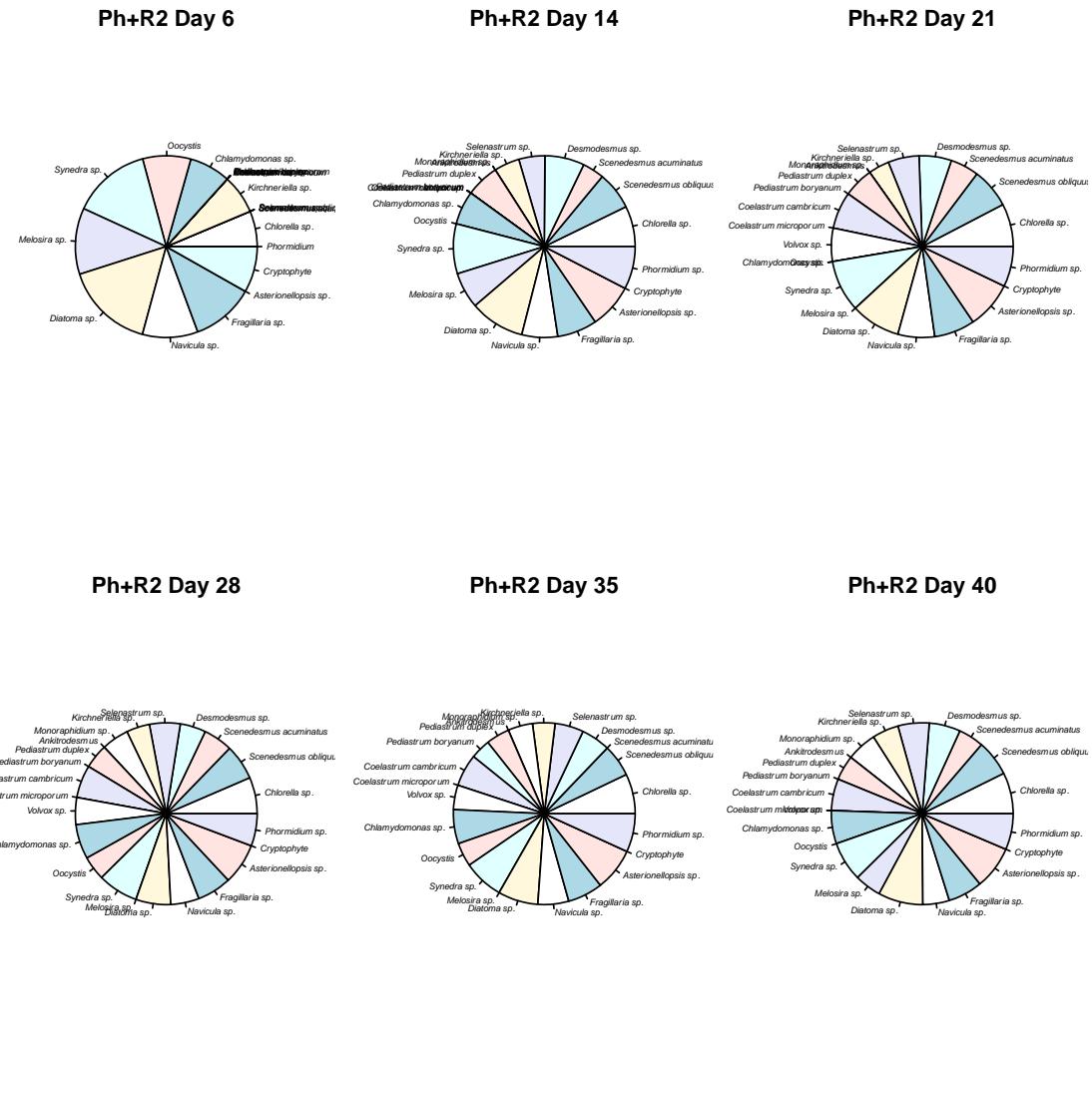
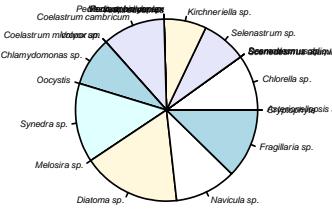


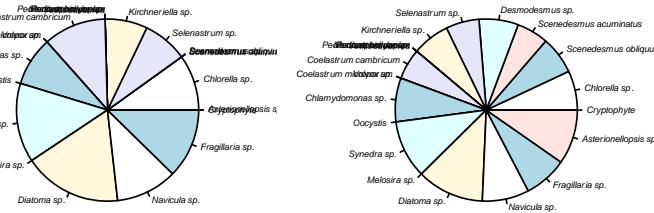
Figure 16: Pie charts (as the logarithm of the abundances in biovolume) for sample Ph+R2 (Phormidium + Rotifers replicate number 2). since day 6 to day 40.

Regulation at the gene expression level of nitrogen acquisition in a nitrogen fixing-cyanobacteria: implications in ecological interactions

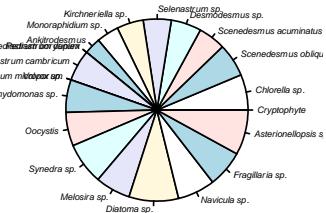
R1 Day 6



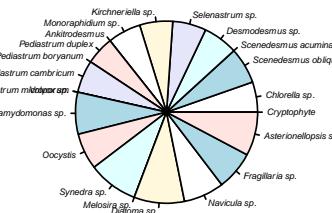
R1 Day 14



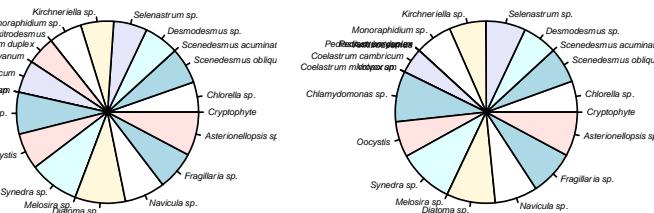
R1 Day 21



R1 Day 28



R1 Day 35



R1 Day 40

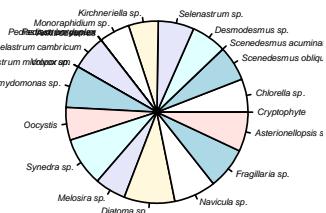


Figure 17: Pie charts (as the logarithm of the abundances in biovolume) for sample R1 (Rotifers replicate number 1). since day 6 to day 40.

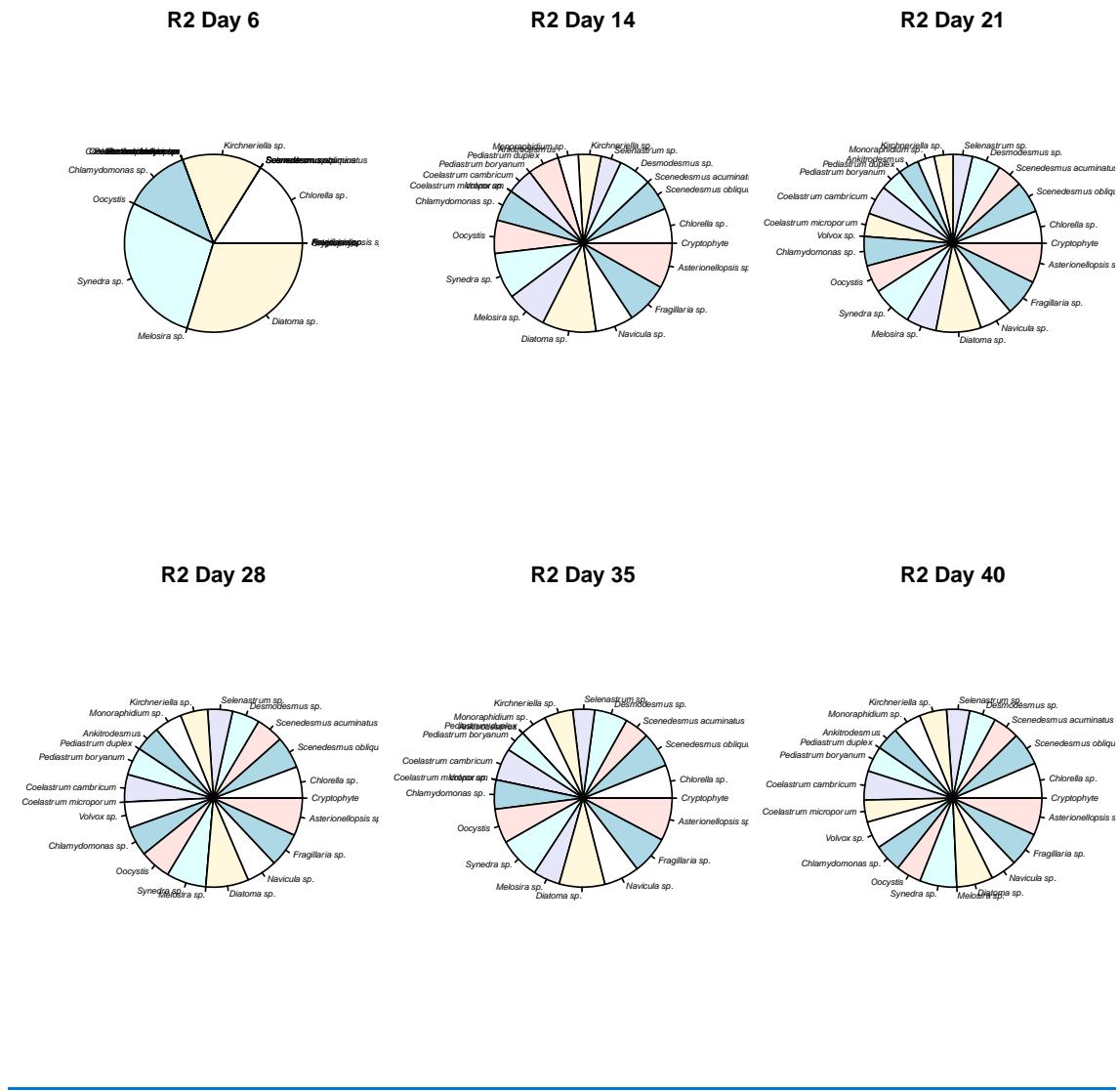


Figure 18: Pie charts (as the logarithm of the abundances in biovolume) for sample R2 (Rotifers replicate number 2). since day 6 to day 40.

