Viral ecology of a shallow eutrophic lake

Virus ecologie in een ondiep eutroof meer
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. W.H. Gispen, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen
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Chapter 1

Introduction

Abstract

This thesis aims to give an insight into the ecology of the viral community in a shallow eutrophic lake. To achieve this, the occurrence, diversity and the decay rates and mechanisms of the viral community in Lake Loosdrecht were studied, as well as the impact of the viral community on plankton mortality and community composition. This chapter provides a general introduction on viruses, their occurrence, and their impact on the plankton community in aquatic ecosystems. Furthermore an introduction to the study area, Lake Loosdrecht, and an outline of the thesis are presented.

Viruses

Viruses are tiny genetic particles causing many diseases of man, animals, plants, algae and bacteria. They range in size between 0.02 and 0.3 µm and are visible by electron microscopy. Bacteriophages are viruses which infect prokaryotes. Bacteriophage literally means 'eater of bacteria', from 'bacteria' and the Greek 'to eat', (φαγειν). Bacteriophages consist of an outer protein capsid, surrounding the genetic material. Many bacteriophages are structurally complex, with head and complex tail structures (Flint et al. 2000). An important characteristic of bacteriophages – as of all viruses – is that they are unable to multiply or express their genes outside a host cell. A phage requires host cell enzymes to aid DNA replication, transcription and translation, making phages obligate parasites. After attachment (or adsorption) to a specific receptor in the bacterial cell wall, the phage genome enters the cell, leaving the protein coat outside. "Head and tail" bacteriophages actively inject their DNA into the host cell. Other icosahedral and helical phages penetrate the bacterial cell wall by adhering to flagella or pilli and being "drawn" into the cell. Once inside, the virus hijacks the cellular machinery of the host to produce viral capsid proteins and progeny RNA/DNA, while host cellular material is degraded. Once enough progeny viruses are produced the host cell bursts open, releasing the new viruses and host-cellular debris (Griffiths et al. 1996, Madigan et al. 2000)(Figure 1.1).

Viral ecology

Bacteriophages have already been described in 1915 by Frederick Twort. After isolation of the first bacteriophages their genetics and biology was intensively studied to obtain a better insight into molecular principles. These bacteriophage isolates were mostly studied in pure cultures contributing to molecular findings such as the discovery of DNA as carrier of genetic information, the definition and mapping of the first gene and the mechanics of gene regulation (Hershey & Chase 1952, Benzer 1955, Ptashne 1991). Since viruses were not believed to occur in high numbers in natural environments, not much attention was paid on their occurrence and role in the environment (Wiggins & Alexander 1985, Bergh et al. 1989). It was not until 1989, when Bergh and colleagues reported high abundances of viruses in aquatic environments (1989), that the interest in the environmental role of viruses was really initiated. Viral abundances were reported ranging from $1 - 6 \times 10^4$ virus like particles (vlp) ml^{-1} in marine

samples during the low productive winter season and 5×10^6 to $1{,}5 \times 10^7$ vlp ml^{-1} during the productive summer season (Bergh et al. 1989). In eutrophic lakes viral abundances as high as $2{,}5 \times 10^8$ vlp ml^{-1} were reported (Bergh et al. 1989). These data show that viral abundances in natural aquatic systems often exceed bacterial abundances, which are generally ~10^6 bacteria per millilitre in productive systems (Fuhrman 1999). After this publication more studies reported high viral abundances in marine environments (Proctor & Fuhrman 1990, Weinbauer et al. 1993), freshwater environments (Hennes & Simon 1995, Mathias et al. 1995) and coastal sediments (Lawrence et al. 2002) (Figure 1.2).

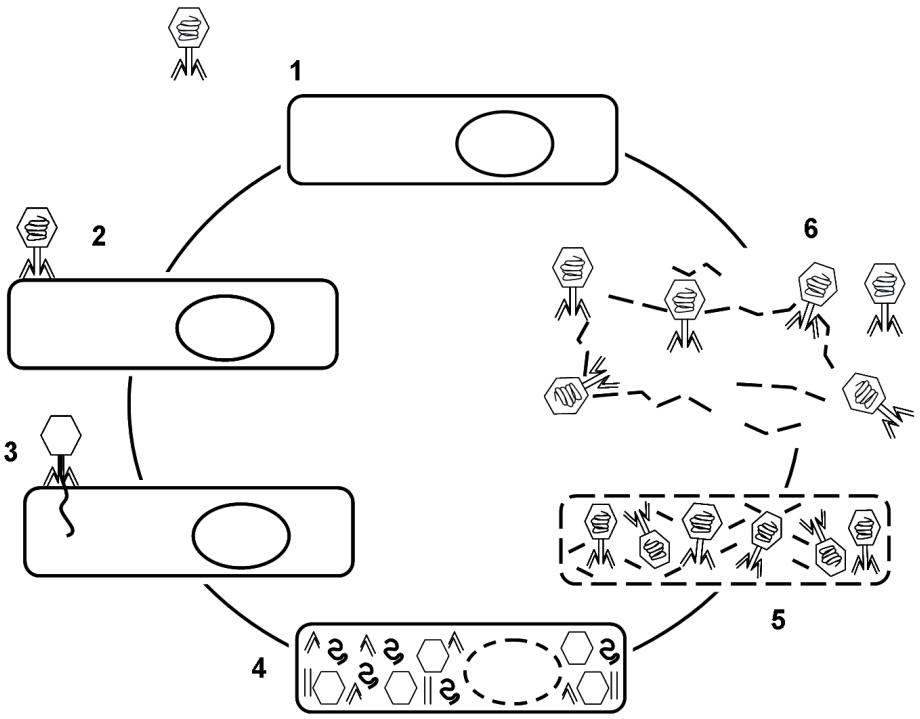

Figure 1.1 Infection cycle of bacteriophages. 1) Free bacterial host and bacteriophages in the aquatic environment. 2) Adsorption of bacteriophages onto bacterial host. 3) Injection of viral genetic content into viral host cell. 4) Viral genome hijacks the cellular machinery of the host to produce viral capsid proteins and progeny RNA/DNA, while host cellular material is degraded. 5) Assembly of progeny viral particles and degradation of cell wall host. 6) Release of progeny of bacteriophages and cellular debris of degraded host cell.

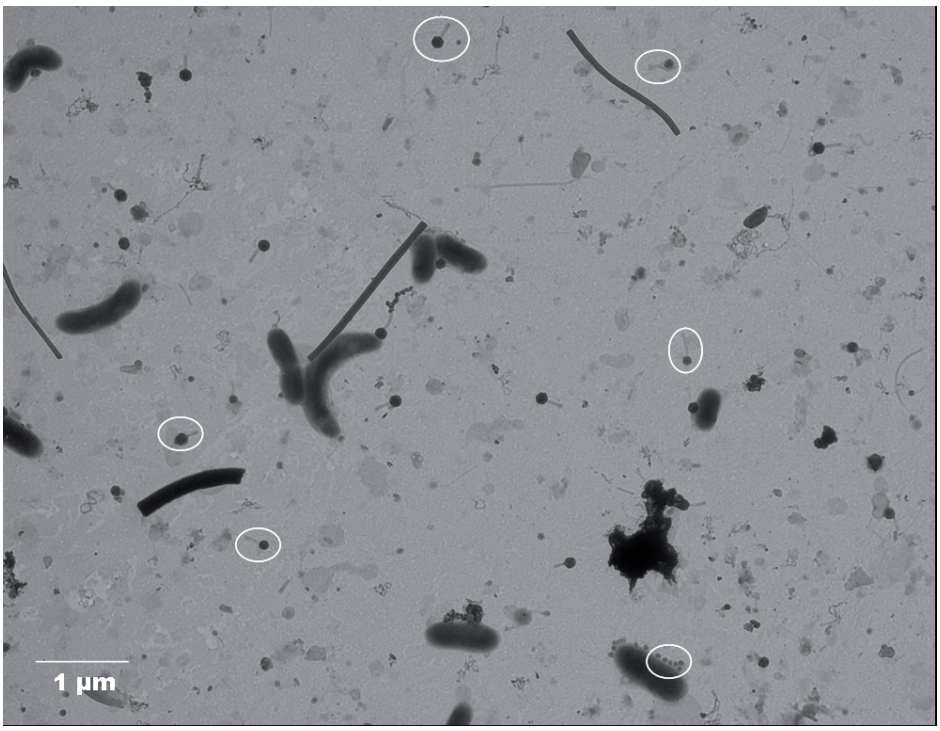

Figure 1.2 Viral particles in Lake Loosdrecht water. Transmission electron miscroscopy image of ultracentrifuged, and negatively stained Lake Loosdrecht water, revealing a variety of different viral particles. White circles indicate different viral morphologies.

The break through in determining abundances of the total viral community in aquatic systems was made by employing a combination of ultracentrifugation and electron microscopy (Bergh et al. 1989). Less laborious, but highly effective enumerations were made by staining viral particles with nucleic acid specific dyes and epifluorescence microscopy (Hennes & Suttle 1995, Noble & Fuhrman 1998). MPN counts (Most Probable Number), classical plaque assay and quantitative PCR can also be used to observe community dynamics of specific viral groups, but require the cultivation of a suitable host organism or sequence information of the viral group of interest (Adams 1959, Hennes & Suttle 1995, Noble & Fuhrman 1998, Tomaru et al. 2004).

Viral diversity

Diversity among viruses can be determined based on host range, morphological (e.g. structure, size of capsid and structure of tail) and molecular characteristics

(e.g. genome size, number of capsid proteins, mol% G-C content, DNA restriction patterns, hybridisation to known DNA fragments and sequence analysis of specific genes). Several studies employing molecular methods such as pulsed field gel electrophoresis (PFGE) and denaturing gradient gel electrophoresis (DGGE) revealed the viral community as a very diverse and dynamic component of the aquatic community (Wommack et al. 1999, Frederickson et al. 2003, Short & Suttle 2003, Dorigo et al. 2004). Metagenome analysis of the viral community in coastal waters indicated that viral diversity is probably even up to an order of a magnitude greater than that of bacteria (Breitbart et al. 2002). Due to the selective and parasitic nature of viruses, viral and host abundances are expected to co-vary (Fuhrman 1999). One would therefore also expect a close linkage over time between host and virus community composition (Hennes et al. 1995, Steward et al. 2000). Viral population dynamics have indeed been reported to be closely linked to microbial and algal population dynamics in aquatic environments (Hennes & Simon 1995, Castberg et al. 2001, Jacquet et al. 2002, Tomaru et al. 2004, Goddard et al. 2005, Muhling et al. 2005). Changes in viral community structure have also been associated with environmental factors such as seasonality, location, water depth, degree of stratification, tide height, salinity and Chl-*a* concentration (Wommack et al. 1999, Frederickson et al. 2003, Short & Suttle 2003, Dorigo et al. 2004).

Virally induced mortality

These high viral abundances in combination with their parasitic nature lead to the realization that viral infection must be an important cause of mortality of both bacteria and algae in aquatic environments, comparable to grazing induced mortality (Bergh et al. 1989, Proctor & Fuhrman 1990). Viruses have indeed been reported to contribute up to 70% of cyanobacterial mortality in marine systems (Proctor & Fuhrman 1990) and up to 90 - 100% of bacterial mortality in freshwater systems (Weinbauer & Hofle 1998, Fischer & Velimirov 2002). Since high host abundances facilitate viral spread and thus the chance of viral infection, it is thought that viruses can regulate the abundances of their host (Murray & Jackson 1992, Hennes et al. 1995). Viral infection has indeed been reported to be responsible for the collapse of complete marine algal blooms, such as the harmful bloom-forming raphidophyte *Heterosigma akashiwo* (Nagasaki et al. 1994, Tomaru et al. 2004), the prymnesiophyte *Phaeocystis globosa* (Brussaard et al. 2005a), and

the haptophyte *Emiliania huxleyi* (Bratbak et al. 1993). Enriching natural seawater with native viruses resulted in a 50% reduction of phytoplankton biomass and primary production (Suttle et al. 1990, Suttle 1992), suggesting a significant effect of viruses on primary production mediated carbon cycling.

Viral impact on community structure

Viral infection is considered to influence microbial community diversity and composition due to its host-specific nature (Fuhrman & Suttle 1993). Changes in marine phytoplankton structure were indeed observed by Suttle (1992) and Peduzzi and Weinbauer (1993), when adding the virus-rich submicron size fraction concentrated from natural seawater. The 'killing the winner' theory postulates that viruses control the abundance of the competitive dominant species. The increased chance of encounter between this numerically dominant species and its specific virus will lead to an increased probability of viral infection, thereby resulting in a down-regulation of the abundance of this competitive dominant. Viral infection is thus predicted to facilitate the co-existence of less competitive species (Thingstad & Lignell 1997, Thingstad 2000) (Figure 1.3). Viral control of the competitive dominant is predicted to promote diversity, by enabling the invasion and co-existence of less competitive species. (Thingstad 2000, Wommack & Colwell 2000). One could thus expect a positive correlation between viral abundances and community diversity. By manipulating viral abundances in marine environments, Hewson (2003) and Winter (2004) indeed observed a positive correlation between viral abundance and community diversity. These results were recently contradicted by Bouvier and Del Giorgio (2007), however, who provided evidence that viral regulation may in fact decrease the overall prokaryotic diversity, by maintaining competitively strong phylogenetic groups at such low densities, that they are below the detection threshold.

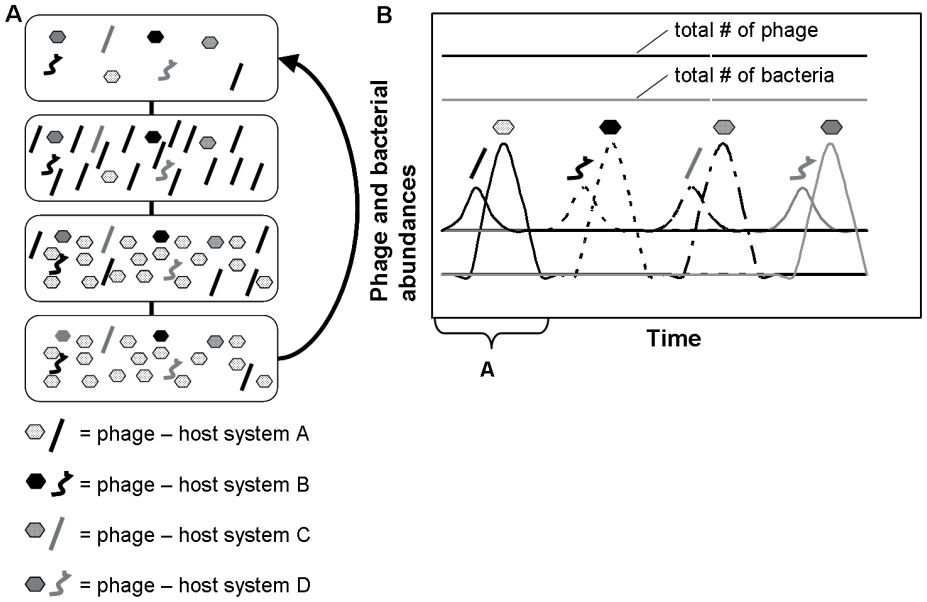

Figure 1.3 The 'killing the winner – principle' (Thingstad & Lignell 1997, Thingstad 2000). A) Schematic representation of viral impact on composition of an aquatic community with 4 different phage – host systems. Bacterium A is competitively superior over the 3 other bacteria and increases in abundances. The high abundances of bacterium A stimulate propagation of its specific phage A, resulting in a reduction in abundances of bacterium A and an increase of phage A abundances. Competitive exclusion is thus prevented by viral infection of the competitive dominant, enabling co-existence of competitively inferior bacteria. B) Changes in environmental conditions over time results in shifts in fitness of the bacteria, resulting in the increase of a different competitive dominant and its subsequent down regulation due to viral infection (Figure copied from Wommack & Colwell 2000).

Viral impact on the food web

Indirectly viruses are thought to impact aquatic communities through the release of cellular debris upon lysis of the host cell, thereby stimulating the respiration and production of heterotrophic bacteria (Bratbak et al. 1992, Gobler et al. 1997, Noble & Fuhrman 1999). Viral lysis thus leads to a recycling of nutrients within the microbial loop, and due to respiratory losses it ultimately results in a decrease of nutrients being transported to higher trophic levels (Fuhrman 1999) (Figure 1.4). Middelboe and colleagues demonstrated in laboratory experiments that viral lysis of a bacterial population can relieve nutrient limitation and increase the metabolic activity, cell production, and dissolved organic carbon uptake of a natural bacterial assemblage (2002). In other studies, radiotracer experiments demonstrated the release of bio-available C, N, P, Fe and Se upon

viral lysis, which was accompanied by an increase in bacterial growth (Gobler et al. 1997). Similar research showed that viral release of Fe could support up to 80% of marine phytoplankton primary production (Poorvin et al. 2004). Since changes in composition and bioavailability of organic nutrients are known to affect chemotrophic bacterial community composition (Lebaron et al. 1999, Van Hannen et al. 1999), the release of organic compounds due to viral lysis is also considered to influence heterotrophic bacterial community composition (Riemann & Middelboe 2002, Brussaard et al. 2005b).

Viral decay

The interest in the mechanisms and rates of viral decay in aquatic environments traditionally mainly concerned the survival of enteric pathogenic viruses in aquatic environments, the processes controlling their survival and the implications for waste- and drinking water treatment (Mitchell & Jannasch 1969, Kapuscinski & Mitchell 1980, Ward et al. 1986). The discovery of viruses as major components of the aquatic food web revived this interest in viral decay. So far, a large variety of mechanisms have been reported to control viral decay rate. Especially UV irradiation, in particular UV-B, is considered to have a strong damaging effect on viral particles (Kapuscinski & Mitchell 1983, Suttle & Chen 1992, Wommack et al. 1996, Noble & Fuhrman 1997, Wilhelm et al. 1998). In the absence of sunlight, adsorption of viral particles onto particulate material and high molecular weight - dissolved organic matter (HMW-DOM) has been reported as an important source of viral infectivity loss (Suttle & Chen 1992, Schneider et al. 1996, Noble & Fuhrman 1997, Lu et al. 2002, Anesio et al. 2004) . Other factors reported to influence viral decay, are enzymatic degradation, adsorption onto and degradation by bacterial cells, temperature, as well as grazing by nanoflagellates (Kapuscinski & Mitchell 1980, Ward et al. 1986, Moebus 1992, Gonzalez & Suttle 1993, Noble & Fuhrman 1997, Bettarel et al. 2005). Previously published turnover times of aquatic viral communities range between 0.036 and 23.9 days (Wommack & Colwell 2000, Weinbauer 2004). These values have been obtained with a range of different methodologies, such as measuring tracer dilution rates using fluorescently labelled viruses as tracer (Noble & Fuhrman 2000), estimating viral production rates (Proctor & Fuhrman 1990, Weinbauer et al. 2002, Wilhelm et al. 2002) or by addition of cyanide to stop viral production and estimation of the loss rate of viral particles (Heldal & Bratbak 1991).

