

UNIVERSITY OF HELSINKI

M. Sc. Thesis

EVOLUTION IN HOST-PARASITE INTERACTION  
BETWEEN NOVEL CYANOPHAGE AND FILAMENTOUS,  
NITROGEN-FIXING CYANOBACTERIUM

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Tiivistelmä — Referat — Abstract  <p>The thesis begins with an introduction to the characteristics of experimental organisms, cyanobacteria and cyanophages, and their role in the marine biogeochemical cycles and food-webs. Subsequently, the methodology of experimental evolution and models of host-parasite dynamics are presented.</p> <p>The aim of the experimental part is to test predictions concerning the effects of host-parasite interactions on the marine nitrogen cycle, food-webs, and host properties. Methods include batch culture growth experiments, liquid chromatography–mass spectrometry, an optical density based phage resistance assay, plaque assay, and microscopy.</p> <p>To the author’s knowledge, this is the first controlled study that demonstrates that viral lysis of a diazotrophic cyanobacterium results in the release of cellular nitrogen to the environment in a form that fuels phytoplankton growth. However, evolution with the phage alters the effect. These observations highlight the importance of host-parasite interactions in biogeochemical cycles and food-webs.</p> <p>Further, a novel phage resistant host genotype with short filaments compared to other sensitive and resistant genotypes was detected, with increased growth ability but decreased buoyancy. Reduced buoyancy is proposed as a novel fitness cost of resistance. Phage-mediated evolution resulted in increased diversity in host filament length, growth ability, and buoyancy, supporting the hypothesis that parasites act as drivers of host diversity.</p>			
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## PREFACE

The master's thesis at hand was completed between October 2014 and April 2015 in the Division of Microbiology and Biotechnology (Department of Food and Environmental Sciences) at the University of Helsinki (UH). The research contributes to the PhD project of M. Sc. Sebastián Coloma (UH) on the role of viruses in the development of toxic cyanobacterial blooms and the nitrogen cycle in the Baltic Sea ecosystem. The work was funded by the Academy of Finland grant no. 1273652 to Kaarina Sivonen. The thesis was supervised by Docent Teppo Hiltunen and Professor Kaarina Sivonen (UH).

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This thesis is dedicated to the continuing effort to understand the fundamental mechanisms of evolution that have in the course of billions of years produced the diversity of life discernible today from a single microbial cell. In Dobzhansky's words, "nothing in biology makes sense except in the light of evolution" (Dobzhansky 1973).

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## LIST OF ABBREVIATIONS

Abi	abortive infection
ANOVA	analysis of variance
ARD	arms-race dynamics
bp	base pair
Cas	CRISPR-associated protein
CAS	Chemical Abstracts Service Registry
CRISPR–Cas	clustered regularly interspaced short palindromic repeats–CRISPR-associated proteins
crRNA	CRISPR RNA
Da	Dalton
DOC	dissolved organic carbon
DOM	dissolved organic matter
DON	dissolved organic nitrogen
ds	double-stranded
EPS	exopolysaccharide
FSD	fluctuating selection dynamics
HGT	horizontal gene transfer
IGS	integenic spacer region
ITS	internally transcribed spacer region
kb	kilobase
LC–MS	liquid chromatography–mass spectrometry
MTase	methyltransferase
<i>m/z</i>	mass-to-charge ratio
LPS	lipopolysaccharide
NRPS	nonribosomal peptide synthetase
OD	optical density
ORF	open reading frame
PAM	protospacer-adjacent motif
phage	bacteriophage
PKS	polyketide synthase
pre-crRNA	precursor CRISPR RNA
R	resistant to phage
RBP	receptor-binding protein
REase	restriction endonuclease
R–M	restriction–modification
RMANOVA	repeated measures ANOVA
S	sensitive to phage
SD	standard deviation
SE	standard error
Sie	superinfection exclusion
TA	toxin-antitoxin
TEM	transmission electron microscope
Tukey's HSD	Tukey's honest significant difference
WGS	whole-genome sequencing

## 1 INTRODUCTION

Hosts and parasites have continued to coexist in nature for billions of years despite having an antagonistic relationship (Avrani et al. 2012). Selection for hosts that are immune or resistant to parasites does not lead to parasite extinction, neither does selection for obligate parasites with higher infectivity lead to host extinction, proposing a paradox. In experimental evolution, a field of study that has emerged since the 1990s, living organisms, often microbial model systems, have been used to study the nature of host-parasite interactions in real time (Buckling et al. 2009). Microbes provide convenient study systems because of short generation times, the possibility to design experiments with large numbers of replicates, and easy storage of live samples in suspended animation. In the previous decade, experimental evolution has been used to design models of host-parasite dynamics that explain the coexistence paradox. These models are currently being tested with an increasing number of organisms, including marine bacteria and cyanobacteria. However, only a few experimental evolution studies have been conducted on cyanobacteria to date, and solely on members of the picocyanobacterial genera *Prochlorococcus* and *Synechococcus* (Martiny et al. 2014).

Cyanobacteria are key members of several marine ecosystems, including the Baltic Sea (Stal et al. 2003). Certain taxonomic groups, such as *Nodularia*, are capable of atmospheric nitrogen fixation. As opposed to photosynthesis, nitrogen-fixation is performed only by bacteria and archaea. Nitrogen-fixation by marine bacteria is essential for maintaining life on Earth as all life is dependent on nitrogenous compounds such as proteins and nucleic acids (Worden et al. 2015). In seeking to understand these processes, marine viruses must also be considered, since viral lysis may remove up to 40% of marine bacteria each day (Editorial 2011). The viral loop is a marine food-web short-circuit proposed to reroute up to 25% of organic matter in the sea (Bratbak et al. 1990, Wilhelm & Suttle 1999). This may increase the amount of dissolved organic matter in the ocean, with important ecosystem consequences, such as stimulation of phytoplankton growth, altering the food-webs through which nutrients flow. An increased understanding of interactions between bacteria and their viruses, bacteriophages (in short, phages), may change perceptions concerning marine nutrient cycles and food-webs.

Based on the above, host-parasite interactions between lytic phages and ecologically important diazotrophic cyanobacteria such as *Nodularia* may have large-scale ecosystem consequences. However, controlled laboratory studies are yet to be conducted on these interactions and effects (Martiny et al. 2014). It is especially uncertain how well models concerning host-parasite dynamics, derived from experiments with well-characterized organisms such as *Escherichia coli* and *Pseudomonas fluorescens* with different genetic and metabolic features, are applicable to marine filamentous cyanobacteria. From another viewpoint, studies on these organisms have the potential to provide novel insights into both the ecosystem effects of interactions between viral parasites and marine nitrogen-fixing cyanobacteria and into the mechanisms that maintain host-parasite coexistence.

The aim of this study was to analyze host-parasite interactions between a viral parasite and the diazotrophic, filamentous cyanobacterium *Nodularia spumigena* strain AV2, both isolated from the Baltic Sea. Specifically, predictions were tested concerning the effect of evolving interactions on the marine nitrogen cycle, planktonic food-webs, and phage and host properties. The work begins with a review of the properties of the organisms and their roles in marine biogeochemical cycles and food-webs. The methodology of experimental evolution and models of host-parasite dynamics are then presented. The subsequent experimental part focuses on two themes. Firstly, ecosystem-level effects of host-parasite interactions were examined by using the phage to challenge sensitive and resistant cells, and analyzing the nitrogen and nodularin content of thus obtained cell-free filtrates. Following this, phytoplankton strains representing green algae, diatoms, and picocyanobacteria were cultured in filtrates. Secondly, the nature of host-parasite interaction was examined by measuring phage sensitive and resistant host properties, including growth ability, filament length, bioactive secondary metabolite production, and buoyancy. Measurements were performed to test model predictions according to which phage resistance is associated with reduced fitness, and phage-mediated evolution is associated with increased host diversity. Finally, a discussion follows on the potential implications of experimental observations concerning both ecosystem effects and the nature of host-parasite interaction.

## **2 LITERATURE REVIEW**

### **2.1 Cyanobacteria and cyanophages**

#### **2.1.1 Cyanobacteria**

The bacterial phylum cyanobacteria consists of the oldest known (> 2.5 billion years) oxygenic phototrophs on Earth distributed worldwide and displaying tremendous physiological and ecological diversity (Lundholm & Moestrup 2006, Paerl & Fulton 2006). Cyanobacteria can be unicellular, colonial or filamentous, and the cells of certain groups have the ability to differentiate into nitrogen-fixing heterocysts or dormant akinetes (Mur et al. 1999). Most cyanobacteria rely on photosynthesis as a mode of energy harvesting, requiring only water, carbon dioxide and inorganic substances for their life processes. However, certain cyanobacteria display ability for heterotrophic nutrition. In addition to both the pelagic and benthic zones of lakes and oceans, cyanobacteria occur in extreme environments such as hot and cold arid deserts (Bahl et al. 2011), hot springs (Miller & Castenholz 2000), cryoconite holes in glaciers (Zakhia et al. 2008), and hypersaline pools (Abed et al. 2011). Cyanobacteria occur independently as well as in symbioses with fungi, plants and animals (Carpenter & Foster 2002, Syiem & Rai 2013).

Cyanobacteria have drawn the attention of the public and researchers due to the frequent formation, by certain taxonomic groups, of toxic planktonic blooms so massive they are visible in satellite images (Kahru et al. 1994). Toxic blooms have been reported in Europe, the Americas, the Middle East, Asia, Australasia, and Africa, including blooms in the Atlantic Ocean, the Indian Ocean, and the Baltic Sea (Codd et al. 1999). Biotoxins from bloom-forming cyanobacterial genera include the neurotoxins anatoxin-a, anatoxin-a(s) and saxitoxins, as well as the hepatotoxins microcystins, nodularins and cylindrospermopsins (Carmichael 2001). These biotoxins have been associated with numerous cases of acute and chronic, occasionally lethal, poisonings of mammals (Sivonen 2009), among others, livestock (Main et al. 1977, Sivonen et al. 1990, Van Halderen et al. 1995), dogs (Edler et al. 1985, Harding et al. 1995, Simola et al. 2012), and humans (Gi-

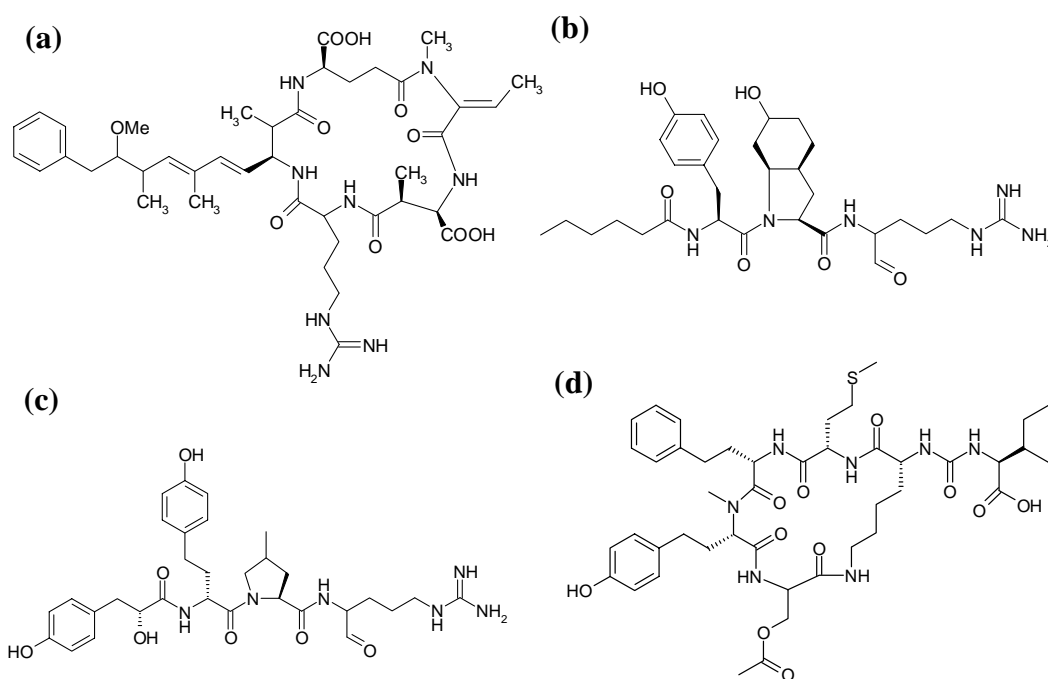
annuzzi et al. 2011). Investigation of cyanobacterial toxins has revealed that cyanobacteria are a prolific source of bioactive secondary metabolites, including compounds that show promise as human drug leads (Singh et al. 2005, Sivonen & Börner 2008, Liu et al. 2014b). The reason for production of these compounds remains unclear (Granéli & Turner 2006). Hypotheses include deterring of grazers, allelopathy, or coincidental bioactivity of compounds primarily associated with other cellular processes such as nitrogen storage.

### **2.1.2 *Nodularia spumigena***

*Nodularia* (order Nostocales) is a genus of filamentous, heterocystous cyanobacteria that occur in both aquatic and terrestrial environments (Baker 1991, Komárek et al. 1993, Janson & Hayes 2006). Numerous species have been morphologically defined. However, several molecular studies suggest that only one planktonic species, *N. spumigena*, is genotypically justified, while moderate support exists for two benthic species, *N. sphaerocarpa* and *N. harveyana*. Molecular approaches have included comparison of 16S rRNA genes, phycocyanin encoding gene and gas vesicle protein A encoding *gvpA* gene intergenic spacer regions (IGS), and rRNA internally transcribed spacer regions (ITS) (Hayes & Barker 1997, Barker et al. 1999, Bolch et al. 1999, Lehtimäki et al. 2000, Laamanen et al. 2001, Lyra et al. 2005, Janson & Hayes 2006). *N. spumigena* is morphologically distinguishable from other *Nodularia* species by breadth of vegetative cells (Baker 1991, Komárek et al. 1993). It can differentiate cells into heterocysts, akinetes or hormogonia (Voß et al. 2013). Hormogonia are short motile filaments that serve as a means of dispersal. *N. spumigena* forms blooms in coastal and estuarine areas across the globe, and belongs to the most frequent bloom-forming cyanobacteria in the Baltic Sea (Sivonen et al. 1989a, Bolch et al. 1999, Stal et al. 2003, Plough et al. 2011). *N. spumigena* blooms have been frequently associated with mammal poisonings, attributed to production of the hepatotoxin nodularin (Edler et al. 1985, Carmichael et al. 1988, Runnegar et al. 1988, Sivonen et al. 1989a, Yoshizawa et al. 1990, Nehring 1993, Harding et al. 1995, McGregor et al. 2012, Simola et al. 2012).

### 2.1.3 Bioactive secondary metabolites

In addition to nodularin, *N. spumigena* is known to produce bioactive secondary metabolites that belong to the aeruginosin, spumigin, pseudoaeruginosin and nodulapeptin peptide families (Fewer et al. 2009, Liu et al. 2014a). Nodularin is a cyclic pentapeptide synthesized nonribosomally by nonribosomal peptide synthetase (NRPS) modules and polyketide synthase (PKS) modules (Moffitt & Neilan 2004) (Figure 1a). These modules are large multienzyme complexes encoded by the 48-kb *nda* gene cluster containing nine open reading frames (ORFs). The Chemical Abstracts Service Registry (CAS) name of nodularin-R ( $m/z$  824 Da), unmodified nodularin with an arginine (R) residue, is cyclo[(2*S*,3*S*,4*E*,6*E*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-deca-dienoyl-D- $\gamma$ -glutamyl-(2*Z*)-2-(methylamino)-2-butenoyl-(3*S*)-3-methyl-D- $\beta$ -aspartyl-L-arginyl] (Rinehart et al. 1988, Sivonen et al. 1989a, IARC 2006). In addition to nodularin-R, at least seven nodularin variants have been identified, consisting of linear nodularin, a geometrical isomer of nodularin-R, three demethylated variants, and two variants with an additional methyl group (Mazur-Marzec et al. 2006).



**Figure 1.** Structures of bioactive secondary metabolites. **(a)** Nodularin-R, **(b)** aeruginosin NAL2 in *Nodularia*, **(c)** spumigin E, **(d)** nodulapeptin C. Courtesy of Jouni Jokela (2015).

Nodularin is a mammal hepatotoxin and hepatocarcinogen (Carmichael et al. 1988, Runnegar et al. 1988, Sivonen et al. 1989a, 1989b). Mammal toxicity has been associated with inhibition of protein phosphatases 1 and 2A (Yoshizawa et al. 1990, Honkanen et al. 1991, An & Carmichael 1994, Ohta et al. 1994). The role of nodularin as a grazer deterrent is undetermined. In one study, the abundances of copepods, rotifers, and cladocerans were found to be unrelated to nodularin-producing *Nodularia* (Repka et al. 2004). Further, the presence of zooplankton grazers has not been found to induce nodularin production (Lundgren et al. 2012, Brutemark & Enström-Öst 2013). The presence of allelopathic effects is uncertain, with contradicting experimental observations (Dokulil & Teubner 2000, Suikkanen et al. 2004, 2006, Karjalainen et al. 2007). Nodularin occurs primarily within the cell, and is released upon cell lysis (Lehtimäki et al. 1997, IARC 2006). In water, nodularin is relatively resistant to biodegradation, decreasing in one study in 21 days to over 70% of the initial concentration in sterile seawater but to 31.2% in non-sterile seawater, possibly due to breakdown by bacteria (Mazur & Pliński 2001). Nodularin has been shown to bioaccumulate in zooplankton, fish, mussels, and shrimp, although rapid detoxification has been observed in some cases (Sipiä et al. 2001, Kankaanpää et al. 2002, Beattie et al. 2003, Kankaanpää et al. 2005, Karjalainen et al. 2008, Vuorinen et al. 2009).

Aeruginosins have been found in various cyanobacterial genera as well as marine sponges (Ersmark et al. 2008) (Figure 1b). Aeruginosins are linear tetrapeptides whose biosynthesis occurs through highly variable NRPS pathways (Fewer et al. 2013). They all possess 2-carboxy-6-hydroxyoctahydroindole as the third unit, with unusual amino acids on the carboxy and amino termini, and a total of fifty-five variants have been described (Ersmark et al. 2008, Liu et al. 2014a). Spumigins have been found in the cyanobacterial genera *Nodularia* and *Anabaena* (Fujii et al. 1997, Liu et al. 2014a). Spumigins are structurally aeruginosin-like linear tetrapeptides synthesized by two NRPSs encoded by the 21-kb *spu* gene cluster containing two ORFs, and a total of twenty variants have been described (Fewer et al. 2009, Liu et al. 2014a) (Figure 1c). Pseudoaeruginosins, found in *N. spumigena*, are hypothesized to be hybrid peptides synthesized through co-operation between the aeruginosin and spumigin biosynthetic pathways, since they have structural features similar to both these compounds (Liu et al. 2014a). Nodulapep-

tins, also found in *N. spumigena*, belong to the diverse cyclic hexapeptide family of anabaenopeptins (Fujii et al. 1997, Mazur-Marzec et al. 2013) (Figure 1d). They are assembled by an NRPS complex, and contain a conserved ureido bond and D-Lys moiety (Rouhiainen et al. 2010).

In animals, aeruginosins, spumigins, pseudoaeruginosins, and nodulapeptins display bioactivity as serine protease inhibitors that affect important physiological processes such as blood coagulation (Ersmark et al. 2008). The ecological function of protease inhibitors is uncertain. However, they have been hypothesized to act as grazing deterrents through inhibiting the catalytic activity of digestive proteases, thereby reducing the digestibility of cyanobacteria (Jüttner & Wessel 2003, Rohrlack et al. 2004, Baumann & Jüttner 2008).

#### **2.1.4 Baltic Sea blooms**

Cyanobacterial blooms occur in the Baltic Sea year-round, especially in the late summer and open sea (Karjalainen et al. 2007). In recent decades, late summer blooms have been observed with increasing frequency. Cyanobacterial bloom formation has been connected to eutrophication through wintertime vertical mixing of river runoffs (Stal et al. 2003, Neumann & Schernewski 2005, Lilover & Stips 2008). Factors contributing to bloom development include rise in temperature, increased availability of light, stability of water column, and an N:P ratio below the Redfield ratio of 16 (otherwise, blooms of non-diazotrophic diatoms are favored) (Stal et al. 2003). Further, iron and phosphorus may be limiting nutrients, and high sulfate concentrations may prevent bloom-formation (Stal et al. 2003, Arrigo 2005, Neumann & Schernewski 2005). Blooms are short-term phenomena, lasting from weeks to several months in the Baltic Sea (Lips & Lips 2008). Surface blooms occur due to buoyancy conferred by gas vesicles. Photosynthetically produced oxygen bubbles have also been associated with cyanobacterial buoyancy (Dervaux et al. 2015).

The predominant diazotrophic cyanobacteria in the Baltic Sea are *N. spumigena*, *Aphanizomenon flos-aquae* and *Anabaena* spp. (Stal et al. 2003). However, they form a minority of the cyanobacterial biomass in Baltic Sea blooms, up to 80% of



which is composed of rapidly growing, small-sized, unicellular, non-diazotrophic picocyanobacteria, mainly *Synechococcus* spp. Passing of fixed nitrogen from the cyanobacterial bloom to nitrogen-limited picocyanobacteria may explain their mass occurrence (Stal et al. 1999, 2003). When a bloom decays, the cyanobacterial biomass is mainly decomposed in the water column but a part of it may reach the seafloor and sediment (Karjalainen et al. 2007).

### 2.1.5 Cyanophages

Cyanophages, viruses that infect cyanobacteria, can be considered to form an important group because of the ecological and evolutionary significance of their hosts whose abundance and diversity they may affect (Mann 2006). Research on cyanophages has focused on viruses infecting marine members of the unicellular picocyanobacterial genera *Synechococcus* and *Prochlorococcus*. In comparison to these, the viruses of freshwater and filamentous cyanobacteria are poorly characterized. Further, most knowledge has been obtained through examination of members of the most commonly observed phage families *Podoviridae* and *Myoviridae*, and considerably less is known about the *Siphoviridae*. The main themes of research have concerned the abundance, diversity and host-range of cyanophages, as well as the role of cyanophages in host photosynthesis and in mediating host abundance. Recently, an increasing amount of knowledge has begun to accumulate in terms of genomic, proteomic and transcriptomic factors, as well as the ecological and evolutionary interactions between cyanophages and their hosts.

The vast majority of known cyanophages belong to the three families of the morphologically defined order of tailed phages, Caudovirales: T4-like or TIM5-like *Myoviridae* with long, contractile tails; T7-like *Podoviridae* with short, non-contractile tails; or  $\lambda$ -like *Siphoviridae* with long, non-contractile tails (Gao et al. 2012, Sabeji et al. 2012) (Table 1). Phenetic classification of viruses is under dispute, since morphologically similar groups can include organisms with little sequence similarity and exclude organisms with high sequence similarity, as has been observed for Caudovirales (Lawrence et al. 2002). In the case of cyanophages, this is contrasted by the detection of a number of core T4-like genes in cyanomyoviruses and T7-like genes in a cyanopodovirus (Hess 2008). All Caudovirales

possess dsDNA genomes, representing group I in the Baltimore classification. Cyanomyoviruses have the largest (37–252 kb) and cyanopodoviruses the smallest genomes (42–48 kb), with cyanosiphoviruses in between (38–108 kb) (Mann 2006, Hess 2008, Sullivan et al. 2009). Cyanopodoviruses have been found to be very host-specific, while cyanomyoviruses tend to have broader host-ranges (Hess 2008). A collection of 17 Baltic Sea cyanophage isolates capable of infecting *N. spumigena* were found to contain only members of *Myoviridae* and *Siphoviridae* (Jenkins & Hayes 2006). Further, they were large in comparison to previously isolated marine cyanophages, and closely related but diverse in terms of morphology and host-range. While most studies have not addressed lysogeny, known to be common among *Myoviridae* and *Siphoviridae*, lysogenic cyanophages belonging to all three groups have been observed, especially among filamentous cyanobacteria (Chen & Lu 2002, Mann 2006, Ohki & Fujita 2008). The genome of *N. spumigena* strain CCY 9414 also encodes a phage integrase, an enzyme that mediates insertion of a lysogenic phage genome into the host genome (Groth & Calos 2004, Voß et al. 2013).