The results of eukaryote phytoplankton counting, and the corresponding community compositions are shown in the figures above, the logarithm for the volumes can be viewed in the table below:

Table 5: Average biovolume for each taxon.

Taxon	Average (μm^3)
<i>Chlorella sp.</i>	26
<i>Kirchneriella sp.</i>	15
<i>Selenastrum sp.</i>	14
<i>S. obliquus</i>	52
<i>S. acuminatus</i>	39
<i>C. cambricum</i>	64
<i>C. microporum</i>	25
<i>P. duplex</i>	42
<i>Monoraphidium sp.</i>	41
<i>Navicula sp.</i>	190
<i>Diatoma sp.</i>	4574
<i>Asterionellopsis sp.</i>	2382
<i>Fragillaria sp.</i>	113
<i>Desmodesmus sp.</i>	154
<i>Ankistrodesmus sp.</i>	22
<i>P. boryanum</i>	34
<i>Volvox sp.</i>	80
<i>Chlamydomonas sp.</i>	12
<i>Oocystis sp.</i>	90
<i>Synedra sp.</i>	2140
<i>Melosira sp.</i>	120
<i>Cryptophyte</i>	64
<i>Phormidium sp.</i>	35
<i>Pyramimonas sp.</i>	173

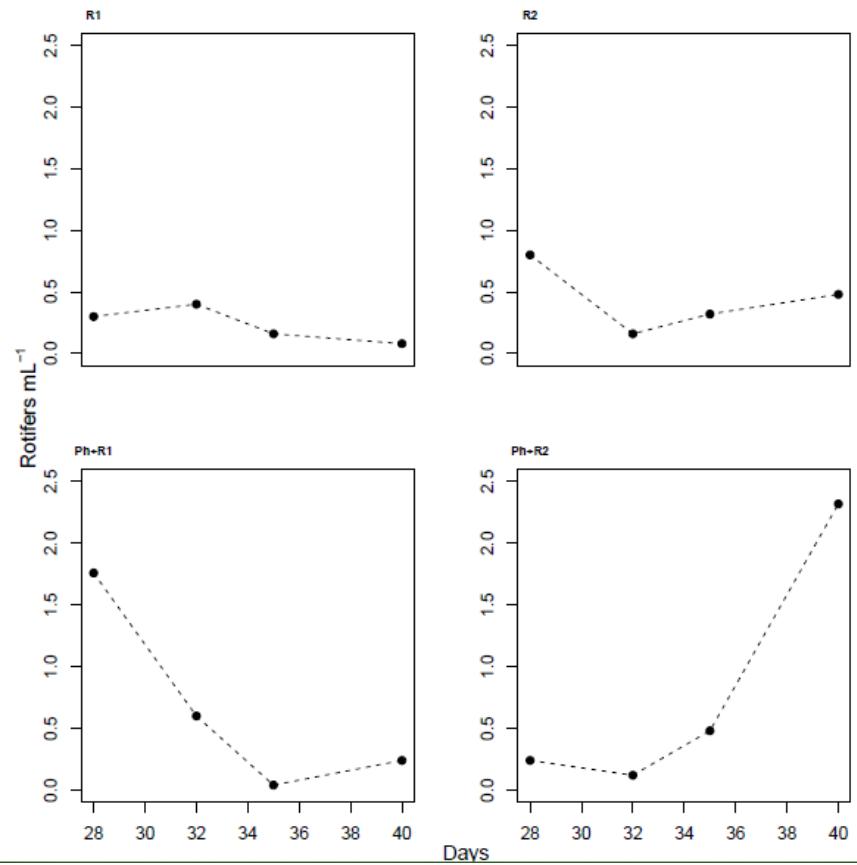


Figure 19: Rotifers abundances with only the pond community ($R1 + R2$) and with the community + *Phormidium* ($Ph+R1$ and $Ph+R2$).

3.2.2. Diversity measures

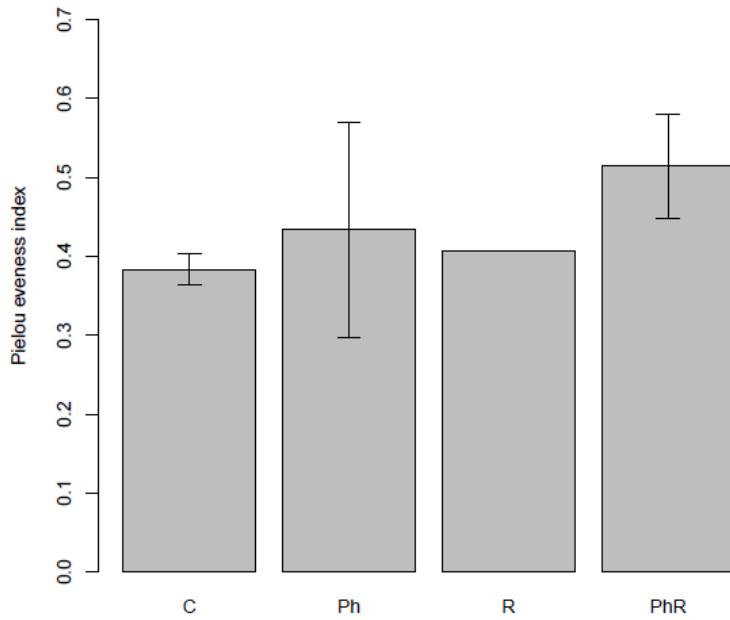


Figure 20: Pielou evenness index. C corresponds to the control with the field community alone Ph the community plus *Phormidium*, R the community plus rotifers and PhR the community plus rotifers and *Phormidium*. Values are the average of the two replicates \pm standard deviation.

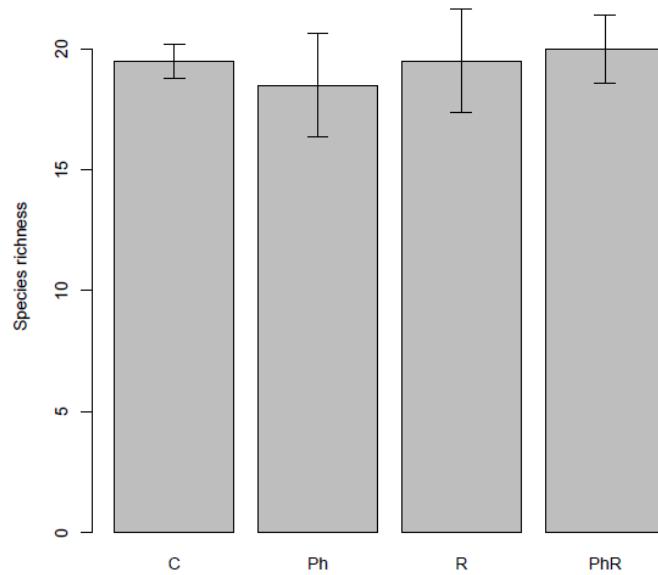


Figure 21: Species richness. Bar identification as in figure above. Values are the average of the two replicates \pm standard deviation.

The measures of diversity calculated are the most common ones for this kind of studies in ecology, estimating the alpha diversity, which is the one of a single community, found in a single sample or a single location (Hill 1973). However, it's clear that some of our measures are biased, which is a common problem in different kinds of microbiology studies (Willis 2019). The species richness, for instance, depends on the ability of detecting all the species. Because we always employed the same volume of sample and the sample counts, the less abundant species were more unlikely to be found in the first days of the experiment. However, they should still be there, even in very small abundances, since they appear after some point in the experiments. The lowest value of richness recorded was for 6 for Ph+R (Fig. 21) replicate 1 at day 6, and the largest was 19 for Control replicate 1 at day 40 (see rarefaction curves in the annex 3, figures 27-32). Then we can conclude that most of our culture should contain a number of species close to 19, but in most of them they were not possible to be detected with our sample size. However, this bias should be equal between treatments and replicates, and, as it can be clearly seen in the rarefaction curves of the last days (annex 3, fig.27-32) at the end of the experiment is very reduced or not present at all. Considering this, for the calculation of each index (each individual sample) we employed the accumulated abundance of all the species from day to day 40. All the diversity indexes employed (Shannon, Simpson, Inverted Simpson and Renyi) agreed in the ranking of treatments (figures 22-25).

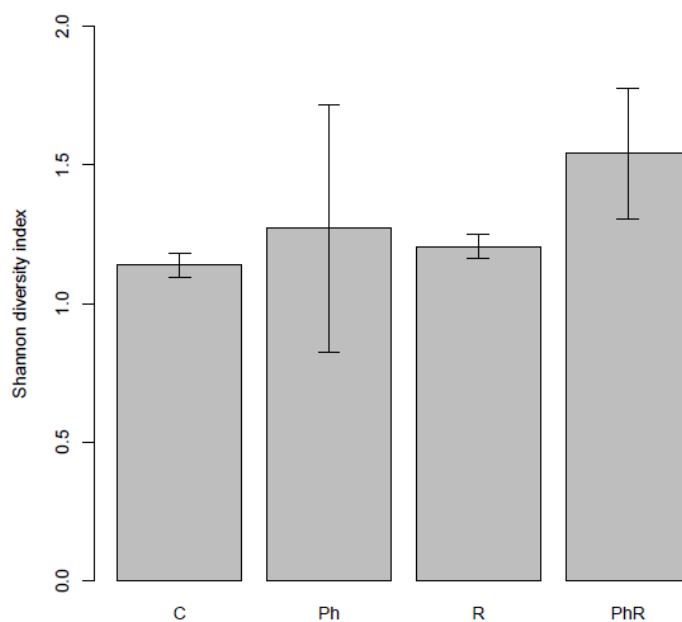


Figure 22: Shannon diversity index. Bar identification as in figure above. Values are the average of the 2 replicates \pm standard deviation.