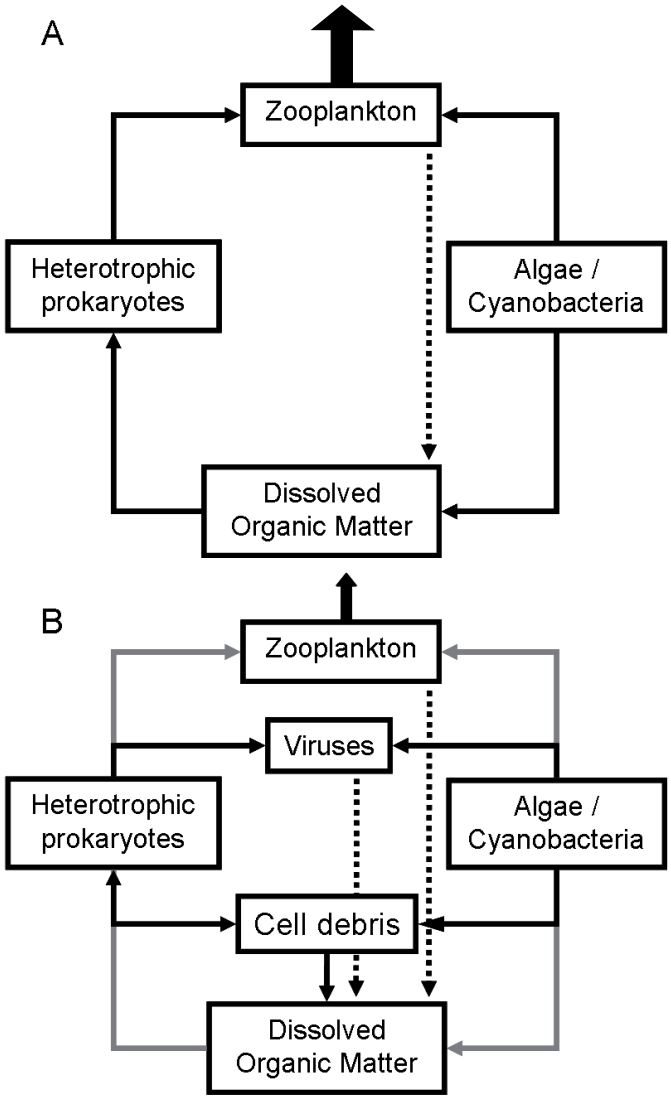

Figure 1.4 Viral impact on the aquatic food web. A) Grazing of bacteria and phytoplankton by zooplankton results in transport of nutrient to higher trophic levels. B) Viral lysis of bacteria and phytoplankton leads to the release of cellular debris, which can stimulate heterotropic bacterial growth. Viral infection therefore results in a recycling of nutrients within the microbial loop, ultimately resulting in a decrease of nutrients being transported to higher trophic levels.

Thesis objectives

Viruses are thus considered to be an important ecological component within aquatic communities. In freshwater environments there is still limited knowledge

to what extent viral community composition reflects changes in environmental conditions and host community composition (Dorigo et al. 2004, Auguet et al. 2006), or the extent to which viral infection influences the food-web and community composition. Within the present PhD research the viral ecology of Lake Loosdrecht was studied (Figure 1.5). Lake Loosdrecht (The Netherlands) is a highly eutrophic (Chl-*a* annual average of ca 60 mg m^{-3}), shallow (mean depth 1.9 m) and turbid (Secchi depth approximately 0.5 m) lake that originated from peat excavation. The lake is dominated by a group of related filamentous cyanobacteria belonging to the *Limnothrix* / *Pseudoanabaena* group (Van Tongeren et al. 1992, Zwart et al. 2004) (Figure 1.6).

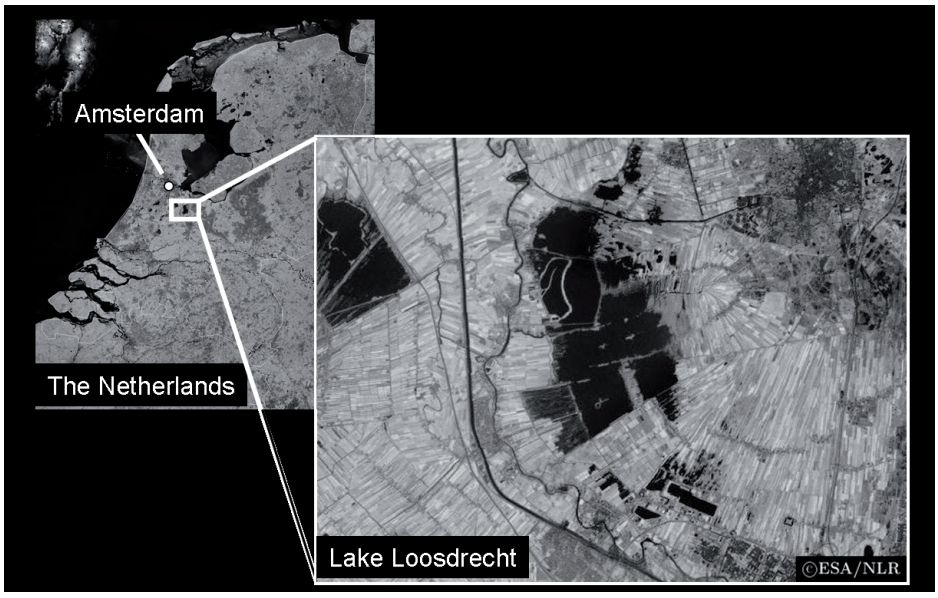

Figure 1.5 Lake Loosdrecht.

The virioplankton community in this lake is particularly interesting since earlier research repeatedly showed a dramatic collapse of the cyanobacterial community associated with viral activity during lake water enclosure experiments (Van Hannen et al. 1999, Gons et al. 2002, Simis et al. 2005). Furthermore it is known from previous research that the zooplankton community largely depends on eukaryotic algae for growth and that grazing only accounts for part of the cyanobacterial mortality (Gulati et al. 1992, Pel et al. 2003). Therefore, the viral community in this lake is thought to play a significant role in cyanobacterial

mortality in this lake (Figure 1.6). Due to their high abundances and based on previous research (Weinbauer & Hofle 1998, Fischer & Velimirov 2002), heterotrophic bacteria are also considered as potential viral host in Lake Loosdrecht. During the present research the population dynamics, diversity, impact, and interactions of the viral community in Lake Loosdrecht were studied by seasonal monitoring and a series of lake water enclosure and other laboratory experiments.

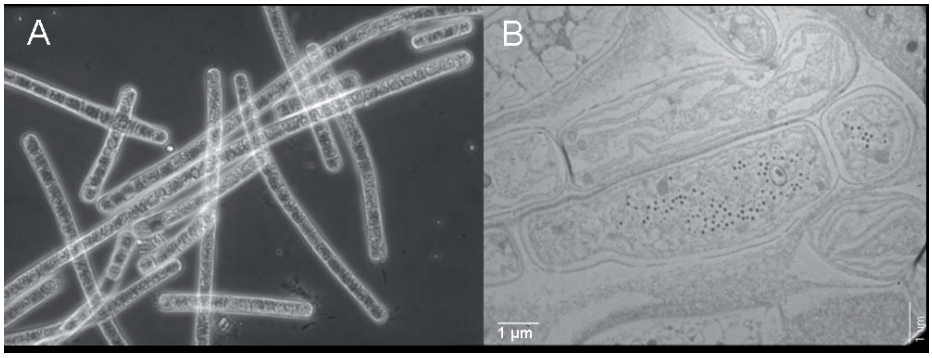

Figure 1.6 Filamentous cyanobacteria. A) The dominant filamentous cyanbacteria in Lake Loosdrecht, which belong to the *Limnothrix* / *Pseudoanabaena* group. B) Thin section of virally infected filamentous cyanobacteria in Lake Loosdrecht as observed with transmission electron microscopy. Black spots within the middle cell are viral particles.

Thesis outline

Chapter 2) **Population dynamics and diversity of viruses, bacteria and phyto-plankton in a shallow eutrophic lake**.

The temporal dynamics of the total viral community in Lake Loosdrecht were studied in relation to environmental parameters and in particular to the community dynamics of the potential viral hosts in the lake, aiming to improve our knowledge of the virioplankton community in eutrophic lakes.

Chapter 3) **Impact of a mass viral lysis event on the microbial community composition in eutrophic lake water**.

Population dynamics and community composition of the viral, chemotrophic bacterial, cyanobacterial and algal communities were studied before, during and after the collapse of the dominant filamentous cyanobacterial population in lake

water enclosure experiments to study the impact of a mass viral lysis event on the composition of the aquatic microbial community.

Chapter 4) **Estimates of potential bacterial and phytoplankton mortality rates due to viral lysis and microzooplankton grazing in a eutrophic lake.**

In this chapter an adapted version of the dilution technique was applied to simultaneously estimate the impact of both viral lysis and zooplankton grazing on the mortality of bacteria, algae, unicellular cyanobacteria, prochlorophytes and especially filamentous cyanobacteria.

Chapter 5) **Mechanisms and rates of viral decay in a shallow, eutrophic lake.**

The rate of viral infectivity loss was studied by adding trace amounts of cyanophages to different treatments of Lake Loosdrecht water. The loss in plaque-forming units was obtained to determine the impact of the factors particulate matter, high molecular weight – dissolved organic matter (HMW-DOM), heat-labile compounds, UV, bacteria and nanoflagellates on the viral decay rate.

Chapter 6) **Summarizing discussion.**

The results presented in this thesis will be summarized and discussed, in addition to an outlook on necessary future research.

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Chapter 2

Population dynamics and diversity of viruses, bacteria and phytoplankton in a shallow eutrophic lake

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Abstract

We have studied the temporal variation in viral abundances and community assemblage in the eutrophic Lake Loosdrecht, through epifluorescence microscopy and pulsed field gel electrophoresis (PFGE). The virioplankton community was a dynamic component of the aquatic community, with abundances ranging between 5.5×10^7 and 1.3×10^8 virus like particles ml^{-1} and viral genome sizes ranging between 30 and 200 kb. Both viral abundances and community composition followed a distinct seasonal cycle, with high viral abundances observed during spring and summer. Due to the selective and parasitic nature of viral infection, it was expected that viral and host community dynamics would covary, both in abundances and community composition. The temporal dynamics of the bacterial and cyanobacterial communities, as potential viral hosts, were studied in addition to a range of environmental parameters, in order to relate these to viral community dynamics. Cyanobacterial and bacterial communities were studied applying epifluorescence microscopy, flow cytometry and denaturing gradient gel electrophoresis (DGGE). Both bacterial and cyanobacterial communities followed a clear seasonal cycle. Contrary to expectations viral abundances were not correlated to abundances of the most dominant plankton

groups in Lake Loosdrecht, the bacteria and the filamentous cyanobacteria, nor could we detect a correlation between the assemblage of viral and bacterial or cyanobacterial communities during the overall period. Only during short periods of strong fluctuations in microbial communities we could detect viral community assemblages to co-vary with cyanobacterial and bacterial communities. In order to detect the more subtle virus-host interactions methods with a higher specificity and resolution are probably needed. Viral abundances did however relate to cyanobacterial community assemblage and showed a significant positive correlation to Chl-*a* as well as prochlorophytes, suggesting that a significant proportion of the viruses in Lake Loosdrecht may be phytoplankton and more specific cyanobacterial viruses. Temporal changes in bacterial abundances were significantly related to viral community assemblage, and *vice versa*, suggesting an interaction between viral and bacterial communities in Lake Loosdrecht.

Introduction

Traditionally, nutrient availability, sedimentation and grazing were considered the major driving forces of microbial and algal communities in aquatic environments. Since the discovery of high viral abundances almost 2 decades ago (Bergh et al. 1989), awareness of the virioplankton community as major player in the aquatic food web has grown (Wommack & Colwell 2000). Reports of viral lysis contributing up to 70% of cyanobacterial mortality in marine systems (Proctor & Fuhrman 1990) and up to 90 - 100% of bacterial mortality in freshwater systems (Weinbauer & Hofle 1998, Fischer & Velimirov 2002) led to the conclusion that viral lysis can be a major mortality cause, comparable to grazing-induced mortality. Several studies employing molecular methods such as pulsed field gel electrophoresis (PFGE) and denaturing gradient gel electrophoresis (DGGE) revealed the viral community as a very diverse and dynamic component of the aquatic community (Wommack et al. 1999, Frederickson et al. 2003, Short & Suttle 2003, Dorigo et al. 2004) Metagenome analysis of the viral community in coastal waters indicated that viral diversity is probably even up to an order of a magnitude higher than bacterial richness (Breitbart et al. 2002).

Due to the selective and parasitic nature of viruses, viral and host abundances are expected to co-vary (Fuhrman 1999). One would therefore also expect

a close linkage over time between host and virus community composition (Hennes et al. 1995, Steward et al. 2000). Viral population dynamics have indeed been reported to be closely linked to microbial and algal population dynamics in aquatic environments (Hennes & Simon 1995, Castberg et al. 2001, Jacquet et al. 2002, Tomaru et al. 2004, Goddard et al. 2005, Muhling et al. 2005). Changes in viral community structure have also been associated with environmental factors such as seasonality, location, water depth, degree of stratification, tide height, salinity and Chl-*a* concentration (Wommack et al. 1999, Frederickson et al. 2003, Short & Suttle 2003, Dorigo et al. 2004). In freshwater environments there is still limited knowledge to what extent viral community composition reflects changes in environmental conditions and host community composition (Dorigo et al. 2004, Auguet et al. 2006).

The present study examined temporal changes in viral community abundance and composition in Lake Loosdrecht, employing epifluorescence microscopy and PFGE. Lake Loosdrecht is a highly eutrophic (Chl-*a* annual average of ca 60 mg m^{-3}), shallow (mean depth 1.9 m) and turbid (Secchi depth approximately 0.5 m) peat lake in the Netherlands. The lake is dominated by a group of related filamentous cyanobacteria belonging to the *Limnothrix* / *Pseudoanabaena* group (Van Tongeren et al. 1992, Zwart et al. 2004). The virioplankton community in this lake is particularly interesting since earlier research repeatedly showed a dramatic collapse of the cyanobacterial community associated with viral activity during lake water enclosure experiments (Van Hannen et al. 1999b, Gons et al. 2002, Simis et al. 2005). Furthermore it is known from previous research that the zooplankton community largely depends on eukaryotic algae for growth and that grazing only accounts for part of the cyanobacterial mortality (Gulati et al. 1992, Pel et al. 2003). Therefore, the viral community in this lake is thought to play a significant role in cyanobacterial mortality in this lake. Chemotrophic bacteria are generally also considered to be an important viral host in freshwater environments (Weinbauer & Hofle 1998, Fischer & Velimirov 2002). During the present study the community dynamics of these potential viral hosts were examined employing flow cytometry and DGGE. If cyanobacteria and chemotrophic bacteria are indeed important viral hosts in Lake Loosdrecht, a close linkage with viral community dynamics would be expected. We thus studied the temporal dynamics of the total viral community in Lake Loosdrecht, in relation to environmental parameters and in particular

to potential viral host community dynamics, aiming to improve our knowledge of the virioplankton community in eutrophic lakes.

Material and Methods

Sampling

Samples were collected from Lake Loosdrecht (The Netherlands) every 2 w from February to November 2003. Upon sampling Secchi-disk depth, pH and water temperature were determined. Surface water was sampled in high density polyethylene-containers and immediately transported to the laboratory. All samples were processed or fixed within 3 h after sampling. Data on chemical and environmental variables was obtained from the Service for Inland Water Management and Wastewater Treatment Amstel, Gooi en Vecht (DWR).

Phytoplankton Enumeration

Different cyanobacterial groups and eukaryotic algae were distinguished using an Epics Elite flow cytometer (Coulter, Miami, USA) equipped with an ion argon laser (excitation 30 mW at 488 nm) and a cell sorter. Fresh samples were analyzed for 5 min at a flow rate of 43 µl min^{-1}. The fluorescence of Chl-*a* was recorded at 675 nm with full width-half maximum (FWHM) of 40 nm. Phycocyanin fluorescence was recorded at 635 nm (FWHM 15 nm). Side scatter was used to trigger count events. A small volume of a known concentration of 1-µm diameter fluorescent beads (Polyscience, Warrington, USA #15702) was added for exact volume determination.

Typically, three major cell clusters of filamentous cyanobacteria, prochlorophytes (Turner et al. 1989), and eukaryotic algae, respectively, were detected (Figure 2.1). This classification was confirmed by light microscopy examination and fatty acids analysis after cell sorting (Pel et al. 2004).

Nanoflagellate Enumeration

Samples were fixed with 0.5 % glutaraldehyde and stored at 4°C in the dark. All samples were counted within a week after sampling applying Utermöhl chambers (Utermöhl 1931) and an inverted Leica, Fluovert microscope, counting at least 200 nanoflagellates. Flagellates <10 µm were counted as nanoflagellates.

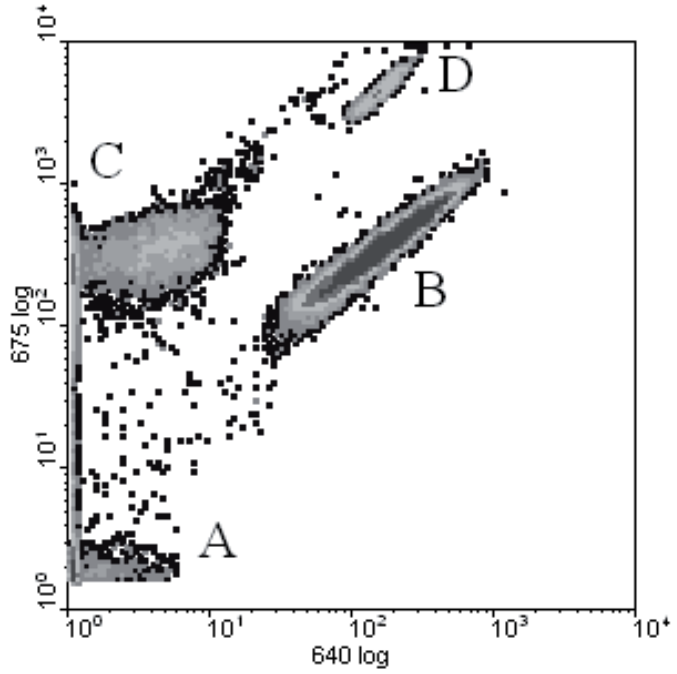


Figure 2.1 Phytoplankton clusters in typical Lake Loosdrecht sample as detected by flow cytometry. (A) bacteria / detritus cluster (B) cyanobacterial cluster (C) prochlorophyte cluster (D) algal cluster. A different flow cytometry protocol was used to obtain bacterial numbers.

Chemotrophic Bacteria and Virus Enumeration

Samples were fixed with 0.02-µm filtered formalin (1% final volume), frozen in liquid nitrogen and stored at -80°C until analysis. Triplicate bacteria and virus counts were obtained following staining of samples with SYBR Green I (Molecular Probes, Europe), as described by Noble and Fuhrman (Noble & Fuhrman 1998). In brief, samples were 100x diluted in 0.02 µm filtered MQ to facilitate counting and 1 ml of this dilution was filtered through a 0.02-µm Anodisc filter (Whatman, Brentford, England). The filters were then placed on 100 µl of a solution of SYBR Green I (1:2500 dilution of original stock) and incubated for 15 min in the dark. Following incubation, the backside of the filters were dried gently on a tissue and mounted onto glass slides with 20 µl of antifading solution (Noble & Fuhrman 1998). At least 200 virus-like particles (vlp) and bacteria were counted using a Zeiss Axiophot epifluorescence microscope at blue light excitation.

Bacterial and Cyanobacterial Community Composition

The diversity of bacterial and cyanobacterial communities was obtained by denaturing gradient gel electrophoresis (DGGE) (Fisher & Lerman 1979) after PCR-amplification applying general bacteria specific as well as cyanobacteria specific primers targeting the small subunit ribosomal RNA gene (SSU rDNA), using the exact protocol as described in (Zwart et al. 1998a, Zwart et al. 2004). We amplified the cyanobacterial SSU rDNA sequences using first a nested procedure with primers Cya-b-F371 and Cya-R783. After this cyanobacteria-selective pre-amplification, a second amplification procedure was performed employing the general bacterial primers F357GC and R518.

The product of both the bacteria and cyanobacteria-specific PCR procedure is a GC clamp containing PCR product of approximately 200-bp long. This product was subjected to DGGE using a clone ladder composed of 23 previously sequenced and described Lake Loosdrecht clones. Of these clones, 11 are of cyanobacterial origin (Zwart et al. 1998a, Zwart et al. 1998b). This procedure enables to directly compare the cyanobacterial community profiles with those of the total bacterial community and was optimized using Lake Loosdrecht water (Zwart et al. 2004).