**Table 1.** Properties of cyanophages belonging to the order Caudovirales.

<b>Family</b>	<b>Tail morphology</b>	<b>Genome size (kb)</b>	<b>Model system</b>
<i>Myoviridae</i>	Long, contractile	37–252	T-even phages
<i>Podoviridae</i>	Short, non-contractile	42–48	T7
<i>Siphoviridae</i>	Long, non-contractile	38–108	$\lambda$

Seawater typically contains  $10^7$  virus particles  $\times$  ml<sup>-1</sup>, and up to  $10^8$  cyanophages  $\times$  ml<sup>-1</sup> have been observed in the upper layers of the ocean (Waterbury & Valois 1993, Suttle & Chan 1994, Wilhelm & Suttle 1999, Marston & Sallee 2003, Hess 2008). The biogeographic distribution of cyanophages seems to mimic that of their hosts, and optimal host growth conditions have been associated with increased phage numbers (Suttle & Chan 1994). However, high host densities do not always correspond with high phage densities, possibly due to development of phage resistance (Waterbury & Valois 1993, Sullivan et al. 2003). The role of cyanophages in regulating host abundance is poorly understood because of difficulties in estimating contact rates between phage and host, host-ranges, burst sizes

and phage decay rates (Mann 2006). These issues have given rise to highly varying infection rate estimates, for example, 0.005–56% in the case of marine *Synechococcus* populations. The contact rate, in particular, must be sufficiently high to cause a significant selection pressure, leading to changes in host community structure and resistant mutants. High selection pressures have been experimentally observed, including association of cyanophages with host bloom decay in marine environments across the world (Mann 2003, 2006, Mühlhling et al. 2005). Freshwater cases are also known, among them a cyanophage-mediated disruption of a food-web and subsequent trophic cascade effect in a lake in Kenya (Mann 2006, Peduzzi et al. 2014).

Studies on the role of phages in altering cyanobacterial dynamics and diversity have primarily focused on lytic phages, and the role of lysogenic phages is largely unknown (Martiny et al 2014). In one study, lysogenic phages were induced by mitomycin C in only 0.6% of *Synechococcus* cells compared to 80% in the case of heterotrophic bacteria (Ortmann et al. 2002). This indicates that temperate viruses may have a lower effect on cyanobacterial mortality than on the mortality of heterotrophic bacteria. Dynamics between cyanobacteria and lytic phages are discussed separately in Section 2.3.5.

## **2.2 Marine biogeochemical cycles and food-webs**

### **2.2.1 Role of cyanobacteria in marine biogeochemical cycles**

Global biogeochemical cycles, most importantly the carbon and nitrogen cycles, enable the sustainment of life on Earth as all organisms require carbon and nitrogen as components of cellular macromolecules. The role of the marine biosphere in these nutrient cycles is critical, accounting for half of global primary production (Worden et al. 2015). While in the terrestrial biosphere, plants are responsible for a large proportion of primary production, in the marine biosphere, photosynthetic microbes as components of marine plankton are considered to be of particular significance (Martínez-Espinosa et al. 2011, Stocker et al. 2013). Algal primary production results in diverse forms of dissolved organic matter (DOM) that have several routes to higher trophic levels, including food-chains associated with

the herbivorous and microbial food-webs, rerouted on occasion through the viral loop (see next section for a discussion of this topic) (Worden et al. 2015). The marine carbon and nitrogen cycles are interconnected processes linked through the stoichiometry of cellular composition. The cycles begin with conversions performed by phytoplankton species: in the carbon cycle, conversion of carbon dioxide to dissolved organic carbon (DOC); and in the nitrogen cycle, conversion of atmospheric diazotrophic nitrogen to dissolved organic nitrogen (DON) (Martínez-Espinosa et al. 2011, Stocker et al. 2013). As opposed to photosynthetic CO<sub>2</sub>-fixation, N<sub>2</sub>-fixation is performed purely by bacteria and archaea. These processes are followed by interconversions of the resulting DOM in the export of nutrients to the deep ocean, including partial remineralization and microbial respiration back into the atmosphere.

Cyanobacteria, prominent components of marine plankton, are considered to account for 20–80% of marine CO<sub>2</sub>-fixation and most of marine N<sub>2</sub>-fixation (Wilhelm & Suttle 1999, Berman-Frank et al. 2007, Gruber 2008). In the Baltic Sea, *N. spumigena* has been shown to play a substantial role in the total carbon flux even at low abundance (Plough et al. 2011). Further, the filamentous cyanobacteria *N. spumigena*, *Aphanizomenon* spp. and *Anabaena* spp. are together considered to be the principal N<sub>2</sub>-fixing organisms in the Baltic Sea (Stal et al. 2003, Plough et al. 2011). They have been demonstrated to fix surplus N<sub>2</sub> which is channeled through the food-web (Plough et al. 2011). *N. spumigena*, in particular, has been shown to contribute substantially to N<sub>2</sub>-fixation and NH<sub>4</sub><sup>+</sup> release during surface blooms. One reason for the dominance of *N. spumigena*, *Aphanizomenon* spp. and *Anabaena* spp. may be the possession of heterocyst cell forms that specialize in nitrogen-fixation. The enzyme nitrogenase used to fix nitrogen is protected in heterocysts from dioxygen-induced inactivation through mechanisms such as a high rate of respiration (Berman-Frank et al. 2003). This gives these organisms the special ability to perform CO<sub>2</sub>-fixation and N<sub>2</sub>-fixation simultaneously during the light period in comparison to non-heterocystous cyanobacterial genera that are only able to perform N<sub>2</sub>-fixation in darkness.

### 2.2.2 Cyanobacterial bloom food-webs

The concept “food-web” is used by ecologists to describe trophic, or feeding, interactions in natural communities, repeatable patterns being termed “food-web types” (Hlaili et al. 2014). Food-web types are defined by dominant trophic pathways identified based on biomass distribution, or material or energy flow among food-web compartments, that is, groups of organisms or chemical compounds with similar trophic characteristics. Other key food-web concepts include trophic levels, or the relationships of organisms to primary energy sources; food-chains, or linear series of organisms that use the previous as a food source; and trophic flows, or rates of transfer of a chemical compound or energy between compartments. Two classical planktonic food-web types have been identified (Cushing 1989). The herbivorous food-web describes a situation in which phytoplankton production is dominated by rapidly bloom-forming large-celled organisms, and grazing of mesozooplankton on phytoplankton forms the primary trophic pathway. The microbial food-web, or microbial loop, describes a situation in which phytoplankton production is dominated by small cells, and grazing of microzooplankton on microbes forms the primary trophic pathway. In an alternative scheme, planktonic trophic pathways are regarded as a continuum from the herbivorous to the microbial food-web, with intermediate stages consisting of varying proportions of both herbivorous and microbial grazing (Legendre & Rassoulzadegan 1995, Mousseau et al. 2001). Hlaili and others (2014) have recently suggested that classical models may need to be further modified to account for higher than expected phytoplankton consumption by microzooplankton.

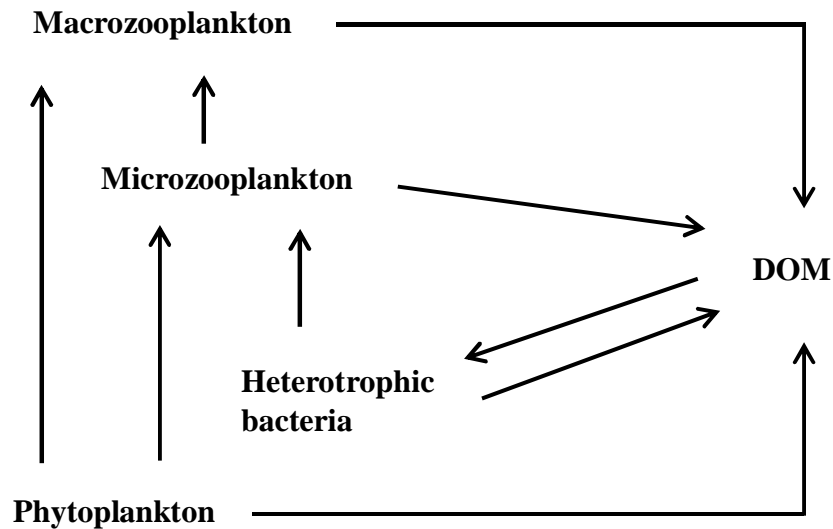
Cyanobacterial blooms can be considered to represent microbial food-webs, in which blooms serve as a food source for microzooplankton species such as flagellates and ciliates (Engström-Öst et al. 2002, Karjalainen et al. 2007, McCarthy et al. 2007). Alternatively, cyanobacterial blooms can be treated as intermediate food-webs in which cyanobacteria and associated phytoplankton groups such as diatoms together provide food for microzooplankton and mesozooplankton (Karjalainen et al. 2007). However, cyanobacterial blooms have a special feature in that they are commonly avoided by planktonic herbivores, possibly associated with lacking long-chain fatty acids required by zooplankton (Holland et al. 2012).

This interferes with the functioning of the primary trophic pathway of the microbial food-web. Recent experimental findings from a study on the assimilation of diazotrophic nitrogen from *N. spumigena* indicate that the primary trophic pathway may instead consist of a heterotrophic food-chain from cyanobacteria through associated bacteria to zooplankton (Woodland et al. 2013).

Another curious feature of cyanobacterial bloom food-webs is the production by cyanobacteria of bioactive secondary metabolites that may affect the functioning of trophic pathways. Some of the compounds, nodularin for example, have been hypothesized to cause decreased reproduction or death of secondary producers including zooplankton and their planktivores such as copepods, marine cladocerans, mysid shrimps, and fish (Karjalainen et al. 2007). Toxic compounds could accumulate in these organisms through grazing, water, or transfer in the food-web. The compounds may also have allelopathic effects on other phytoplankton species. Further, they may have an indirect effect through altering the behavior of secondary producers or competition for nutrients with other primary producers. This may result in an increase in the N:P ratio of the water, causing visual and mechanical interference of feeding and altering the spatial structure of the pelagic environment. Alterations in spatial structure may, in turn, generate vertical segregation of phytoplankton and contribute to a dynamical vertical structure in the food-web environment (Hajdu et al. 2007).

Food-web models have generally focused on free-living species, and the possibility of parasites playing a significant role has only recently achieved serious consideration (Dunne et al. 2013, Cirtwill & Stouffer 2014). However, because of their contribution to the diversity and mortality of marine bacteria, early attempts have been made to incorporate phages in marine food-webs (Wilhelm & Suttle 1999, Stoddard et al. 2007). The viral loop model proposed by Bratbak and others (1990) especially relates to cyanobacteria-phage interactions because of the prominent role of cyanobacteria in marine primary production. According to the model, phage-induced lysis of host-cells diverts the flow of nutrients from secondary consumers to the pool of DOM in the ocean (Figure 2). DOM is subsequently utilized by bacteria, which eventually return a part of it back into the food-web. It has been suggested that as much as a quarter of marine organic matter flows

through the viral loop (Wilhelm & Suttle 1999). The process has several potential ecosystem effects, including stimulation of phytoplankton growth, causing an increase in community respiration, and causing a reduction in the flow of matter and energy to higher trophic levels through the microbial food-web (Martiny et al. 2014).



**Figure 2.** The viral loop in marine food-webs. Lysis of host-cells diverts the flow of nutrients from secondary consumers (macrozooplankton) to the dissolved organic matter (DOM) pool in the ocean. DOM is subsequently utilized by heterotrophic bacteria, in turn consumed by primary consumers (microzooplankton). Up to 25% of marine organic matter is considered to flow through the loop. Adapted from Wilhelm and Suttle (1999).

## 2.3 Studying host-parasite interactions with bacteria-phage systems

### 2.3.1 Experimental evolution with microbial model systems

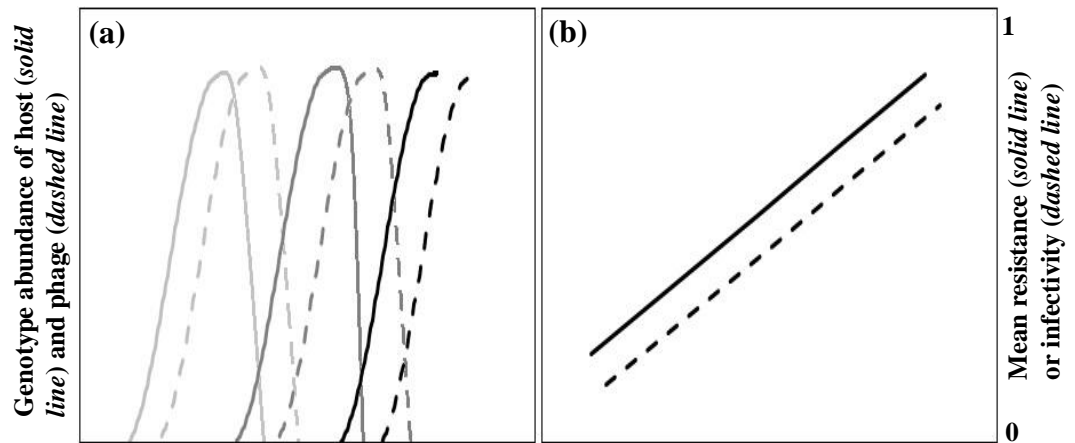
Experimental evolution is the study of evolution in real time through propagating replicate populations of organisms in laboratory conditions (Buckling et al. 2009, Schlötterer et al. 2014). Since the early 1990s, an increasing number of studies have appeared employing this approach. While multicellular organisms such as nematodes and mice have also been used, microorganisms are considered to be the most productive models. Reasons for this include large population sizes, short

generation times, easy storage of live samples in suspended animation, and well-characterized genomes, allowing rapid evolution and identification of genetic targets for selection. Although generalization of experimental observations to natural communities and larger organisms remains a disputed issue, experimental evolution with microbial model systems has produced a wealth of knowledge concerning fundamental ecological and evolutionary phenomena. These include the development of diversity, social behavior and sexual reproduction, as well as mechanisms of coevolution between organisms.

### **2.3.2 The host-parasite coexistence paradox**

Despite having an antagonistic relationship, hosts and parasites are known to have coexisted in nature for billions of years (Avrani et al. 2012). The Red Queen hypothesis posits that host-parasite coexistence requires constant adaptation, or “running,” from organisms in response to opposing organisms just to maintain their ecological position (Stenseth 1979, Lively 2010). However, how this running occurs is uncertain. Experiments with bacteria-phage systems have recently been used in an effort to answer this question. As obligate parasites, lytic viruses depend on host-cells for replication, causing death of the host in the process. This gives rise to the question why have they not caused their own extinction by killing all hosts. Experimental evolution studies with *Pseudomonas fluorescens* and phage  $\phi 2$ , in particular, have provided insights into the evolutionary dynamics that may explain bacteria-phage antagonistic coexistence (Buckling & Rainey 2002, Brockhurst et al. 2007, Hall et al. 2011). Initial experiments yielded evidence in favor of rapid arms-race dynamics (ARD) type coevolution (Figure 3). Host-phage cultures were serially transferred for hundreds of generations, after which the host was challenged by the phage from different time-points (Buckley & Rainey 2002). Average resistance was found to increase in the host, and infectivity to increase in the phage. In ARD type coevolution, host and parasite genotypes with wider resistance and infectivity ranges, respectively, are constantly being selected (Figure 3a). This results in a succession of genotypes, with directional evolution toward increased average resistance and infectivity (Figure 3b).





**Figure 3.** Arms-race dynamics (ARD) type coevolution. **(a)** In ARD, host (*solid line*) and phage (*dashed line*) genotypes (*different colors*) with higher resistance and infectivity ranges, respectively, are constantly being selected. **(b)** This results in directional evolution toward universal resistance (*solid line*) or infectivity (*dashed line*). Adapted from Martiny and others (2014).

### 2.3.3 Molecular basis of infectivity and resistance

Arms-race dynamics type coevolution is based on molecular mechanisms of resistance and infectivity, many of which have been characterized in detail. The mechanisms may target any phase of infection: phage adherence, genome ejection, or intracellular replication. Phage adherence relies on interaction between the phage receptor-binding protein (RBP) and host cell surface receptor (Samson et al. 2013). Receptors include proteins, polysaccharides and lipopolysaccharides (LPS). Bacteria can develop resistance by altering the structure and decreasing the expression of receptors (Labrie et al. 2010). Bacteria can also produce exopolysaccharides (EPSs) to create a physical barrier between phages and receptors. In turn, phages can modify RBPs to new receptors, develop recognition of altered receptors, and hydrolyse EPSs (Samson et al. 2013). Due to selection pressures, genes involved in host recognition are among the most rapidly evolving phage genes.

If phage adherence is successful, superinfection exclusion (Sie) systems, consisting of membrane-associated proteins often encoded by genes found in prophages, can be used to prevent the entry of phage DNA into host cells from specific receptors (Labrie et al. 2010). Phage genomes that manage to enter the cell may be digested by restriction–modification (R–M) or clustered regularly interspaced short

palindromic repeats–CRISPR-associated proteins (CRISPR–Cas) systems. In R–M systems, restriction endonucleases (REases) digest foreign DNA at specific recognition sites, while the host DNA is protected by modification of recognition sites by a partner methyltransferase (MTase) enzyme (Labrie et al. 2010, Samson et al. 2013). Type I REases cleave DNA at variable distances from the recognition site, Type II REases cleave DNA at fixed positions, and Type III REases cleave DNA 25–27 nucleotides away from the recognition site (Loenen et al. 2014). Type IV REases, which cleave DNA at variable distances from the recognition site, differ from the other types in that they recognize modified, usually methylated DNA. Passive antirestriction mechanisms of phages include modification of dsDNA by host MTase, as well as orientation, distance between and low number of restriction sites in genome (Samson et al. 2013). Active mechanisms include production of MTase, DNA-binding proteins that mask recognition sites, DNA-mimicking proteins that interfere with the R–M system, enzymes that stimulate the activity of host MTase, and enzymes that hydrolyze R–M cofactors (Westra et al. 2012, Samson et al. 2013).

The CRISPR-Cas system targets and digests foreign DNA through three steps: adaptation, expression and interference (Westra et al. 2012). Adaptation consists of acquiring *c.* 30 bp stretches of foreign DNA known as spacers between similar-sized direct repeats in CRISPR loci. During expression, *cas* genes are expressed, and CRISPRs are transcribed into precursor CRISPR RNAs (pre-crRNA) that are cleaved into mature crRNA containing a single spacer sequence. During interference, crRNA guides Cas proteins to digest homologous sequences (protospacers) (Westra et al. 2012, Samson et al. 2013). CRISPR-Cas systems are divided into three types (Westra et al. 2012). Pre-crRNA is cleaved by a Cas6 homolog in Type I and Type III systems and by RNase III and Cas9 in Type II systems. Further, in Type I and Type II systems, a conserved sequence known as the protospacer-adjacent motif (PAM), located downstream and upstream of the protospacer, respectively, is required for spacer acquisition and interference. Anti-CRISPR–Cas mechanisms of phages include single-nucleotide substitution in and deletion of the protospacer region or PAM, and production of anti-CRISPR proteins that interfere with the system (Samson et al. 2013). Phages can also encode their own CRISPR-Cas systems that inactivate the host antiviral defense system.

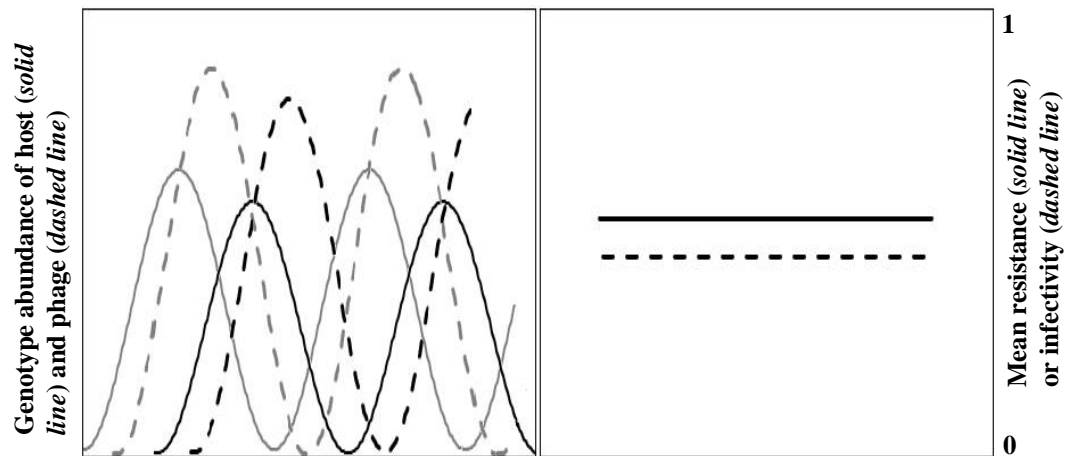
Finally, abortive-infection (Abi) systems inhibit phage replication and induce death of the host upon infection, presumably to allow non-infected bacteria to escape infection (Labrie et al. 2010, Samson et al. 2013). Abi systems include toxin-antitoxin (TA) systems. In TA systems, a toxin is kept ineffective by an antitoxin during normal bacterial growth but under stress, the antitoxin is degraded and the toxin induces dormancy or cell death. Anti-Abi mechanisms of phages include mutations in genes that prevent activation of Abi systems and encoding antitoxins that neutralize host toxins (Samson et al. 2013).

#### **2.3.4 Effect of antagonistic coexistence on host fitness and diversity**

Further experiments with the *Pseudomonas fluorescens*–phage  $\phi 2$  system, however, indicated that ARD is limited to a few cycles, as common genotypes eventually began to be replaced by rare genotypes without higher resistance or infectivity (Hall et al. 2011). The discontinuity of directional evolution has been explained by genetic constraints for the development of reinfectivity in the phage and metabolic constraints on fitness associated with phage resistance in the host (Hall et al. 2011, Koskella et al. 2011, Avrani et al. 2012). Firstly, it has been argued that bacteria-phage coevolution is constrained by a mutational asymmetry in favor of bacteria (Lenski & Levin 1985, Koskella & Brockhurst 2014). This is because bacteria have various mutational routes to the development of phage resistance while phages must evolve specific binding to modified or new receptors. Secondly, if resistance is brought about by a configurational change in a receptor molecule on the surface of the bacterial cell (interfering with phage adsorption), reduced rates of resource uptake may occur as a side-effect (Lennon et al. 2007). Cost of resistance has most commonly been observed in terms of decreased resource uptake and, consequently, decreased growth ability (Koskella & Brockhurst 2014). According to this line of reasoning, instead of super-resistant hosts, selection favors host genotypes with an optimal balance in terms of the positive selection pressure from resistance acquisition and negative selection pressure from fitness reduction (Martiny et al. 2014).