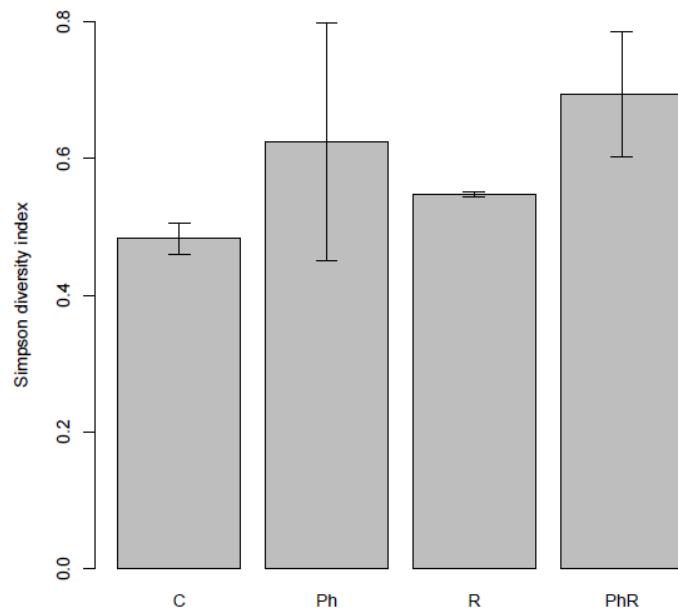


Figure 23: Simpson diversity index. Bar identification as in figures above. Values are the average of the two replicates \pm standard deviation.

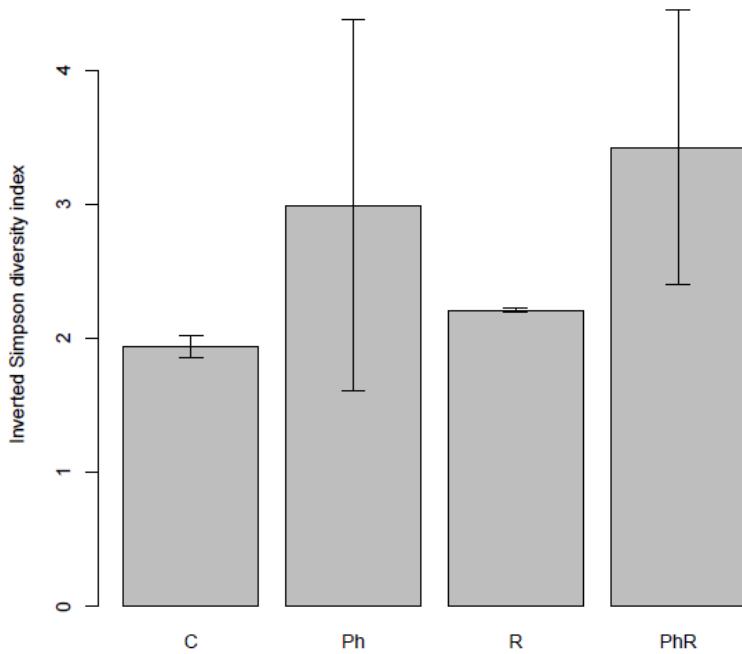


Figure 24: Inverted Simpson diversity index. Bar identification as in figures above. Values are the average of the two replicates \pm standard deviation.

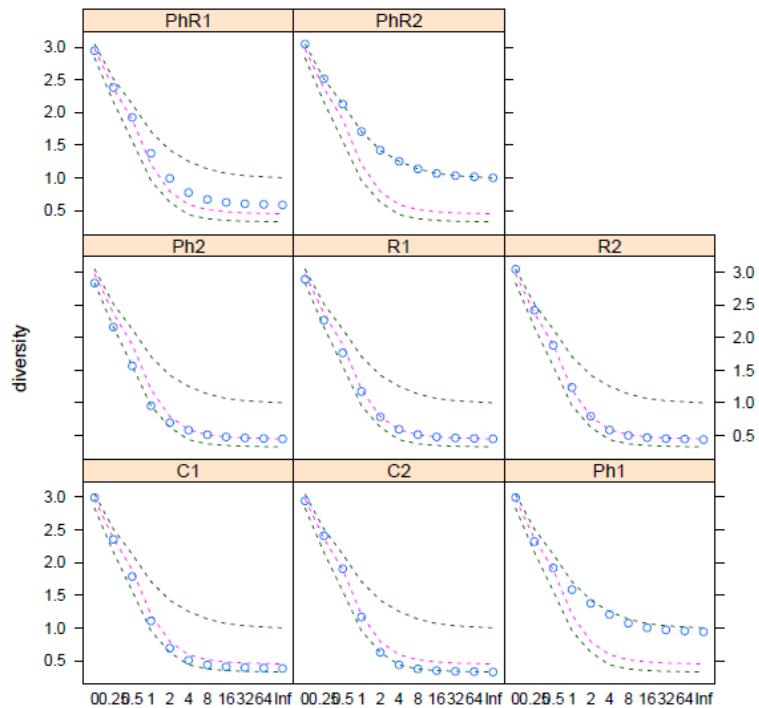


Figure 25: Renyí diversity index. Each point is diversity on a different degree. Samples with higher diversity in all degrees have higher diversity. Bar identification as in figures above. Values are the average of the two replicates \pm standard deviation.

The controls showed the lower diversity. The presence of *Phormidium* sp. and rotifers increased the diversity relative to the control, independently, but also in an additive way when they were together (Ph+R treatment). Sample 2 of the Ph treatment, however, showed abnormally lower diversity than the other one, leading to a large standard deviation of the estimated indexes. This was probably because this sample contained lower amounts of *Phormidium* sp. (see Table 6 in the annex).

All the diversity indexes combine a measure of species richness and evenness. The differences in diversity detected between our treatments were not shown in species richness (Fig. 21) which was about the same in all treatments. The observed differences in diversity were due to differences in species evenness (Fig 20).

The number of replicates (2 per treatment) is too low to perform statistical analysis comparing the results of diversity between treatments. Obviously, the low number of replicates is due to a logistic limitation. It is not possible to keep many chemostats running at the same time in a culture room and sample them.

However, except for the Ph treatment, the replicates showed low standard deviation. When standard deviations do not overlap, it is very likely that significant differences exist. The nature of these experiments, which consist in a long-term follow of species dynamics is a feature that makes classical statistical inference less important, since the results are based on analysis performed during many days in controlled conditions. This makes our results very robust. This situation is completely opposed to obtaining a random sample from a community on a single day.

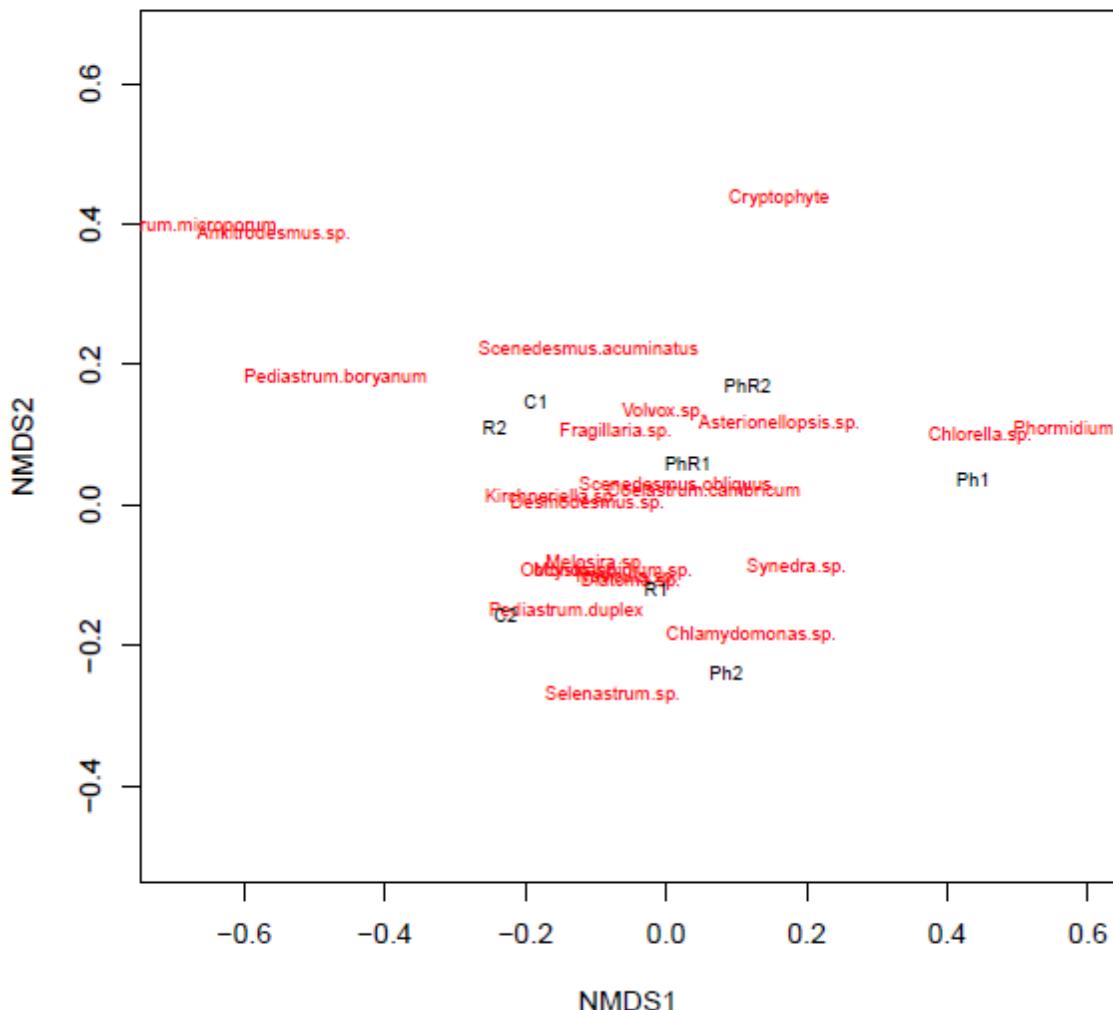


Figure 26: Non-metric multidimensional scaling (MDS). Samples identifications as in Figs 11-18.