DGGE gels were stained using ethidium bromide and gel images were analyzed and documented using the ImaGo imaging system (Isogen life science). Since this method is designed to compare the cyanobacterial and total bacterial communities, the latter DGGE pattern also contains bands corresponding to cyanobacteria. Because the same primers have been used to generate the final product for both DGGE patterns, cyanobacterial bands in the total bacterial DGGE pattern migrate to the same position as their corresponding bands in the cyanobacterial DGGE. Such cyanobacterial bands were subtracted from the total bacterial DGGE pattern and omitted from further analysis for comparing the communities of chemotrophic bacteria and cyanobacteria.

Viral Community Composition

The diversity of the total viral community based on differences in total genome length was obtained with pulsed field gel electrophoresis (PFGE) using a modified version of an existing protocol (Larsen et al. 2001). For PFGE analysis, 2 l of water was pre-filtered over a low-protein binding 0.45-µm filter (Durapore, Millipore, Billerica, MA, USA). The viral community was then concentrated to ca. 50 ml using

the VivaFlow 200 assembly (VivaFlow, Vivasciences, Hannover, Germany; 30.000 MWCO PES membrane). After adding 10% Tween 80 to separate viral particles from other particles in solution (1:1350 final concentration), the sample was centrifuged for 25 min at 15,500 x g at 4˚C to remove bacteria. The supernatant (45 ml) was stored at -80˚C until further processing. When thawed quickly at 35°C before further processing, storage at -80˚C does not result in a significant loss in viral particles. This primary concentrate was further concentrated to a volume of 1 - 5 ml using centrifugal ultrafiltration units (30,000 MWCO, centricon-plus-20, Millipore, Billerica, MA, USA). The final volume was doubled by adding SM buffer (0.1M NaCl, 10mM $MgS0_4$, 50mM Tris, 0.005% glycerol, pH 8) and 0.2 µm filtered Na-azide (0.1% final volume) for optimal storage and preservation, overnight at 4˚C in the dark. For a final round of concentration of the viral community the samples were ultra-centrifuged for 2 h at 135000 x g at 8˚C (Sorvall Discovery M120 SE; swinging bucket rotor S52-ST). After dissolving the virus pellet overnight at 4˚C in the dark in SM buffer, the virus concentration was determined as described above.

Table 2.1 Ranges of physical, chemical and biological parameters observed in surface water of Lake Loosdrecht in 2003.

Parameter		Range		
Mean depth	m	1.9	to	1.9
Secchi depth	m	0.4	to	0.6
Temperature	˚C	3	to	23
Chl-*a*	µg/l	44.0	to	88.8
O_2	mg/l	9.3	to	13.5
PO_4^{3-}	mg/l	<0.005	to	0.218
NO_3^-	mg/l	<0.1	to	0.24
NH_4^+	mg/l	<0.05	to	0.218
N:P ratio	w/w	19.5	to	41.3

Agarose plugs were prepared according to (Larsen et al. 2001), using InCert agarose (1.5 % final volume, FMC, Rockland, Maine) taking care to load 5 x 10^9 viruses per plug. The plugs were digested overnight at 30˚C in the dark with a lysis buffer (250 mM EDTA pH 8.0; 1% SDS; 1 mg/ml Proteinase K (Sigma-Aldrich, Zwijndrecht, the Netherlands). After repeated washings with TE buffer the plugs

were loaded onto and run on a 1.0 % (w/v) Seakem GTG agarose gel (FMC, Rockland, Maine, USA) prepared in 1 x TBE gel buffer (90 mM Tris-Borate and 1 mM EDTA, pH 8.0). Samples were electrophorised for 21 h at 14˚C; with pulse ramp from 1 to 6 s at 6V cm^{-1} using the Bio-Rad CHEF-DR II PFGE apparatus. As a molecular weight ladder, 5kb and λ concatamers (Bio-Rad, Richmond, CA, USA) were used. The used PFGE settings enabled optimal separation of marker and PFGE bands in the size regions of interest and allowed the detection of viruses with genome sizes between 10 and 200 kb. Gels were stained with Sybr Green I (Molecular Probes, Leiden, the Netherlands) for 1 h, de-stained for 30 min in distilled water and documented as is described for the DGGE gels.

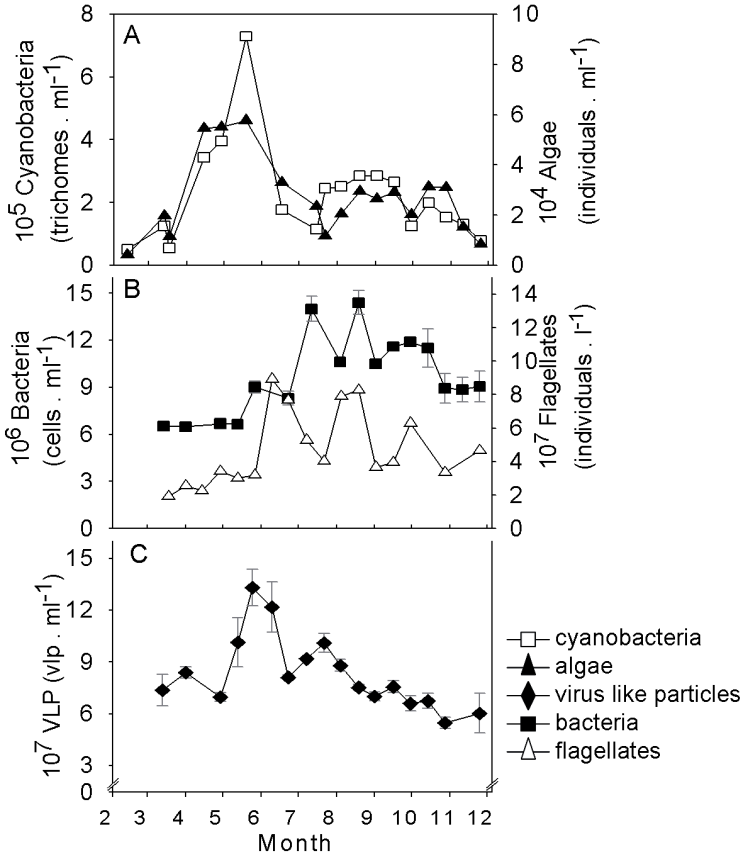


Figure 2.2 Algal, cyanobacterial, bacterial, nanoflagellate and viral abundances during the study period. (A) Flow cytometric counts of total cyanobacterial (including prochlorophytes) and algal communities. (B) Epifluorescence microscopic counts of chemotrophic bacterial community and light-microscopic counts of nanoflagellate community. (C) Epifluorescence microscopic counts of viral community. Error bars show standard deviation (n = 3).

Data Analysis

Correlations between different variables were determined using linear correlation (Pearson r) with Statistica (StatSoft Inc., Tulsa, OK, USA).

Both the DGGE and the PFGE gels were analyzed using Phoretix 5.00 (Nonlinear Dynamics, Ltd, United Kingdom). Background noise was subtracted using the rolling ball algorithm with a radius of 50 pixels and peaks smaller than 2% of the maximum peak were discarded. The band matrices obtained with Phoretix were further analyzed using Primer 5.2.0.9 (Clarke & Warwick 2001) to obtain similarity matrices of the different data sets using presence - absence analysis of bands. Similarity coefficients were calculated using the Bray-Curtis coefficient (Bray & Curtis 1957). Dendrograms were constructed based on these similarity matrices using hierarchical clustering with group-average linking. Dissimilarity matrices of untransformed environmental variables were obtained using Euclidean distances. Mantel tests, applying spearman rank's correlation coefficient ρ as the test statistic and 999 permutations, were used to test the null hypothesis of 'no relationship between matrices' in order to determine the presence of a seasonal cycle in the community profiles and if the similarity matrix of the viral community was related to the matrices of the chemotrophic bacterial and the cyanobacterial communities. To analyse the similarity matrices for the occurrence of a seasonal cycle, they were compared to a ranked matrix of sampling points in time representing a seasonal cycle in which adjacent months are most similar and samples 6 months apart least similar. To investigate which combination of environmental variables related best to the viral, cyanobacterial and chemotrophic bacterial community assemblages, the Bio-Env procedure from Clarke and Ainsworth was used (Clarke & Ainsworth 1993). The spearman rank correlations (ρ_w) between the Bray-Curtis dissimilarity matrix for the community composition and the Euclidean distance matrix for all possible different subsets of the environmental variables were determined, tabulating the highest coefficients at each level of complexity, which can be interpreted as the 'best explanatory environmental variables' of the community assemblage. Environmental variables tested were viral, bacterial, cyanobacterial, prochlorophyte, algal and flagellate abundances, as well as the abiotic factors pH, water temperature, and concentrations of oxygen, phosphate, ammonium, nitrate and bicarbonate.

Results

Abiotic Conditions

The environmental parameters given in Table 2.1 reflect the nature of Lake Loosdrecht as a highly eutrophic and turbid shallow lake (Carlson 1977). Concentrations of the potentially growth-limiting nutrients dissolved reactive phosphate (DRP), nitrate and ammonium were highest in March and below detection limits for a large part of the study period (Table 2.1). The pH and water temperature were both highest in summer, whereas Secchi-disk depth was highest in March and lowest in August.

Microbial and Viral Abundance

From March onwards algae and in particular cyanobacteria increased in numbers (Figure 2.2A). The plankton community was dominated by large filamentous cyanobacteria, with average concentrations of 2.2×10^5 cyanobacterial trichomes ml^{-1}, which corresponds to about 1×10^7 cyanobacterial cells ml^{-1}. Highest filamentous cyanobacterial abundances (7.3×10^5 trichomes ml^{-1}) were detected in May, after which cyanobacterial numbers showed a rapid 6.3 fold decrease in June. Relatively high cyanobacterial numbers re-occurred from the end of July until the onset of autumn. The eukaryotic algae followed this pattern, however at much lower abundances. Cyanobacterial numbers showed a strong positive correlation to algal numbers and a negative correlation with Secchi-disk depth (Table 2.2).

The chemotrophic bacterial community started to increase in late spring, when the cyanobacterial community was already peaking (Figure 2.2B). This bacterial community reached high abundances of 14.0 and 14.4×10^7 bacterial cells ml^{-1} in July and August, respectively, and showed a 2.3-fold fluctuation during the study period. Bacterial numbers were negatively correlated with viral richness (number of PFGE bands) and positively with flagellate abundances (Table 2.2). Nutrients were not significantly correlated to any of the other measured variables in Lake Loosdrecht (data not shown).

The viral community in Lake Loosdrecht showed a 2.5-fold fluctuation during the study period (Figure 2.2C). The most pronounced change in viral numbers was observed between April and May, when a 1.9 fold increase in VLP was observed up to a maximum number of 1.3×10^8 VLP ml^{-1}. Another marked increase in viral numbers was observed in July, when viral numbers reached 1.0×10^8 VLP

ml^{-1}. Viral numbers were observed to gradually decrease towards the end of the experimental period, with 6.0 x 10^7 VLP ml^{-1} observed in November. High viral numbers in May coincided with maximum cyanobacterial numbers, whereas the viral increase in July closely followed high bacterial numbers. Throughout the study period viral numbers were positively correlated to prochlorophytes and Chl-*a*. Viral numbers were negatively correlated to cyanobacterial richness (number of DGGE phylotypes) and Secchi-disk depth (Table 2.2).

Table 2.2 Pearson linear correlation coefficients and level of significance for different variables. *= p<0.05, **= p<0.01, ***= p<0.001.

Lake Loosdrecht 2003						
Variable	Viruses	Flagellates	Cyanobacteria	Prochlorophytes	Algae	Bacteria
Viruses		0,10	0,25	0,59*	0,16	-0,09
Flagellates	0,10		-0,25	0,29	-0,23	0,58*
Cyanobacteria	0,25	-0,25		0,61*	0,79***	-0,37
Prochlorophytes	0,59*	0,29	0,61*		0,22	0,18
Algae	0,16	-0,23	0,79***	0,22		-0,35
Bacteria	-0,09	0,58*	-0,37	0,18	-0,35	
Chl-a	0.52*	0.45	0.48	0.70**	0.36	0.11
Secchi	-0,58*	-0,40	-0,53*	-0,60*	-0,54*	-0,14
Temperature	0,48	0,67**	0,13	0,75**	0,00	0,40
Cyanobacterial richness	-0,73*	-0,28	-0,47	-0,61	-0,30	0,14
Bacterial richness	-0,24	-0,17	0,01	-0,20	0,31	-0,20
Viral richness	0,02	-0,25	0,64	0,21	0,38	-0,82*

The nanoflagellate community in Lake Loosdrecht showed large seasonal fluctuations, reaching high numbers of 8.94 x 10^7 l^{-1} in June after a 2.8 fold increase; 8.26 x 10^7 l^{-1} in August after a 2.1 fold increase and 6.3 x 10^7 l^{-1} at the end of September after a 1.6 fold increase (Figure 2.2B). High nanoflagellate numbers in June and July coincided with decreasing viral and bacterial numbers during these periods. Flagellate numbers were correlated with both bacterial abundances and water temperature (Table 2.2).

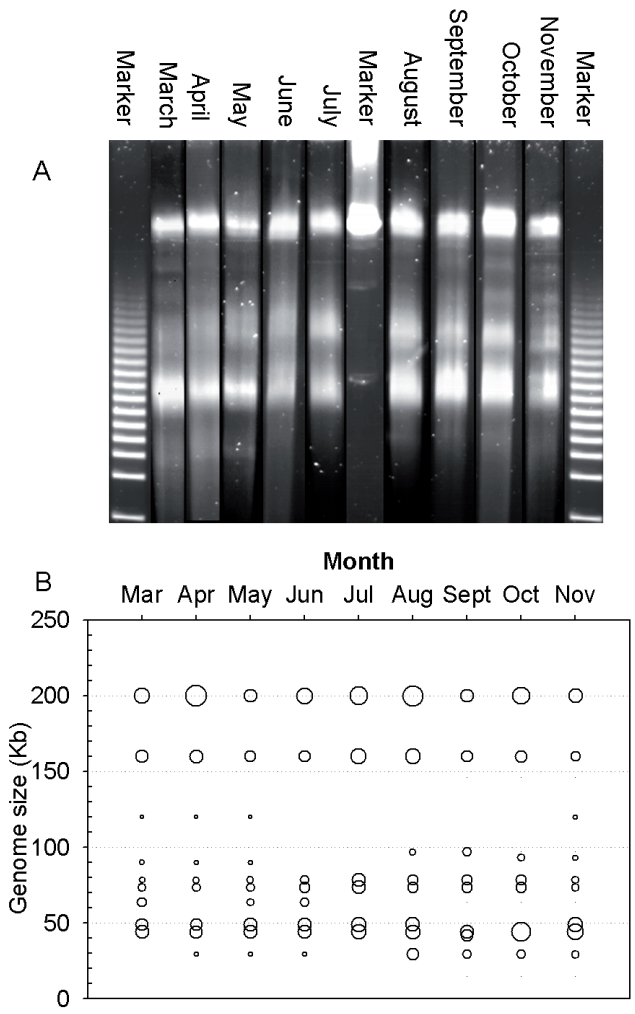


Figure 2.3 PFGE gel of viral population (A), and bubble plot representation of PFGE gel (B). Viral diversity was determined using samples pooled per month for the study period.. Day when samples were taken from experiment is indicated on x-axis. M = marker. Bubble position indicates position PFGE band and corresponding genome size, bubble size indicates relative band intensity. The DNA standards used in the PFGE were a 5 kb and a λ marker.

Viral and Microbial Community Composition

The viral diversity in Lake Loosdrecht was determined monthly based on differences in total viral genome size using PFGE. Difficulties were experienced when applying the PFGE technique on eutrophic lake water, probably due to high

concentrations of polymeric materials. This methodological problem resulted in low band resolution and high background staining of the PFGE gels (Figure 2.3). To improve the gel quality, cleaning procedures were tested as reported by Riemann and Middelboe (2002). These cleaning procedures improved the gel quality but also led to a high loss of viral numbers, especially in the larger viral size classes and were therefore chosen not to be applied in this study. Finally, it should be noted that the high background staining on the gels may have caused incomplete detection of small, faint bands and thus to an underestimation of viral diversity.

Throughout the experimental period a total of 13 different viral populations were observed with genome sizes varying between 30 and 200 kb (Figure 2.3). Genome sizes of 44, 49, 74, 78 and 163 kb were most abundant with average relative abundances of 17, 15, 12, 11 and 14 %, respectively. The high band intensities at 200 kb were due to a combination of genomes larger than 200 kb, which were not separated with the PFGE settings that we used. The number of observed viral genome sizes varied per month with highest virioplankton richness (10 genome sizes) during the peak in viral numbers in May *(Figure 2.4A). Viral richness decreased along with the cyanobacterial numbers, reaching a minimum of 6 viral genome sizes during the second viral peak in July. Viral genome sizes of > 200, 160, 78, and 44 kb varied in intensity but persisted throughout the study period.

Cluster analysis based on Bray-Curtis similarity indices of the monthly PFGE banding patterns puts the viral community in September on a separate branch, implying low similarity to the viral community of all other samples *(Figure 2.5A). This difference in viral community composition was mainly due to the absence of a PFGE band of 49 kb and the presence of bands in the 40 and 97-kb range (Figure 2.3). The successive months May and June clustered separately, indicating changes in viral community composition during this period. The separate clustering of spring, summer and autumn samples suggests a seasonal trend in viral community composition. Mantel tests indeed confirmed the presence of a significant annual cycle in viral community composition ($\rho = 0.542$, $p = 0.002$).

Table 2.3 Bio-Env procedure. Combinations of variables which give rise to the largest rank correlations (ρ_w) between the community assemblage and environmental variable dissimilarities, as the size of the subset of environmental variables (k) increases. Bold variables indicate the environmental variable(s), which best match the community dissimilarity matrix. Bact: bacterial abundances; Temp: water temperature; Flag: nanoflagellate abundances.

Spearman rank correlation	
Viral community structure **(PFGE)**	
k	Best variable combinations (ρ_w)
1	**Bact** (**.37**) pH (.28) Temp (.12) NH_4 (.11) ...
2	Bact, pH (.35) Bact, Temp (.29) Bact, HCO_3 (.29) pH, NH_4 (.27) …
3	Bact, Temp, pH (.34) Bact, pH, NH_4 (.30) Bact, pH, NO_3 (.30) …
4	Bact, pH, NO_3, NH_4 (.34) Bact, Temp, pH, NO_3 (.30) …
Chemotrophic bacterial community structure **(DGGE)**	
1	VLP (.37) Algae (.22) Flag (.21) Cyano (.10) …
2	VLP, Algae (.445) VLP, Flag (.41) VLP, O_2 (.38) VLP, NH_4 (.34) …
3	**VLP, Flag, Algae** (**.448**) VLP, Algae, NH_4 (.43) VLP, Algae, NO_3 (.43) ...
4	VLP, Algae, NO_3, NH_4 (.42) VLP, Flag, Algae, O_2 (.39) …
Cyanobacterial community structure **(DGGE)**	
1	Algae (.59) VLP (.27) Flag (.23) Bact (.13) …
2	VLP, Algae (.61) Flag, Algae (.56) Algae, Bact (.55) VLP, Bact (.47) ...
3	**VLP, Algae, Bact** (**.70**) VLP, Flag, Algae (.59) Flag, Algae, Bact (.57) …
4	VLP, Flag, Algae, Bact (.66) VLP, Algae, Bact, PH (.53) …

A Spearman rank correlation was performed to test which (combination of) environmental variables correlated best with the viral community assemblages observed in Lake Loosdrecht throughout the experimental period (Table 2.3). The environmental variable which best grouped the months, in a manner consistent with the viral community pattern, was bacterial abundance (ρ = 0.37); next best was the pH value. A Mantel test indeed confirmed that the overall pattern in bacterial abundances and viral community composition were significantly correlated (p = 0.024). Increasing the subset of environmental variables (k) did not raise the matching coefficient (ρ; Table 2.3).