Models assuming specific fluctuation patterns in population compositions due to alterations in fitness between host genotypes under different selection pressures

have been used to explain prolonged coexistence between bacteria and phages in the absence of rapid arms-race dynamics. These include fluctuating selection dynamics (FSD) and the “kill the winner” hypothesis. In FSD, a rare resistant host genotype increases in frequency when the dominant genotype is infected by a phage (Hall et al. 2011, Avrani et al. 2012) (Figure 4a). Once the sensitive host has decreased to a low number, phage numbers begin to decrease as well, reducing the selection pressure imposed by the phage. Since the resistant host has lower fitness compared to the sensitive host, the sensitive host eventually rises to dominate again in the absence of phage-imposed selection. This completes one fluctuation cycle. The process results in a fluctuation between rare resistant and common sensitive host genotypes, with no change in average resistance or infectivity (Figure 4b). A similar model, the kill the winner hypothesis simply holds that phages control dominant genotypes, allowing sustainment of diversity among the host population (Thingstad 2000). Unlike FSD, the kill the winner hypothesis does not assume fluctuation between particular genotypes.



**Figure 4.** Fluctuating selection dynamics (FSD) type coevolution. **(a)** In FSD, sensitive host (*solid line*) genotypes (*different colors*) with higher fitness compared to rare resistant genotypes, and their phage (*dashed line*) genotypes, fluctuate in a frequency-dependent manner. **(b)** This results in non-directional evolution without a change in average resistance or infectivity. Adapted from Martiny and others (2014).

Fluctuating selection models assume a trade-off between phage resistance and fitness (Avrani et al. 2012, Våge et al. 2013). This has primarily been assumed to

imply reduced growth rate. However, while reduced growth ability is a common experimental observation, there are several cases in which it has not been detected (Lennon et al. 2007, Meyer et al. 2010, Avrani et al. 2011). Lack of sufficient experimental conditions may not explain all such instances. Resistance-conferring mutations associated with LPS and cell-wall biosynthesis genes instead of nutrient uptake associated genes may not result in decreased competitive ability in resource utilization (Avrani et al. 2012). Alternatively, a growth ability cost may be eliminated by compensatory mutations. Cost of resistance may involve not only a growth ability cost but also complex trade-offs between various host properties associated with fitness. These include increased infectivity by other phages, as proposed by Avrani and others (2011, 2012) based on experimental observations and the assumption of a specificity continuum between bacteria and phages. The reasoning is supported by previous observations of varying degrees of host specificity in phages (Bohannan & Lenski 2000), and by a theoretical specificity continuum model (Agrawal & Lively 2002).

Host-parasite coexistence through fluctuations in population composition predicts sustainment or increase in phage and host diversity in terms of species richness and genomic information (Weinbauer & Rassoulzadegan 2004, Avrani et al. 2012). Experimental observations widely demonstrate that phages act to maintain host diversity within genomes, populations and communities (Koskella & Brockhurst 2014). At the genomic level, diversity is brought about by acquirement of resistance and infectivity factors, phage-mediated horizontal gene transfer (HGT), including assortments of genes encoded within genomic islands, and sequence diversity obtained through the use of CRISPR-Cas systems (Banfield & Young 2009, Paterson et al. 2010, Avrani et al. 2011, Westra et al. 2012). At the population level, phages can increase allopatric diversity through rapid arms-race dynamics and net diversity through fitness trade-offs between phage resistance, growth rate, and competitive ability (Brockhurst et al. 2004, Benmayor et al. 2008, Marston et al. 2012). The latter especially applies to extreme environments and homogeneous environments with lack of diversification through resource competition. At the community level, factors such as differences between evolved phage resistant genotypes, level of resistance (dependent on phage-specificity), associated fitness costs and ability of phage to coevolve reinfectivity all have the potential

to alter population dynamics and thereby community structure and dynamics (Bohannan & Lenski 2000, Koskella & Meaden 2013).

### 2.3.5 Experiments with marine cyanobacteria-phage systems

According to ballpark estimates, there are up to  $1.3 \times 10^{29}$  bacteria and  $10^{30}$  viruses (primarily phages) in the ocean (Weinbauer & Rassoulzadegan 2004, Suttle 2007, Editorial 2011). This includes up to  $2 \times 10^6$  bacterial and archaeal species, of which only  $2 \times 10^4$  are represented by archaea (Weinbauer & Rassoulzadegan 2004). Viral diversity seems to be higher, with a potential ratio of 10 specific phages per bacterial species. In the ocean,  $1 \times 10^{23}$  viral infections occur per second, removing 20–40% of bacteria on a daily basis (Editorial 2011). These activities may play important roles in major biological processes, such as global biogeochemical cycles, marine food-webs, and the evolution of bacterial and viral genetic and functional diversity (Wilhelm & Suttle 1999, Suttle 2007, Martiny et al. 2014). Among marine bacteria, cyanobacteria are particularly important (see Section 2.2). Antagonistic interactions between marine cyanobacteria and their phages can therefore be considered to have wide-ranging implications. However, these interactions are still relatively poorly understood (Martiny et al. 2014). An overview is presented here of the main findings in the field to date.

Observations concerning the effect of phage infection and resistance on the dynamics of natural cyanobacterial communities are inconclusive. Waterbury and Valois (1993) isolated 10 clonal *Synechococcus* strains and 7 clonal *Myoviridae* strains from the same water sample collected from Woods Hole Harbor, USA. An infection assay demonstrated that the majority of *Synechococcus* isolates were resistant to most of the phages. This gave rise to the hypothesis that cyanobacterial communities are dominated by resistant cells, and that phages are maintained by infection of rare sensitive cells. On a similar note, McDaniel and others (2006) isolated 24 *Synechococcus* strains and 35 cyanophage (33 *Myoviridae* and 2 *Podoviridae*) strains, and detected 11–100% resistance to co-occurring phages. Suttle and Chan (1994) used cyanophage-containing seawater samples from the western Gulf of Mexico and Aransas Pass, USA, to infect five marine *Synechococcus* strains. Based on infectivity results they estimated that in nearshore waters, 80%

of *Synechococcus* cells are contacted daily by infectious phages, and only 1% of contacts needs to result in infection to balance virus removal rates. This, conversely, indicates that cyanophages may lyse a significant proportion of *Synechococcus* populations. Mühling and others (2005) examined the seasonal abundances of *Synechococcus* and co-occurring cyanophage genotypes. Covariation was detected between the abundance and genetic diversity of host and phage, as well as a significant relationship between host and phage assemblage structures. In line with McDaniel and others (2006), they conclude that cyanophage infection plays a significant role in picophytoplankton succession.

More recently, experimental evolution studies have emerged addressing the nature of cyanophage resistance to co-occurring phages. Support has especially been found for arms-race dynamics type directional coevolution. Stoddard and others (2007) cultured 4 marine *Synechococcus* strains with 32 *Myoviridae* isolates for a prolonged period to select for phage resistant mutant strains. Subsequently, they used the same phages in an infection assay with both ancestral and mutant strains. Resistant mutant strains were found to acquire cross-infectivity to an average of 8 other cyanophages. Similarly, Marston and others (2012) detected multiple co-evolved phage and host genotypes in a 6-month-long chemostat experiment with a marine *Synechococcus-Myoviridae* system. This was taken to indicate continued arms-race dynamics type coevolution, as no ARD end phase was observed. Coevolution was also found to increase the average resistance of *Synechococcus* to 31 other, genetically distinct myovirus strains, as well as increasing the ability of the phage to infect other *Synechococcus* strains.

Studies on the possibility of a fitness cost of phage resistance in marine cyanobacteria have produced varying results. Lennon and others (2007) used 4 phylogenetically distinct strains of marine *Synechococcus* and 32 *Myoviridae* isolates to produce 22 mutant strains resistant to one or more of the phages (i.e. with variable total resistance). Resistant strains were then assayed for differences in maximum growth rate and competitive ability against a *Synechococcus* reference strain. A cost of resistance was detected as a *c.* 20% reduction in relative fitness in only half of the experiments, and was associated with a few of the phages. The cost was not found to increase proportionally with total resistance according to coevo-

lutionary theory. Avrani and others (2011) compared the growth rates of 23 phage resistant mutant strains of *Prochlorococcus*, and found that 11 of them grew significantly more slowly than ancestral strains. This included all 4 mutants in which resistance-associated mutations had been detected in core genes, suggesting that mutations in core genes may be more strongly associated with a fitness cost than mutations in non-core genes. Closer examination of resistant strains with a high fitness cost indicated that they exhibited a trajectory of evolution toward improved growth rate and a narrower resistance range, primarily as a result of compensatory mutations (Avrani & Lindell 2015). This is suggested to explain the appearance of resistant cells in nature with high growth rates despite an initial growth ability cost of resistance. Further, increased sensitivity to other phages was found to be associated with resistance and proposed as a novel form of cost of resistance (see also Section 2.3.4) (Avrani et al. 2011). Despite an average increase in total resistance, individual *Synechococcus* strains with increased sensitivity to other phages as a result of phage resistance were also identified by Stoddard and others (2007) and Marston and others (2012). The latter also observed among their coevolution experiments high co-occurrence of host phenotypes with highly differing degrees of phage resistance, and suggested that this may be a result of a fitness cost of resistance.

Phages have been found to maintain or increase cyanobacterial diversity at several levels. At the genomic level, development of phage resistance has been attributed to prevention of phage adsorption by alteration of phage receptor sites, such as the O antigen component of the LPS (Xu et al. 1997, Stoddard et al. 2007). Recently, CRISPR–Cas adaptive immunity systems have also been detected in cyanobacteria (Kuno et al. 2012, Chan et al. 2014). Further, a number of studies indicate reciprocal genome evolution through phage-mediated horizontal gene transfer (HGT) of photosynthesis and other metabolism genes between cyanobacteria (Lindell et al. 2004, Zeidner et al. 2005, Sullivan et al. 2006, Lindell et al. 2007, Avrani et al. 2011). At the population level, ARD type coevolution has been observed to lead to a rapid increase in the phenotypic diversity of a marine *Synechococcus* strain (Marston et al. 2012). A correlation has also been observed between the genetic diversity of marine *Synechococcus* and their phages (Mühling et al. 2005, Mann 2006). At the community level, the effect of coevolution on the abil-



ity of other phages to infect the cyanobacterial host may result in an interruption of pairwise coevolution (Marston et al. 2012). This dynamic might prevent the dominance of specific genotypes, facilitating fluctuations in population composition that enable antagonistic coexistence.

### 3 RESEARCH OBJECTIVES

The objective of this project was to examine the ecosystem effects and nature of evolving host-parasite interactions between the viral parasite 2AV2 and the filamentous, atmospheric nitrogen fixing cyanobacterial host *N. spumigena* strain AV2. Specifically, the following predictions were experimentally tested:

- i. host-parasite interactions affect the release of cellular nitrogen and nodularin to the environment
- ii. by affecting release of cellular compounds, host-parasite interactions indirectly affect phytoplankton growth
- iii. arms-race dynamics lead to coevolution of reinfectivity in the phage
- iv. phage resistance is associated with a fitness cost, detected as decreased growth ability, bioactive compound production, or buoyancy
- v. phage-mediated evolution increases the diversity of the host, detected as increased variability in the above properties

## 4 EXPERIMENTAL PART

### 4.1 Materials and methods

#### 4.1.1 Strains used in this study

The filamentous, atmospheric nitrogen fixing host species, *N. spumigena* strain AV2, isolated from the Baltic Sea in August of 1987, was obtained from the University of Helsinki Culture Collection HAMBI/UHCC (Sivonen et al. 1989a). The cyanophage 2AV2, identified as a member of *Siphoviridae* based on morphology observed with transmission electron microscopy (TEM), was isolated from the surface water in the Gulf of Finland in June of 2010 after the disappearance of a *Nodularia* bloom (Coloma et al. 2015). The identity and origin of the eleven phytoplankton strains used in this study are listed in Table 1.

**Table 2.** Phytoplankton strains used in this study.

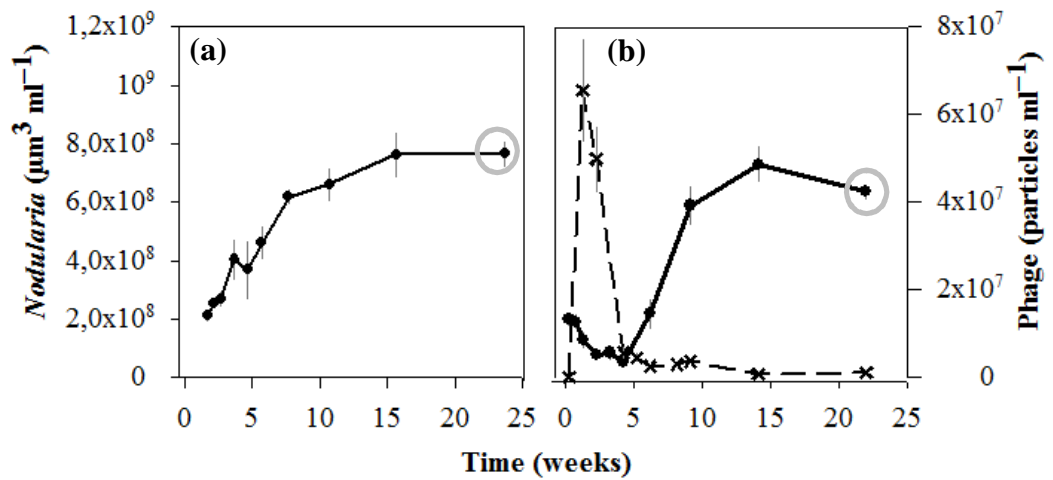
Strain identity	Phylum	Origin	Source
<i>Chlamydomonas reindardtii</i> UTEX 89	Chlorophyta	MA, USA	1
<i>Chlorella pyrenoidosa</i> TV 216	Chlorophyta	Baltic Sea	2
<i>Chlorella vulgaris</i> UTEX 26	Chlorophyta	CZ	1,3
<i>Phaeodaktylum tricornerutum</i> TV 335	Bacillariophyta	Baltic Sea	2
<i>Rhodomonas</i> sp. Crypto07-B1	Cryptophyta	Baltic Sea	4
<i>Scenedesmus obliquus</i>	Chlorophyta	Unknown	5
<i>Synechococcus</i> sp. CCY 0417	Cyanobacteria	Baltic Sea	6
<i>Synechococcus</i> sp. CCY 0435	Cyanobacteria	Baltic Sea	6
<i>Synechococcus</i> sp. TV65	Cyanobacteria	Baltic Sea	2
<i>Synechocystis</i> sp. UHCC 0318	Cyanobacteria	Baltic Sea	7
<i>Thalassiosira pseudonana</i> TV5	Bacillariophyta	Baltic Sea	2

1 UTEX = The Culture Collection of Algae at the University of Texas at Austin, 2 Tvärminne Zoological Station of the University of Helsinki (described in Hällfors & Hällfors 1992), 3 Yoshida et al. 2004, 4 courtesy of Anke Kremp, Tvärminne Zoological Station, 5 Max-Planck Institute für Limnologie, 6 Haverkamp et al. 2009 (CYY = Culture Collection Yerseke), 7 University of Helsinki Culture Collection.

#### 4.1.2 Sample collection and isolation of clonal strains

The populations of *N. spumigena* AV2 studied here were the result of a 22-week-long microcosm experiment consisting of two treatments, host alone and host with

the phage 2AV2, with three biological replicates in both treatments (Coloma et al. 2015). In the viral treatment, a mass mortality event was followed by an increase in the host after week 5, indicating selection for phage resistant genotypes (Figure 5). The *N. spumigena* population that evolved alone, assumed to be dominated by phage sensitive genotypes, is hereafter referred to as the naïve population. The population that evolved with the phage, assumed to be dominated by phage resistant genotypes, is hereafter referred to as the evolved population.



**Figure 5.** Evolutionary history of experimental populations (mean  $\pm$  standard error, SE). Samples were collected from week 22 (grey circles). (a) The phage sensitive genotype dominated population consists of the host *N. spumigena* AV2 (solid line) that has evolved alone. (b) The phage resistant genotype dominated population consists of the host that has evolved together with the phage 2AV2 (dashed line).

Samples were collected at the end of the experiment (week 22). To test the effect of host-parasite dynamics on the release of cellular compounds and phytoplankton growth ability, *N. spumigena* was precultured for 3 weeks in Z8 medium with salt and without nitrogen (Kótai 1972, Lehtimäki et al. 1994) (Appendix 1). The medium was prepared in glass containers in type-2 analytical grade water (ELIX® water purification system, Millipak® 40 0.22  $\mu\text{m}$  filter, Merck Millipore, Billerica, MA, USA) and sterilized by autoclaving. Culturing was performed in a volume of approximately 500 ml in a 850 ml tissue culture flask with a 0.22  $\mu\text{m}$  hydrophobic filter vented cap (VWR Standard Line Cell Culture Flasks, PA, USA) at  $25 \pm 2^\circ\text{C}$  and a continuous light intensity of  $5\text{--}8 \mu\text{mol m}^{-2} \text{s}^{-1}$ . To test the effects of host-

parasite dynamics on host fitness and diversity, approximately 20 clonal strains were isolated from each of the three biological replicates in both treatments ( $n = 118$ ). Strains were isolated and purified by culturing samples in 0.55% agarose plates containing Z8 medium with salt and without nitrogen. Upon plating, individual filaments were marked under a light microscope (Olympus CK2, Tokyo, Japan), allowed to grow into colonies, and transferred to fresh medium. Purified isolates were cultured in Z8 medium with salt and without nitrogen in the same conditions as above, except that culturing was performed in a volume of approximately 20 ml in a 75 ml tissue culture flask (Standard TC Flask T75, Sarstedt, Nümbrecht, Germany) with a loosely sealed cap to permit exchange of gases.

#### **4.1.3 Phage resistance and coevolution**

The phage resistance of isolates was determined by a previously used optical density (OD) based method (Torres-Barceló et al. 2014). A volume of 20  $\mu\text{l}$  (*c.*  $10^7$  phage particles) of phage stock containing approximately  $4.5 \times 10^7$  phage particles, or the same stock inactivated by autoclaving, was added to 200  $\mu\text{l}$  of clonal culture ( $n = 118$ ), with 4 biological replicates for both treatments. OD at 750 nm (UV-1800 spectrophotometer, Shimadzu, Japan) was recorded after 7 and 16 days. Phage resistance was analyzed as a quantitative trait as the difference in OD obtained with active and inactivated phage added. Phage sensitive and resistant isolates are hereafter referred to as sensitive and resistant genotypes based on the assumption that there is a genetic basis for the distinct phenotypic difference. This is in accordance with the custom commonly used in ecology and evolutionary biology (Jones et al. 2009, Becks et al. 2010).

Coevolution of reinfectivity in the phage against isolates from the evolved population ( $n = 58$ ) during the 22-week-long microcosm experiment was determined by using a modified version of the OD method. To produce evolved phage stocks, supernatant from evolved population cultures (three biological replicates) from week 22 of the microcosm experiment was obtained by centrifugation (7 min at  $7,000 \times g / 4^\circ\text{C}$ ), and stored in darkness at  $+4^\circ\text{C}$ . The supernatant was subsequently used to challenge the ancestral culture collection host strain by adding 100  $\mu\text{l}$  of

supernatant to approximately 200 ml of recently refreshed host culture. After 10 days, culture supernatant was obtained by centrifugation (7 min at  $7,000 \times g / 4^{\circ}\text{C}$ ), and stored in darkness at  $+4^{\circ}\text{C}$ . Phage counts were performed by a modified plaque assay from 1 ml subsamples collected in cryotubes and stored in darkness at  $+4^{\circ}\text{C}$  (Appendix 2). Phage particle numbers were determined from the number of plaque forming units (PFU) on a seeded agarose plate (Z8 medium with salt and without nitrogen), containing a base layer with 0.5% agarose and upper layer with a mixture of sample and host in high density in soft 0.25% agarose. Plates were cultured for 6 days in the same conditions as before (Section 4.1.2), after which plaques were counted from plate replicates from two different dilutions. Subsequently,  $8.5 \times 10^6$  ancestral or evolved phage particles (20–60  $\mu\text{l}$ ) were added to 200  $\mu\text{l}$  host culture, with 8 biological replicates for both treatments. The evolved phage was added from the same microcosm replicate culture from which the clonal strain had been isolated. In addition to phage-evolved clonal strains, ancestral and evolved phage particles ( $n = 4$ ) were used to challenge the ancestral culture collection host strain to determine evolution of infection efficiency. OD was subsequently recorded after 0, 1, 2, 3, 4, 7, 9 and 11 days. Coevolution was analyzed as the difference in OD obtained with ancestral and evolved phage added.

#### **4.1.4 Release of cellular nitrogen and nodularin**

Both naïve and evolved populations were challenged by the phage for 4 days, after which viral lysis had removed most of the naïve population. A volume of 50 ml phage stock was added to 550 ml recently refreshed host cultures with leveled densities to equalize the initial concentrations of compounds. Host and phage counts were performed daily. Total nitrogen and nodularin were quantified at the beginning and end of the experiment, and daily, respectively, from 0.22  $\mu\text{m}$  filtrates. Host counts were performed by light-microscopy (CKX41 Olympus Inverted Microscope, Tokyo, Japan) with a  $10\times$  objective for 1 ml samples collected in cryotubes, preserved in 2% Lugol's solution, and stored in darkness at  $+4^{\circ}\text{C}$ . Filaments were measured from images taken with an Olympus SC30 digital camera using the CellSens standard v. 1.7 (Olympus) software. Phage counts were performed as before (Section 4.1.3). Total nitrogen was determined from 50 ml samples stored in 50 ml falcon tubes at  $-20^{\circ}\text{C}$  by a third party (MetropoliLab, Helsin-

ki, Finland) using the standard SFS-EN ISO 11095-1 that follows a previously described method (Grasshoff 1976).

Nodularin analysis was performed from a 1 ml sample collected in a cryotube, freeze-dried and extracted with 1 ml of 75% HPLC grade methanol (Fisher Scientific, USA) for 1 h at 80°C. Cell debris was centrifuged (2 min at 20,000 × g), and liquid chromatography–mass spectrometric (LC–MS) measurements were performed on the supernatant. Fresh, freeze-dried or eluted samples were stored at –80°C. Extracts were analyzed (injection volume 10 µl) with an Agilent 1100 Series LC/MSD Trap System high-performance liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA), which has an XCT Plus model ion trap as a mass detector. The ionization method used was electrospray ionization (ESI) in positive mode. The column used in high-performance LC was Phenomenex Luna C18 (150 by 2.0 mm, 5 µm) (Phenomenex, Torrance, CA, USA). The mobile phase was composed of 0.1% formic acid in water (A) and 0.1% formic acid in 2-propanol (B) The gradient run was from 5% to 100% B over 50 min at a flow rate of 0.15 ml min<sup>-1</sup> at 40°C. In the ion source nebulizer gas (N<sub>2</sub>) pressure was 207 kPa, desolvation gas flow rate 8 liters/min, and the desolvation temperature was 350°C. The capillary voltage was set to 3,270 V, the capillary exit offset was 152.4 V, the skimmer potential was 40.0 V, and the trap drive value was 106.1. Spectra were recorded 50 to 1,200 *m/z* and a scanning rate of 26,000 *m/z* s<sup>-1</sup>. Samples were measured between two identical sets of standard dilutions. A nodularin stock solution with a concentration of 8.835 ng × ml<sup>-1</sup> was prepared by dissolving 1.767 µg of nodularin in 200 µl of 75% HPLC grade methanol (Fisher Scientific, USA). A dilution series was prepared from the stock solution, and eight dilutions ranging from 0.432 to 55.219 ng × ml<sup>-1</sup> were measured. Data analysis was performed from extracted ion chromatogram (EIC) peak areas using the LC/MSD Trap Software 5.2 Data Analysis program (Agilent Technologies, USA). Nodularin was identified based on the unit mass 825.7 from the ion trap, roughly corresponding to mass-to-charge ratio (*m/z*), and sample concentrations were determined from a standard curve created from standard dilution peak areas.