The non-metric multidimensional scaling (MDS, Figure 26) showed a differentiation between the replicates in the first dimension that seems to be positively correlated with the presence of *Phormidium* sp. The second dimension is less clear and might be related with aspects of species composition that are not related with diversity. The presence of rotifers does not seem to have a clear effect on the results of this MDS.

According to our data, both allelopathy and predation by rotifers contributed to increase eukaryote phytoplankton community diversity. And they did it in an additive way, which means that when both factors are crossed, their respective contributions to an increased diversity add up independently.

Theoretical analysis and some experimental studies suggested long ago that allelopathy and predation could be factors that promote phytoplankton community

diversity (Sih 1985; Chao & Levi 1981; Greig & Travisano 2008) but so far only one experimental demonstration exists for allelopathy (Felpeto, et al. 2018) but in a two-species system, and hence, a very different scale of community complexity than the one in this work. Regarding the effect of predation, only field studies existed so far, which lack the close control of several variables that can be set in laboratory experiments.

The effect of allelopathy, however, could be contradictory depending on its strength. Strong allelopathy is expected to reduce diversity because of a large inhibitory effect on the community, whereas weak allelopathy is not expected to influence diversity (Felpeto, et al. 2018). In the experimental system employed in the present work, we knew that nitrogen availability was the factor controlling *Phormidium* sp. allelopathy, and hence, we chose a nitrate concentration for our experiments that was expected to produce an allelopathic effect of intermediate strength effect, according to our previous experience (Felpeto, et al. 2018) and (Barreiro et al. unpublished results).

The present work is the first one demonstrating the effect of these two factors (allelopathy and predation) alone and in interaction, in promoting phytoplankton diversity, and hence contributing to explain the paradoxical diversity of planktonic communities. And this was performed with an experimental system that combines advantages of laboratory works (strongly controlled conditions and accurate long-term monitoring) and still working with communities almost as complex as those found in the field.

4. Conclusion

The initial hypothesis for first part of this work was to determine if *Phormidium* sp could regulate the nitrogen fixation and nitrate uptake according to the presence of absence of another competitor. We tested the expression levels of the genes that are responsible for nitrogen fixation and nitrate uptake in a monoculture environment and in an environment with a competitor (*Ankistrodesmus falcatus*). However, as seen and discussed above, the results of the RT-PCR on the housekeeping gene, secA, showed that this was not expressed in all samples, compromising the remaining results, and thus making it not possible to conclude whether *Phormidium* sp can regulate these fixations and uptakes in response to a competitor.

For the second part of the experiment, we conclude that:

- Diatoms were more resistant to *Phormidium* sp. allelopathy than chlorophytes.
- Rotifers increase community evenness and reduce the advantage of diatoms under the allelopathy effect.
- *Phormidium* sp. allelopathy and rotifer grazing contribute to increase eukaryote phytoplankton community diversity in an additive way.

4.1. Future perspectives

The results from the RT-PCR were inconclusive due to the lack of global expression of the housekeeping gene secA, which leaves room for further studies attempting to obtain this expression..

The portoamides and nitrate analysis will contribute to clarify our conclusions.

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6. Annexes

Annex 1: Genes for qPCR in LEGE 05292

nifH_nitrogenase reductase _LEGE 05292

TTAGACAGTAACCTTGCCTGCTCGCTCGAGTGCTTTGGTATTCGTCTCACC
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CATTGTTGGCTGTCGGTGCAATTGTTAACAGTCATCCGACGTAGTTCTGCG
TGTTGAACAATGTTGTCACGAGGAACAAAGTGAATCATTGGGTGTTCAGACGTT
TGCCAAGGTTCGATTAGTCAGTTCCGGTCAACTTACGGCTGTTGCAAATCA
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GTTGTTAGCTGCGTACATGCCATCATTCAACCAGAGGTAACGATGTAGATTCTT
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TACGTCATAAGATAACGAAATCGAGGTCTCATAAGGCCATTCTCTAAGAAGT
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GATGCGTTGGCCCATTCTGCCATTGCTGCGATCGTATTGAGAAGTTGTTGGATT
TGCCGATACCGCCTTGCCTAGAACGCAATCTGTCTAATTTCGTCAGTCAT

narB_nitrate reductase _LEGE 05292

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ntcA_global nitrogen regulator_LEGE 05292

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cphA_cyanophycin synthase_LEGE 05292

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CGCAGTCCCAGTCATTCCGCCAATTAGGATAAAGCAGGAACAGGTTTGATAA
GTGATAGTAGCTAAAGTTGGTTCTGGTTGAATTCTACCCGTTCTTGCTTC
GATCGCTTGGGTAAACCATCACTCCAGCGCCGTCCGGGAAGTACCCCTCCGGTA
AATTCAACAACTCACTACTCGTCATTGCGAACTATATAGTTACGTCTTAATA
AACAGTTCTTGCCTTAATTGCATTAAAAACGTAGTGTGCCAAGGATCATTGGG
GTCGTAAGGTCTTAACCTCTAACATAATTCTGCTTCCGCAAAACCTCATCGGA
TAGGATAACGTTACGAGCTTTCATCTACTCATAATGCTCGTCCTATTCAATCC
AGCTGCTACTTCGGAAGCACGGATATATTGACTAGGACGTTACTTGACCGG
AAATAATTAAACGGAGTCCGGGCTCGTCAATTAAACGAGTCAACTCGTCAATA
ATGCAGTAATTAAAGGGACGTTGCACGACTCTGACATGGCAACTGCCATATTAC
GCGCAGGTAGTCAAAACCAAGTCGCTGTTAGTACCGTAAGTAATGTCGCAGGCG

TAGTTTTTGCCTCGGGAGGAGACATTCTGTTGGATTAATCCAACGTAA
GCCTAACGAAACGATGAATTGTCCCATTCTCGTCCGACGCGCAAGGTAA
TCGTTAACAGTTACGACGTGAACGCCTTGCCGAAATCGCATTAGTAAGAAG
GTAGTGTGGCAACTAAGGTTTCCTCACCTGTTCATTCGGCAATTGTCCG
GTGTGCAGGATAATTCCGCCAAGTAATTGAACATCAAATGTCGCATTCCAAGAC
GCGCCGACCTGCTCTGGACGACGGAAAAGCTCTGGGAGAATTCAAGT
AATTCTTCTCATGTGCCAGGTTTGGCAGCTTGAGTTTGTTAAATTCTGCT
GTTTGCCTCTTAACCTTGATCTGAAAGTGCTTGATTCTTCTCCAGTAAGTT
ATTCGGTAATGTAAGGTTGGTATTTTTAGCTTACGAGCGTTGGATGCCTAG
AAGGGTTTTAACAT

Annex 2: Absolute numerical values of the phytoplankton community

Table 6: Absolute numerical values of phytoplankton communities

Treatment	Day	<i>Chlorella</i> sp.	<i>Scenedesmus obliquus</i>	<i>Scenedesmus acuminatus</i>	<i>Desmodesmus</i> sp.	<i>Selenastrum</i> sp.	<i>Kirchneriella</i> sp.	<i>Monoraphidium</i> sp.	<i>Ankistrodesmus</i> sp.
C1	6	4576	3330,93376	0	28336	224	1340,8348 8	0	0
C1	14	7072	7078,23424	0	78848	9072	8410,6915 2	0	0
C1	21	27872	89102,4781	8655,73632	40656	19712	14261,607 4	980,80752	352
C1	28	6656	77860,5766	2163,93408	55440	14112	13774,031	0	0
C1	35	30576	119080,882	16074,9389	22176	30800	64360,074 2	12096,6261	6512
C1	40	74880	74529,6429	2473,06752	17248	10976	57290,217 6	14385,177	6336
C2	6	1040	0	0	8624	224	1340,8348 8	0	0
C2	14	17888	0	0	51744	13440	2925,4579 2	5884,84512	0
C2	21	24336	64953,2083	0	89936	175840	487,57632	0	0
C2	28	30576	54127,6736	927,40032	0	149520	1706,5171 2	0	0
C2	35	143936	91600,6784	0	19712	235200	6216,5980 8	19943,0862	0
C2	40	130000	182368,623	4946,13504	24640	205520	8410,6915 2	17654,5354	0

Ph1	6	4368	0	0	0	0	0	0	0
Ph1	14	416	1665	1237	0	112	0	0	0
Ph1	21	12064	1665	0	4928	1232	0	0	0
Ph1	28	416	30811	0	4928	2688	1219	0	0
Ph1	35	221520	9576	0	0	4368	488	981	0
Ph1	40	3914560	73281	0	0	15120	244	327	0
Ph2	6	16848	0	0	0	448	243,78816	0	0
Ph2	14	1456	1665,46688	0	0	0	121,89408	326,93584	0
Ph2	21	4160	8327,3344	0	11088	3808	853,25856	0	0
Ph2	28	8736	33309,3376	0	0	0	0	0	0
Ph2	35	24544	25398,3699	0	18480	36848	3778,71648	6865,65264	0
Ph2	40	184080	56625,8739	0	0	96880	7923,1152	28443,4181	0
R1	6	1456	0	0	0	336	243,78816	0	0
R1	14	7488	4996,40064	1236,53376	7392	1792	4875,7632	0	0
R1	21	70096	32476,6042	4946,13504	12320	6272	3778,71648	980,80752	176
R1	28	4992	27480,2035	0	18480	12880	11701,8317	11115,8186	0
R1	35	2080	6661,86752	0	4928	19040	8654,47968	9481,13936	0
R1	40	15600	28312,937	0	28336	35504	5485,2336	7519,52432	0
R2	6	1248	0	0	0	0	609,4704	0	0
R2	14	27664	8327,3344	0	14784	336	731,36448	326,93584	0
R2	21	86736	61205,9078	6800,93568	22176	784	731,36448	326,93584	1232
R2	28	64064	74946,0096	13911,0048	14784	5600	14383,501	15365,9845	7216