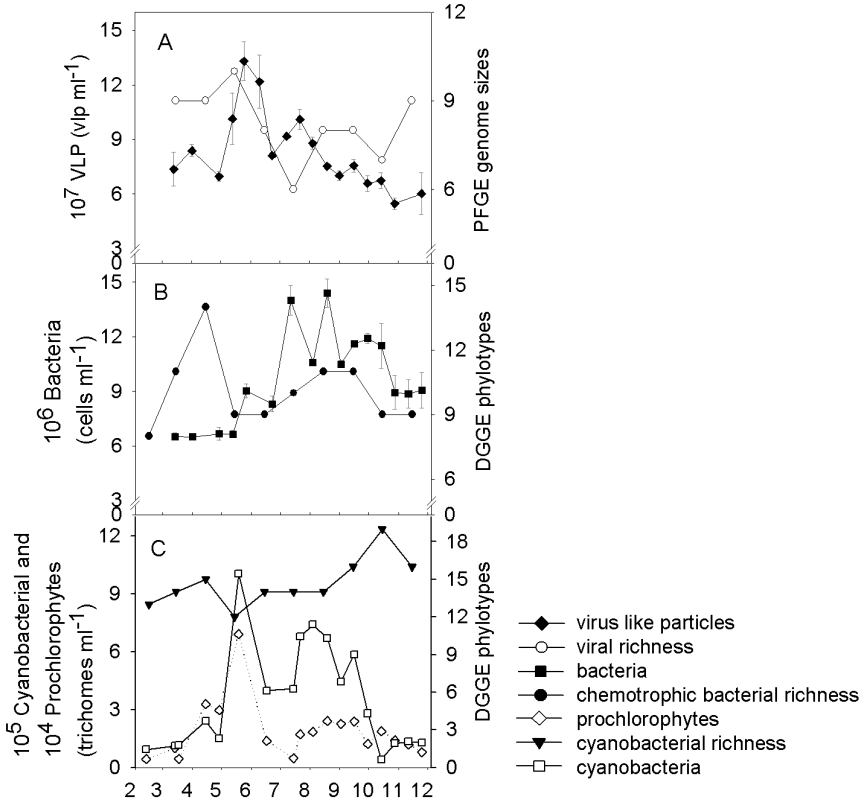


Figure 2.4 Viral, bacterial and cyanobacterial abundances and richness over the study period. (A) Number of viral genome sizes per month observed with PFGE and viral abundances. (B) Number of chemotrophic bacterial DGGE phylotypes observed per month and bacterial abundances. (C) Number of cyanobacterial DGGE phylotypes observed per month and cyanobacterial abundances. The flow cytometric cyanobacterial counts are split up in the phycocyanin-containing filamentous cyanobacterial species (cyanobacterial) and filamentous cyanobacteria not containing phycocyanin (prochlorophytes). Error bars show standard deviation (n = 3).

Analysis of the chemotrophic bacterial community composition revealed a diverse bacterial community with a total of 18 different DGGE phylotypes observed throughout the experimental period. The highest bacterial richness of 14 DGGE phylotypes was observed in April, when the bacterial abundance was still low (Figure 2.4B). Analysis of the monthly DGGE profiles revealed separate clustering of the late spring to early summer samples from the rest of the year, indicating that changes in the bacterial community structure occurred in both May and August (Figure 2.5B). A Mantel test confirmed a significant seasonal cycle in the bacterial community profile (ρ = 0.483, p = 0.006). Virus like particle abundance was the variable which, on its own, best grouped the months in a manner consistent with the chemotrophic community composition (ρ = 0.37; Table 2.3); second best was algal abundance (ρ = 0.22). The subset of VLP, flagellate and algal abundances correlated best with chemotrophic bacterial community structure (ρ = 0.448). A Mantel test indicated that patterns in chemotrophic community composition were significantly correlated to VLP abundance (p = 0.028) but not to changes in viral community composition (ρ = 0.122; p = 0.25).

The cyanobacterial community structure in Lake Loosdrecht was very diverse with in total 25 different DGGE phylotypes (Figure 2.6). The highest cyanobacterial richness was recorded in October when cyanobacterial abundances decreased (Figure 2.4C). At the time of the cyanobacterial peak in May the cyanobacterial richness was observed to be at its minimum, with 12 different phylotypes. Analysis of the monthly cyanobacterial DGGE profiles revealed that the cyanobacterial community in April and May differed from communities in the previous and subsequent months (Figure 2.5C). Changes in cyanobacterial community composition were observed from June to July as well. A significant annual cycle could also be detected in the cyanobacterial community pattern (Mantel test; ρ = 0.32, p = 0.04). Of all the environmental variables tested, the pattern in algal abundances provided the best match to cyanobacterial community structure (ρ = 0.59; Table 2.3); VLP abundances provided the second best match (ρ = 0.27). The subset of VLP, algal and bacterial abundances 'explain' most of the pattern in cyanobacterial community composition (ρ = 0.7). No significant relation could be detected between the cyanobacterial community profile and VLP abundances (p = 0.078) or VLP community profile (ρ = 0.01, p = 0.44).

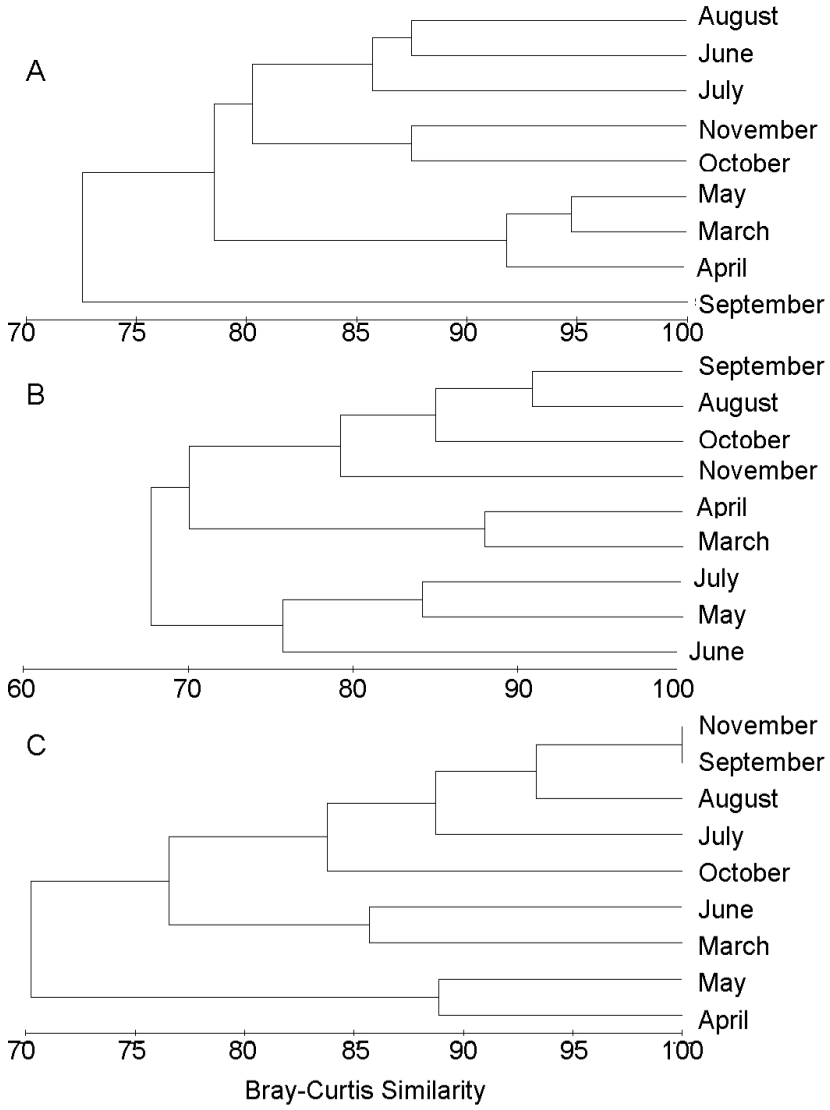


Figure 2.5 Similarity analysis of the viral, bacterial and cyanobacterial community profiles. All dendrograms were constructed using group-average linking. Bray-Curtis similarity matrices were obtained based on presence – absence analysis of bands. (A) Dendrogram showing degree of similarity between viral community profiles of the different months based on PFGE analysis. (B) Dendrogram showing degree of similarity between chemotrophic bacterial community profiles of the different months based on DGGE analysis. (C) Dendrogram showing degree of similarity between cyanobacterial community profiles of the different months based on DGGE analysis.

Discussion

Due to the selective and parasitic nature of viral infection, it is expected that viral and host community dynamics will co-vary, both in abundances and community composition (Fuhrman 1999). We aimed to study the temporal dynamics of the total viral community in relation to environmental parameters and in particular to bacterial and cyanobacterial community dynamics, as potential viral hosts in Lake Loosdrecht.

The viral community in Lake Loosdrecht showed marked temporal changes in abundance and composition, indicating viruses to be a dynamic component of the aquatic community. By applying the Sybr Green I dye, which has a high sensitivity for dsDNA, we especially observed dynamics of dsDNA viruses during the present study. Viral abundances observed during spring and summer were significantly higher compared to the rest of the year (student t-test, $p = 0.01$), indicating that viral abundances followed a seasonal pattern. Especially between May and August strong fluctuations in viral abundances could be observed. The most pronounced increase in viral numbers in May coincided with a peak in filamentous cyanobacterial abundances, and the viral increase in July closely followed high bacterial numbers, suggesting a tight interaction between the viral and both the cyanobacterial and bacterial communities during this period. In spite of this, no significant correlation could be detected when comparing the temporal fluctuations in viral abundances with fluctuations in bacterial and dominant filamentous cyanobacterial abundances observed throughout the experimental period.

Viral abundances did show a significant positive correlation to Chl-*a* concentrations, suggesting that a significant proportion of the viruses in Lake Loosdrecht may be phytoplankton viruses. A significant correlation between viral abundances and Chl-*a* has previously been reported for a variety of freshwater environments (Taylor et al. 2003, Vrede et al. 2003, Madan et al. 2005), including eutrophic shallow lakes (Liu et al. 2006). Viral numbers also showed a significant correlation to the second most abundant group of filamentous cyanobacteria present in Lake Loosdrecht, the filamentous prochlorophytes (Dignum 2003, Pel et al. 2004). Prochlorophytes are filamentous cyanobacteria lacking phycobilins, but possessing Chl -*b* as accessory pigment (Turner et al. 1989). Viruses have been isolated and identified for the marine prochlorophyte *Prochlorococcus* (Sullivan

et al. 2003), but for freshwater environments no viruses of prochlorophytes have been reported, so far. During the present study, prochlorophytes reached maximum numbers in May, which was followed by a rapid decrease in June, as observed for the phycobilin-containing cyanobacteria (Figure 2.4C). The prochlorophyte community in Lake Loosdrecht is mainly composed of *Prochlorothrix hollandica*, but recently the presence of a taxon closely related to *P. hollandica* has been described by Zwart et al. (2004). It is unknown if this taxon is included in our flow cytometric prochlorophyte counts, since it does not have a cultivated representative, and we do not yet know if its pigmentation and shape resembles *P. hollandica*.

High nanoflagellate numbers in June and July coincided with decreasing viral numbers in these periods, suggesting that flagellate grazing might have played a role in the observed viral decay. Previous research has shown that grazing of viral particles by nanoflagellates could be a source of viral decay in both marine and freshwater environments (Gonzalez & Suttle 1993, Bettarel et al. 2005). Besides the above association, no significant correlation between viral and nanoflagellate abundances could be detected during the present study however. Nanoflagellate abundances did reflect changes in bacterial abundances, suggesting bacteria as probable food source for the nanoflagellate community in Lake Loosdrecht.

Microbial Community Composition

To describe the bacterial and cyanobacterial diversity in Lake Loosdrecht we applied a PCR-DGGE protocol that has been thoroughly tested and described for Lake Loosdrecht water (Zwart et al. 1998a, Zwart et al. 1998b, Zwart et al. 2004). To enable the comparison between the chemotrophic bacterial community and the cyanobacterial community we omitted bands corresponding to cyanobacterial band positions in the total bacterial DGGE pattern from further analysis. This procedure could have led to an underestimation of the chemotrophic bacterial diversity since chemotrophic bacterial and cyanobacterial bands may migrate to the same position. This situation was not encountered however, when testing the protocol and sequencing a subsample of excised DGGE bands (Zwart et al. 2004).

Changes in chemotrophic bacterial community composition followed a striking seasonal pattern. Seasonality in freshwater bacterial assemblages has also been reported for the eutrophic Lake Pluβsee (Hofle et al. 1999) and two

hypereutrophic lakes in Belgium (Van der Gucht et al. 2001). Two marked shifts in chemotrophic bacterial community composition were apparent in Lake Loosdrecht, the first shift was mainly due to a decrease in chemotrophic bacterial richness during the cyanobacterial peak in May. The second shift occurred between July and August, implying that the two bacterial peaks observed during these months differed in community composition. It might very well be that these shifts in community assemblage were due to changes in the phytoplankton community, since Van Hannen and co-workers (Van Hannen et al. 1999a, Van Hannen et al. 1999b) demonstrated that the bacterial community structure in Lake Loosdrecht is tightly linked to phytoplankton community composition. Our data indeed indicates algal and cyanobacterial abundances among the four environmental variables which, on their own, best relate to the chemotrophic bacterial community assemblage, as well as viral and flagellate abundances. Viral abundance was the only variable that significantly related to chemotrophic bacterial community composition, however, suggesting an interaction between bacterial and virioplankton communities. It could thus also be that the observed correlation between Chl-*a* and VLP was actually due to an interaction between viruses and chemotrophic bacteria which are associated with phytoplankton. No significant correlation between viral and bacterial abundances could be detected during the present study, though.

Cyanobacterial richness was higher than the richness observed for the chemotrophic bacterial community, which was probably due to the higher overall cyanobacterial abundances (up to 3.3×10^7 cyanobacterial cells ml^{-1}) and to the difference in resolution of the two applied DGGE protocols, since the cyanobacterial DGGE protocol was specifically designed to detect cyanobacteria. The cyanobacterial community followed an annual cycle, with the largest shifts in community composition occurring during spring and early summer. The low number of DGGE phylotypes and the joint clustering of April and May indicate that the cyanobacterial peak in May was probably due to the numerical increase of cyanobacterial phylotypes that were already present in April. Analysis of the DGGE gel confirmed that in May no new DGGE phylotypes appeared, while 3 phylotypes disappeared and 7 phylotypes increased in band intensity as compared to the pattern in April. After the cyanobacterial decline in May a marked shift in cyanobacterial community assemblage occurred, with several new DGGE phylotypes appearing. Both flow cytometric counts and

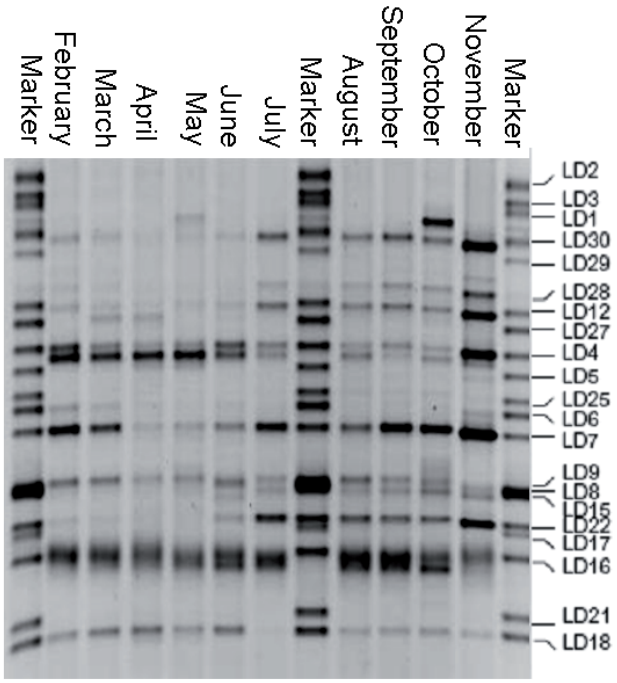
DGGE analysis showed that several of these newly appearing phylotypes can be attributed to prochlorophytes.

Zwart et al. (2004) already demonstrated that the dominant, seemingly uniform filamentous cyanobacterial community in Lake Loosdrecht is actually a diverse assemblage of different cyanobacteria, composed of at least 5 different taxa belonging to the *Limnothrix* / *Pseudoanabaena* group. It might be that viral infection enables the stable co-existence of this assemblage of different cyanobacteria, in accordance with the 'killing the winner' theory. This theory predicts that viral infection can control the abundance of the competitive dominant, thereby enabling the stable co-existence of less competitive species and enhancing microbial diversity (Thingstad & Lignell 1997, Thingstad 2000). Viral abundances were indeed found to be among the environmental variables which best explained the cyanobacterial community assemblage, but no significant correlation between viral and cyanobacterial community composition could be detected. In addition, the viral community did not seem to enhance microbial diversity during the present study, since viral abundance was negatively correlated to bacterial and especially cyanobacterial richness. Bouvier and Del Giorgio (Bouvier & del Giorgio 2007) recently provided evidence that viral regulation may in fact decrease the overall prokaryotic diversity, by maintaining competitively strong phylogenetic groups at such low densities, that they are below the detection threshold.

Viral Community Composition

PFGE is now a commonly applied method in aquatic ecology for analysis of total viral community diversity based on differences in total genome size (Wommack et al. 1999, Steward et al. 2000, Larsen et al. 2001, Riemann & Middelboe 2002). This study is one of the first applying PFGE to describe viral diversity in a freshwater setting (Auguet et al. 2006). With the PFGE settings used in this study we were able to detect viral genome sizes between 30 and 200 kb, the size region in which most bacteriophages can be found (Ackermann & DuBow 1987). We therefore did not detect the large genome size viruses which are often attributed to algal viruses (Larsen et al. 2001, Larsen et al. 2004). PFGE studies to date report most viral DNA to be present in the smaller genome size range between 30 and 70 kb (Steward et al. 2000, Wommack & Colwell 2000, Larsen et al. 2001, Auguet et al. 2006). Also in the present study most viral

genome sizes clustered within this range, around 45 kb. A second cluster of genome sizes was observed around 75 kb, thus somewhat larger than the 70 kb cluster reported by Larsen and colleagues [22].



Clone	Cyanobacterium
LD4	*Limnothrix* sp.
LD5	*Prochlorothrix* sp.
LD25	*Prochlorothrix* sp.
LD6	*Limnothrix* sp
LD7	*Prochlorothrix* sp.
LD9	*Synechococcus* sp.
LD8	*Aphinozomenon* sp.
LD15	*Prochlorothrix* sp.
LD22	*Prochlorothrix* sp.
LD16	*Prochlorothrix* sp.
LD18	*Planktothrix* sp.

Figure 2.6 Denaturing gradient gel electrophoresis (DGGE) gel showing cyanobacterial community profiles over the study period. M = marker composed of cyanobacterial clone library from lake Loosdrecht. Labels indicate the corresponding clone label of the marker band. Clone-labels of marker bands corresponding to cyanobacteria are identified.

A substantial 39 % of the detected viral genome sizes persisted throughout the experiment, only varying in viral numbers per genome size (detected as relative PFGE band intensity) between the different months. Stability of virioplankton genome groups has also been reported for a range of marine environments (Steward et al. 2000, Riemann & Middelboe 2002) and in the Charente Estuary (Auguet et al. 2006), when applying PFGE. It might very well be that the actual viral community is more dynamic than detected with PFGE, since different viruses can have the same genome size and would therefore not be detected as different using PFGE. Even though an underestimation of viral richness due to low quality PFGE gels may have occurred in this study, between 6 to 10

different PFGE bands could be observed per sample, which is in the range of other studies (Steward et al. 2000, Larsen et al. 2004) and higher than the viral richness observed by PFGE in the Charente river (Auguet et al. 2006). There is still room for further improvement of the applied PFGE technique on highly eutrophic lake water, however.