#### 4.1.5 Growth ability of phytoplankton strains

To examine the large-scale effect of host-parasite interactions on other members of the phytoplankton community, strains representing green algae, diatoms, and picocyanobacteria were cultured in filtrates from naïve and evolved population cultures following phage-exposure. After host populations had been cultured with the phage for 4 days (see previous section), cultures were sterile-filtered to obtain filtrates of medium containing dissolved compounds from cyanobacterial cells. Cultures were centrifuged (7 min at  $7,000 \times g / 4^{\circ}\text{C}$ ) and filtered through a  $70 \mu\text{m}$  nylon cell strainer (Falcon®, NY, USA), followed by filtration through  $0.22 \mu\text{m}$ . To allow diatom growth and equalize culturing conditions for all phytoplankton strains,  $\text{Na}_2\text{SiO}_3 \times 9\text{H}_2\text{O}$  was added by filtration of stock solution through  $0.22 \mu\text{m}$  to a final concentration of 106 mM. Filtrates were collected in glass bottles that had been acid-washed with 2% HCl and sterilized by autoclaving, and stored in darkness at  $+4^{\circ}\text{C}$ . Phytoplankton strains (Section 4.1.1) were precultured in Z8 medium with salt and 106 mM  $\text{Na}_2\text{SiO}_3 \times 9\text{H}_2\text{O}$  in a volume of approximately 100 ml in a 250 ml tissue culture flask (PE Vented Cap, Sarstedt) in the same conditions as before (Section 4.1.2). Prior to beginning the experiment, phytoplankton strains were nitrogen-starved (from dissolved nitrogen in medium) for 3 days by centrifugation (7 min at  $7,000 \times g / 4^{\circ}\text{C}$ ), rinsing with 50 ml Z8 medium with salt and without nitrogen, and repeating the process, with resuspension in 100 ml medium. The process was repeated on day 2, with resuspension in 40 ml medium.

The experiment was performed by adding phytoplankton strains in three replicates to a final cell density of  $10^4 \text{ cells ml}^{-1}$  in 40 ml of Z8 medium with salt and without nitrogen in a 75 ml tissue culture flask (PE Vented Cap, Sarstedt). Culturing was performed in the same conditions as before, and cells were enumerated each week until maximum cell density had been reached (Appendix 3). For cell enumeration, 0.5 ml samples were collected in cryotubes, fixed with 2% glutaraldehyde and stored in darkness at  $+4^{\circ}\text{C}$ . Cell enumeration was performed using a compound microscope (Zeiss Axioskop 2 plus, Oberkochen, Germany) with a  $40\times$  objective and a haemocytometer counting chamber (Improved Neubauer, Marienfeld, Germany). Eukaryotic phytoplankton strains were determined by light microscopy and picocyanobacterial strains (*Synechococcus* and *Synechocystis*) by



epifluorescence microscopy using a tritic fluorescence filter and HBO 100 W mercury vapor short-arc lamp. Cell densities below  $10^3 \text{ ml}^{-1}$  were not determined. To avoid underestimating the cell densities of samples that were not enumerated, their cell densities were assumed to be  $10^3 \text{ ml}^{-1}$ . Biovolumes were also determined, since phytoplankton cell biovolume is an important ecological parameter related to various other parameters, including cell size, carbon content, and cell physiology (Hillebrand et al. 1999, Sun & Liu 2003). Biovolumes were calculated using species-specific standardized geometric formulas and size-classes (Olenina et al. 2006).

#### **4.1.6 Filament length, cell size and heterocyst density**

Visual and light-microscopic observations of resistant isolates indicated that isolates from the evolved host population contained a significant proportion of a phenotype with a considerably shorter mean filament length compared to other isolates. To confirm the observation, mean filament length was determined for 34 randomly selected isolates from the naïve population and all isolates from the evolved population ( $n = 58$ ). Samples (1 ml) from clonal cultures were collected in cryotubes, preserved in 2% Lugol's solution, and stored in darkness at  $+4^\circ\text{C}$ . For each isolate, the length of 20 filaments, selected based on being representative of filament length distribution, was measured using the same microscope, objective and software as in host counts (Section 4.1.4). Resistant genotypes are hereafter referred to as either long-filamentous genotypes, with similar filament length compared to sensitive genotypes, or short-filamentous genotypes. In addition, the length and width of vegetative cells was measured using a  $40\times$  objective for 3 randomly selected isolates from the following groups: sensitive genotype, long-filamentous resistant genotype, and short-filamentous resistant genotype ( $n = 9$ ). Further, the number of heterocysts in 1 mm filament was determined using the same objective for 6 randomly selected isolates from the same groups ( $n = 18$ ).

#### **4.1.7 Growth ability of *Nodularia spumigena***

To examine the effect of phage-mediated evolution on host growth ability, a growth experiment was performed in Z8 medium with salt and without nitrogen

with limiting concentrations of iron or phosphorus. Nutrient limitations were used since they increase competition for resources (Yoshida et al. 2004), revealing differences in fitness associated with uptake of limiting nutrients. Iron and phosphorus are among the key limiting nutrients for cyanobacteria (Kelly et al. 2013, Biller et al. 2015). Concentrations of 2  $\mu\text{M}$  P and 4  $\mu\text{M}$  Fe were selected since *N. spumigena* is estimated to have reduced growth ability at these concentrations (Lehtimäki et al. 1997, Paczuska & Kosakowska 2003). Chosen concentrations were achieved by adjusting the amount of  $\text{FeCl}_3 \times 6\text{H}_2\text{O}$  for Fe-limiting medium and replacing a part of  $\text{K}_2\text{HPO}_4 \times 3\text{H}_2\text{O}$  with KCl for P-limiting medium. The experiment was performed in both media for the following groups of randomly selected isolates: 3 sensitive genotype isolates from each naïve population biological replicate, and 3 long-filamentous and 3 short-filamentous resistant genotype isolates from each evolved population biological replicate ( $n = 27$ ).

Prior to beginning the experiment, isolates were nutrient-starved by centrifugation, rinsing with nutrient-limiting medium, repeating the process, and culturing for 7 days in nutrient-limiting medium in the same conditions as before (Section 4.1.2). The experiment was performed at a low initial density by adding 100  $\mu\text{l}$  of clonal strain culture to 20 ml medium and culturing in the same conditions as before (Section 4.1.2). Replicate samples of 2 ml culture were collected each week in cryotubes, preserved in 2% Lugol's solution, and stored in darkness at +4°C. Cultures were maintained by replacing lost volume with fresh medium. Cells were enumerated each week as before (Section 4.1.4) (Appendix 4). Maximum growth rate was calculated as

$$r_{max} = \ln \frac{N_{t1}}{N_t}$$

where  $r_{max}$  is the population growth rate (per week) and  $N_t$  and  $N_{t1}$  are population sizes on successive weeks when the highest growth was observed. In a recent growth experiment that started with a low cell density, *N. spumigena* was shown to grow exponentially from day 10 to day 20, supporting weekly instead of daily cell density comparisons (Rakko & Seppälä 2014). In addition, the maximum biovolume reached before cultures entered stationary phase was calculated for the P-

limiting treatment from the geometrical formula for cylinder ( $V = \pi r^2 h$ ) using a standardized size-class (Olenina et al. 2006).

#### 4.1.8 Bioactive secondary metabolite production

Determination of relative amount of production of the bioactive secondary metabolites nodularin, aeruginosin, nodulapeptin, and spumigin in isolates was performed to test for reduced production associated with phage resistance. The experiment was performed for 5 randomly selected isolates from each of the three replicates and two treatments of the microcosm experiment ( $n = 30$ ). Prior to sample collection, 200  $\mu\text{l}$  of culture was inoculated in 2.3 ml Z8 medium with salt and without nitrogen in a 24-well tissue culture plate (Flat Bottom Suspension Cells, Sarstedt), and cultured for 5 days in the same conditions as before (Section 4.1.2) for cultures to reach exponential growth phase. Cell densities were enumerated as before (Section 4.1.4) for 1 ml samples collected in cryotubes, preserved in 2% Lugol's solution, and stored in darkness at  $+4^\circ\text{C}$ . For LC-MS, 1 ml culture was collected in a cryotube and stored at  $-80^\circ\text{C}$ .

Samples were freeze-dried and extracted with 1 ml of 75% HPLC grade methanol (Fisher Scientific, USA) at room temperature to avoid heat degradation of aeruginosin, nodulapeptin, or spumigin. Cells were disrupted with 0.5 mm glass beads (Scientific Industries, USA) in a FastPrep<sup>®</sup>-24 Instrument (MP Biomedicals, USA) for 15 s at  $6.5 \text{ m s}^{-1}$ . The protocol was continued as previously (Section 4.1.3), with the following exceptions: The column used in high-performance LC was Phenomenex Luna C8 (2) (150 by 2.0 mm, 5  $\mu\text{m}$ ) (Phenomenex, Torrance, CA, USA). The gradient run was from 5% to 100% B over 35 min at a flow rate of  $0.15 \text{ ml min}^{-1}$  at  $40^\circ\text{C}$ . The capillary voltage was set to 5,000 V, the capillary exit offset was 300.0 V, the skimmer potential was 85.0 V, and the trap drive value was 144.0. Spectra were recorded 2000 to 1,100  $m/z$  and a scanning rate of  $26,000 \text{ m/z s}^{-1}$ . Samples were measured without standards. The following unit masses from the ion trap ( $[\text{M}+\text{H}]^+$ ), were used for compound identification: 825.7 for nodularin-R; 587.5 and 589.5 for the aeruginosin aldehyde-alcohol pair NAL2 and NOL3, respectively; 902.8 for nodulapeptin; and 597.5 and 599.5 for the aldehyde-alcohol pair spumigin F and D (Fewer et al. 2009, 2013). Nodularin con-

centrations were determined from the standard curve. Differences in the amount of nodularin, aeruginosin (combined peak area of alcohol-aldehyde pair), nodula-peptin and spumigin (combined peak area) between sensitive and resistant genotypes were determined by comparing mean peak areas per cell.

#### **4.1.9 Buoyancy**

Observation of cultures indicated that short-filamentous resistant genotypes remained at the bottom of the culture flask when left unperturbed, exhibiting reduced buoyancy compared to other strains. The ability of strains to remain in suspended form in liquid medium was tested for 3 randomly selected sensitive genotypes from each of the naïve population biological replicates, and for 3 long-filamentous and 3 short-filamentous resistant genotypes from each of the evolved population biological replicates ( $n = 27$ ). A volume of 2 ml from cultures with cell densities that did not differ significantly was added to a 24-well tissue culture plate (Flat Bottom Suspension Cells, Sarstedt), and cultured for 7 days in the same conditions as before (Section 4.1.2) without perturbation. The culture was subsequently removed by a pipette, with special care taken to avoid stirring medium, and the amount of filaments per area in the bottom of the well was counted using the same microscope, objective and software as for cell enumeration (Section 4.1.4). In connection with reduced buoyancy, lack of visually detectable accumulation of photosynthetically produced oxygen bubbles was observed among the short-filamentous resistant genotype. Formation of these bubbles has been shown to be associated with increased buoyancy in cyanobacteria (Dervaux et al. 2015). To confirm the observation, culture flasks containing clonal strains at high densities and exhibiting growth were placed in an upright position for 7 days, after which the presence of visually detectable oxygen bubbles was recorded.

#### **4.1.10 Statistical analyses**

The effect of host-parasite dynamics on the release of cellular compounds was analyzed with repeated measures ANOVA (RMANOVA) for host and phage counts and nodularin concentrations, and with a *t*-test for total nitrogen concentrations. The effect on phytoplankton growth was analyzed with RMANOVA. Phage

resistance of clonal isolates was analyzed with a *t*-test, and coevolution was analyzed with RMANOVA. Differences between clonal isolates in filament length, cell size, heterocyst density, growth ability, ability to remain in suspended form in medium, and bubble-formation were analyzed with one-way ANOVA, and differences in bioactive secondary metabolite production were determined with a *t*-test. The Tukey's honest significant difference (HSD) test was used for post hoc analyses. One-way ANOVA, RMANOVA, and post hoc analyses were performed with SPSS Statistics v. 22 (IBM SPSS Statistics, Chicago, IL).

## **4.2 Results**

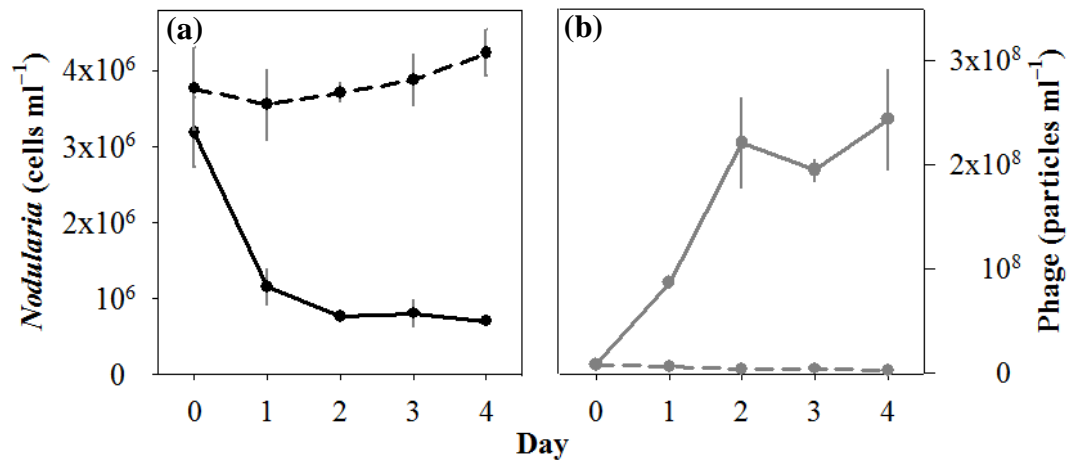
### **4.2.1 Phage resistance and coevolution**

All isolates from the naïve host population ( $n = 60$ ) differed significantly (*t*-test:  $p < 0.05$ ) between active and inactivated phage treatments, indicating sensitivity to the phage. None of the isolates from the evolved host population ( $n = 58$ ) differed between treatments, indicating resistance to the phage.

In the coevolution experiment, no significant differences were observed between treatments with ancestral or evolved phage, indicating that reinfectivity had not evolved against phage resistant isolates. No difference was observed in infection efficiency between the ancestral and evolved phages against the ancestral culture collection host strain.

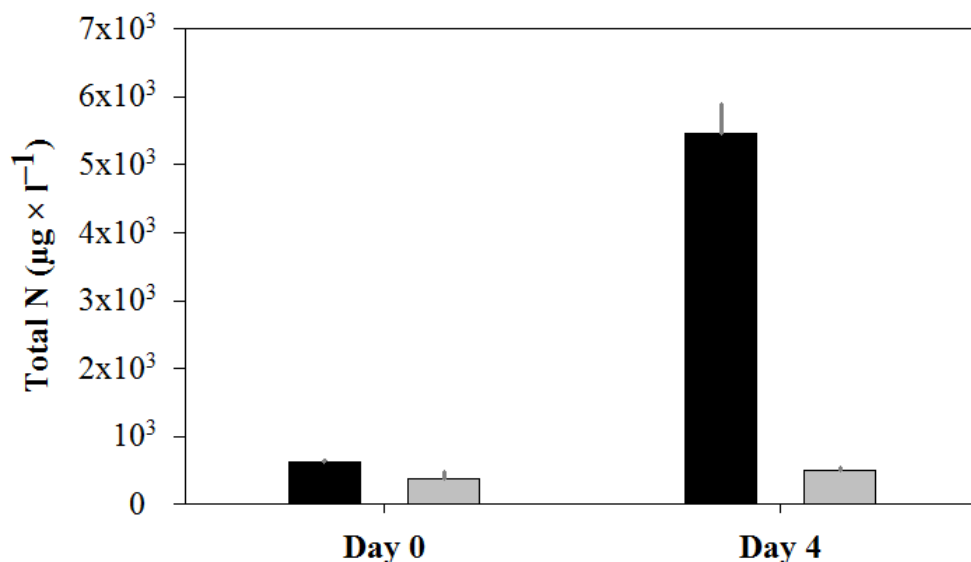
### **4.2.2 Release of cellular nitrogen and nodularin**

In the naïve population, phage addition resulted in a mass mortality event among the host and substantial increase in phage particle number (Figure 6). In the evolved population, phage addition did not result in a change in either host or phage numbers. The treatments differed significantly for both host ( $F_{1,20} = 151$ ,  $p < 0.001$ ) and phage ( $F_{1,20} = 123$ ,  $p < 0.001$ ).



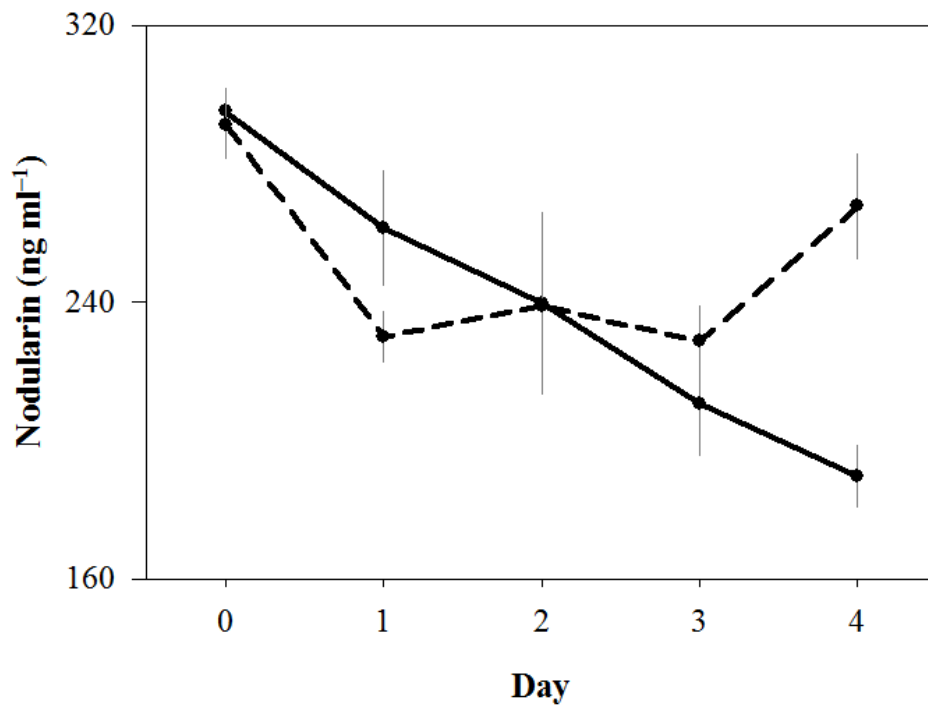
**Figure 6.** Host and phage population dynamics in experimental treatments following phage addition (mean  $\pm$  SE). **(a)** A mass mortality event took place among the naïve host population (*black solid line*), while the evolved host population (*black dashed line*) remained at a high density. **(b)** The phage with naïve host (*grey solid line*) increased rapidly, while the phage with evolved host (*grey dashed line*) remained at a low particle number.

Total nitrogen increased significantly (*t*-test:  $p = 0.011$ ) in the cell-free filtrate from the naïve population (Figure 7). No difference was observed (*t*-test:  $p = 0.464$ ) in the cell-free filtrate from the evolved population.



**Figure 7.** Total nitrogen concentrations in filtrates from experimental treatments at the beginning and end of 4 day phage exposure (mean  $\pm$  SE). Total nitrogen increased significantly in the cell-free filtrate from the naïve host population (*black bar*) while remaining at the same level in the filtrate from the evolved host population (*grey bar*).

The concentration of nodularin did not increase in filtrates during the experiment. A significant change was not observed between filtrates from naïve and evolved populations ( $F_{1,4} = 1.774$ ,  $p = 0.198$ ) (Figure 8).



**Figure 8.** Nodularin concentrations in experimental treatments (mean  $\pm$  SE). No difference was observed between the nodularin concentration of filtrates from the naïve (*solid line*) and evolved host populations (*dashed line*).

#### 4.2.3 Growth ability of phytoplankton strains

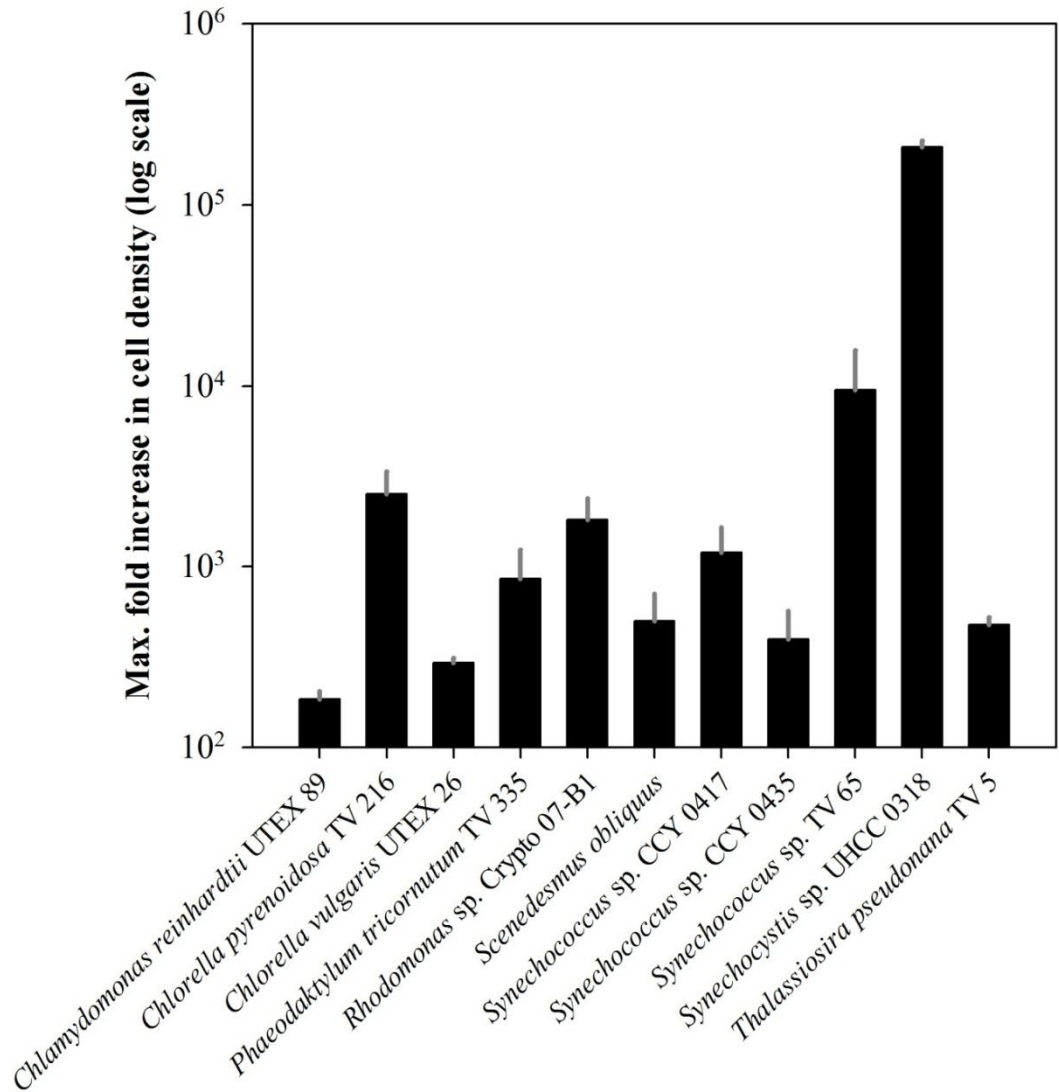
All eleven studied phytoplankton strains grew highly significantly ( $p < 0.001$ ) better in the filtrate from the naïve population compared to the evolved population.  $F$  values from RMANOVA are listed in Table 2.