							4			
R2	35	74256	135319,184	3709,60128	68992	1568	15358,654 1	7519,52432	0	
R2	40	609440	237745,397	18548,0064	19712	5376	28279,426 6	37270,6858	5632	
Ph+R1	6	2704	0	0	0	0	121,89408	0	0	
Ph+R1	14	109408	7910,96768	2473,06752	48048	0	0	0	0	
Ph+R1	21	153712	6661,86752	1236,53376	6160	672	365,68224	0	0	
Ph+R1	28	67808	68700,5088	9892,27008	22176	448	3291,1401 6	2615,48672	0	
Ph+R1	35	124384	98262,5459	2473,06752	56672	1568	3291,1401 6	13404,3694	0	
Ph+R1	40	457600	109088,081	1236,53376	9856	784	3900,6105 6	20923,8938	0	
Ph+R2	6	208	0	0	0	0	365,68224	0	0	
Ph+R2	14	54704	27063,8368	309,13344	41888	1008	853,25856	0	0	
Ph+R2	21	90688	37889,3715	1854,80064	4928	3584	243,78816	0	0	
Ph+R2	28	326976	226503,496	24730,6752	7392	42000	3047,352	12423,5619	0	
Ph+R2	35	795600	54544,0403	0	17248	16128	1950,3052 8	3923,23008	0	
Ph+R2	40	1237600	206517,893	4946,13504	39424	37856	7435,5388 8	26154,8672	0	
Treatment	Pediastrum duplex	Pediastrum boryanum	Coelastrum cambricum	Coelastrum microporum	Volvox sp.	Chlamydomonas sp.	Oocystis sp.	Synedra sp.	Melosira sp.	
C1	0	0	0	0	0	2496	2880	445120	10560	
C1	2034,5001 6	0	15942,6775	0	0	56256	8640	513600	21120	

C1	4408,0836 8	0	16456,9574	0	0	57312	23760	1403840	6720
C1	0	0	31371,0751	0	0	211200	30960	1831840	0
C1	7798,9172 8	4896	79199,1077	0	0	44832	37440	2003040	28800
C1	3729,9169 6	8432	21085,4767	0	0	34560	15840	1061440	56640
C2	0	0	1542,83976	0	0	1536	1440	119840	6720
C2	0	0	6171,35904	0	0	14688	5760	308160	42240
C2	37638,253	8704	17485,5173	0	0	331200	2880	736160	3840
C2	16954,168	0	15428,3976	0	0	296640	27360	410880	0
C2	24753,085 3	0	23656,8763	0	10240	617280	46800	205440	19200
C2	120374,59 3	2176	13885,5578	0	12800	824160	26640	342400	4800
Ph1	0	0	0	0	0	0	0	239680	3840
Ph1	0	0	0	0	0	6336	0	308160	0
Ph1	0	0	0	0	0	168000	1440	2978880	0
Ph1	0	0	11314	0	0	247680	7200	2704960	0
Ph1	0	0	11314	0	5120	183360	0	770400	0
Ph1	4069	0	23143	0	0	132960	0	753280	0
Ph2	0	0	0	0	0	288	2880	17120	8640
Ph2	0	0	0	0	0	6816	1440	530720	26880
Ph2	6442,5838 4	0	0	0	0	116640	0	2003040	18240
Ph2	0	0	0	0	0	152640	23760	2362560	9600
Ph2	2034,5001 6	0	29313,9554	0	0	184800	3600	2961760	3840

Ph2	0	0	41142,3936	0	0	133440	10080	2585120	13440
R1	0	0	3599,95944	0	0	576	0	34240	0
R1	0	0	1028,55984	0	0	20928	0	616320	0
R1	0	0	17485,5173	0	0	26496	36720	513600	57600
R1	3051,7502 4	0	9771,31848	0	0	146880	28800	1694880	0
R1	0	0	514,27992	0	0	290880	6480	1095680	0
R1	0	0	26228,2759	0	0	217920	13680	1780480	7680
R2	0	0	0	0	0	192	0	256800	0
R2	9494,3340 8	0	2057,11968	0	0	9408	15840	770400	125760
R2	0	2176	26742,5558	3151,65312	0	28512	14400	1472320	36480
R2	0	16320	14399,8378	0	8960	27456	52560	1249760	0
R2	0	1360	46285,1928	0	0	18336	106560	1352480	7680
R2	0	13600	52456,5518	3742,58808	26880	34080	12960	1147040	0
Ph+R1	0	0	0	0	0	288	0	17120	0
Ph+R1	0	0	19028,357	0	0	7584	6480	1129920	48960
Ph+R1	0	0	19542,637	0	0	0	19440	564960	32640
Ph+R1	14919,667 8	2176	38056,7141	0	0	71520	27360	1540800	0
Ph+R1	0	0	49885,1522	0	0	24864	6480	1284000	0
Ph+R1	0	544	70970,629	0	0	94560	0	1078560	0
Ph+R2	0	0	0	0	0	480	1440	171200	24960
Ph+R2	7459,8339 2	0	0	0	0	7776	0	736160	16320
Ph+R2	2712,6668 8	0	17485,5173	0	6400	0	0	975840	0

Ph+R2	4747,1670 4	0	86399,0266	0	11520	176544	4320	1472320	0
Ph+R2	2712,6668 8	816	53999,3916	0	3840	101280	2880	941600	0
Ph+R2	4408,0836 8	0	45256,633	0	0	74880	0	770400	7680
Treatment	<i>Diatoma sp.</i>	<i>Navicula sp.</i>	<i>Fragillaria sp.</i>	<i>Asterionellops is sp.</i>	<i>Cryptophyt e</i>	<i>Phormidiu m sp.</i>			
C1	6513673,43	27324,380 2	13524	0	1536	0			
C1	5818393,68	91081,267 2	1803,2	0	512	0			
C1	8050607,61	62238,865 9	177615,2	971815,2	4096	0			
C1	6586860,77	74383,034 9	98274,4	76220,8	0	0			
C1	1829683,55	47058,654 7	222695,2	1619692	0	0			
C1	1683308,86	39468,549 1	238924	1200477,6	0	0			
C2	731873,419	6072,0844 8	0	0	0	0			
C2	1024622,79	68310,950 4	7212,8	0	0	0			
C2	3476398,74	147248,04 9	0	209607,2	0	0			
C2	3842335,45	144212,00 6	3606,4	19055,2	0	0			
C2	9733916,48	36432,506 9	18933,6	228662,4	0	0			

C2		54648,760				
	1610121,52	3	6311,2	457324,8	0	0
Ph1	1244185	22770	902	0	512	5600
Ph1	1061216	4554	6311	285828	0	5600
Ph1	109781	1518	6311	838429	0	219240
Ph1	292749	6072	20737	0	0	74760
Ph1	0	10626	3606	1905520	0	67760
Ph1	0	13662	9918	705042	0	72240
Ph2		1518,0211				
	292749,368	2	0	0	0	52360
Ph2		3036,0422				
	1390559,5	4	2704,8	171496,8	0	5040
Ph2		56166,781				
	8709293,69	4	11720,8	0	0	12320
Ph2		36432,506				
	7428515,2	9	6311,2	0	0	19320
Ph2		47058,654				
	2744525,32	7	28851,2	152441,6	0	30520
Ph2		3036,0422				
	1646715,19	4	16228,8	95276	0	13440
R1		3036,0422				
	475717,722	4	9016	0	0	0
R1		44022,612				
	5635425,33	5	18032	266772,8	0	0
R1		124477,73				
	6477079,76	2	103684	1429140	0	0
R1		101707,41				
	1829683,55	5	61308,8	247717,6	0	0
R1	182968,355	37950,528	68521,6	57165,6	0	0

R1	3256836,72	217077,02	193844	95276	0	0
R2	731873,419	0	0	0	0	0
R2		66792,929				
	8709293,69	3	192942,4	590711,2	0	0
R2		113851,58				
	8562919	4	359738,4	1162367,2	0	0
R2		45540,633				
	4244865,83	6	149665,6	571656	0	0
R2	5598831,66	162428,26	343509,6	1886464,8	0	0
R2		18216,253				
	914841,774	4	401212	1143312	0	0
Ph+R1	73187,3419	0	1803,2	0	0	1120
Ph+R1		144212,00				
	10173040,5	6	76636	533545,6	0	10920
Ph+R1		22770,316				
	2305401,27	8	30654,4	266772,8	0	97720
Ph+R1		89563,246				
	2085839,24	1	45981,6	0	0	127680
Ph+R1	1024622,79	75901,056	81144	2000796	0	22960
Ph+R1	841654,432	75901,056	68521,6	1753078,4	0	24080
Ph+R2		4554,0633				
	951435,445	6	14425,6	0	1024	0
Ph+R2		15180,211				
	2927493,68	2	44178,4	171496,8	0	93240
Ph+R2		16698,232				
	585498,735	3	48686,4	285828	0	41160
Ph+R2		19734,274				
	182968,355	6	149665,6	1829299,2	0	75320
Ph+R2	841654,432	34914,485	137944,8	2134182,4	0	459200