Changes in viral community composition followed a clear seasonal pattern, reflecting the seasonality detected in bacterial and cyanobacterial community composition. Viruses are generally believed to follow the seasonal distribution of their host organisms, due to their host specificity (Wommack & Colwell 2000). Seasonal changes in viral community composition have previously been demonstrated in both freshwater and marine studies (Wommack et al. 1999, Dorigo et al. 2004, Muhling et al. 2005, Auguet et al. 2006).

The viral peak detected in May differed in community composition from the viral peak detected in July. This suggests that the viral community in May consisted for a considerable part of viruses infecting different hosts than the viral community present in July. This shift in viral community assemblage between May and June was accompanied by marked changes in viral richness and abundances, and co-occurred with shifts in bacterial and especially cyanobacterial community composition. Strong fluctuations in viral abundance and community composition in spring and summer were thus in accordance with changes observed in bacterial and cyanobacterial communities, suggesting a tight linkage between viral and microbial community dynamics during that period of time. Shifts in viral community composition were not always correlated to shifts in the microbial community, however. The separate clustering of the viral community in September compared to the other months was not accompanied by marked changes in bacterial or cyanobacterial community composition. Neither was it reflected by strong shifts in viral richness or abundances. When comparing temporal changes in viral community composition with changes in cyanobacterial and bacterial community assemblages over the entire experimental period, no significant relationship could be detected. We could thus only detect a tight linkage between viral and microbial community composition during periods of strong fluctuations in the bacterial and cyanobacterial community. Most virus-host dynamics in natural aquatic environments have so far been demonstrated during extreme situations, such as during the collapse of phytoplankton blooms (Tarutani et al. 2000, Castberg et al. 2001, Larsen et al. 2001).

Bacterial abundance was the only environmental variable which was significantly correlated with the temporal variation in viral community assemblage during the present study, suggesting a tight interaction between viruses and bacteria in Lake Loosdrecht. The tested environmental variables could only explain a small part of the fluctuations in viral community composition (best variable combination: $\rho = 0.37$), however. It could of course be that the majority of the viral community in Lake Loosdrecht infects non bacterial and cyanobacterial hosts and is not affected by the environmental variables measured in this study. Since cyanobacteria and bacteria are highly abundant in Lake Loosdrecht, and the chance of viral propagation is linearly dependent on the density of susceptible hosts (Murray & Jackson 1992, Hennes et al. 1995), this would be unlikely however. An alternative explanation would be that viral infection is not as host specific as always assumed. The extent of host specificity of viral infection is indeed under discussion. Some viruses have been reported to exhibit a wide range of hosts (Mann 2003). For example, phages of *Synechococcus* can infect up to 10 different strains (Waterbury & Valois 1993). Also, some cyanomyoviridae have been observed by Sullivan et al. (2003) to cross-infect hosts of different cyanobacterial genera.

A probable explanation for the lack in correlation between viral and microbial community composition would be that we were not able to detect the more subtle virus – host interactions with the applied technique to detect viral diversity, and more specific techniques with a higher resolution might be necessary to reveal virus-host interactions in natural assemblages. Applying a cyanophage specific DGGE protocol; Mühling et al. (Muhling et al. 2005) demonstrated a significant relationship between cyanophage and *Synechococcus* community composition over an annual cycle in the Red Sea.

In conclusion, the virioplankton was found to be a dynamic component of the aquatic community, with changes in viral community composition following a distinct seasonal pattern. Nutrients related very poorly to temporal changes in viral, bacterial and cyanobacterial community abundances and composition. Contrary to expectations viral abundances were not correlated to abundances of the most dominant plankton groups in Lake Loosdrecht, the bacteria and the filamentous cyanobacteria. In addition, we could only detect a linkage between the composition of viral and both bacterial and cyanobacterial community during periods of strong fluctuations in plankton abundances, whereas during

the overall period no such correlation could be detected. Viral abundances did however relate well to cyanobacterial community assemblage and showed a significant positive correlation to Chl-*a* and prochlorophytes, suggesting that a significant proportion of the viruses in Lake Loosdrecht may be phytoplankton and more specific cyanobacterial viruses. A significant correlation between bacterial abundances and viral community assemblage, and *vice versa*, also indicates bacteria as potential viral hosts in the lake.

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Chapter 3

Impact of a mass viral lysis event on the microbial community composition in eutrophic lake water

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Summary

Viral lysis is not only a significant mortality source of aquatic microorganisms; it is also thought to have a strong impact on aquatic community composition and diversity. The extent to which viral infections influence the aquatic food-web and community composition is still not quite clear, however. A series of enclosure experiments with eutrophic lake water was used to study the impact of mass viral lysis of the cyanobacterial population on the composition of the aquatic microbial community. Population dynamics of the viral, chemotrophic bacterial, cyanobacterial and algal communities were assessed by means of epifluorescence microscopy (EM), transmission electron microscopy (TEM), flow cytometry (FCM) and light microscopy (LM) before, during and after the collapse of the dominant filamentous cyanobacterial population. The molecular diversity of the viral, and of the chemotrophic bacterial and cyanobacterial community was investigated using pulsed field gel electrophoresis (PFGE) and denaturing gradient gel electrophoresis (DGGE), respectively. A dramatic collapse of the cyanobacterial community was observed in all enclosures. High percentages (between 24 and 43%) of virally-infected filamentous cyanobacterial cells and increasing viral numbers during the collapse indicated that viruses were the major cause of mortality. The lysis of the cyanobacterial bloom not only led to a reduction in cyanobacterial biomass, but was also accompanied by a change in

community composition of both cyanobacteria and chemotrophic bacteria. In addition, green algae increased after the cyanobacterial collapse and became the predominant phytoplankton group in one of the enclosures. These results imply that viral infection can control cyanobacterial blooms and has a marked impact on the structure of plankton communities in eutrophic lakes.

Introduction

Viruses are the most abundant biological entities in aquatic environments. They are recognized as an important cause of mortality of both bacteria and algae (Bergh et al. 1989, Proctor & Fuhrman 1990). An important ecological question, therefore, is the influence of viral infection on the aquatic foodweb and community structure (Weinbauer & Rassoulzadegan 2004, Winter et al. 2005, Bouvier & del Giorgio 2007). Since high host abundances facilitate viral spread and thus the chance of viral infection, it is thought that viruses can regulate the abundances of their host (Murray & Jackson 1992, Hennes et al. 1995). Viral infection has indeed been reported to be responsible for the collapse of complete algal blooms (Bratbak et al. 1993, Nagasaki et al. 1994, Tomaru et al. 2004, Brussaard et al. 2005a).

In addition, viral infection is considered to influence microbial community diversity and composition due to its host-specific nature (Fuhrman & Suttle 1993). The 'killing the winner' theory postulates that viruses can control the abundance of the competitive dominant species. The increased chance of encounter between this numerically dominant species and its specific virus will lead to an increased probability of viral infection, thereby resulting in a down-regulation of the abundance of this competitive dominant. Viral infection is thus predicted to facilitate the co-existence of less competitive species and to enhance microbial diversity (Thingstad & Lignell 1997, Thingstad 2000).

Indirectly viruses are thought to impact aquatic communities through the release of cellular debris upon lysis of the host cell, thereby stimulating the growth of heterotrophic bacteria (Bratbak et al. 1992, Gobler et al. 1997, Noble & Fuhrman 1999, Middelboe & Lyck 2002, Poorvin et al. 2004). This release of organic compounds due to lysis not only leads to the recycling of nutrients within the microbial loop but is also considered to influence heterotrophic

bacterial community composition (Riemann & Middelboe 2002b, Brussaard et al. 2005b). Viruses are, therefore, believed to strongly affect aquatic environments. The extent to which viral infections influence the food-web and community composition is especially in freshwater environments yet clear, however.

We studied the impact of a mass viral lysis event on the aquatic community in a series of enclosures containing water from the shallow and eutrophic Lake Loosdrecht (the Netherlands). The lake is predominated by a group of closely related filamentous cyanobacteria belonging to the *Limnothrix* / *Pseudoanabaena* group (Van Tongeren et al. 1992, Zwart et al. 2005). During previous enclosure studies with water from Lake Loosdrecht, filamentous cyanobacterial blooms showed a dramatic collapse, which was attributed to viral lysis (Van Hannen et al. 1999, Gons et al. 2002). The present study aimed to give more insight into these cyanobacterial mass mortality events, substantiating viruses as the main mortality cause during the collapse and assessing the community dynamics and composition of the viral, chemotrophic bacterial as well as the cyanobacterial populations before, during and after the cyanobacterial collapse. Based on these results, we discuss the extent to which viral lysis influences planktonic communities in eutrophic lakes.

Material and Methods

Experimental design

The laboratory-scale enclosures (LSE) used in this study were especially designed to mimic the physical environment of Lake Loosdrecht (52° 11.7'N, 5° 3.1'E), the Netherlands (Rijkeboer et al. 1990). One enclosure comprises a 2-m long perspex cylinder with a volume of 130 L. The experiments were run at a 16:8 h light-dark cycle, using 360 µmol m^{-2} s^{-1} of photosynthetically active radiation (PAR). The synthetic medium used in the LSE for phytoplankton contained nitrogen and phosphate as macronutrients (158.4 µmol $NaNO_3$ and 3.3 µmol K_2HPO_4 per litre LSE volume were added daily to the LSE) but no silica and carbon were added. The experimental conditions were same as used by Simis et al. (2005): temperature, 20°C; pH < 9.5; and O_2 saturation < 120%.

Two experiments were conducted, i.e. one each in November 2002 and March 2003, the last one was performed in triplicate. Surface water from eutrophic

Lake Loosdrecht was used to fill the enclosures (Table 3.1). Lake Loosdrecht is highly eutrophic (annual average chlorophyll *a* (Chl-*a*) approximately 60 mg m^{-3}), shallow (mean depth, 1.9 m), peaty, and turbid (Secchi-disc transparency of approximately 0.5 m) lake (Van Liere & Gulati 1992). Samples were taken from the enclosures every 2-5 days and up to twice daily during the cyanobacterial lysis event, and processed or fixed directly.

Table 3.1. Experimental and starting conditions in Lake Loosdrecht for the enclosure experiments. POM = particulate organic matter.

Experiment (LSE)	Date	Number of enclosures	Duration (days)	Lysis period (day)	Starting conditions			
					T (°C)	pH	Chl *a* (mg m^{-3})	POM (g m^{-3})
November	06-11-2002	1	44	12 - 18	9.5	8.3	86	14.6
March	24-03-2003	3	18	9 - 13	9.5	8.3	50	13.2

Phytoplankton and chemotrophic bacteria enumeration

Different cyanobacterial groups and eukaryotic algae were distinguished using an Epics Elite flow cytometer (Coulter corp. Miami, USA) and the protocol as described by Pel et al.(Pel et al. 2003). Typically, cell clusters of filamentous cyanobacteria, prochlorophytes (Turner et al. 1989), and eukaryotic algae could be distinguished (Figure 3.1). The classification of these clusters was confirmed by light microscopy examination and fatty acids analysis after cell sorting (Pel et al. 2004).

Samples for flow cytometric bacterial counts were fixed with 1% formalin, frozen in liquid nitrogen and stored at -80°C until analysis. The samples for bacterial counts were first briefly sonicated to remove clumps without loss of bacterial cells, before they were 5-10X diluted in 1% PBS solution with 0.05% Tween 80. They were subsequently incubated for 2 hrs at room temperature after which PicoGreen (Molecular Probes, Europe) was added at 0.1% final volume. After an hour of incubation, bacteria were detected at 525 nm (FWHM 20 nm) using side scatter to trigger count events.

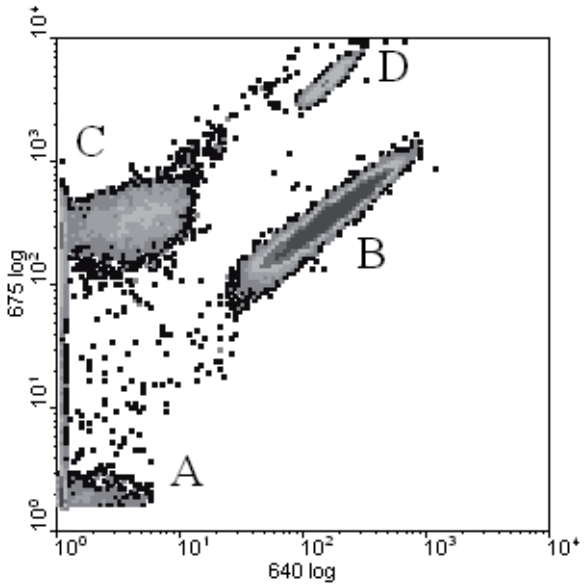


Figure 3.1 Phytoplankton clusters in typical Lake Loosdrecht sample as detected by flow cytometry. (A) bacteria / detritus cluster (B) cyanobacterial cluster (C) prochlorophyte cluster (D) algal cluster. A different flow cytometry protocol was used to obtain bacterial numbers.

Virus Enumeration

For the counting of viruses the samples were fixed with 0.02-µm filtered formalin (1% final volume), frozen in liquid nitrogen and stored at -80˚C until analysis. Virus counts were obtained with a Zeiss, Axiophot epifluorescence microscope, following staining of samples with SYBR Green I (Molecular Probes, Europe), as described by Noble and Fuhrman (1998).

Transmission electron microscopy

During the cyanobacterial collapse in the November experiment (day 14) a sample was taken to determine the frequency of infected cyanobacterial cells. The sample was fixed with 2% glutaraldehyde and stored at 4°C for preparing thin sections for TEM using a modification of the protocol described by Jacquet et al. (2002) to determine the frequency of visibly infected cyanobacterial cells (FVIC). The cyanobacterial cells were harvested in a swing-out rotor at 800 g for 12 min, mixed with a drop of 2% alginate solution and concentrated by centrifugation for 10 min. at 1000 g. After centrifugation, one ml 0.1M $CaCL_2$ was added and the sample was stored overnight for gelification at 4°C. The

alginate embedded cells were washed with 0.1M cacodylate buffer overnight at 4°C and post-fixed for 1.5 hrs with 2% OsO_4. The post-fixed cells were washed 3 times with 0.1M cacodylate buffer. Cells were dehydrated using three, 30 min incubations in ethanol with increasing strength (70, 85 and 96%). Cells were incubated in LR white resin (Agar scientific) overnight at 4°C after which the LR white resin was refreshed and polymerized for 6 hrs at 50°C.

Thin sections were prepared using an ultramicrotome with a Diatome diamond knife. These sections were placed onto 200 mesh Cu-grids (thin bar grids, Agar Scientific, Essex, England) supported with carbon-coated formvar film, and stained with 2% uranyl acetate and Reynolds lead citrate. They were then examined using TEM (Jeol 100s and Philips EM400T) at 10,000 – 50,000 magnification. Since mature viral particles are only visible at the end of the latent period, we used conversion factors from literature to calculate the actual frequency of infected cyanobacterial cells (FIC):

FIC = 4 * FVIC (Suttle 2000)

FIC = 7.11 * FVIC (Weinbauer et al. 2002)

FIC = 7.1 * FVIC – 22.5 $FVIC^2$ (Binder 1999)

Additionally, viral morphology was determined on some samples prepared for TEM (Bratbak & Heldal, (1993). Cells were harvested onto 400 mesh Ni-grids with carbon-coated formvar film at 35,000 rpm for 30 min at 20°C in a L8-70M ultracentrifuge (Beckman SW 41). The grids were stained with 2% uranylacetate and samples were examined using a Jeol 100S TEM at a magnification of 10,000 to 50,000. Viral capsid sizes were determined using an image analysis program (Scion Image).

Bacterial and Cyanobacterial Diversity

The diversity of bacterial and cyanobacterial communities was obtained by denaturing gradient gel electrophoresis (DGGE) of PCR-amplified general bacterial as well as cyanobacterial small subunit ribosomal RNA (SSU rRNA) using the exact protocol as described in (Zwart et al. 1998a, Zwart et al. 2005). We amplified the cyanobacterial SSU rDNA sequences using first a nested procedure with primers Cya-b-F371 and Cya-R783. After this cyanobacteria-selective pre-amplification, a second amplification procedure was performed employing the general bacterial primers F357GC and R518.

The product of both the bacteria and cyanobacteria-specific PCR procedure is

a GC clamp containing PCR product of approximately 200-bp long. This product was subjected to DGGE using a clone ladder composed of 23 previously sequenced and described Lake Loosdrecht clones. Of these clones, 11 are of cyanobacterial origin (Zwart et al. 1998a, Zwart et al. 1998b). This procedure enables to directly compare the cyanobacterial community profiles with those of the total bacterial community and was optimized using Lake Loosdrecht water (Zwart et al. 2005).

DGGE gels were stained using ethidium bromide and gel images were analyzed and documented using the ImaGo imaging system (Isogen life science). Since this method is designed to compare the cyanobacterial and total bacterial communities, the latter DGGE pattern also contains bands corresponding to cyanobacteria. Because the same primers have been used to generate the final product for both DGGE patterns, cyanobacterial bands in the total bacterial DGGE pattern migrate to the same position as their corresponding bands in the cyanobacterial DGGE. Such cyanobacterial bands were subtracted from the total bacterial DGGE pattern and omitted from further analysis for comparing the communities of chemotrophic bacteria and cyanobacteria.

Viral Diversity

The diversity of the total viral community based on differences in total genome length was obtained with pulsed field gel electrophoresis (PFGE) using a modified version of an existing protocol (Larsen et al. 2001). For PFGE analysis, 2 l of water was pre-filtered over a low-protein binding 0.45-µm filter (Durapore, Millipore, Billerica, MA, USA). The viral community was then concentrated to ca. 50 ml using the VivaFlow 200 assembly (VivaFlow, Vivasciences, Hannover, Germany; 30.000 MWCO PES membrane). After adding 10% Tween 80 (1:1350 final concentration) the sample was centrifuged for 25 min at 15,500 x g at 4˚C to remove bacteria. The supernatant was stored at -80˚C until further processing. This primary concentrate was further concentrated to a volume of 1 - 5 ml using centrifugal ultrafiltration units (30,000 MWCO, centricon-plus-20, Millipore, Billerica, MA, USA). The final volume was doubled by adding SM buffer (0.1M NaCl, 10mM $MgS0_4$, 50mM Tris, 0.005% glycerol, pH 8). It was preserved with 0.2 µm filtered Na-azide (0.1% final volume) and stored overnight at 4˚C in the dark. For a final round of concentration of the viral community the samples were ultra-centrifuged for 2 h at 135000 x g at 8˚C, using a swing-out rotor.

After dissolving the virus pellet overnight at 4°C in the dark in SM buffer, the virus concentration was determined as described above.

Agarose plugs were prepared according to (Larsen et al. 2001), using InCert agarose (1.5 % final volume, FMC, Rockland, Maine) taking care to load 5 x 10^9 viruses per plug. The plugs were digested overnight at 30°C in the dark with a lysis buffer (250 mM EDTA pH 8.0; 1% SDS; 1 mg/ml Proteinase K (Sigma-Aldrich, Zwijndrecht, the Netherlands). After repeated washings with TE buffer the plugs were loaded onto and run on a 1.0 % (w/v) Seakem GTG agarose gel (FMC, Rockland, Maine, USA) prepared in 1 x TBE gel buffer (90 mM Tris-Borate and 1 mM EDTA, pH 8.0). Samples were electrophorised for 21 h at 14°C; with pulse ramp from 1 to 6 s at 6V cm^{-1} using the Bio-Rad CHEF-DR II PFGE apparatus. As a molecular weight ladder, 5kb and λ concatamers (Bio-Rad, Richmond, CA, USA) were used. The used PFGE settings allowed the detection of viruses with genome sizes between 10 and 200 kb. Gels were stained with Sybr Green I (Molecular Probes, Europe) for 1 h, de-stained for 30 min in distilled water and documented as is described for the DGGE gels.