**Table 3.** RMANOVA  $F$  values for phytoplankton strains cultured in filtrates obtained 4 days after challenging naïve and evolved host cultures with the phage 2AV2.

<b>Strain identity</b>	<b><math>F_{4,50}</math></b>
<i>Chlamydomonas reinhardtii</i> UTEX 89	435
<i>Chlorella pyrenoidosa</i> TV 216	345
<i>Chlorella vulgaris</i> UTEX 26	207
<i>Phaeodaktylum tricornutum</i> TV 335	82.7
<i>Rhodomonas</i> sp. Crypto07-B1	28.6
<i>Scenedesmus obliquus</i>	139
<i>Synechococcus</i> sp. CCY 0417	47.4
<i>Synechococcus</i> sp. CCY 0435	81.2
<i>Synechococcus</i> sp. TV65	395
<i>Synechocystis</i> sp. UHCC 0318	147
<i>Thalassiosira pseudonana</i> TV5	48.3

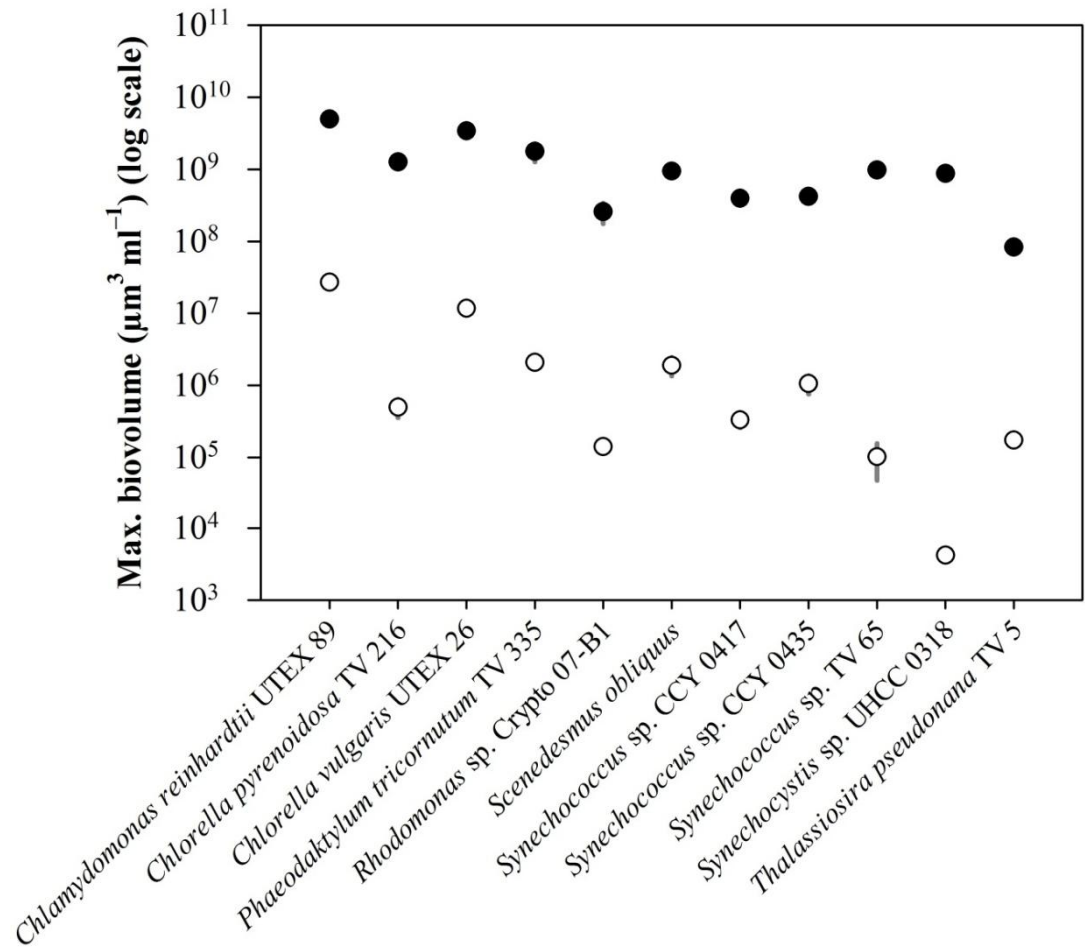
The maximum fold increase in phytoplankton cell density in filtrates from the naïve population compared to the evolved population ranged from  $1.9 \times 10^2$  fold (*Chlamydomonas reinhardtii* UTEX 89) to  $2.1 \times 10^5$  fold (*Synechocystis* sp. UHCC 0318) (Figure 9).





**Figure 9.** Maximum fold increase in phytoplankton cell density (mean  $\pm$  SE). Phytoplankton cultured in filtrate from the naïve population is compared to the evolved population.

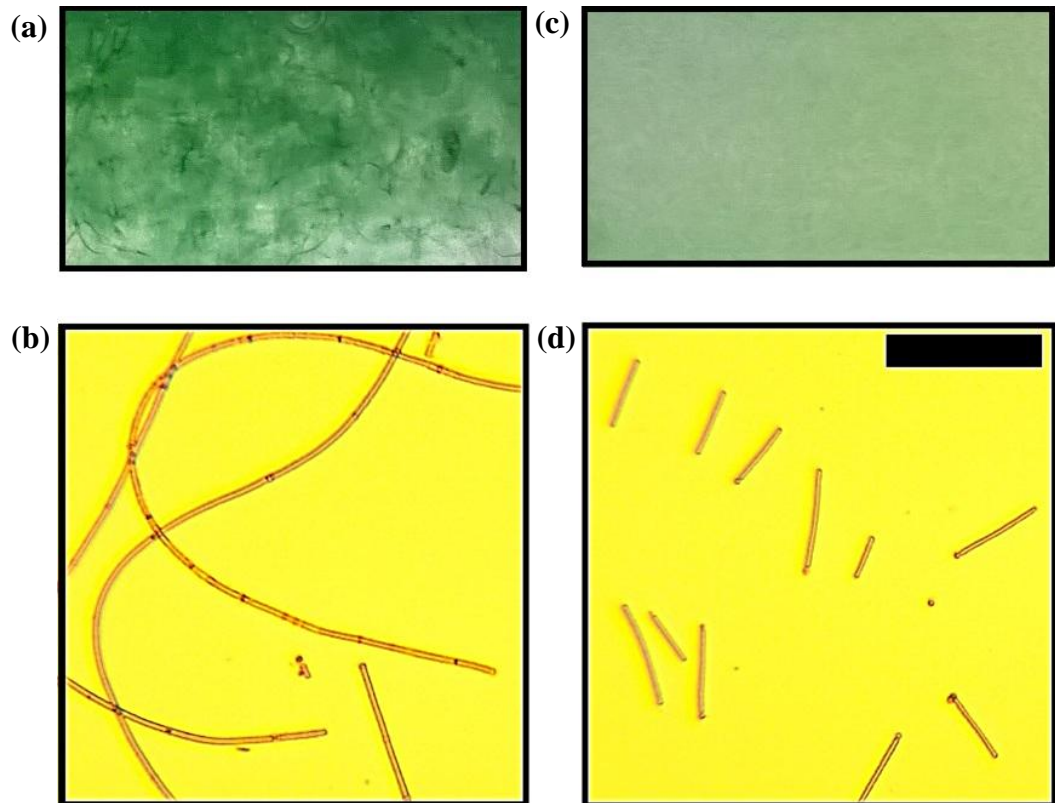
The maximum phytoplankton biovolume the filtrate was able to sustain ranged from  $8.3 \times 10^7$  (*Thalassiosira pseudonana* TV 5) to  $5.0 \times 10^9 \mu\text{m}^3 \text{ml}^{-1}$  (*Chlamydomonas reinhardtii* UTEX 89) in filtrates from the naïve population, and from  $4.2 \times 10^3$  to  $2.7 \times 10^7 \mu\text{m}^3 \text{ml}^{-1}$  (*Chlamydomonas reinhardtii* UTEX 89) in filtrates from the evolved population (Figure 10).



**Figure 10.** Maximum phytoplankton biovolume sustained by experimental filtrates (mean  $\pm$  SE). The biovolumes of phytoplankton strains cultured in the filtrate from the naïve population (*black dots*) are  $1.9 \times 10^2$  to  $2.1 \times 10^5$  higher (*Chlamydomonas reinhardtii* UTEX 89 and *Synechocystis* sp. UHCC 0318, respectively) compared to the evolved population (*white dots*).

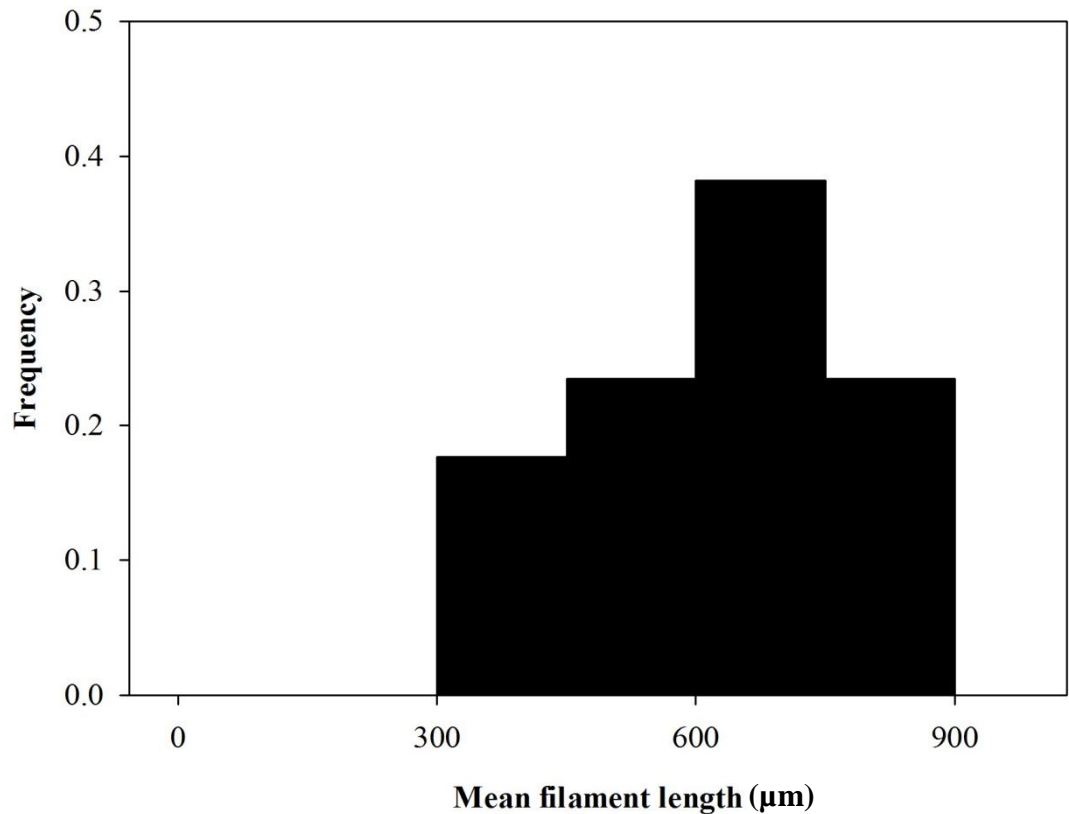
#### 4.2.4 Filament length, cell size and heterocyst density

Two filament-length genotypes were observed among phage resistant isolates (Figure 11). A novel short-filamentous resistant genotype was present in 20.0–57.9% ( $40.0 \pm$  standard deviation, SD 19.0) of isolates from evolved population microcosm replicates, and was not detected among the sensitive genotype. The morphotype remained the same for the duration of the experiment (5 months), did not exhibit gliding motility when cultured on a 0.25% agarose plate, and was only present among resistant isolates, suggesting a genetic basis instead of representing hormogonia.



**Figure 11.** Genotypes observed among phage resistant isolates. **(a–b)** Visual appearance of culture after mixing (a), and light micrograph of long-filamentous genotype (b). **(c–d)** Novel short-filamentous genotype. Scale bar for light micrographs: approx. 200  $\mu\text{m}$ .

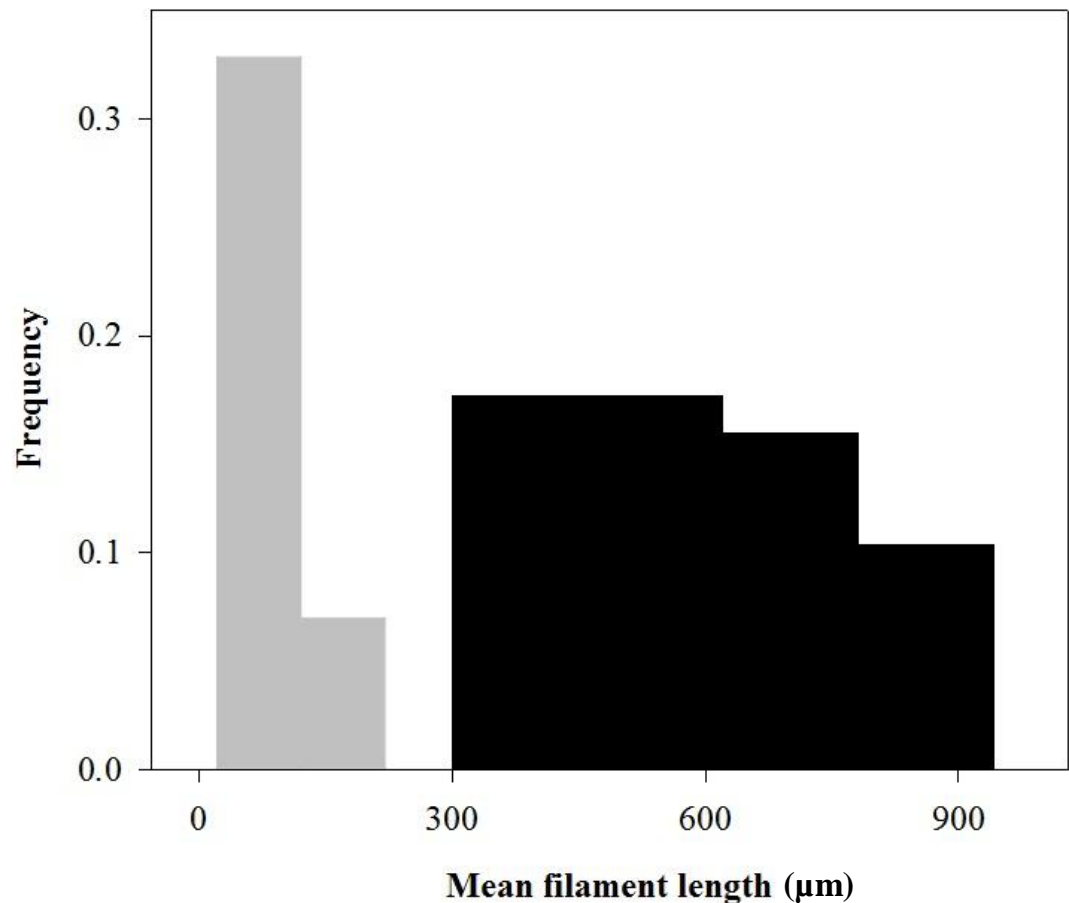
The mean filament length of the sensitive genotype was  $623 \pm 153 \mu\text{m}$ , ranging from 353 to 880 (Figure 12).



**Figure 12.** Filament length frequency distribution among sensitive genotype isolates ( $n = 34$ ).

The mean filament length of the resistant genotype was  $376 \pm 276 \mu\text{m}$  (Figure 13). Two distinct groups were detected: a long-filamentous genotype with a mean filament length of  $576 \pm 157 \mu\text{m}$ , ranging from 306 to 923  $\mu\text{m}$ ; and a short-filamentous genotype with a mean filament length of  $58.8 \pm 35.9 \mu\text{m}$ , ranging from 39.2 to 202  $\mu\text{m}$ . The short-filamentous genotype had a highly significantly lower mean filament length compared to both the sensitive genotype (Tukey's HSD:  $p < 0.001$ ) and the resistant long-filamentous genotype (Tukey's HSD:  $p < 0.001$ ; ANOVA:  $F_{89,2} = 130$ ,  $p < 0.001$ ). The long-filamentous resistant genotype did not differ from the sensitive genotype (Tukey's HSD:  $p = 0.339$ ).

The vegetative cells of the short-filamentous resistant genotype were narrower ( $4.94 \pm 0.461 \mu\text{m}$ ) compared to the sensitive genotype ( $6.55 \pm 0.139 \mu\text{m}$ ) (Tukey's HSD:  $p = 0.003$ ) and the resistant long-filamentous genotype ( $6.60 \pm 0.030 \mu\text{m}$ ) (Tukey's HSD:  $p = 0.004$ ; ANOVA:  $F_{6,2} = 26.9$ ,  $p = 0.002$ ). Cell length ( $3.13 \pm 0.467 \mu\text{m}$ ) did not differ between groups (ANOVA:  $F_{6,2} = 1.82$ ,  $p = 0.254$ ).



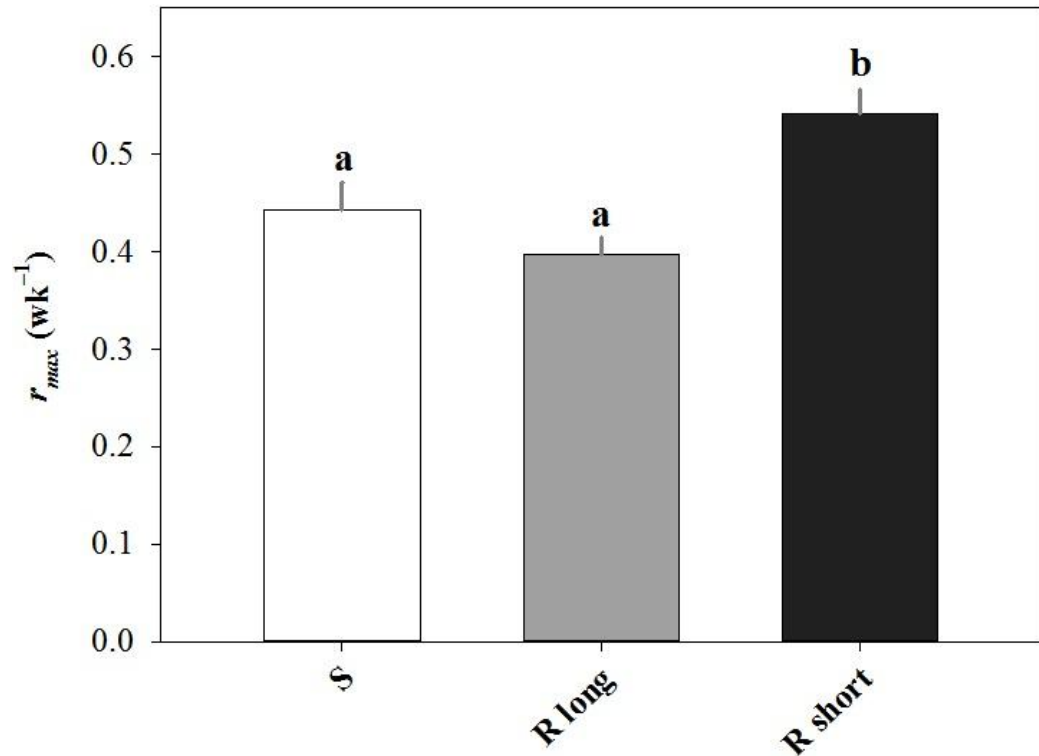
**Figure 13.** Filament length frequency distribution among resistant genotype isolates ( $n = 58$ ). There are two distinct length classes: long-filamentous (*black bars*) and short-filamentous (*grey bars*).

Heterocysts occurred in filaments at an interval of  $91.9 \pm 34.8 \mu\text{m}$ . The interval did not differ between the sensitive genotype, resistant short-filamentous genotype, and resistant long-filamentous genotype (ANOVA:  $F_{15,2} = 0.946$ ,  $p = 0.410$ ). In resistant short-filamentous isolates, heterocysts did not occur in all filaments. When they occurred, they occurred frequently at the end of the trichome but were also found within the trichome.

#### 4.2.5 Growth ability of *Nodularia spumigena*

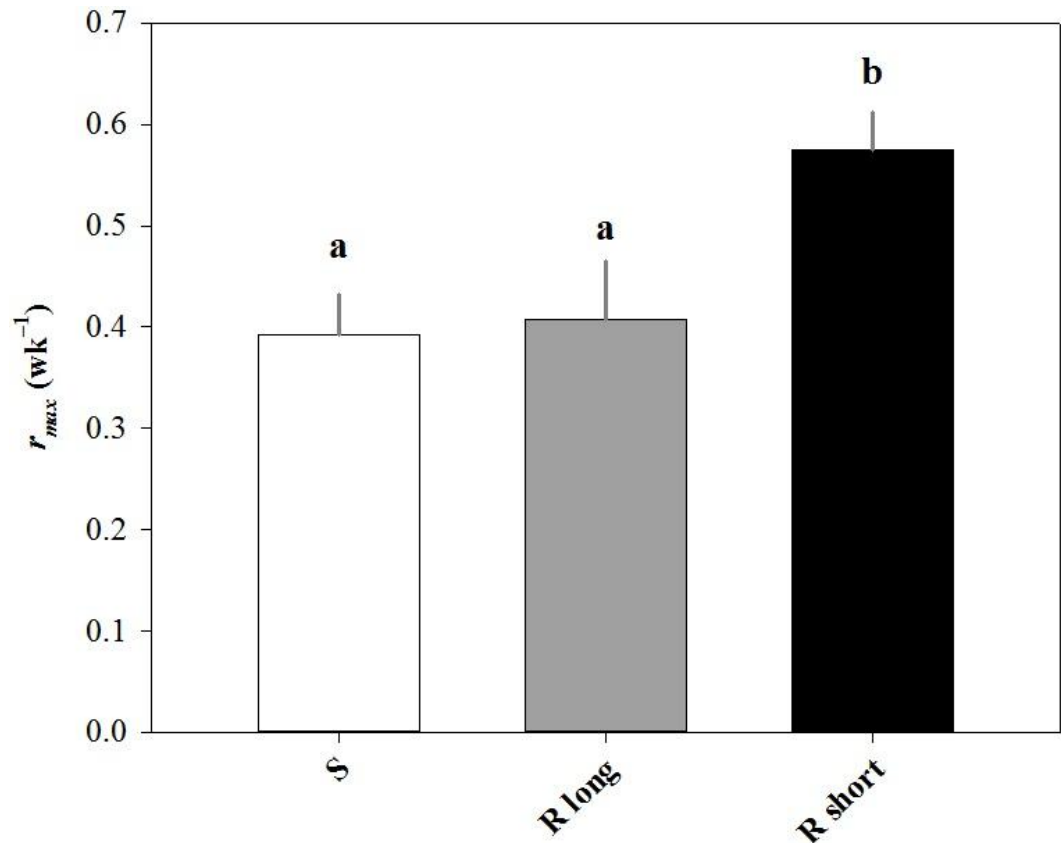
In iron-limited Z8 medium, the short-filamentous resistant genotype had a higher maximum growth rate compared to both the sensitive genotype (Tukey's HSD:  $p = 0.026$ ) and the long-filamentous resistant genotype (Tukey's HSD:  $p = 0.002$ ; ANOVA:  $F_{21,2} = 8.75$ ,  $p = 0.002$ ) (Figure 14). No difference was observed be-

tween the sensitive and resistant long-filamentous genotypes (Tukey's HSD:  $p = 0.430$ ).