		8				
Ph+R2	4647396,21	7590,1056	116306,4	2439065,6	0	208600

Annex 3: Rarefaction curves for all samples

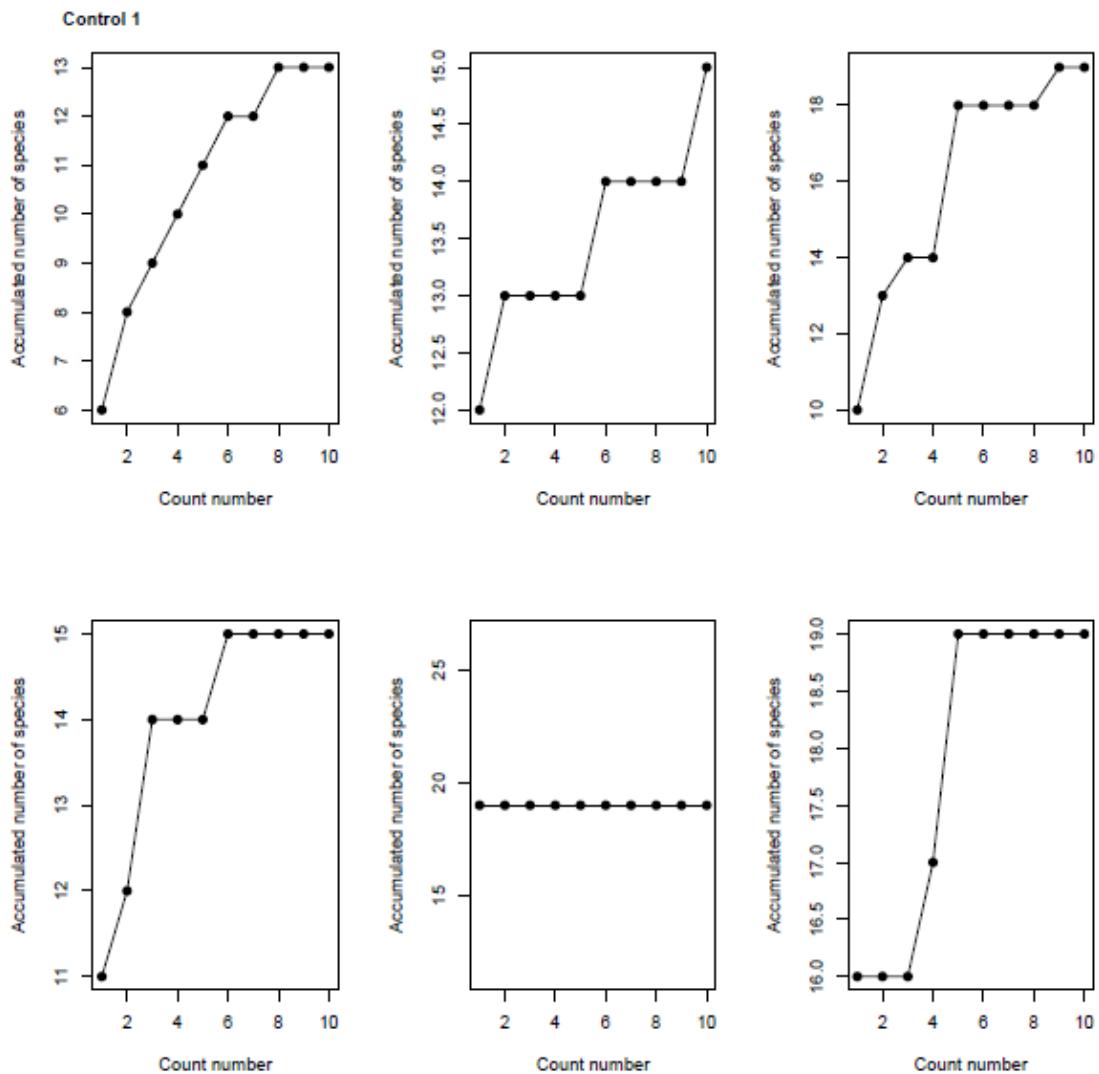


Figure 27: Rarefaction curve from culture Control 1

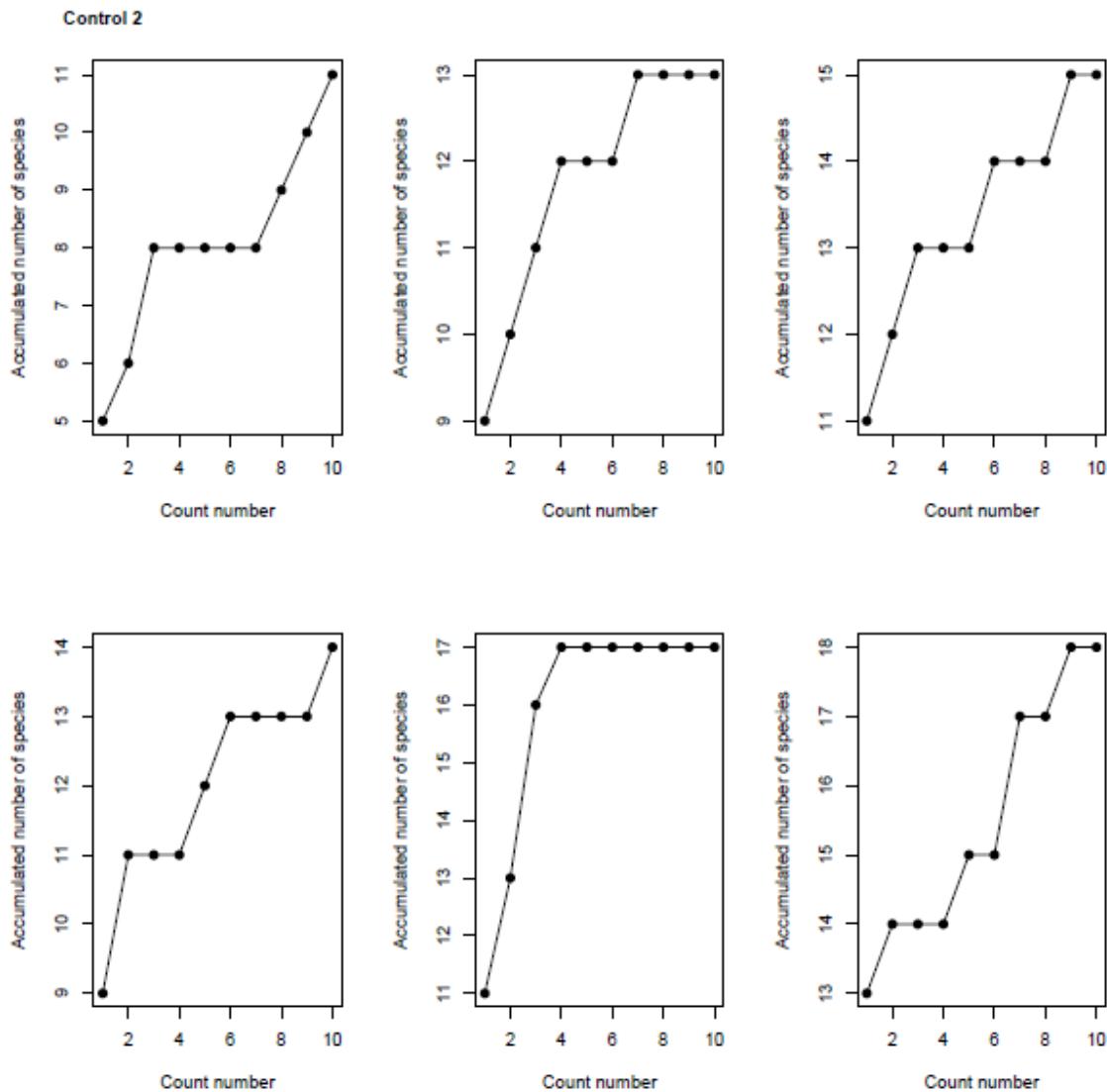


Figure 28: Rarefaction curve from culture Control 2