Data Analysis

Both the DGGE and the PFGE gels were analyzed using Phoretix 5.00 (Nonlinear Dynamics, Ltd, United Kingdom). The band matrices obtained with Phoretix were further analyzed using Primer 5.2.0.9 (Clarke & Warwick 2001) to obtain Bray-Curtis similarity matrices of the different data sets using presence - absence analysis of bands. Non-metric multi-dimensional scaling (MDS) configurations were constructed based on these similarity matrices.

Correlations between different variables were determined using linear correlation (Pearson r) with Statistica (StatSoft Inc., Tulsa, OK, USA). Algal abundances were log transformed before further analysis.

Results

Enclosure experiments

Two enclosure experiments using water from Lake Loosdrecht, were conducted in November 2002 and March 2003 (Table 3.1). A cyanobacterial lysis event was observed within 18 days in all enclosures. The November enclosure was run

for an additional 24 days (for a total of 42 days; Table 3.1) in order to monitor population dynamics after the cyanobacterial collapse. During both experiments the abundances of viruses, chemotrophic bacteria, cyanobacteria and eukaryotic algae were measured. During the November experiment the frequency of virus infected cyanobacterial cells (FIC) was determined as well as the molecular diversity of the viral, chemotrophic bacterial and cyanobacterial communities.

Plankton dynamics

At the start of both experiments, cyanobacteria belonging to the *Limnothrix* / *Pseudoanabaena* group (Van Tongeren et al. 1992, Zwart et al. 2005) predominated the lake phytoplankton with abundances of 6.5×10^4 cyanobacterial and 2.9×10^4 prochlorophyte trichomes ml^{-1} in February and 8.7×10^4 cyanobacterial and 8.6×10^3 prochlorophyte trichomes ml^{-1} in March. Flow cytometric analysis (FCM) showed that in both experiments the populations of these filamentous cyanobacteria increased during the first week (Figure 3.1 and 3.2A & B). After cyanobacterial abundances reached 2.1 to 2.5×10^5 trichomes ml^{-1}, they plummeted within 5 to 6 days by more than 80%.

Interestingly, the population of green algae increased markedly after the cyanobacterial collapse, which was especially clear in the November experiment due to its extended post-lysis experimental period (Figure 3.2A & C). Light microscopy showed a diverse assemblage of species of algae in the November enclosure with equal abundances of green algae (mainly *Scenedesmus* sp. and *Ankistrodesmus* sp.) and diatoms (mainly *Melosira* sp., *Diatoma* sp. and *Nitzschia* sp.) before the collapse, but with > 90 % green algae after the collapse (data not shown). *Scenedesmus* sp. and *Ankistrodesmus* sp. were the dominant greens during peak densities at day 26; they comprised up to 50 and 39% of the algal populations, respectively. However, from day 26, the algal population declined rapidly (Figure 3.2C). Statistical analysis indicated that algal abundances were negatively correlated with cyanobacterial and bacterial abundances in the November experiment (Table 3.2). During the March experiment, the algal community increased also during the short post-lysis period in this experiment (Figure 3.2D), but the overall correlation between algal and cyanobacterial abundance was positive (Table 3.2).

The crash in cyanobacterial numbers was accompanied by a sharp increase in total viral numbers in all enclosures, as determined by epifluorescence microscopy

(EM) (Figure 3.2A & B). Viral increase during the collapse in November was only slightly stronger than during the March experiments, by a factor of 2,5 and 2,3 respectively, which corroborates observations of previous experiments (Gons et al. 2002). The collapse of the cyanobacterial population was with 87% also slightly stronger during the November experiment as during the March experiment (82%). Highest viral numbers in November were concurrent with and after the algal peak (days 28 and 40; Figure 3.2A & C). Statistical analysis indicated a significant negative correlation between viral abundance and cyanobacterial abundance in both experiments (Table 3.2). Viral abundance was also significantly correlated with bacterial and algal abundances during the November experiment and negatively correlated to algal abundances during the March experiments (Table 3.2).

Bacterial numbers were determined using FCM after DNA staining. Bacterial numbers showed a marked increase during the cyanobacterial collapse in all three March enclosures (Figure 3.2D). In the November enclosure bacterial numbers decreased upon the mass mortality event and increased only after the decline of the algal population (Figure 3.2C). During the November experiment bacterial abundance was negatively correlated to both viral and algal abundances and positively to cyanobacterial abundances. During the March experiments bacterial abundance was negatively correlated to algal abundances (Table 3.2).

Frequency of virally infected cells

Transmission electron microscopy (TEM) analysis of thin-sectioned filamentous cyanobacteria revealed that 6.1 % of the cells (n = 99 cells) was visibly infected (FVIC) during the cyanobacterial collapse (day 14, November experiment: Figure 3.3). Since mature viral particles are detectable only during the final stage of viral infection, a correction factor has to be used to calculate the actual frequency of virally infected cells (FIC) from the FVIC. For this, we used 3 correction factors based on previous studies of the viral infection cycle using: (1) Binder's model (1999); (2) natural bacterial communities (Weinbauer et al. 2002); and (3) cyanobacterial cultures (Suttle 2000). Thus, we obtained an actual FIC ranging from 24 to 43 % during the cyanobacterial collapse, revealing viral infection as an important mortality cause during the cyanobacterial collapse.

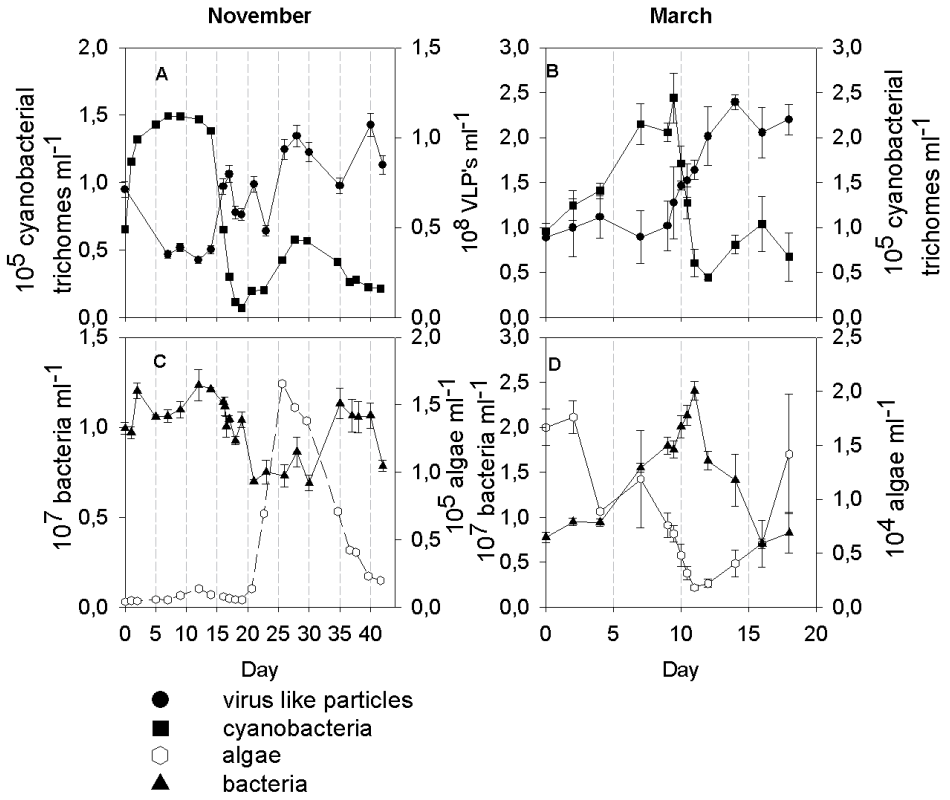


Figure 3.2 Population dynamics of cyanobacteria and viral populations during November and March experiments (A & B) bacterial and algal population dynamics (C & D). Black circles represent virus like particles; black squares cyanobacteria; white circles algae and black triangles represent bacteria. Error bars November experiment: standard deviation of triplicate analysis. Error bars March experiment: standard deviation of average values of triplicate enclosure experiments. Note the difference in experimental period between the two experiments.

Cyanobacterial community composition

The community composition of the cyanobacteria during the November experiment was analysed employing denaturing gradient gel electrophoresis (DGGE), using a nested PCR procedure with general bacterial and cyanobacteria specific primers (Zwart et al. 1998a, Zwart et al. 2005) (Figure 3.4). At the start of the experiment, a diverse cyanobacterial community with 20 different cyanobacterial DGGE bands (phylotypes) could be distinguished (Figure 3.5A). During the cyanobacterial collapse richness decreased to 15 cyanobacterial phylotypes.

Table 3.2 Pearson correlation coefficients and level of significance for different variables. Community richness was determined as the product of bacterial, cyanobacterial richness (number of DGGE phylotypes) and algal richness (number of species observed by LM) per sample. *= p<0.05, **= p<0.01, ***=p<0.001, NS = not significant.

November experiment				
Variable	Cyanobacteria	Bacteria	log Algae	Community richness
Viruses	-.6964**	-.4942*	.5252*	.5574*
Bacteria	.5833**	---	-.5141*	NS
log Algae	-.4990*	-.5141*	---	NS
Cyanobacteria	---	.5833**	-.4990*	-.8253***
March experiment				
Viruses	-.5305**	NS	-.4934**	---
Bacteria	NS	---	-.6763***	---
log Algae	.3421*	-.6763***	---	---
Cyanobacteria	---	NS	.3421*	---

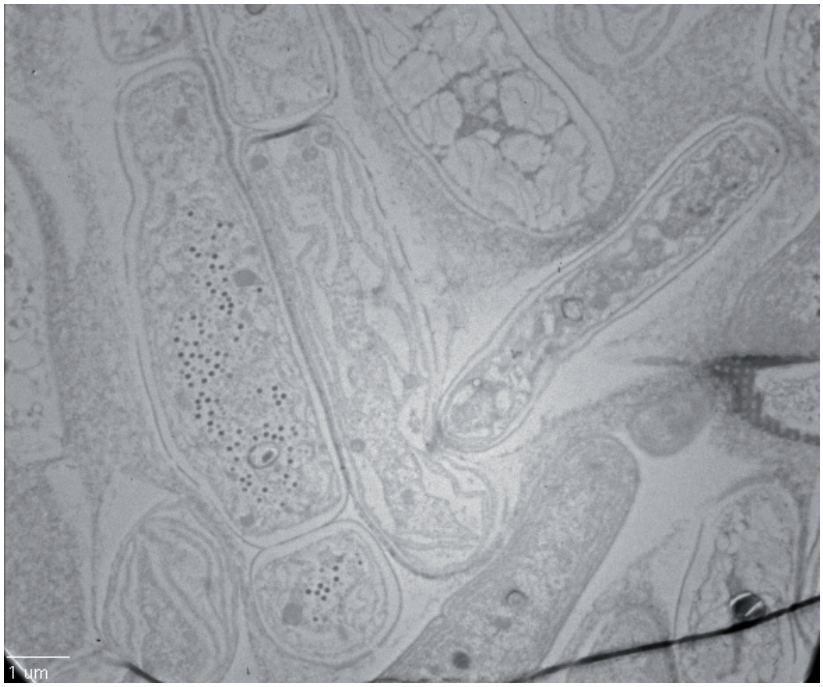
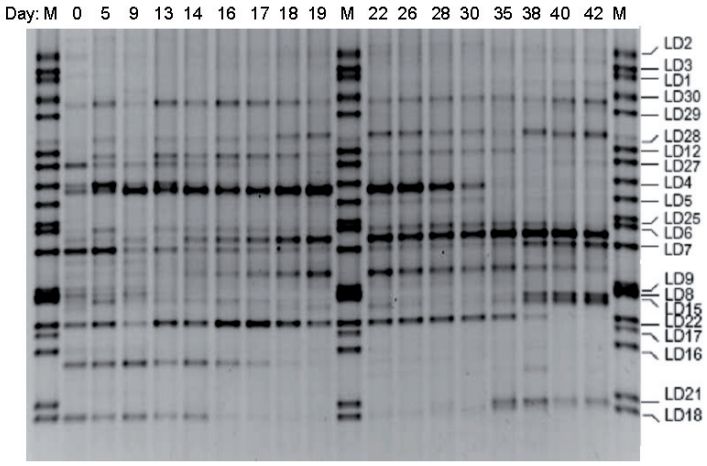


Figure 3.3 Thin-section of filamentous cyanobacteria observed with transmission electron microscopy, showing virally infected cell. The capsid diameter of the viral particles ranged from 60 to 80 nm.



Clone	Cyanobacterium
LD4	*Limnothrix* sp.
LD5	*Prochlorothrix* sp.
LD25	*Prochlorothrix* sp.
LD6	*Limnothrix* sp
LD7	*Prochlorothrix* sp.
LD9	*Synechococcus* sp.
LD8	*Aphinozomenon* sp.
LD15	*Prochlorothrix* sp.
LD22	*Prochlorothrix* sp.
LD16	*Prochlorothrix* sp.
LD18	*Planktothrix* sp.

Figure 3.4 Cyanobacterial diversity as depicted by DGGE during November experiment. M = marker composed of cyanobacterial clone library from lake Loosdrecht. Labels indicate the corresponding clone label of the marker band. Clone-labels of marker bands corresponding to cyanobacteria are identified.

Some of the disappearing phylotypes during the mass mortality event were bands that co-migrated with bands in the clone-ladder, which have previously been sequenced and attributed to *Aphanizomenon* sp., *Planktothrix* sp., *Limnothrix* sp., *Prochlorothrix* sp. and *Synechococcus* sp. by Zwart et al. (1998 a & b; Figure 3.4, cyanobacterial clones LD 8, 18, 4 , 16 and 9, respectively). At the end of the collapse cyanobacterial richness increased again up to 19 phylotypes (day 18), which was mainly due to the appearance of new phylotypes which were not detected earlier during the experiment. One of these newly appearing phylotypes co-migrated with marker band LD5, corresponding to *Prochlorothrix* sp. After the mass mortality event cyanobacterial richness did not recover to the levels observed at the start of the experiment (Figure 3.5A).

Similarity analysis and non-metric multidimensional scaling (MDS) were used to compare the different cyanobacterial DGGE profiles, resulting in ordination plots in which samples similar in composition are plotted close to one another (Figure 3.6A). The cyanobacterial community profiles before the collapse (days 0, 5 and 9) clustered separately from those during the collapse (days 13, 14, 16, 17 and 18), indicating changes in community composition during the collapse. The largest shift in cyanobacterial community composition between

successive days was discernible at the end of the lysis period (days 17 - 18). Bray-Curtis similarity analysis indicated 86% similarity between the community profiles of these two successive days. Strong shifts in cyanobacterial community composition also occurred after the cyanobacterial collapse (Figure 3.6A) with less than 50% similarity between the DGGE profiles of days 26 and 42.

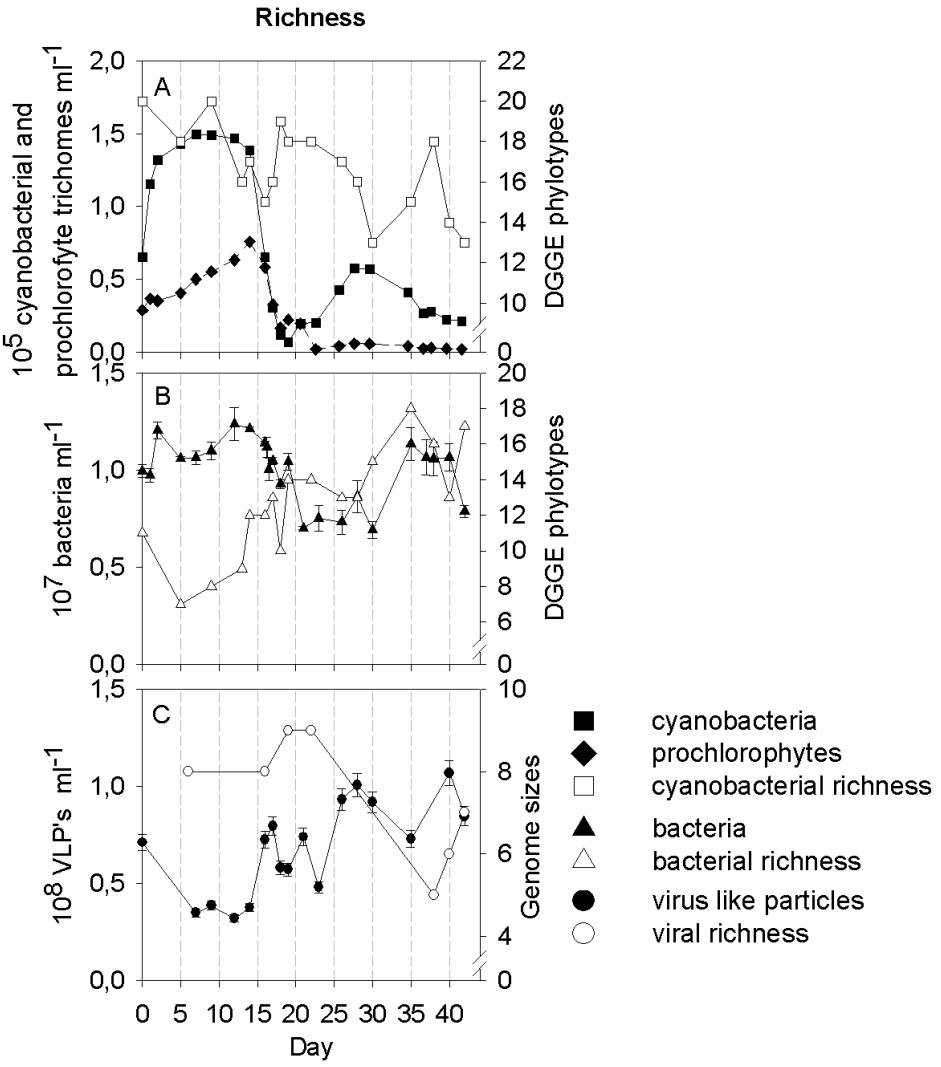


Figure 3.5 Richness during November experiment of cyanobacteria (A), chemotrophic bacteria (B) and viruses (C). Cyanobacterial and chemotrophic bacterial richness (A & B) are depicted by number of DGGE-phylotypes, and viral richness (C) by the number of PFGE bands observed per sample.