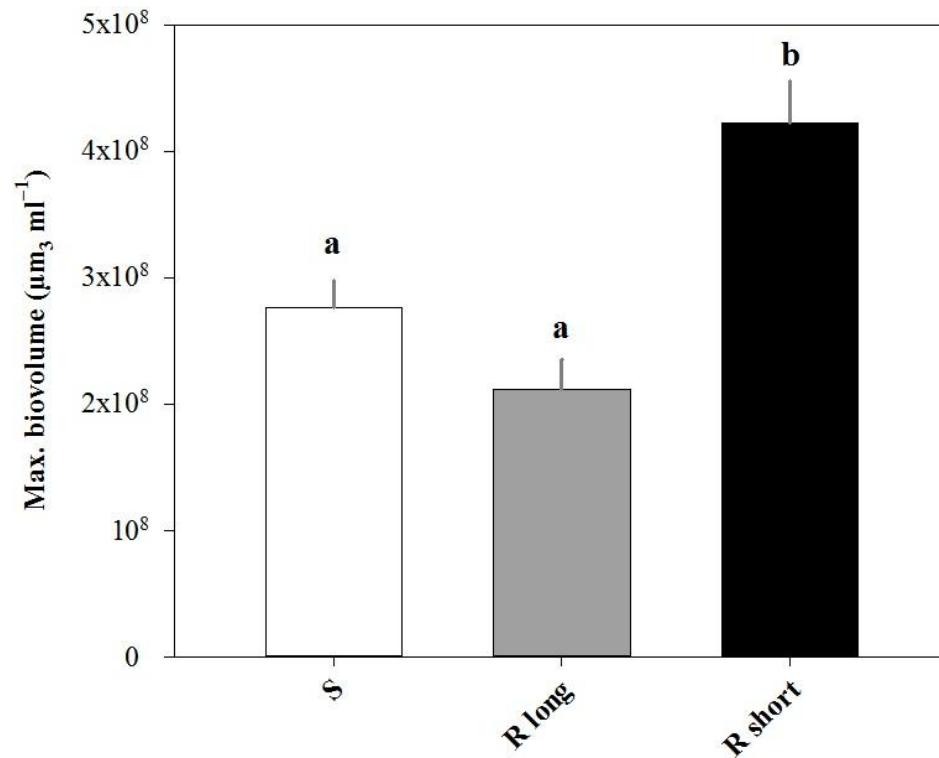
**Figure 14.** Maximum growth rates ( $r_{max} \pm SE$ ) of *N. spumigena* genotypes cultured in iron-limited ( $4 \mu M$  Fe) Z8 medium with salt and without nitrogen. Bar color indicates host phage sensitivity and filament length: *S* = sensitive genotype (*white bar*), *R long* = long-filamentous resistant genotype (*grey bar*), *R short* = short-filamentous resistant genotype (*black bar*). Different letters indicate significant differences between genotypes (Tukey's HSD:  $p < 0.05$ ).

In P-limited medium, the short-filamentous resistant genotypes had a higher maximum growth rate compared to both the sensitive genotype (Tukey's HSD:  $p = 0.026$ ) and the long-filamentous resistant genotype (Tukey's HSD:  $p = 0.046$ ; ANOVA:  $F_{21,2} = 4.81$ ,  $p = 0.019$ ) (Figure 15). No difference was observed between the sensitive and resistant long-filamentous genotypes (Tukey's HSD:  $p = 0.971$ ).



**Figure 15.** Maximum growth rates ( $r_{max} \pm SE$ ) of *N. spumigena* genotypes cultured in phosphorus-limited ( $2 \mu M$  P) Z8 medium with salt and without nitrogen. Bar color indicates host genotype: *S* = sensitive genotype (*white bar*), *R long* = long-filamentous resistant genotype (*grey bar*), *R short* = short-filamentous resistant genotype (*black bar*). Different letters indicate significant differences between genotypes (Tukey's HSD:  $p < 0.05$ ).

In addition, the maximum biovolume reached in phosphorus-limited medium was higher compared to both the sensitive genotype (Tukey's HSD:  $p = 0.002$ ) and the long-filamentous resistant genotype (Tukey's HSD:  $p < 0.001$ ; ANOVA:  $F_{21,2} = 16.5$ ,  $p < 0.001$ ) (Figure 16). No difference was observed between the sensitive and resistant long-filamentous genotypes (Tukey's HSD:  $p = 0.222$ ).



**Figure 16.** Maximum biovolume ( $\mu\text{m}^3 \text{ml}^{-1} \pm \text{SE}$ ) of *N. spumigena* genotypes cultured in phosphorus-limited ( $2 \mu\text{M P}$ ) Z8 medium with salt and without nitrogen. Bar color indicates host phage genotype: *S* = sensitive genotype (white bar), *R long* = long-filamentous resistant genotype (grey bar), *R short* = short-filamentous resistant genotype (black bar). Different letters indicate significant differences between genotypes (Tukey's HSD:  $p < 0.05$ ).

#### 4.2.6 Bioactive secondary metabolite production

No significant differences were observed in bioactive secondary metabolite production between sensitive and resistant genotypes (Table 3).

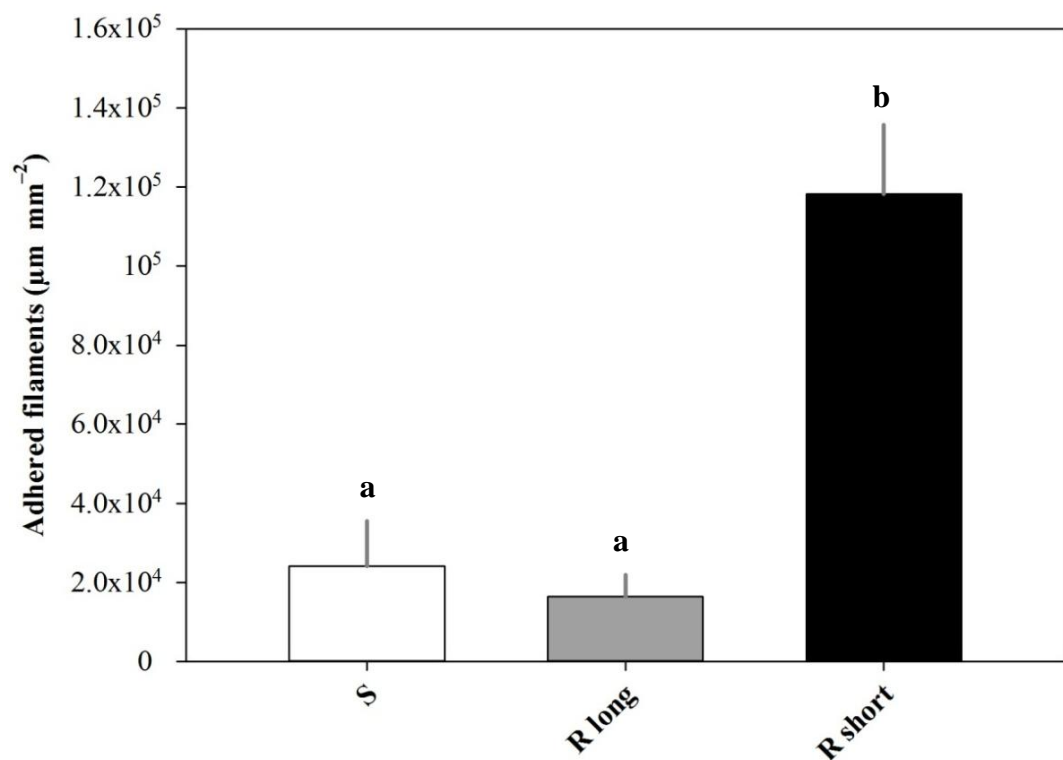
**Table 4.** Relative bioactive secondary metabolite production in phage sensitive (*S*) and resistant (*R*) genotypes.

Secondary metabolite	S/R	Mean peak area cell <sup>-1</sup>	SE	<i>p</i> ( <i>t</i> -test)
Aeruginosin NOL3–NAL2	S	656	135	0.179
	R	908	117	
Nodulapeptin	S	39.5	10.2	0.170
	R	22.6	2.85	
Nodularin-R	S	1753	416	0.423
	R	2094	229	
Spumigin D–F	S	861	260	0.273
	R	1113	85.3	



#### 4.2.7 Buoyancy

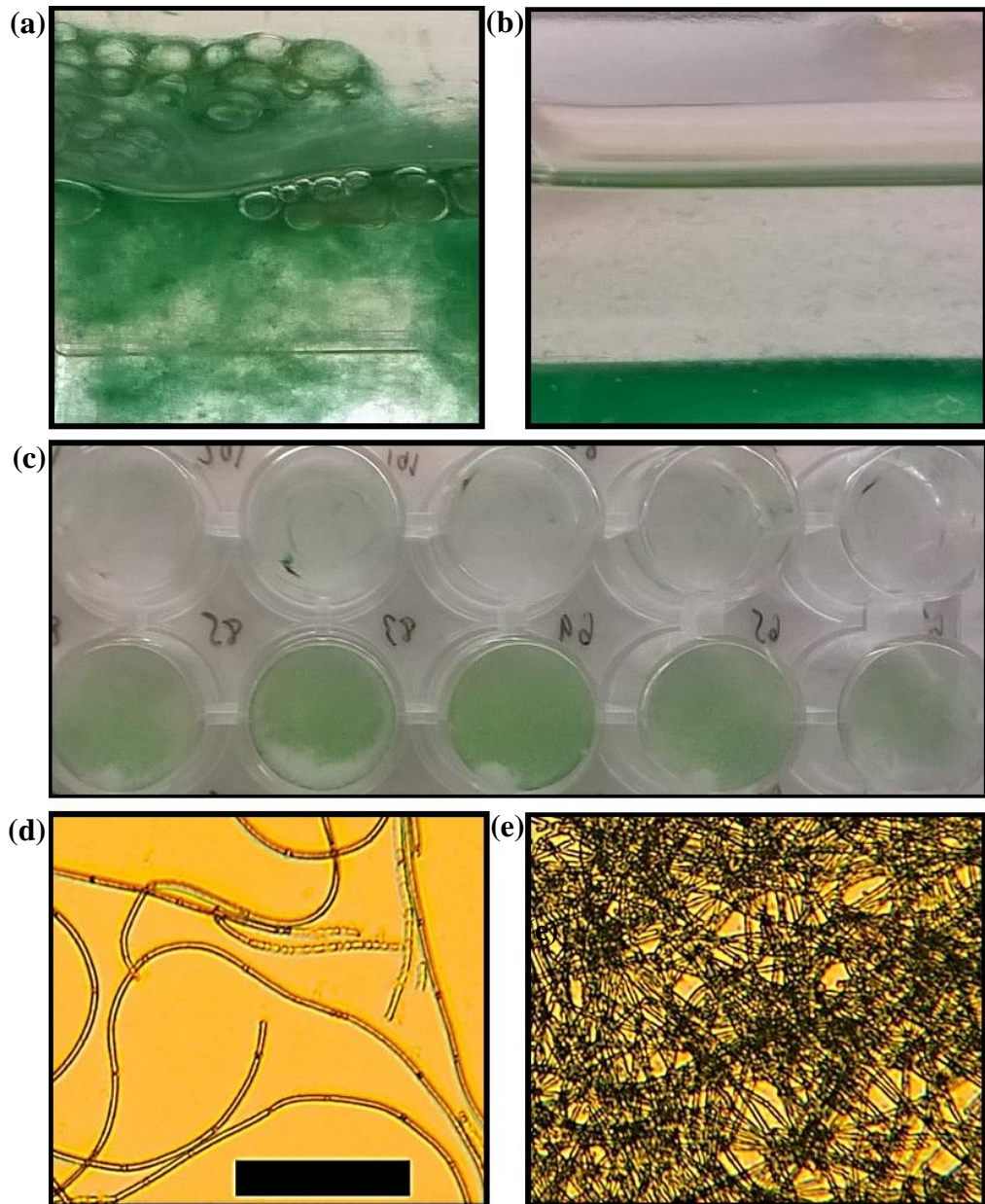
The ability of the short-filamentous resistant genotype to stay in suspension in liquid medium was lower compared to both the sensitive genotype (Tukey's HSD:  $p < 0.001$ ) and the long-filamentous resistant genotype (Tukey's HSD:  $p < 0.001$ ; ANOVA:  $F_{24,2} = 20.7$ ,  $p < 0.001$ ) (Figures 17 & 18a–c). The long-filamentous resistant genotype did not differ from the sensitive genotype (Tukey's HSD:  $p = 0.903$ ).



**Figure 17.** Amount of *N. spumigena* filaments from different genotypes that adhered to surface after culturing for 7 days without mixing ( $\mu\text{m mm}^{-2} \pm \text{SE}$ ). Bar color indicates host genotype: *S* = sensitive genotype (*white bar*), *R long* = long-filamentous resistant genotype (*grey bar*), *R short* = short-filamentous resistant genotype (*black bar*). Different letters indicate significant differences between genotypes (Tukey's HSD:  $p < 0.05$ ).

Visible oxygen bubble formation in the filament matrix was observed in 97% of sensitive genotype cultures, 100% of long-filamentous resistant genotype cultures, and in none of short-filamentous resistant genotype cultures, differing from the

others (Tukey's HSD:  $p < 0.001$ ; ANOVA:  $F_{114,2} = 904$ ,  $p < 0.001$ ) (Figure 18a,b). The long-filamentous resistant genotype did not differ from the sensitive genotype (Tukey's HSD:  $p = 0.651$ ).



**Figure 18.** Differences in buoyancy and adhesion between resistant genotypes. (a–b) Visual appearance of long-filamentous (a) or short-filamentous genotype (b) in culture flask after being unperturbed for 7 days. (c) Appearance of bottom surface of 2 ml culture well containing long-filamentous genotype (*top row*) or short-filamentous genotype (*bottom row*) after culturing for 7 days without perturbation, followed by careful removal of medium and suspended cells. (d–e) Light micrographs from bottom surface of culture well containing long-filamentous genotype (d) or short-filamentous genotype (e). Scale bar for light micrographs: approx. 200  $\mu\text{m}$ .

## 4.3 Discussion

### 4.3.1 Effect of host-parasite interactions on the marine nitrogen cycle

This study is among the first to provide evidence, in controlled laboratory conditions, supporting the hypothesis that viral lysis of marine diazotrophic bacteria causes release of cellular nitrogen to the planktonic environment (Bratbak et al. 1990, Wilhelm & Suttle 1999, Coloma et al. 2015). This observation is in line with the hypothesis that the viral loop is responsible for rerouting a significant proportion of organic matter in the ocean. The presence of high numbers of viruses in seawater, approximately  $10^7$  ml<sup>-1</sup>, including up to  $10^8$  cyanophages ml<sup>-1</sup>, indicates that these findings are related to ongoing processes in marine ecosystems (Waterbury & Valois 1993, Suttle & Chan 1994, Marston & Sallee 2003, Hess 2008). This is further supported by numerous observations linking cyanophages to cyanobacterial bloom decay across the globe (Mann 2003, 2006, Mühling et al. 2005, Peduzzi et al. 2014).

The ongoing lysis of cyanobacterial blooms can have large-scale ecosystem effects because cyanobacteria are the principal nitrogen-fixing organisms in several marine environments (Berman-Frank et al 2007, Gruber 2008). This includes *N. spumigena* in the Baltic Sea (Plough et al. 2011). The findings in this study may also have significant implications concerning the marine carbon cycle, which is closely connected to the nitrogen cycle through nutrient stoichiometry (Worden et al. 2015). In the Baltic Sea, *N. spumigena*, *Aphanizomenon* spp. and *Anabaena* spp. together account for up to 90% of pelagic primary production (Stal et al. 2003), and cyanobacteria in general are estimated to account for 20–80% of CO<sub>2</sub>-fixation in the marine phytoplankton (Wilhelm & Suttle 1999, Worden et al. 2015), highlighting the potential significance of these processes.

In this study, the nitrogen content of the medium did not change when resistant genotypes were challenged by the phage, indicating disappearance of the ecosystem effect following development of phage resistance. Evolution of phage resistance has commonly been observed among marine cyanobacterial communities (Waterbury & Valois 1993, McDaniel et al. 2006). This suggests that evolution in

host-parasite interactions between cyanobacteria and phages may contribute to a dynamic nutrient landscape in marine planktonic ecosystems by causing temporal and spatial changes in nutrient availability.

#### **4.3.2 Community-level effects on planktonic food-webs**

All eleven phytoplankton strains used in this study grew considerably better in filtrates obtained after using the phage to challenge sensitive host cultures compared to resistant host cultures. The strains represent different taxonomic groups of common marine phytoplankton species, including green algae, diatoms, and picocyanobacteria. This supports the hypothesis that the viral loop stimulates phytoplankton growth (Wilhelm & Suttle 1999, Martiny et al. 2014). To the author's knowledge, this is the first time this phenomenon has been demonstrated with a clear chain of evidence based on controlled laboratory experiments.

The findings may have several consequences concerning marine planktonic food-webs. Firstly, as suggested in the previous section, host-parasite interactions between cyanobacteria and phages have the potential to cause temporal and spatial alterations in nutrient availability in planktonic food-webs. Secondly, temporally increased nutrient availability due to viral lysis of cyanobacterial hosts may increase the competitive ability of phytoplankton species against nitrogen-fixing cyanobacteria that benefit from low total nitrogen levels (Dokulil & Teubner 2000). This supports the treatment of cyanobacterial bloom food-webs as intermediate forms between the herbivorous and microbial food-web (Legendre & Rassoulzadegan 1995, Mousseau et al. 2001, Hlaili et al. 2014), and indicates that the viral loop may mediate shifts between these planktonic food-web extremes. Thirdly, results support the hypothesis that passing of fixed nitrogen from cyanobacterial blooms to nitrogen-limited picocyanobacteria may explain their mass occurrence in association with nitrogen-fixing cyanobacteria (Stal et al. 1999, 2003). Although the issue was not examined in this study, high phytoplankton growth suggests that nitrogenous compounds released from cyanobacterial hosts may also be utilized by associated heterotrophic bacteria. In this case, the viral loop may stimulate the heterotrophic food-chain from cyanobacteria through associated bacteria to zooplankton (Woodland et al. 2013).

It has been hypothesized that the bioactive compounds produced by cyanobacteria may have allelopathic effects against other phytoplankton species (Dokulil & Teubner 2000, Suikkanen et al. 2004, Karjalainen et al. 2007), raising the question whether viral lysis mediates possible inhibitory effects. In this study, the concentration of the bioactive secondary metabolite nodularin did not increase in the medium following viral lysis. Further, the possible inhibitory effects of other cellular bioactive compounds were not strong enough to prevent the rapid growth of phytoplankton species in cell-free filtrates containing high levels of cellular nitrogen released from the cyanobacterial host.

#### **4.3.3 Effect of phage resistance on host fitness**

Several observations were made in this study concerning the effect of phage resistance on host fitness. Firstly, no cost of phage resistance in host growth ability was detected in competition for nutrients dissolved in the culture medium. It is possible that a growth ability cost exists but that experimental conditions were insufficient to reveal it, as has been suggested in other studies with a similar outcome (Lennon et al. 2007, Meyer et al. 2010, Avrani et al. 2011). It must also be noted that only six phage resistant isolates were examined, which may be too low a number to bring out differences, especially because this number consisted of only 3 individuals belonging to each of the two observed resistant genotypes (long-filamentous and short-filamentous). For example, Avrani and others (2011) found reduced growth ability in only 11 of 23 phage resistant *Prochlorococcus* genotypes and Lennon and others (2007) found reduced growth ability in only half of 22 phage resistant *Synechococcus* genotypes. This indicates that relatively high numbers of phage resistant genotypes may need to be examined to reveal reduced growth ability when it only appears in a limited proportion of the resistant population. However, it is also possible that resistance-conferring mutations in the host used in this study are not associated with genes involved in nutrient uptake, or that compensatory mutations have eliminated a growth ability cost (Avrani et al. 2012). In addition to growth ability, no cost of resistance was observed in terms of reduced production of the bioactive secondary metabolites aeruginosin, nodulapeptin, nodularin or spumigin, providing the first experimental evidence concerning this question.

Secondly, the discovery of a novel resistant genotype with short and narrow filaments may have several implications concerning host fitness. Avrani and others (2011, 2012) have proposed that other forms of cost of resistance, such as increased sensitivity to other phages, may also explain antagonistic coexistence between hosts and parasites without the necessity of a metabolic cost of resistance. In this study, reduced buoyancy was clearly observed in the short-filamentous resistant genotype. This was detected visually as significantly decreased buoyancy in culture flasks and lack of oxygen-bubble formation in the filament matrix, suggested to be involved in conferring buoyancy to cyanobacteria together with gas vesicles (Dervaux et al. 2015). Further, aggregation of filaments in the bottom of culture flasks was tested and found to be considerably higher for the short-filamentous resistant genotype compared to the other resistant and sensitive genotypes. Buoyancy is considered to provide a competitive advantage to *N. spumigena* in the Baltic Sea by allowing wintertime survivors to float up to the surface (thermocline) when it melts in the spring, and allowing upward movement in the vertical light gradient during calm periods (Walsby et al. 1995). Further, during the growth period, buoyancy allows cyanobacteria to form blooms that increase their ability to compete for light both directly and indirectly through overshadowing competing phototrophs. Reduced buoyancy may therefore be considered to represent a novel fitness cost of phage resistance in filamentous cyanobacteria, enabling bacteria-phage antagonistic coexistence. Interestingly, the short-filamentous genotype possessed an increased growth ability compared to the other sensitive and resistant genotypes. This may be caused by a higher surface-to-volume ratio compared to genotypes with longer and wider filaments, resulting in increased nutrient uptake via insertion of greater numbers of nutrient transport complexes in the cell membrane (Young 2006). In this context, a fitness trade-off may be considered to occur between increased growth ability and reduced buoyancy, with reduced overall fitness.