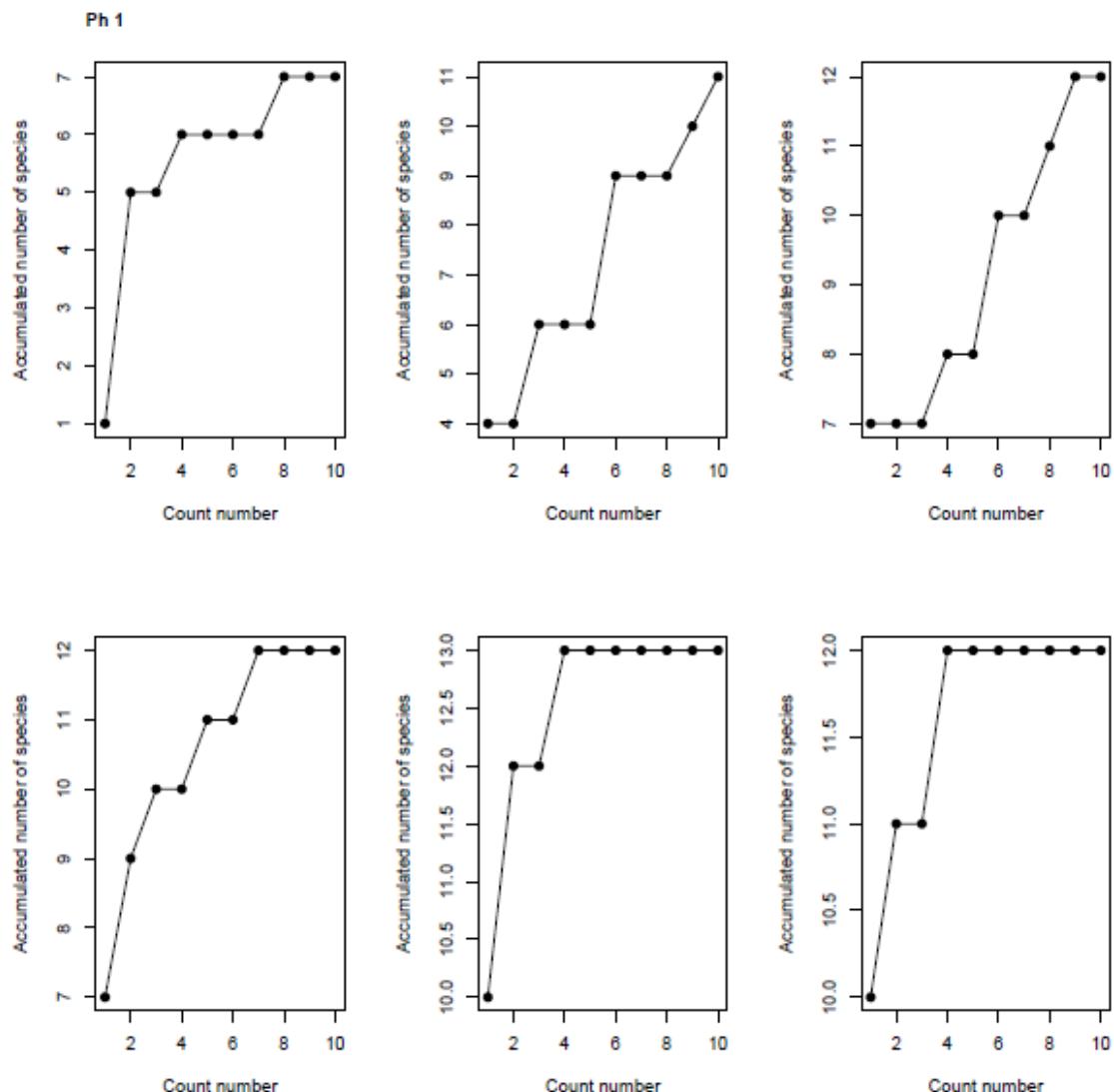


Figure 29: Rarefaction curve from culture *Phormidium 1*

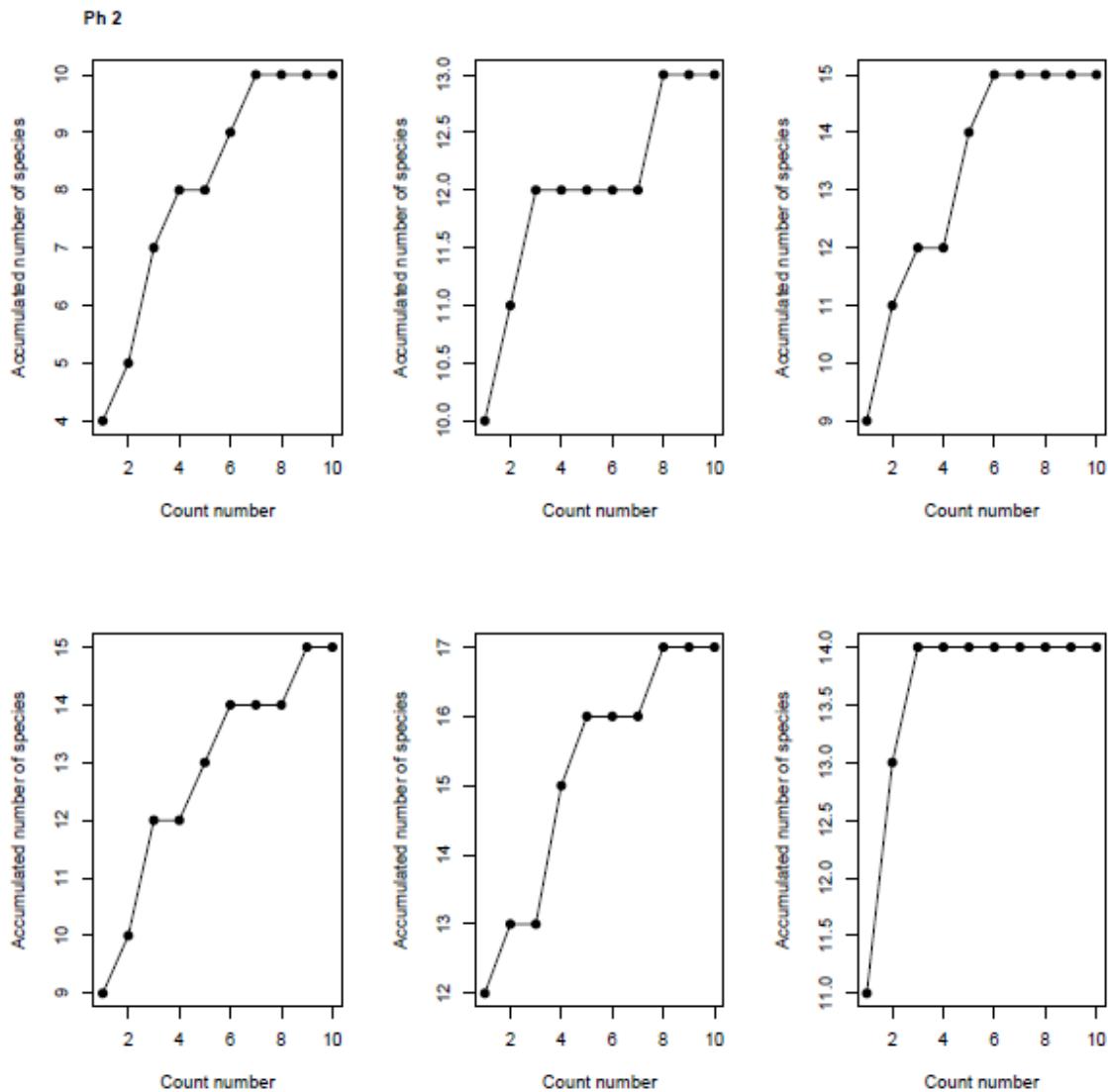


Figure 30: Rarefaction curve from culture *Phormidium 2*

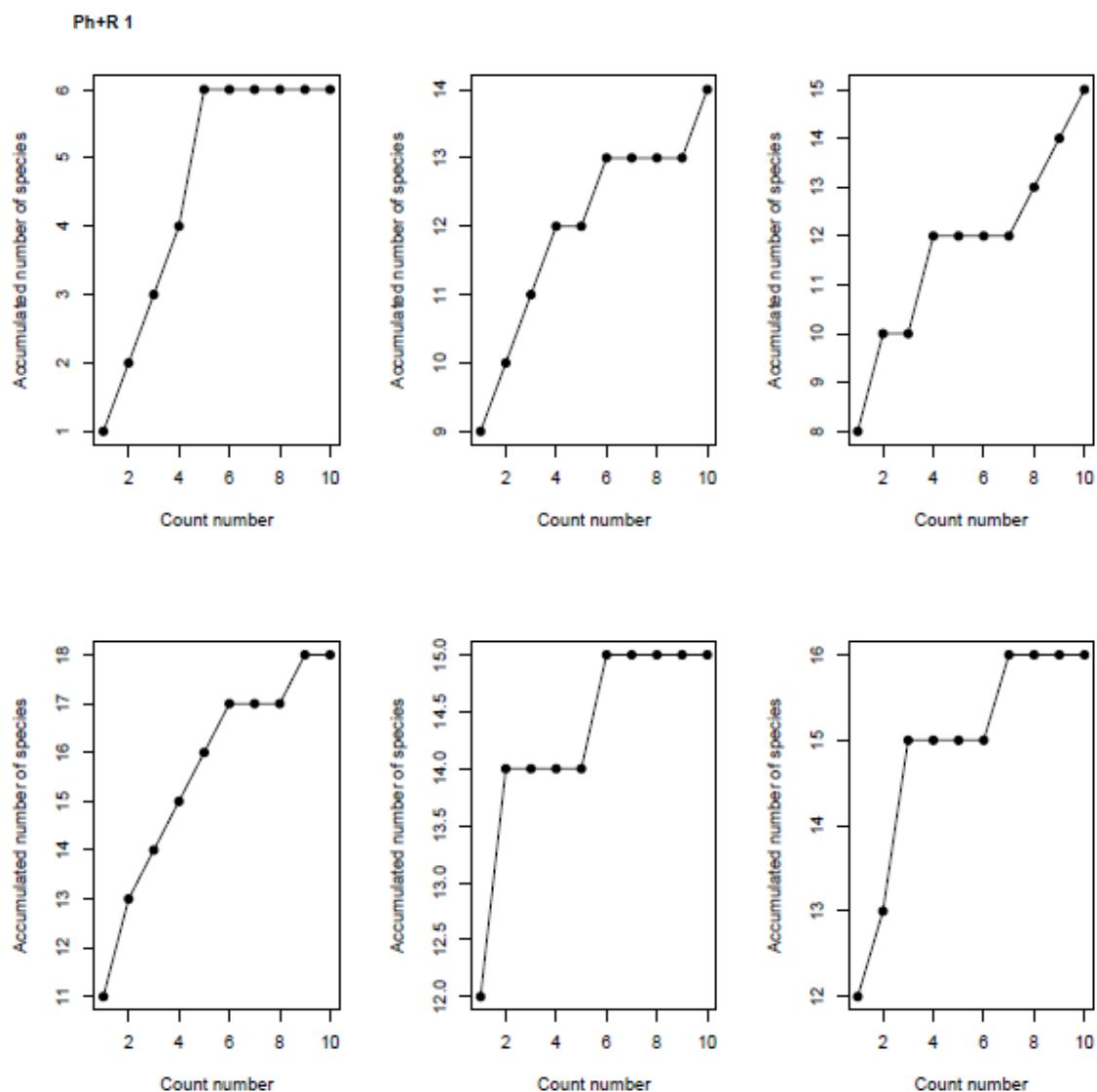


Figure 31: Rarefaction curve from culture Phormidium + rotifers 1