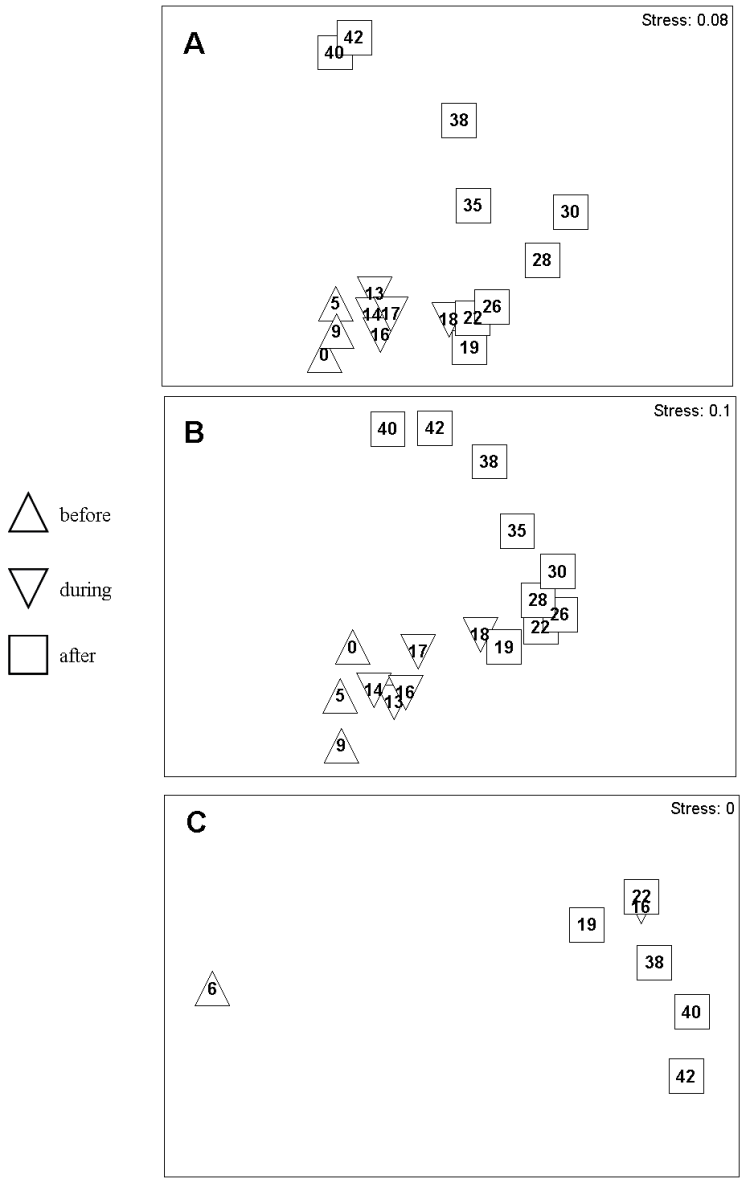


Figure 3.6 Changes in community composition during November experiment. A) cyanobacterial community; B) chemotrophic bacterial community; and C) viral community. MDS plots are based on similarity matrices obtained from the presence-absence analysis of DGGE or PFGE banding patterns. Numbers represent experimental day when samples were taken. Upward triangles, downward triangles and squares represent samples taken before the lysis event (day 0-11), during the lysis event (day 12-18) and taken after the lysis event (day19-42), respectively. Stress values are used to estimate the adequacy of the MDS representation, stress values of 0.1 and lower correspond to a good ordination with no real prospect of a misleading interpretation.

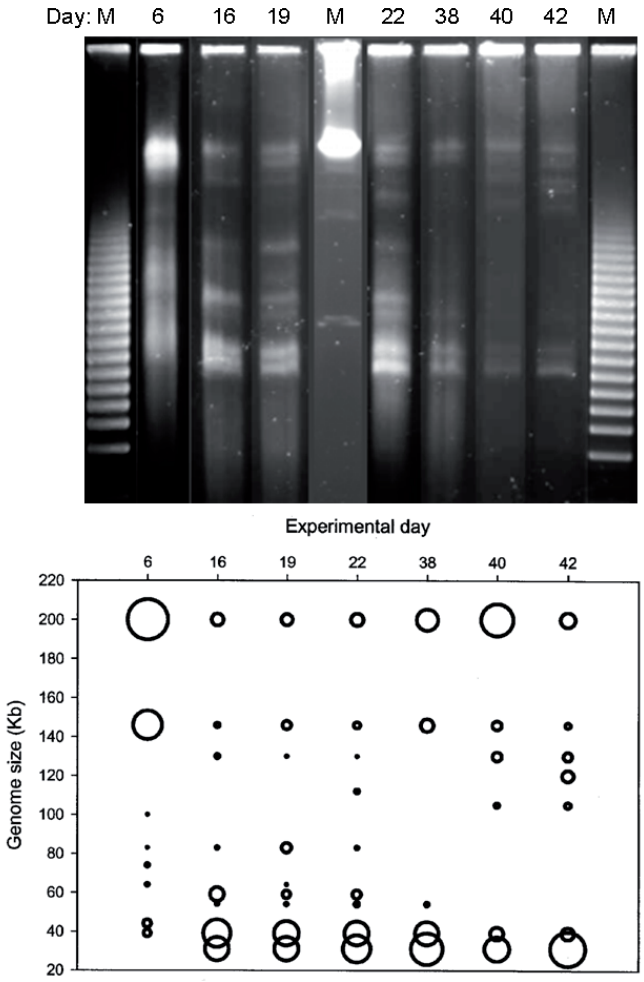


Figure 3.7 PFGE gel of viral population (A), and bubble plot representation of PFGE gel (B). Viral diversity was determined using PFGE during November experiment. Day when samples were taken from experiment is indicated on x-axis. M = marker. Bubble position indicates position PFGE band and corresponding genome size, bubble size indicates relative band intensity.

Bacterial community composition

DGGE analysis of chemotrophic bacterial community composition showed a diverse population with a total of 28 different phylotypes during the entire November experiment (Figure 3.5B). Chemotrophic bacterial richness increased from 8 to 13 phylotypes during the cyanobacterial collapse. In contrast to the diversity, bacterial numbers were decreasing during that period. MDS clustering

revealed that the strongest shift in bacterial community composition between successive days occurred at the end of the lysis period, representing 78% similarity between the successive days 17 and 18 (Figure 3.6B). Strong shifts in community composition also occurred after the cyanobacterial collapse, with less than 60 % similarity when comparing days 26 and 42.

Viral community diversity

The viral diversity in the November experiment was determined based on differences in capsid size using TEM, and on differences in total viral genome size using pulsed field gel electrophoresis (PFGE; Figure 3.7). The capsid sizes of the viral particles observed inside thin-sectioned cyanobacterial cells ranged between 60 and 80 nm, which is within the most prominent viral size category observed during this experiment (Figure 3.3 & Table 3.3). During the strong decrease of the algal population (day 35) almost half (49%) of the virus population had a capsid size smaller than 60 nm. At the same time large viruses with capsid sizes ranging between 90 and 150 nm reached maximum abundances, composing one quarter of the virus population (Table 3.3).

Table 3.3 Viral capsid size distribution per day, observed by TEM during November experiment.

Day	Viral capsid size (%)			
	< 60 nm	60 - 90 nm	90 - 150 nm	> 150 nm
17	32	55	9	4
23	25	57	18	0
28	28	60	11	1
35	49	26	25	0
42	21	71	8	0

Difficulties were experienced when applying the PFGE technique on eutrophic lake water, probably due to high concentrations of polymeric materials. This methodological problem resulted in low band resolution and high background staining of the PFGE gels. Therefore, especially samples taken at the start of the experiment could not be used in further analysis. To improve the gel quality, cleaning procedures were tested as reported by Riemann and Middelboe (2002a). These cleaning procedures improved the gel quality but also led to a high loss of viral numbers, especially in the larger viral size classes and were

therefore chosen not to be applied in this study. Finally, it should be noted that the high background staining on the gels may have caused incomplete detection of small, faint bands and thus to an underestimation of viral diversity.

Despite these limitations, PFGE revealed a total of 15 different viral populations with genome sizes varying between 30 and 200 kb during the entire November experiment (Figure 3.7). Genome sizes of 31, 39, 59 and 146 kb were most abundant, with average relative intensities of 30, 20, 12 and 13%, respectively. High band intensities were also observed at 200 kb, which was probably due to a combination of genomes larger than 200 kb, which were not separated with the PFGE settings that we used. The first PFGE pattern available for analysis was day 6, when 8 viral genome sizes were observed (Figure 3.5C). Viral richness increased during the cyanobacterial collapse to 9 viral genome sizes just after the lysis event (day 19). Viruses with genomes sizes of 55, 83, 132 kb, and especially 31, 39 and 59 kb increased in numbers during the cyanobacterial collapse. Fifty-five per cent of the viruses had capsid sizes between 60 and 90 nm and 32 % had capsid sizes <60 nm during the lysis event (Table 3.3). The changes in viral community composition were most marked between day 6 and day 16, with 60% similarity between these two days (Figure 3.6C). Viral community composition changed only weakly after the lysis event, even though changes in viral numbers and richness were marked (Figures 3.2A, 3.5C & 3.6C).

Discussion

Enclosure experiments with eutrophic lake water were used to study the viral community and the impact of a mass viral lysis event on the planktonic community composition and succession. The filamentous cyanobacterial population rapidly collapsed after a period of steady growth in all 4 enclosures. TEM analysis of the November experiment revealed a high fraction of virally infected filamentous cyanobacterial cells (FIC), ranging from 24 to 43%, indicating viruses as major mortality cause during the cyanobacterial collapse. This FIC value indicates that viral infection could indeed account for the cyanobacterial decay of 23% per day observed in the November enclosure between day 14 and day 16. In addition, the FIC value almost exactly matches the FIC value predicted in a model simulation of cyanobacterial population dynamics in these enclosures during

a cyanobacterial collapse with viruses as the single causal factor for mortality (Gons et al. 2006). Furthermore, viral and cyanobacterial abundance showed a significant negative correlation in all enclosures, due to the strong increase in viral numbers during the cyanobacterial collapse. Alternative mortality factors for the detected cyanobacterial decay would be predatory bacteria (Shilo 1971, Holfeld 1998, Rashidan & Bird 2001) and chytrid infection (Holfeld 1998). However, we have no microscopic evidence of chytrid infection during the enclosure experiments (Dr. M. Kagami, personal communication), nor did we find proof for significant impact of predatory bacteria. Nutrient depletion and zooplankton grazing seem to have played a role in the cyanobacterial collapse neither, since nutrients were supplied in surplus and no elevated numbers of grazers capable of consuming filamentous cyanobacterial, such as the cladoceran *Bosmina* spp. (Pel et al., 2003(Gulati et al. 1992) were observed during the collapse. Based on (1) the strong increase in viral numbers and (2) the presence of 24 – 43% virally infected filamentous cyanobacterial cells, we conclude that the cyanobacterial collapse was virally induced.

Experimental set-up

Even though the enclosures are sufficiently large (130 L) and designed to mimic the physical environment in Lake Loosdrecht, there are some substantial differences between lake and enclosures. First, the absence of a sediment layer and continuous mixing in the enclosures excluded the effect of wind resuspension of sediment particles and phytoplankton mortality due to sedimentation, which are both considered to play an important role in lakes like Lake Loosdrecht (Gons et al. 1991). In addition, the synthetic growth medium supplied in these experiments contained an unlimited supply of nitrogen and phosphate, but lacked a source of organic carbon. Cyanobacterial exudates were therefore considered to be an important carbon source for the chemotrophic bacterial community in the enclosures before the collapse of the cyanobacterial community (Brock & Clyne 1984). These differences between enclosures and Lake Loosdrecht might explain the reduction in chemotrophic bacterial and viral richness at the start of the present experiment.

Many studies on the impact of viral lysis on community composition, report that viral impact can be partly masked because confinement was found to impact community richness (Hewson et al. 2003, Schwalbach et al. 2004, Winter et al.

2004, Bouvier & del Giorgio 2007). Enclosure effect may therefore also explain the reduction in chemotrophic bacterial and viral richness at the start of our experiments. The incorporation of a no-virus control in which viral abundances have been actively reduced by filtration should be added in future experiments to confirm that the observed community changes are solely due to the virally-induced collapse of the cyanobacterial community.

The prevalence of optimal conditions in the enclosures concerning temperature, nutrient availability and physical mixing clearly stimulated cyanobacterial growth to reach bloom concentrations. High concentrations of cyanobacteria in combination with thorough mixing are assumed to have lead to elevated encounter rates between host and virus in the enclosures, facilitating epidemic spread of the viral infection that caused the cyanobacterial population to crash. Strikingly, green algae, which are normally not a dominant group in Lake Loosdrecht, dominated phytoplankton in the November enclosure after the lysis event. This unusual situation probably explains the large changes observed in both the cyanobacterial and the chemotrophic bacterial community composition in the enclosure after the cyanobacterial collapse (between days 26 and 42). We would also like to point out the reproducibility of the cyanobacterial collapse and associated dynamics of viral, bacterial and algal communities with this experimental set-up, as was nicely shown by the triplicate March experiments, corroborating the validity of the obtained and previous Lake Loosdrecht enclosure results (Van Hannen et al. 1999, Gons et al. 2002).

Viral dynamics

PFGE is a commonly applied technique enabling the detection of the total viral community and providing a direct estimate of the abundances of specific viral genome size groups, since it does not require selective pre-amplification (Wommack et al. 1999, Steward et al. 2000, Larsen et al. 2001, Riemann & Middelboe 2002a). Despite poor band resolutions and high background staining of the PFGE gel during the present study, we detected between 5 to 9 different genome sizes per sample, which is within the range of viral diversity observed in other studies (Wommack et al. 1999, Larsen et al. 2004).

Large changes occurred in the viral community composition between days 6 and 16; 50% of the viral genome sizes detected at day 6 could not be detected at day 16. The cyanophages responsible for the cyanobacterial collapse are expected

to be represented by the PFGE bands that newly appeared or increased in intensity between day 6 and 16. Viruses with genomes sizes of 31, 39, 55, 59, 83 and 132 kb increased in numbers at the time of the cyanobacterial collapse. The middle four of these fall into the genome size range of 36 to 100 kb described for isolated cyanophages (Wilson et al. 1993, Suttle 2000, Lu et al. 2001).

Interestingly, the cyanobacterial collapse affected different genera of filamentous cyanobacteria, all of which were observed to lyse within the same period. When we study Figure 3.5A more closely however, it becomes clear that prochlorophytes were still increasing when the collapse of the other filamentous cyanbacteria already started (day 12). The same is true for the March experiments (data not shown). The DGGE profile confirmed that even though several cyanobacterial strains were lost from the community during the lysis period, there were some variations in the exact time of their disappearance. Phylotypes at the same position as the *Synechococcus* sp. marker band (LD9) for example, disappeared earlier from the community profile than phylotypes at the position of the *Prochlorothrix* sp. marker band (LD16).

Since viral infection is generally believed to be host-specific and even strain-specific (Ackermann & DuBow 1987), the collapse of different cyanobacterial genera entails involvement of a variety of viruses. PFGE analysis indeed revealed that a diverse viral population was present during the cyanobacterial collapse. The scenario might have been that the ideal growth conditions in the enclosures selected for filamentous cyanobacterial strains with high growth and metabolic rates, enabling these strains to reach high densities. High densities of these strains allow for rapid viral replication of their specific viruses, since viruses propagate as a function of host density (Murray & Jackson 1992, Hennes et al. 1995). Viral infection of these strains might have been further accelerated bacause cells with higher growth and metabolic rates generally have higher virus yields and are more susceptible to viral infection (Lenski 1988, Middelboe 2000).

The extent of host specificity of viral infection is under discussion, however. The myoviridae have been reported to exhibit a wide range of hosts (Mann 2003). For example, phages of *Synechococcus* can infect up to 10 different strains (Waterbury & Valois 1993). Also, some cyanomyoviridae have been observed by Sullivan et al. (2003) to cross-infect hosts of different cyanobacterial genera. It is quite possible that in our study a broad-host-range virus induced the simultaneous collapse of different cyanobacteria. Classical plaque assay,

quantitative PCR (Fuller et al. 1998, Muhling et al. 2005) and denaturing gradient gel electrophoresis (DGGE) using primers developed for the *g20* gene (Short & Suttle 1999, Wilson et al. 2000, Frederickson et al. 2003, Dorigo et al. 2004) or other major capsid protein genes (Baker et al. 2006) can be applied to describe population dynamics of cyanophages specifically, and could give us more insight into this topic in the future.

Viral impact on aquatic community composition

The release of organic compounds and inorganic nutrients due to viral lysis of the host cell can stimulate growth of heterotrophic bacteria. This will lead to a recycling of nutrients within the microbial loop and eventually result in the transport of less nutrients to higher trophic levels within the aquatic food web (Bratbak et al. 1994, Fuhrman 1999). During the March experiments we observed an increase in bacterial growth upon lysis, whereas we could not detect such an increase during the November experiment. We did observe a strong shift in the chemotrophic bacterial community composition upon the lysis event in this enclosure, however. Changes in composition and bioavailability of organic nutrients upon mass lysis are known to affect chemotrophic bacterial community composition (Lebaron et al. 1999, Van Hannen et al. 1999) and are likely to be the main causes for the shift in community compositon observed during cyanobacterial lysis in the present experiment.

The viral lysis event not only resulted in a reduction of cyanobacterial biomass, but also in a marked shift in cyanobacterial community composition, as observed in the November experiment. Changes in phytoplankton community structure were also observed by Suttle (1992) and by Peduzzi and Weinbauer (1993) when adding a concentrated virus-rich submicron size fraction to natural seawater. Viruses are thought to influence community composition by increased infection of competitively superior host species that have high numbers and are fast growing (Thingstad & Lignell 1997, Thingstad 2000, Wommack & Colwell 2000). In doing so, the viruses facilitate co-existence of many species. In other words, by keeping in check the superior competitors, viruses prevent competitive exclusion of inferior species. In the present experiments we indeed observed the removal of the dominant filamentous cyanobacterial community due to lysis, which initially led to a decrease in cyanobacterial richness. The subsequent increase in cyanobacterial richness at the end of the lysis period might thus

have been due to the increase of cyanobacteria that profited from relaxed competitive pressure owing to the collapse of the dominant cyanobacteria.
Within the 'killing the winner' context, the increase of the green algae after the cyanobacterial collapse is an interesting phenomenon, especially in the November enclosure. In the turbid and shallow Lake Loosdrecht, light will normally be the growth limiting factor during the experimental period (November – March), for which cyanobacteria and algae are competing. Since in the enclosures the nutrients were provided in excess, light is also expected to become the limiting factor in the enclosures. Water transparency increased in the enclosures after the virally induced collapse (Simis et al. 2005), probably enabling the green algae to benefit of the improved light conditions and outcompete other species. Interestingly, the algal community started decaying rapidly on reaching high densities. The algal peak in the November experiment coincided with high viral numbers with relatively large genome- and capsid sizes, which are often attributed to algal viruses (Reisser 1993). We did not collect electron microscopic data to confirm the presence of virally-infected algae at that time, however, so it remains to be investigated whether also the green algal collapse has been caused by viral lysis.
Viral control of the competitive dominant is predicted to promote diversity, by enabling the invasion and co-existence of less competitive species. (Thingstad 2000, Wommack & Colwell 2000). One could thus expect a positive correlation between viral abundances and community richness. In the present experiment community richness was used as a measure of diversity, using DGGE and light microscopy to determine richness of bacterial and algal communities, respectively (Table 3.3). Viral abundance was indeed positively correlated with bacterial and algal richness ($r = 0.5560$ $p<0.05$; and $r = 0.8345$ $p<0.001$, respectively), but negatively correlated with cyanobacterial richness ($r = -0.5239$ $p<0.05$). Cyanobacterial abundances could even explain 68% of the temporal variation in total community richness (product of algal, cyanobacterial and bacterial richness; Table 3.2), which is in accordance with the hypothesis that viral control of the competitive dominant has a stimulating effect on community diversity, and corroborates recent results obtained in marine environments (Hewson et al. 2003, Winter et al. 2004). Our results imply that viral infection can potentially control cyanobacterial blooms and thereby has a marked impact on the plankton community composition in eutrophic lakes.