In the dispersal form hormogonia, vegetative cells of *N. spumigena* can differentiate into short and narrow filaments (Voß et al. 2013). However, several key characteristics of hormogonia are lacking in the short, narrow filaments observed in this study. Hormogonia are typically transient, lasting for 24–72 hours (Meeks et al. 1999), which is contrasted by the persistence of the short, narrow resistant

morphotype for the entire 5-month-long duration of the experiment, corresponding to *c.* 50 generations. Hormogonia are also characterized by gliding motility of filaments lacking heterocysts. However, gliding motility on 0.25% agarose plates was not observed for the short genotype, while heterocysts were observed. Further, hormogonia involves cessation of DNA replication and biomass increase, and decreased carbon and nitrogen assimilation rates, in turn contrasted by the observation in this study of increased growth ability of the short phenotype compared to long-filamentous isolates. These observations indicate that instead of hormogonia, filament fragmentation may be the result of resistance-conferring mutations in genes associated with filament formation. Filaments, or trichomes, are chains of individual cells with a cytoplasmic membrane and peptidoglycan layer that share a continuous outer membrane. Cyanobacterial filament-formation has been associated with genes encoding for septal proteins (e.g. SepJ), transpeptidases involved in peptidoglycan elongation, or enzymes involved in the biosynthesis of lipid A, an LPS component (Burnat et al. 2014). Resistance-conferring mutations in the long-filamentous resistant isolates are presumably not associated with key genes involved in filament formation. The presence of both long-filamentous and short-filamentous isolates therefore indicates the potential presence of more than one resistance mechanism among the population.

Several adaptive scenarios may also be hypothesized in which short filaments or reduced buoyancy confers a selective advantage to the host, resulting in a decreased contact rate with the phage. Phage adsorption to and subsequent lytic infection of adjacent cells in a filament is more probable than successful infection of a new filament. The less cells a filament possesses, the smaller the damage to the population when one filament becomes infected. This, in turn, results in a decreased contact rate. Reduced buoyancy may also be hypothesized to increase host fitness by allowing the host to spatially escape the lytic viral parasite into the deep ocean. Since the rate of phage adsorption is proportional to phage and host concentrations (Ellis & Delbrück 1939), spatial separation from the parasite would rapidly reduce contact rates with the host. Spatial separation as a viral defense strategy may provide an alternative to spatial clustering as an algal defense strategy, as observed, for example, in the unicellular eukaryotic alga *Chlamydomonas* (Becks et al. 2010). The latter strategy has the opposite effect of decreasing the

surface-to-volume ratio, thereby decreasing the competitive ability of defended genotypes.

#### **4.3.4 Effect of interactions on host and phage diversity**

In the cyanobacterium-phage system studied here, increased diversity in growth ability, filament length and buoyancy was detected among phage resistant genotypes compared to sensitive genotypes. These observations agree with previous experimental findings indicating that bacteria-phage interactions lead to increased host diversity at all levels from genomes and populations to communities and ecosystems (Weinbauer & Rassoulzadegan 2004, Koskella & Brockhurst 2014). The observed diversity is potentially related to fitness trade-offs between phage resistance and competition for nutrients and light, the latter of which is a key aspect of competition among phytoplankton species (Brockhurst et al. 2004, Huisman et al. 2004, Benmayor et al. 2008, Marston et al. 2012). This is the first time, to the author's knowledge, that increased diversity has been recorded in a filamentous, nitrogen-fixing cyanobacterium as a result of phage-mediated evolution.

As opposed to previous studies with picocyanobacteria and their phages (Stoddard et al. 2007, Marston et al. 2012), rapid arms-race dynamics type directional coevolution was not observed in this study system. During the 22-week-long microcosm experiment, corresponding to approximately 50 host generations, the phage 2AV2 had not evolved reinfectivity against any of approximately 20 isolates obtained from each of the three biological replicates of the phage treatment. The observation supports the mutational asymmetry hypothesis as well as the hypothesis that ARD type dynamics alone may be insufficient to explain the antagonistic coexistence paradox (Lenski & Levin 1985, Koskella & Brockhurst 2014).

This study indicates that phage-mediated evolution can lead to the development of clearly distinct morphotypes and phenotypes among filamentous cyanobacteria. In several cases, molecular analyses of cyanobacteria have demonstrated that morphological properties are a weak indicator of phylogenetic relatedness, challenging previous morphology-based taxonomical assignments (Sihvonen et al. 2007, Halinen et al. 2008, Acinas et al. 2009). This includes *Nodularia* in the Baltic Sea



(Hayes & Barker 1997, Lehtimäki et al. 2000). Based on the findings in this study, it is possible that this disconnect is in some cases explained by rapid morphological diversification of genetically nearly identical hosts through phage-mediated evolution. Development of large phenotypic differences may also lead to expansion into new ecological niches, such as from planktonic to benthic zones as is imaginable in the case of reduced buoyancy, facilitating genetic divergence between morphotypes through adaptive radiation (Rainey & Travisano 1998). In the system examined in this study, adaptive radiation may be enhanced by fitness differences between phage resistant genotypes. In this scenario, the long-filamentous genotype has a fitness advantage in the planktonic zone because of buoyancy, while the short-filamentous genotype has a fitness advantage in the benthic zone because of a higher growth rate. These findings may have implications concerning the evolutionary history and diversification of filamentous nitrogen-fixing cyanobacteria.

Host-parasite interactions can alter community structure and dynamics, resulting in increased diversity (Bohannan & Lenski 2000, Koskella & Meaden 2013, Koskella & Brockhurst 2014). In the context of this experiment, it may be hypothesized that the dynamic ecosystem effects of interactions between viral parasites and nitrogen-fixing cyanobacteria may sustain higher diversity in planktonic communities compared to a situation where the primary trophic pathway is fixed (see Section 4.3.2). Cyanobacteria-phage interactions may therefore function to maintain diversity in marine planktonic communities.

## 5 CONCLUSIONS

In this study, several novel observations were made concerning host-parasite interactions between a marine viral parasite and nitrogen-fixing, filamentous cyanobacterium. A manuscript is currently being prepared from the observations (Cairns et al. 2015). In light of the experimental predictions that were tested (Section 3), the following results were obtained:

- i. host-parasite interactions affected the release of cellular nitrogen: nitrogen was released from sensitive host-cells but not from resistant host-cells after challenging cells with the phage; release of cellular nodularin was not detected
- ii. host-parasite interactions affected phytoplankton growth: strains grew far better in cell-free filtrates (from above experiment) from sensitive host-cells compared to filtrates from resistant host-cells
- iii. coevolution of reinfectivity was not observed in the phage
- iv. phage resistance was associated with a fitness cost in buoyancy but not in growth ability or bioactive secondary metabolite production
- v. phage-mediated evolution resulted in the appearance among the resistant population of a novel short-filamentous genotype, increasing the diversity of host filament length, growth ability, and buoyancy

Several aspects of the study system demand further consideration. Firstly, the molecular basis of infectivity and resistance has not yet been established. Whole-genome sequencing (WGS) of phage sensitive and resistant genotypes is underway at the University of Helsinki to answer this question. Secondly, WGS will also be used to study the basis of filament fragmentation among resistant isolates. Thirdly, an experiment utilizing stable isotope techniques has been planned to determine the precise nature of the flow of nitrogen between organisms in the food-web as a consequence of viral lysis. Fourthly, an experiment has been designed to test the community-level effects of interactions between the phage and cyanobacterial host at higher trophic levels, including phytoplankton species and microzooplankton grazers. These analyses are expected to provide precise explanations and additional insights concerning the observations made in this thesis.

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## APPENDICES

## Appendix 1: Z8 medium

## Components

*Z8I stock solution (original)*

NaNO <sub>3</sub>	47.6 g
Ca(NO <sub>3</sub> ) <sub>2</sub> × 4H <sub>2</sub> O	5.9 g
MgSO <sub>4</sub> × 7H <sub>2</sub> O	2.5 g
H <sub>2</sub> O	1 l

*Z8IX stock solution (combined N omitted)*

MgSO <sub>4</sub> × 7H <sub>2</sub> O	2.5 g
CaCl <sub>2</sub> × 2H <sub>2</sub> O	3.7 g
NaCl	32.1 g
H <sub>2</sub> O	1 l

*Z8II stock solution (phosphorus)*

K <sub>2</sub> HPO <sub>4</sub> × 3H <sub>2</sub> O	4.1 g
Na <sub>2</sub> CO <sub>3</sub>	2.1 g
H <sub>2</sub> O	1 l

*Z8III stock solution (iron)*

<i>Fe solution</i>	
FeCl <sub>3</sub> × 6H <sub>2</sub> O	2.8 g
0.1 N HCl	100 ml
<i>EDTA solution</i>	
EDTA	3.9 g
0.1 N NaOH	100 ml
1) mix 10 ml Fe sol. in 900 ml deionized H <sub>2</sub> O	
2) add 9.5 ml EDTA solution and fill to 1 l	

*Trace element solution*

1. Na <sub>2</sub> WO <sub>4</sub> × 2H <sub>2</sub> O	0.330 g / 100 ml
2. (NH <sub>4</sub> ) <sub>6</sub> × Mo <sub>7</sub> O <sub>24</sub> × 2H <sub>2</sub> O	0.880 "
3. KBr	1.200 "
4. KJ	0.830 "
5. ZnSO <sub>4</sub> × 7H <sub>2</sub> O	2.870 "
6. Cd(NO <sub>3</sub> ) <sub>2</sub> × 4H <sub>2</sub> O	1.550 "
7. Co(NO <sub>3</sub> ) <sub>2</sub> × 6H <sub>2</sub> O	1.460 "
8. CuSO <sub>4</sub> × 5H <sub>2</sub> O	1.250 "
9. (NH <sub>4</sub> ) <sub>2</sub> Ni(SO <sub>4</sub> ) <sub>2</sub> × 6H <sub>2</sub> O	1.980 "
10. Cr(NO <sub>3</sub> ) <sub>3</sub> × 9H <sub>2</sub> O	0.410 "
11. V <sub>2</sub> O <sub>5</sub>	0.089 g/l
12. Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> K <sub>2</sub> SO <sub>4</sub> × 24H <sub>2</sub> O	4.740 g / 100 ml
13. H <sub>3</sub> BO <sub>3</sub>	31.0 g
14. MnSO <sub>4</sub> × 4H <sub>2</sub> O	22.3 g (/ MnSO <sub>4</sub> × H <sub>2</sub> O 16,4 g)
1) mix 1 ml 1–10 & 12 and 10 ml 11 & 13 in 700 ml deionized H <sub>2</sub> O	
2) fill to 1 l; store away from light in brown glass bottle	

*Z8 salt*

NaCl	87.5 g
MgSO <sub>4</sub> × 7H <sub>2</sub> O	37.5 g
H <sub>2</sub> O	1 l

## Preparation

*Liquid medium (1 l)*

**Z8:** 10 ml Z8I + 10 ml Z8II + 10 ml Z8III + 1 ml trace element solution; fill to 1 l with 1:1 ratio of 10 min CO<sub>2</sub>-bubbled and non-bubbled deionized RO-quality H<sub>2</sub>O

**Z8 with salt and without nitrogen:** 10 ml Z8IX + 10 ml Z8II + 10 ml Z8III + 1 ml trace element solution + 100 ml Z8 salt; fill to 1 l with 1:1 ratio of 10 min CO<sub>2</sub>-bubbled and non-bubbled deionized RO-quality H<sub>2</sub>O

*Agarose medium, 0.55% (1 l)*

- 1) mix 5.5 g of ultra-pure molecular grade agarose with 0.25 l tap water and 0.25 l deionized water; autoclave
- 2) add components for 1 l medium to 0.5 l of 10 min CO<sub>2</sub>-bubbled deionized RO-quality H<sub>2</sub>O; autoclave
- 3) combine autoclaved solutions, mix, and pour plates

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**Appendix 2: Modified plaque assay**

- 1) Prepare 0.55% Z8 agarose medium with salt and without nitrogen
- 2) Prepare 0.25% soft Z8 agarose medium with salt and without nitrogen; keep autoclaved soft agarose in water bath at 45°C
- 3) Prepare a dilution series of samples in autoclaved RO-quality deionized H<sub>2</sub>O
- 4) Prepare high-density host culture by centrifuging 2 × 50 ml of dense culture (7 min at 7,000 × g / 4°C), removing part of the supernatant and combining culture concentrates to obtain desired volume
- 5) Combine 1 ml high-density host culture, 100 µl sample, and 3 ml soft agarose in 10 ml glass test tube
- 6) Vortex, pour contents on 0.55% Z8 agarose medium plate, and gently swirl 4–5 times to spread out soft agarose well in the plate
- 7) Allow soft agarose layer to solidify, taking care to avoid perturbation (results in an uneven surface from which results cannot be read)
- 8) Culture for approximately 6 days in desired conditions
- 9) Count plaque forming units (PFU) in the two dilutions with countable numbers (generally, 30–300 PFU)
- 10) Calculate PFU ml<sup>-1</sup> with the following formula:

$$\frac{\text{total plaques on plates}}{\text{pipetted volume (0.1 ml)} \times \text{dilution}}$$

**Example:** 248 PFU in 10<sup>-4</sup> dilution and 31 PFU in 10<sup>-5</sup> dilution:

$$\frac{248 + 31}{0.1 \times 10^{-4} + 0.1 \times 10^{-5}} = 2.5 \times 10^7 \text{ PFU ml}^{-1}$$

**References**

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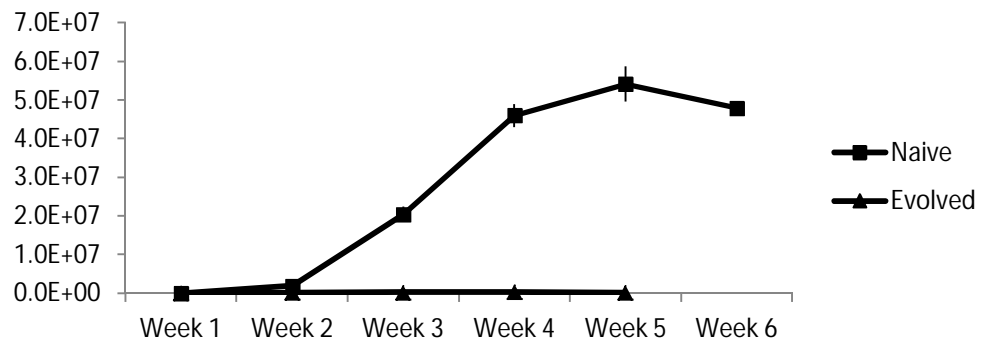
### Appendix 3: Phytoplankton growth curves

Curves are from the phytoplankton growth ability experiment (cells ml<sup>-1</sup> ± SE based on three biological replicates).

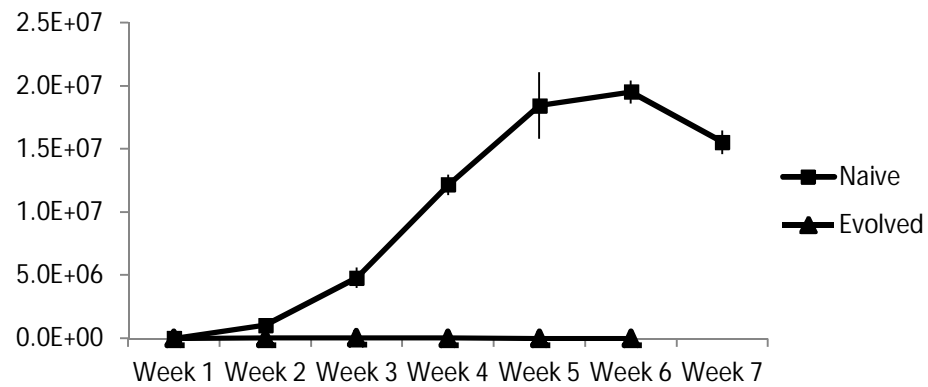
**Naïve:** cultured in cell-free filtrate from phage sensitive *N. spumigena* population culture after 4 day phage exposure

**Evolved:** cultured in cell-free filtrate from phage resistant *N. spumigena* population culture after 4 day phage exposure

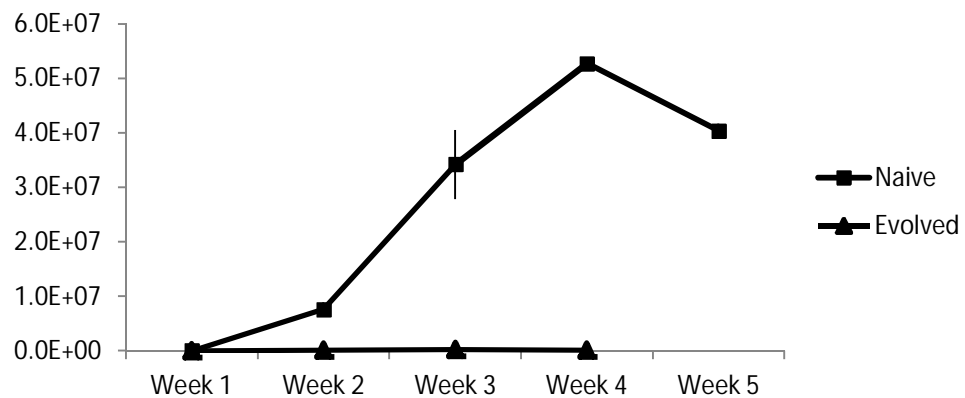
#### *Chlamydomonas reinhardtii* UTEX 89

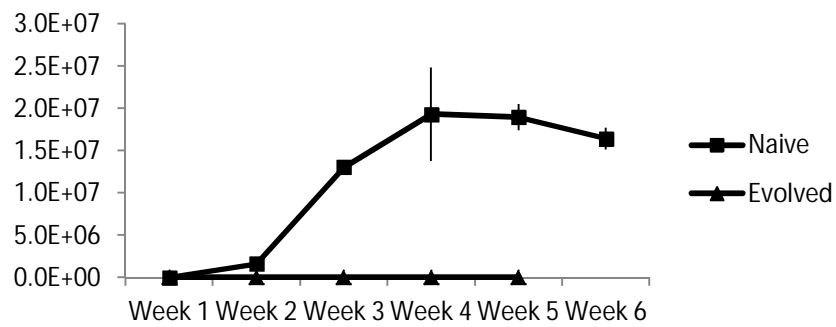
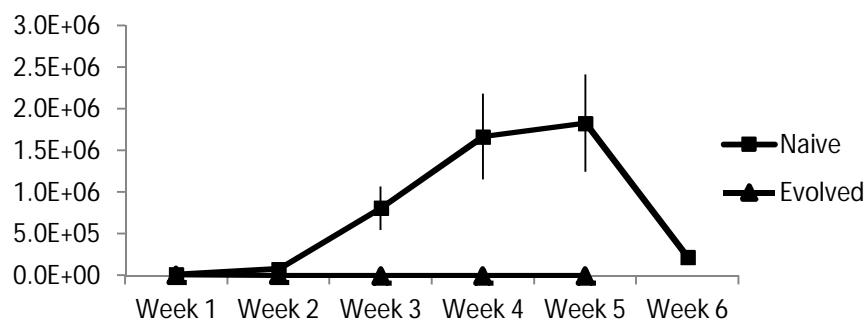
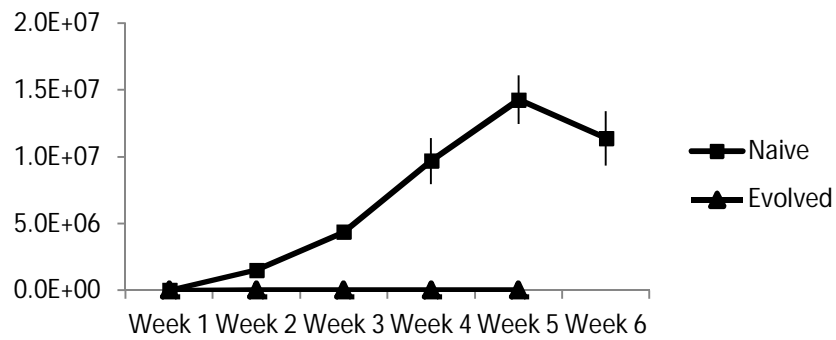
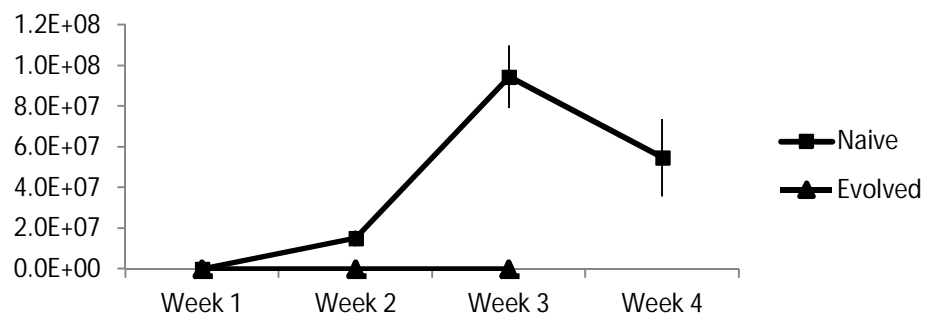


#### *Chlorella pyrenoidosa* TV 216

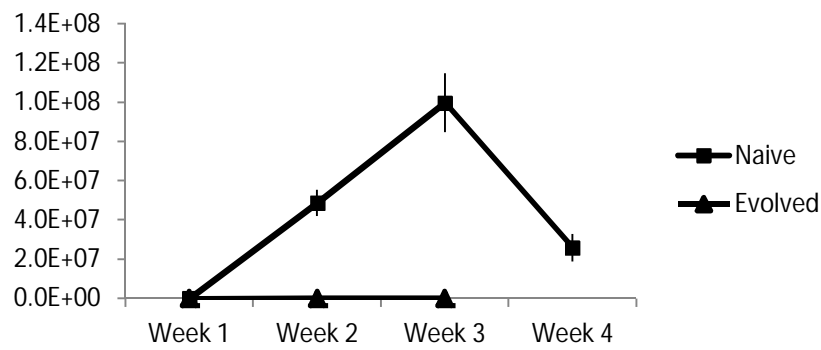
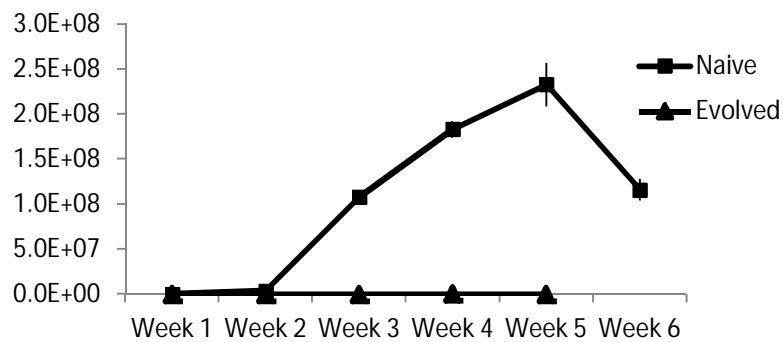
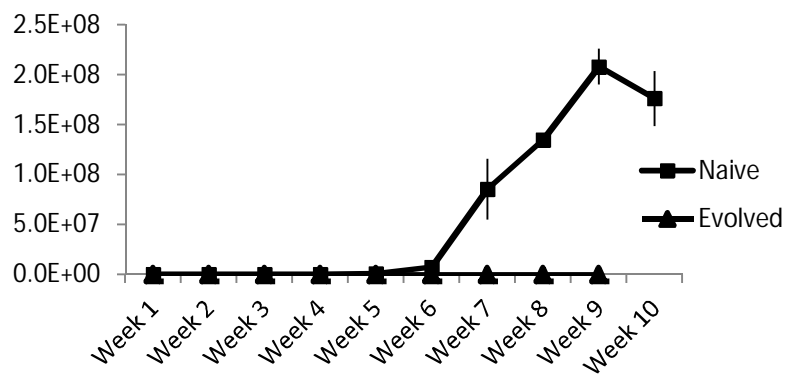
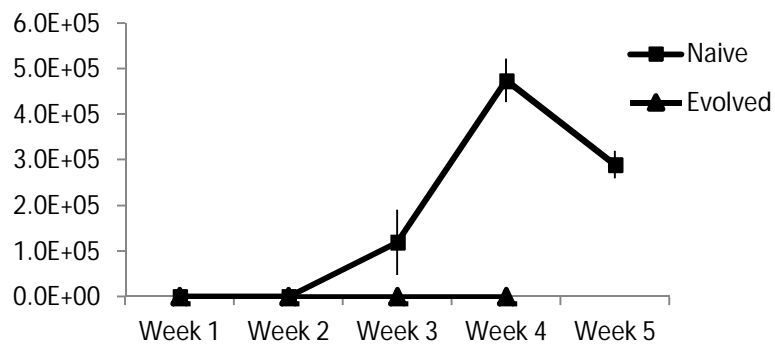


#### *Chlorella vulgaris* UTEX 26



*Phaeodaktylum tricornutum* TV 335*Rhodomonas* sp. Crypto07-B1*Scenedesmus obliquus**Synechococcus* sp. CCY 0417



*Synechococcus* sp. CCY 0435*Synechococcus* sp. TV65*Synechocystis* sp. UHCC 0318*Thalassiosira pseudonana* TV5

**Appendix 4: *Nodularia spumigena* growth curves**

Curves are from weeks 2–5 of the host growth ability experiment.