Ph+R 2

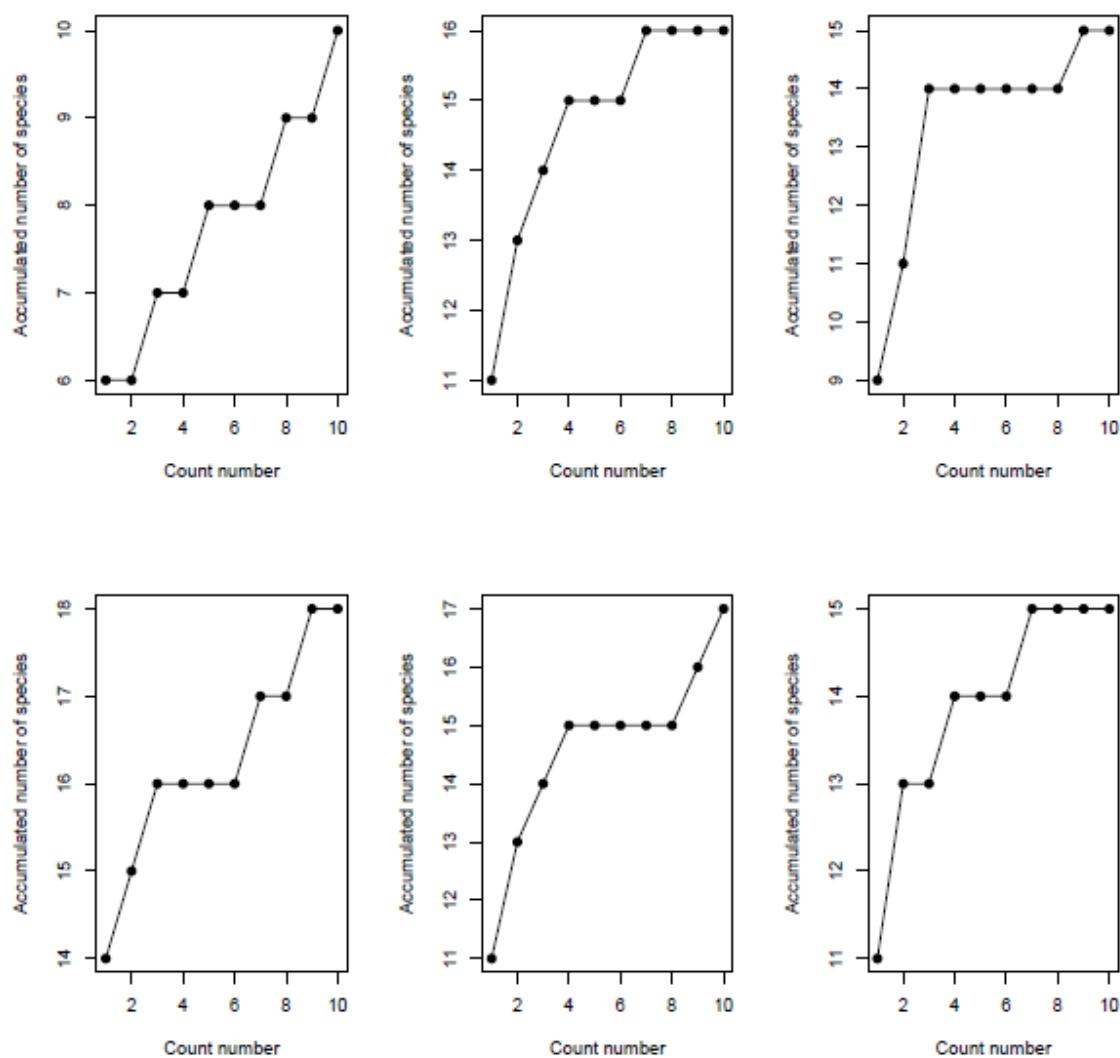


Figure 32: Rarefaction curve from culture Phormidium + rotifers 2

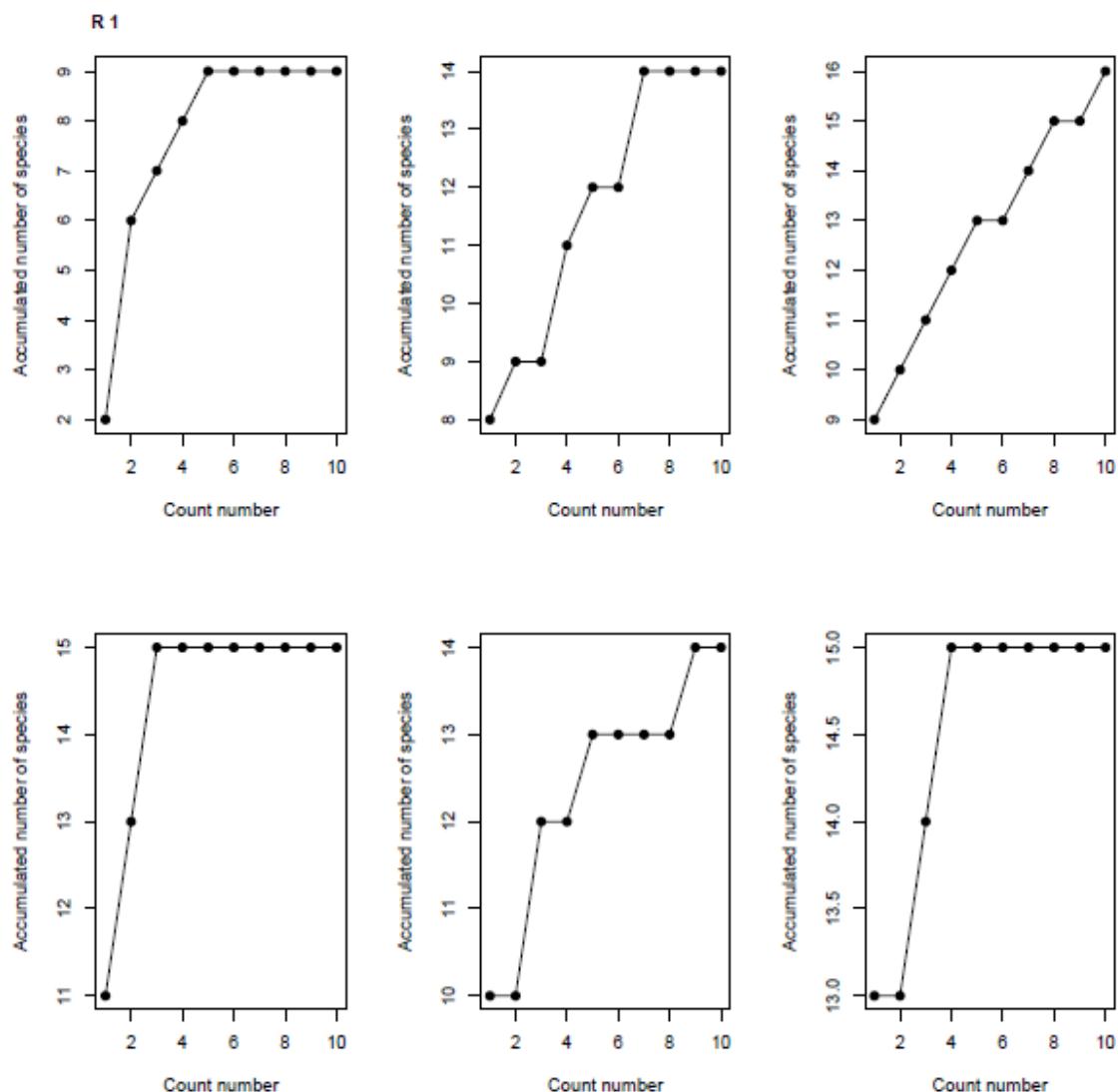


Figure 33: Rarefaction curve from culture Rotifers 1

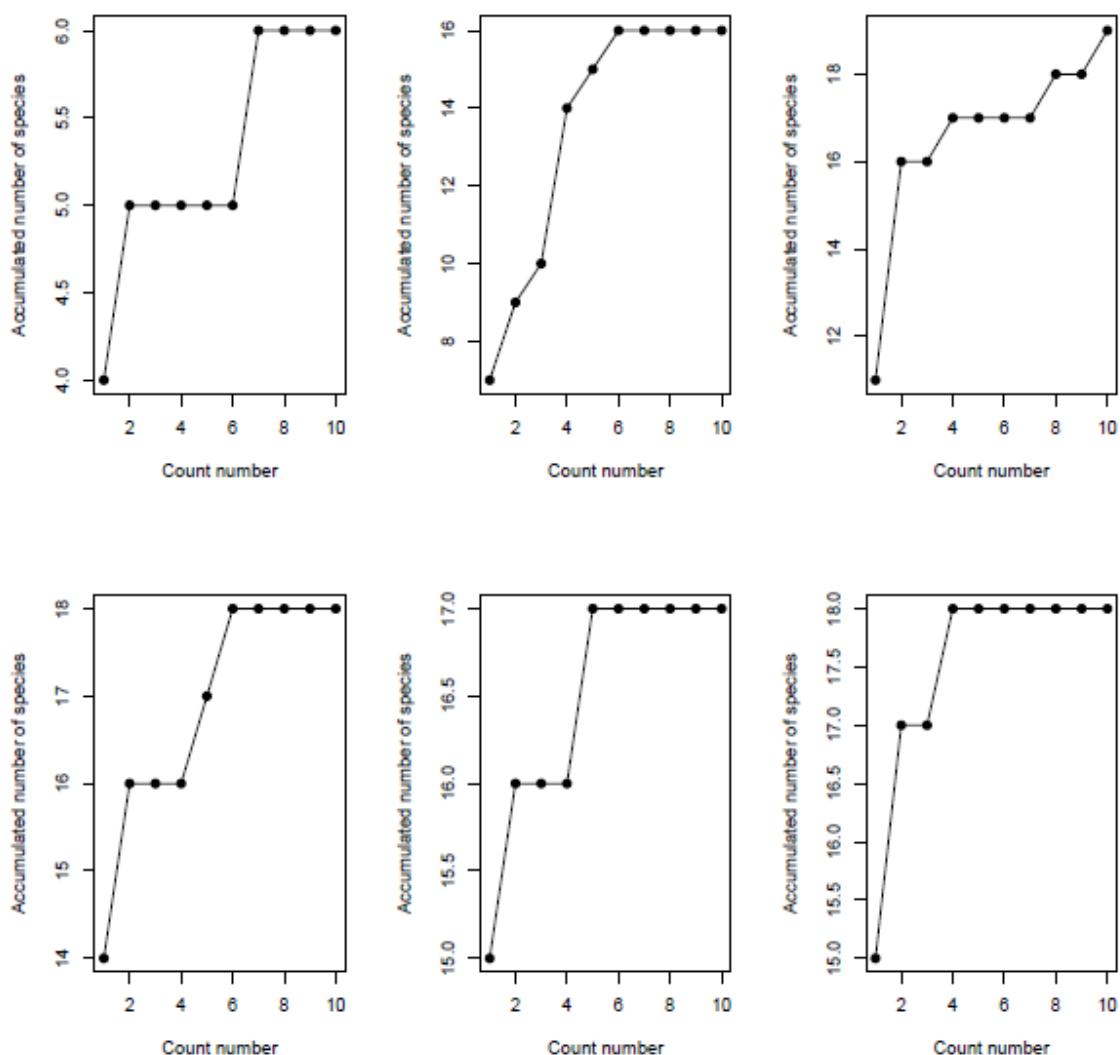


Figure 34: Rarefaction curve from culture Rotifers 2