Samples are in order of isolate identity, phage sensitivity and filament length as follows:

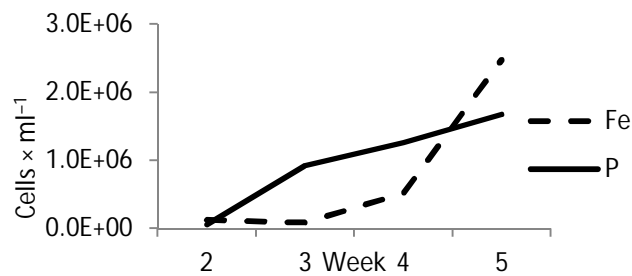
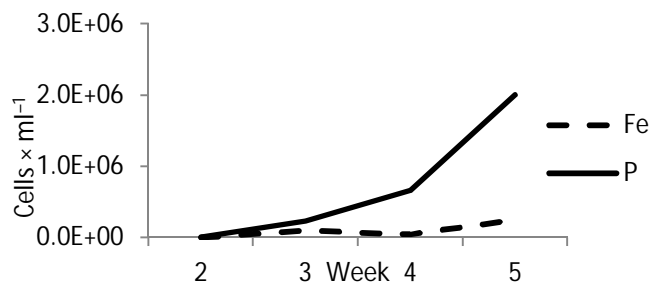
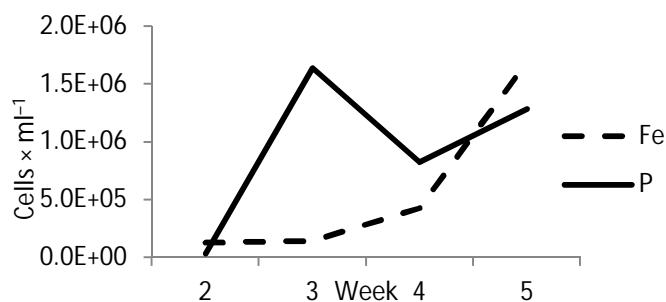
**Phage sensitive genotype:** 1, 2, 3, 21, 22, 23, 41, 42, 43

**Phage resistant genotype with long filaments:** 61, 62, 64, 81, 82, 84, 101, 102, 103

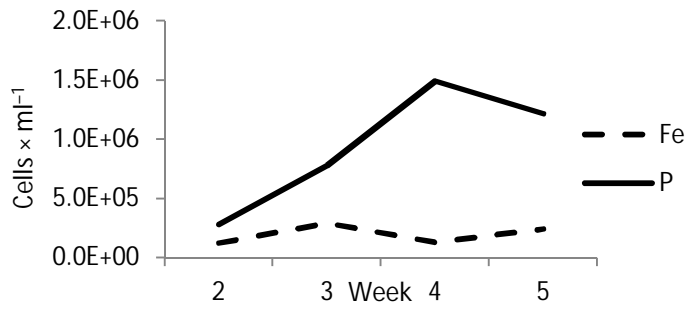
**Phage resistant genotype with short filaments:** 63, 65, 69, 83, 85, 86, 116, 117, 120

**P:** Cultured in P-limited (2  $\mu\text{M}$  phosphorus) Z8 medium with salt and without nitrogen

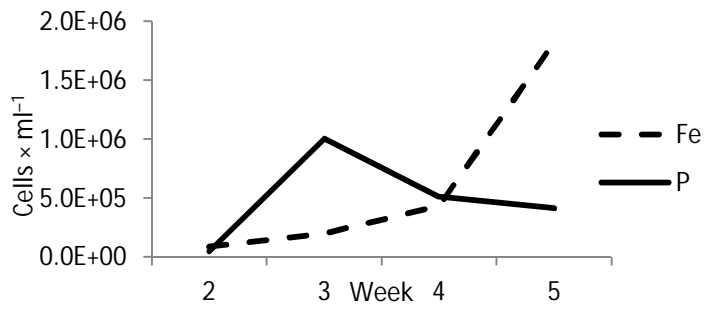
**Fe:** Cultured in Fe-limited (4  $\mu\text{M}$  iron) Z8 medium with salt and without nitrogen

**Isolate 1****Isolate 2****Isolate 3**

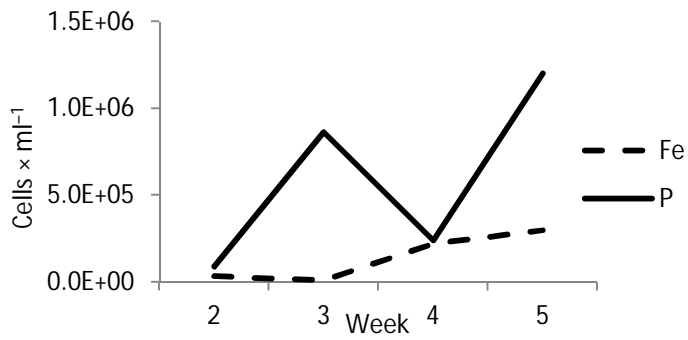
Isolate 21



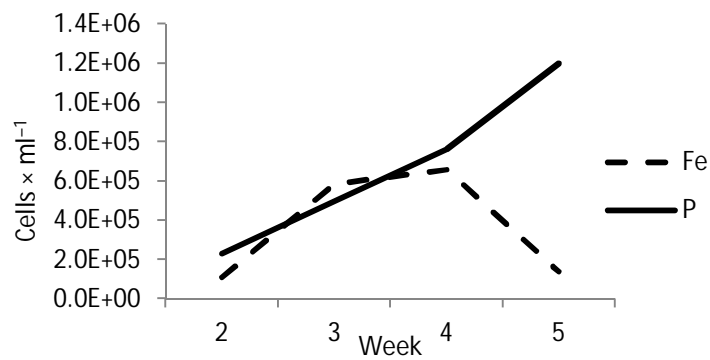
Isolate 22



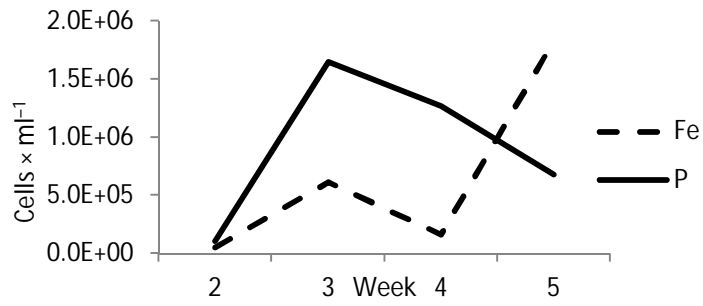
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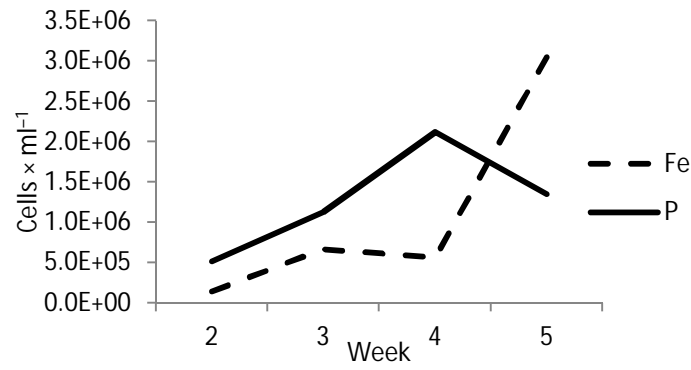
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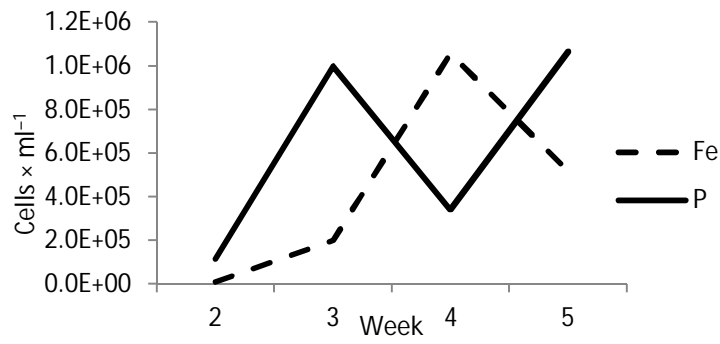
Isolate 42



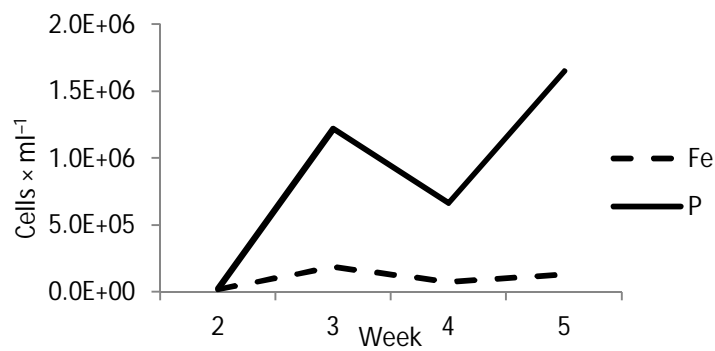
Isolate 43



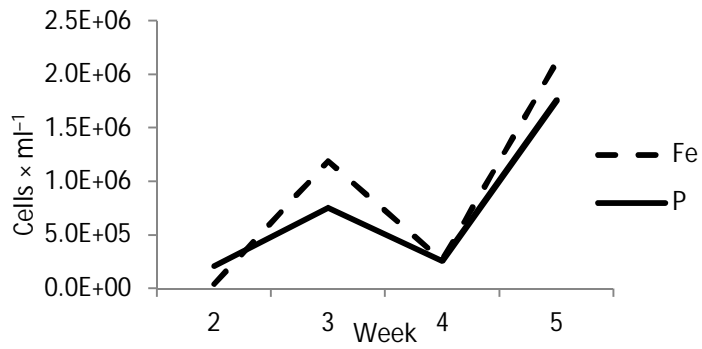
Isolate 61



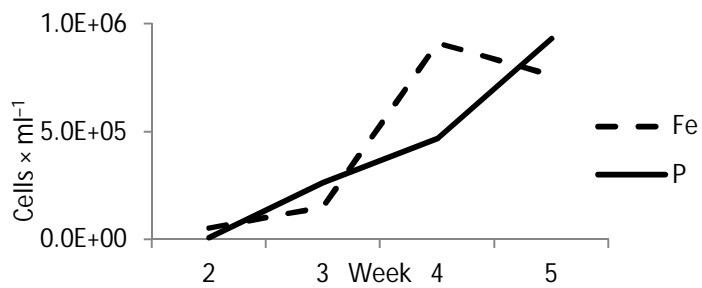
Isolate 62



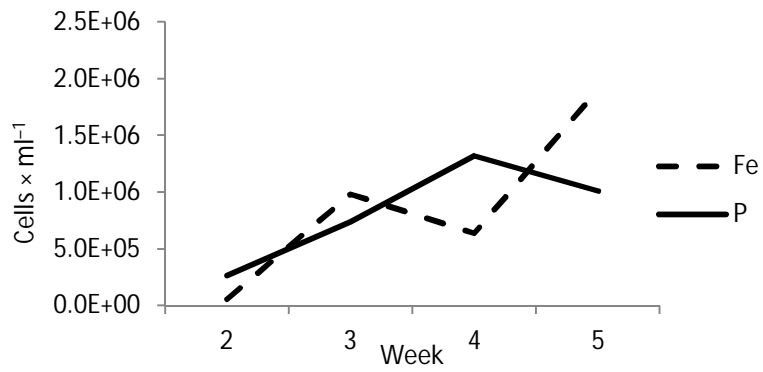
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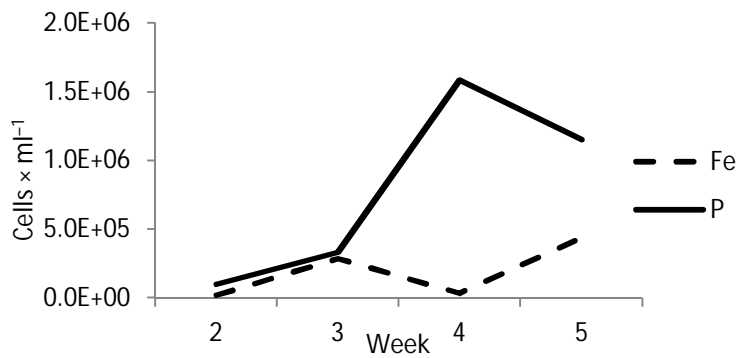
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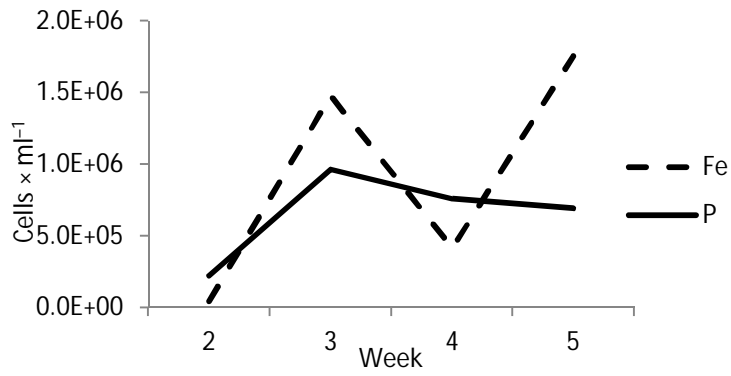
Isolate 82



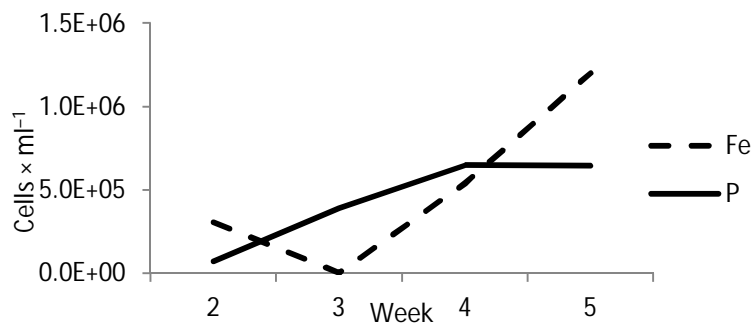
Isolate 84



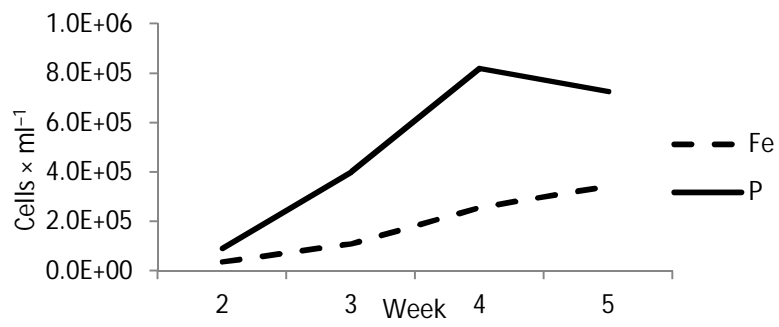
Isolate 101



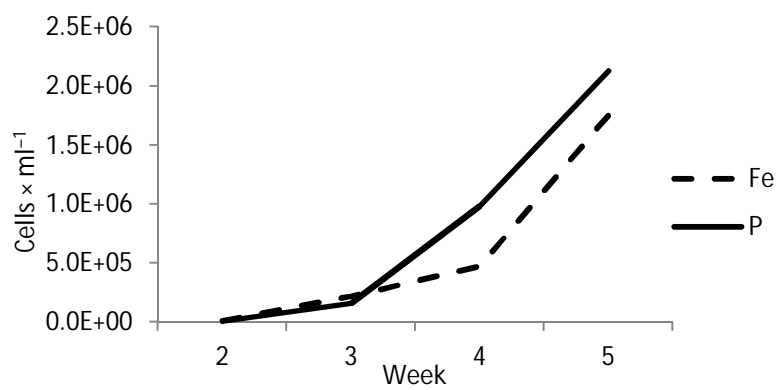
Isolate 102



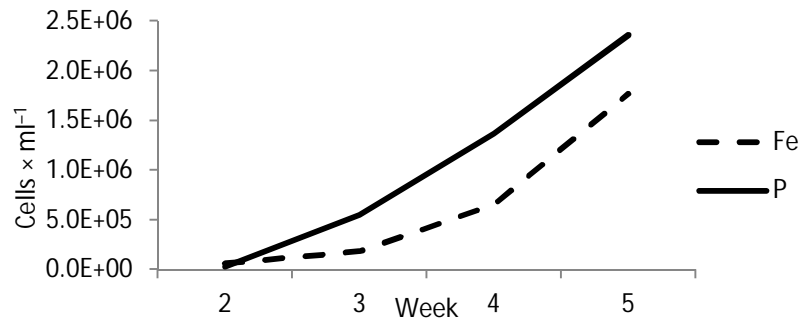
Isolate 103



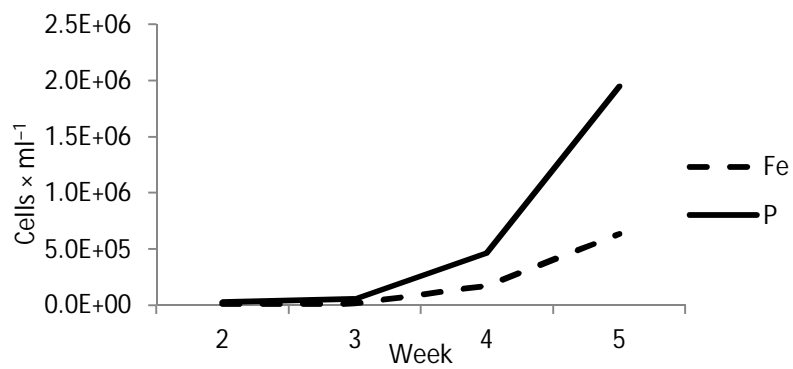
Isolate 63



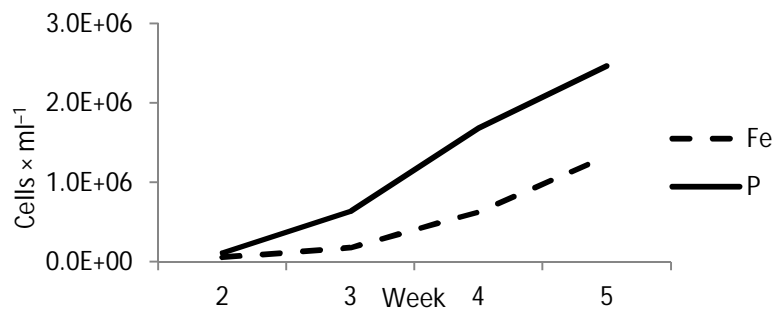
Isolate 65



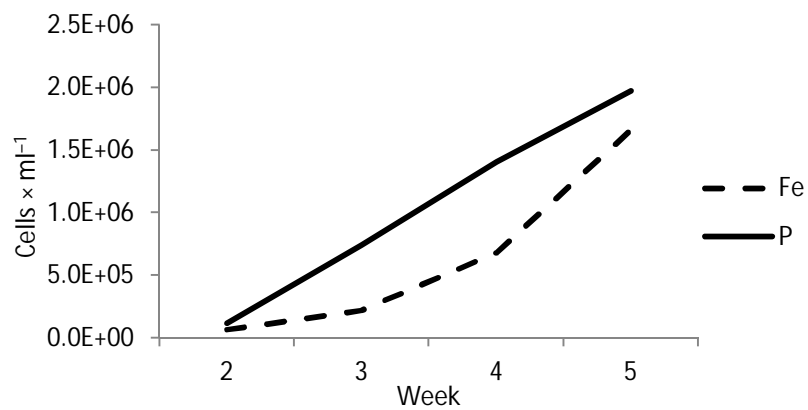
Isolate 69



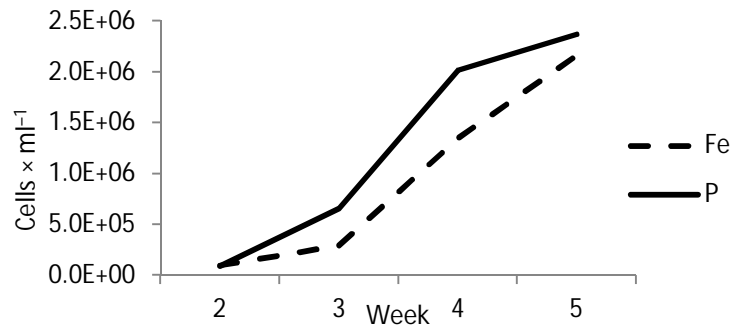
Isolate 83



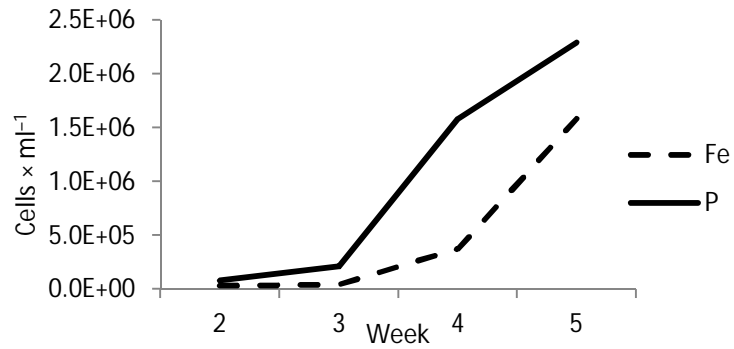
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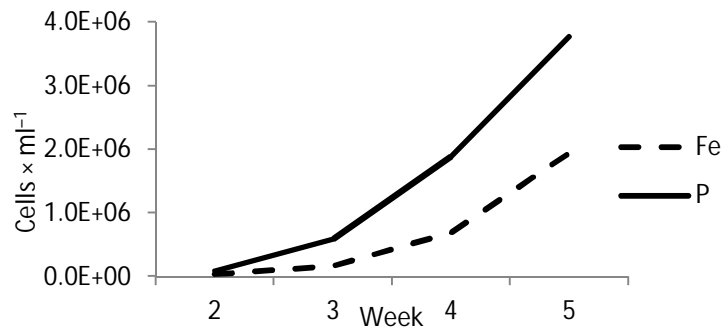
Isolate 86



Isolate 116



Isolate 117



Isolate 120