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Chapter 4

Estimates of potential bacterial and phytoplankton mortality rates due to viral lysis and microzooplankton grazing in a eutrophic lake

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Summary

Since viral lysis and zooplankton grazing differ in their impact on the aquatic food web, it is important to assess the relative importance of both mortality factors. In this study an adapted version of the dilution technique was applied to simultaneously estimate the impact of both viral lysis and zooplankton grazing on the mortality of bacteria, algae, unicellular cyanobacteria, prochlorophytes and especially filamentous cyanobacteria in the shallow eutrophic Lake Loosdrecht. We report the results of 5 dilution experiments; 1 performed in autumn 2004, 2 in winter 2004 / 2005 and 2 performed in spring 2006. Viral and bacterial abundances were obtained using epifluorescence microscopy (EM) and abundances of different phytoplankton groups were obtained using flow cytometry (FCM) and light microscopy (LM). Viral lysis was identified as the main mortality source during the winter experiments, removing between 84 and 97% of the potential filamentous cyanobacterial production and up to 101% of the potential bacterial production. Microzooplankton grazing removed between 90 and 99% of the potential unicellular production and up to 46% of the potential bacterial production in spring, when viral lysis had a significant

impact on algal growth.In some cases no significant impact of viral lysis or zooplankton grazing could be detected. Contrary to expectations, the apparent growth rate of filamentous cyanobacteria was even sometimes observed to decrease significantly upon dilution of microzooplankton.The dilution technique gives a valuable insight into the impact of both zooplankton grazing and viral lysis on the mortality of different plankton groups but care should be taken when interpreting results and to optimize the technique for application in different environments and on a variety of plankton groups.

Introduction

Since the discovery of high viral numbers in 1989 (Bergh et al.), viral infection has been recognized as an important process in aquatic ecosystems (Fuhrman 1999) Reports of viral lysis contributing up to 70% of cyanobacterial mortality in marine systems (Proctor & Fuhrman 1990) and up to 90 - 100% of bacterial mortality in freshwater systems (Weinbauer & Hofle 1998, Fischer & Velimirov 2002) led to the conclusion that viral lysis can be a major mortality cause for aquatic microorganisms, comparable to grazing-induced mortality (Brussaard 2004).The effect of these two mortality causes on the aquatic food web is quite different however, since grazing leads to transport of nutrients to higher trophic levels (Wetzel 2001), whereas viral lysis leads to a recycling of nutrients within the microbial loop (Bratbak et al. 1994). Cellular debris is namely released upon lysis of the virally infected host cell, which can be readily taken up by heterotrophic bacteria (Gobler et al. 1997). Furthermore, viral infection is thought to influence the microbial community composition, due to the host-specific and density-dependent nature of viral infection (Fuhrman & Suttle 1993). Since viral infection is often species- and even strain-specific (Ackermann & DuBow 1987), viruses are thought to have a stronger selective impact on the aquatic community composition than selective grazing by zooplankton (Thingstad & Lignell 1997, Thingstad 2000). It is therefore of great importance to estimate both virally and grazing induced mortality to obtain a better insight into the driving forces and the nutrient flow within an aquatic food web.

Evans et al. (2003) introduced an adapted version of the dilution technique to simultaneously estimate the impact of viral lysis and microzooplankton

grazing on the mortality of the phytoplankter *Micromonas pusilla.* The original dilution technique, introduced by Landry and Hasset (1982), yields estimates of both the specific growth rate of the phytoplankton and specific grazing rate of the microzooplankton. With this technique the original water sample is serially diluted with filtered water from the same location, step-wise reducing the probability of encounter between microzooplankton (the predator) and phytoplankton (the prey). The more dilute samples are thus assumed to suffer less grazing impact, allowing a high apparent growth rate (k) of the phytoplankton in these samples. The specific growth rate (μ) of the phytoplankton is given as the apparent growth rate extrapolated to 100 % dilution; the growth rate when no grazers are present. The difference between this true growth rate and the observed apparent growth rate in the undiluted treatment is then taken as the grazing mortality rate (M_g).

The dilution technique is now commonly applied to a wide variety of prey and environments (Ducklow & Hill 1985, Campbell & Carpenter 1986, Weisse & Scheffel-Moeser 1990, Worden & Binder 2003). To also enable the estimation of mortality due to viral lysis (M_v), Evans et al. (2003) applied an additional dilution series in which both viruses and microzooplankton are diluted. This adapted dilution technique has been successfully applied to estimate the impact of viral lysis on *Phaeocystis globosa* in temperate coastal waters (Baudoux et al. 2006) and on the bacterioplankton community in a mesotrophic lake in France (Jacquet et al. 2005). Even though several viruses of freshwater eukaryotic algae and cyanobacteria have been isolated and described (Van Etten et al. 1991, Suttle 2000a), little is known about the mortality impact of viral lysis on these phytoplankton groups in freshwater environments.

In the present study, the dilution technique was applied to estimate the impact of both microzooplankton grazing and viral lysis on the mortality of the bacterioplankton, algae, unicellular cyanobacteria and especially filamentous cyanobacteria in the Dutch eutrophic Lake Loosdrecht. Lake Loosdrecht is a shallow peat lake, which is predominated by a group of related filamentous cyanobacteria belonging to the *Limnothrix* / *Pseudoanabaena* group (Van Tongeren et al. 1992, Zwart et al. 2004). It is known from previous research that the zooplankton community in this lake largely relies on eukaryotic algae for growth and that grazing only accounts for part of the filamentous cyanobacterial mortality (Gulati et al. 1992, Pel et al. 2003). It is therefore expected that the

virioplankton community could play an important role in the mortality of the filamentous cyanobacterial community in the lake, which is supported by earlier observations of a dramatic collapse of the cyanobacterial community associated with viral activity during lake water enclosure experiments (Van Hannen et al. 1999, Gons et al. 2002, Simis et al. 2005). The current study aims to elucidate the phyto- and bacterioplankton mortality rate due to both viral lysis and microzooplankton grazing in Lake Loosdrecht by comparing the results of 5 different dilution experiments.

Methods

Study site

Lake Loosdrecht is a highly eutrophic (Chl-*a* annual average of ca 60 mg m^{-3}; P_{total} = 40 – 60 µg L^{-1}; and N_{total} = 1.4 – 1.9 mg L^{-1}), shallow (mean depth 1.85 m) and turbid (Secchi depth approximately 0.5 m) peat lake in the Netherlands. The lake is predominated by a group of related filamentous cyanobacteria belonging to the *Limnothrix* / *Pseudoanabaena* group (Van Tongeren et al. 1992, Zwart et al. 2004), with concentrations ranging from 0.5 x 10^5 to 3 x 10^5 trichomes ml^{-1} (De Kloet et al. 1990), M. Tijdens, unpubl. data). The second most abundant population of filamentous cyanobacteria closely resembles *Prochlorothrix hollandica,* a cyanobacterium which does not contain phycobilins but does possess Chl *b* (Burger-Wiersma et al. 1986). The concentrations of prochlorophytes in Lake Loosdrecht range from 0.4 x 10^4 to 9.5 x 10^4 trichomes ml^{-1}(Van Liere et al. 1989). The population of eukaryotic algae is relatively low and ranges from 0.2 x 10^4 to 4.5 x 10^4 cells ml^{-1}, comprising mainly diatoms and green algal species (De Kloet et al. 1990). Bacterial and viral concentrations in the lake range from 1.0 x 10^6 to 9.0 x 10^6 ml^{-1} and from 6.0 x 10^7 to 13.5 x 10^7 ml^{-1}, respectively (M. Tijdens, unpubl. data). The zooplankton community in the lake is dominated by small-bodied cladocerans, mainly *Bosmina* spp.; cyclopoid copepods; and small rotifers, such as *Keratella, Anuraeopsis, Filinia* and *Polyarthra* spp. (Gulati et al. 1992, Ooms-Wilms et al. 1999), although during summer large bodied taxa such as *Asplanchna* and *Euchlanis* can be prominent too (Gulati et al. 1992). Concentrations of nanoflagellates range from 1.0 x 10^4 to 9.0 x 10^4 ml^{-1}(M. Tijdens, unpubl. data).

Viral and bacterial enumeration

For the counting of viruses and bacteria the samples were fixed with 0.02-µm filtered formalin (1% final concentration), frozen in liquid nitrogen and stored at -80˚C until analysis. Samples were 10 x diluted in 0.02µm filtered MQ before staining them with SYBR Green I (Molecular Probes, Europe), following the protocol of Noble and Fuhrman (1998).Viral and bacterial counts were obtained with a Zeiss, Axiophot epifluorescence microscope (EM) making sure to count at least 200 virus like particles (VLP) and bacteria.

Cyanobacterial and algal enumeration

Samples for flow cytometry (FCM) were fixed with 0.01% paraformaldehyde and 0.1% glutaraldehyde (final volume), and stored at 4°C in the dark no longer than 2 weeks until processing. Different cyanobacterial groups and eukaryotic algae were distinguished using a MoFlo flow cytometer (Dako, Ft. Collins, Co., USA) equipped with a 70 micron flowcell and running at 60 psi (100 mW excitation; 488 nm). A multitriggerboard was used for triggering on sidescatter, orange (630/30 nm) and red (670/30 nm) fluorescence, combined in the AND-setting. Typically, cell clusters of filamentous cyanobacteria, prochlorophytes (Turner et al. 1989), and eukaryotic algae were distinguished by their clustering in orange and red fluorescence. The classification of these clusters was confirmed by light microscopy examination and fatty acids analysis after cell sorting (Pel et al. 2004). Particles were further processed for size, using the time they need for passing the laser beam (beads of known sizes and a culture of *Leptolyngbya boryana* were used for calibration). A small volume of a known concentration of 1-µm diameter fluorescent beads (Polyscience, Warrington, USA #15702) was added for exact volume determination.

For light microscopy counts (LM) the samples were fixed with lugol (1% final concentration) and stored in the dark at 4°C for maximum 2 weeks until analysis. Filamentous cyanobacterial trichomes were enumerated using Utermöhl chambers and a Zeiss inverse microscope. Cyanobacterial trichome lengths were measured for 50 to 100 filamentous cyanobacterial cells per sample using image analysis (Cell'D, Olympus, the Netherlands).

Dilution experiments

To assess the impact of viral lysis as well as microzooplankton grazing on the

mortality of phytoplankton and bacteria, an adapted version of the dilution technique was used (Landry & Hassett 1982, Evans et al. 2003). A series of 5 dilution experiments (Table 4.1) were performed in the autumn of 2004, the winter of 2004 / 2005 and in the spring of 2006 using Lake Loosdrecht water. Upon sampling water transparency was measured using a Secchi disk and water temperature and pH were assessed. Surface water was sampled, transported to the laboratory and processed within 4 h.

Table 4.1 Experimental conditions during each dilution experiment. The first 3 late autumn and winter experiments were incubated at lower light and temperature conditions than the last 2 spring experiments.

Date (dd-mm-yy)	Temp (°C)	Light ($\mu E\ m^{-2}\ s^{-1}$)	Light : dark cycle (hrs)	Duration (hrs)	Replicates (dilution)
02-11-04	15	20	7 : 20	24	2
07-12-04	15	20	7 : 20	36	2
11-01-05	15	20	7 : 20	48	3
23-03-06	18	45	12 : 12	40	3
04-04-06	18	45	12 : 12	40	3

Three different treatments were prepared from the water sample. One part of the water was filtered over a 200 µm plankton filter to remove large-bodied zooplankton; this filtrate will be called the whole lake water fraction from hereon. From the second treatment all zooplankton was removed by filtration over a 0.2 µm filter and both viruses and zooplankton were removed from the third water treatment by filtration over a 30 kDa filter. To obtain the 0.2µm filtrate the lake water was first prefiltered gently with glass fiber filters (GF/F, Whatman, San Diego, USA.) and 0.45 µm polycarbonate filters (47 mm, Whatman) before gentle filtration over 0.2 µm polycarbonate filters (47 mm, Whatman). Filters were replaced frequently to prevent blockage and filtration was performed gently, to prevent loss of VLP (virus like particles) and infectivity. This filtration procedure was tested for loss of viral infectivity using the cultured cyanophage LPP-1 and its host *Leptolyngbya boryana* (formerly characterized as *Plectonema boryanum*; (Safferman & Morris 1963), but no significant loss in plaque forming units was observed (data not shown). To obtain the 30 kDa filtrate the water was carefully pumped over a 30 kDa polyether sulfone ultrafiltration membrane

(VivaFlow, Vivasciences, Hannover, Germany) after gentle prefiltration over glass fiber filters (GF/F, Whatman) and 0.45 µm polycarbonate filters (47 mm, Whatman). To check if all virus particles had indeed remained in the 0.2 µm-filtered fraction and were removed from the 30 kDa-filtered fraction, samples from all 3 treatments were taken for viral counts as described above. These 3 different lake water fractions were used to prepare 2 different dilution series (Figure 4.1):

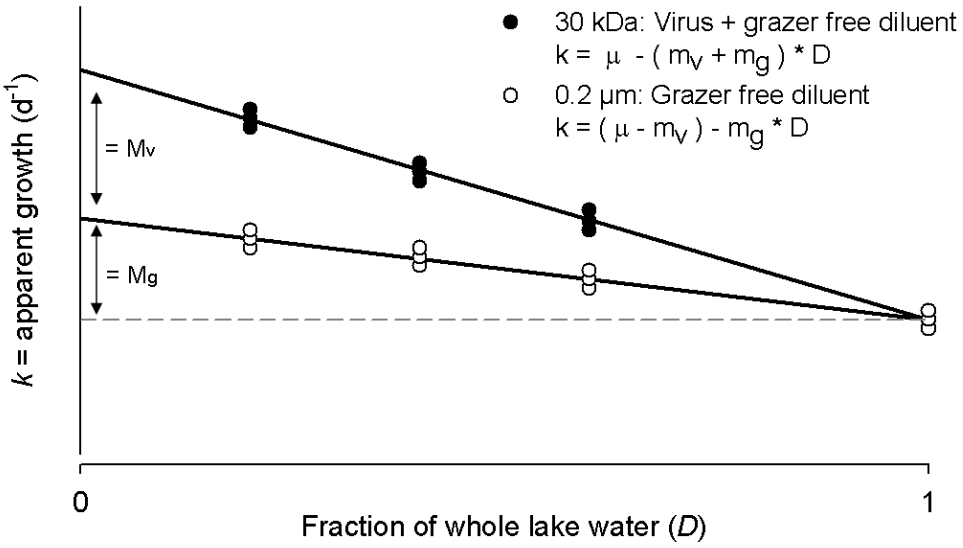


Figure 4.1 Schematical representation of the dilution method. Two dilution series are prepared from the whole lake water (D = 1); 1) a 30 kDa dilution series to measure impact of both viral lysis and microzooplankton grazing ($M_v + M_g$), 2) a 0.2 µm dilution series to measure impact of microzooplankton grazing (M_g) on phytoplankton mortality. The difference between the slopes of the regression lines represents the phytoplankton mortality rate due to viral lysis (M_v). The *y*-axis intercept of the 0.2 µm diluent gives the apparent growth rate (k) of the phytoplankton when no grazing occurs. The *y*-axis intercept of the 30 kDa diluent gives the instantaneous growth rate (µ) of the phytoplankton when no grazing or viral lysis occurs.

1) A 0.2 µm dilution series to measure the impact of microzooplankton grazing on plankton mortality. For this dilution series the whole lake water was serially diluted using the 0.2 µm-filtered lake water.
2) A 30 kDa dilution series to measure the impact of both microzooplankton grazing and viral lysis on plankton mortality. For this dilution series the whole lake water was serially diluted using the 30 kDa-filtered lake water.

For both dilution series 4 different dilutions were prepared containing 20, 40, 60 and 100% of the whole lake water. During the spring 2006 experiments the 60% whole lake water dilution was replaced by a 70% whole lake water dilution. To prevent nutrient limitation, 33 µM of P (as K_2HPO_4) and 357 µM of N (as $NaNO_3$) were added to the dilutions. In order to test if the plankton in the lake was nutrient-limited an additional bottle of 100% whole lake water was prepared which received no nutrient addition.

Only the autumn 2004 experiment did not contain a no-nutrients treatment. The different dilutions were divided over replicate, sterile, 250 ml-erlenmeyers and incubated with sufficiently high light and temperature conditions to prevent growth limitation (see table 4.1 for complete overview of experimental conditions). Samples for viral, bacterial, cyanobacterial and algal counts were taken at the start and at the end of the experiment (Table 4.2).Viral counts (EM) were used to check if the desired level of dilution was achieved and maintained during the experiment.

Table 4.2 Plankton groups observed during experiments and methodology used. ND = not determined; EM = epifluorescence microscopy; LM = light microscopy and FCM = flow cytometry.

Date (dd-mm-yy)	Viruses	Bacteria	Algae	Cyanobacteria		
				filamentous	unicellular	prochlorophytes
02-11-04	ND	ND	ND	LM	ND	ND
07-12-04	EM	EM	ND	LM	ND	ND
11-01-05	EM	EM	ND	LM	ND	ND
23-03-06	EM	EM	FCM	FCM	FCM	FCM
04-04-06	EM	EM	FCM	FCM	FCM	FCM

Interpretation results dilution experiments

Apparent plankton growth rate (k, d^{-1}) was determined based on counts of measured variables (N) at the start and the end of the experiment assuming an exponential model (Landry & Hassett 1982)

$$k = \frac{1}{t} \ln \frac{N_{t0}}{N_t} \qquad (1)$$

where t is the duration of the experiment, and t_0 is the start of the experiment. Microzooplankton grazing rate (M_g) and the mortality rate due to both microzooplankton grazing and viral lysis ($M_{(g+v)}$) were estimated using linear regression on a plot of computed apparent growth rates (k) vs. the fraction of whole lake water (D) (Figure 4.1). Subsequently, mortality rate due to viral lysis (M_v) could be calculated as follows (Evans et al. 2003):

$$M_v = M_{(g+v)} - M_g \qquad (2)$$

Whenever nonlinearity of microzooplankton feeding kinetics was observed due to saturated microzooplankton grazing in the high whole lake water fractions, the approach of Gallegos (1989) was applied to estimate grazing rates. First the instantaneous growth rate (μ) was estimated by linear regression of apparent growth rate against D at the 2 to 3 lowest whole lake water fractions, where D and k were linearly related. The average apparent growth rate in the range of saturated microzooplankton grazing (k_s) and μ were used to estimate the grazing rate, assuming microzooplankton net growth rate (k_z) to be zero (Gallegos & Jordan 1997):

$$e^{ks} = e^{m} - M_g \frac{1 - e^{m}}{-m} \qquad (3)$$

Statistical analyses

The significance of the regression analysis was tested using an analysis of variance (ANOVA). An F-test (Sokal & Rohlf 1995) was used to test if the slopes of regression lines were significantly different. A factorial ANOVA with the Fisher LSD Post Hoc analysis (t-test) was applied to test if variables differed between treatments, dilutions or between t_0 and t. For statistical analysis the STATISTICA 7.0 software was used (StatSoft Inc, Tulsa, USA) and a probability <0.05 was considered significant.

Results

Lake water samples

Five dilution experiments were performed, one in late autumn 2004, 2 in winter 2004 / 2005 and 2 in spring 2006. The physical and biological parameters of Lake Loosdrecht at the start of each dilution experiment are given in table 4.3. Viral numbers in Lake Loosdrecht at the start of the experiments ranged from 7.3 x 10^7 to 9.3 x 10^7 VLP ml^{-1}. Highest filamentous cyanobacterial numbers in the lake were observed in November 2004 (11.4 x 10^4 trichomes ml^{-1}) and the lowest numbers in April 2006 (4.7 X 10^4 ml^{-1}). The number of unicellular cyanobacteria in the lake dropped by a factor of 9 between the experiments of March and April in 2006.

Table 4.3 Physical and biological parameters in lake Loosdrecht at start of dilution experiments. ND = not determined.

Date	Temp	Secchi	Viruses	Bacteria	Algae	Cyanobacteria		
(dd-mm-yy)	(°C)	(cm)	(10^7 vlp's ml^{-1})	(10^6 cells ml^{-1})	(10^4 cells ml^{-1})	(10^4 cells or trichomes ml^{-1})		
						Unicellular	Filamentous	Prochlorophytes
02-11-04	9.5	49	ND	ND	0.66	ND	11.40	ND
07-12-04	5.2	59	7.27	8.46	1.02	ND	8.20	ND
11-01-05	7.5	48	7.29	1.27	1.02	ND	8.00	ND
23-03-06	6.0	60	9.29	8.03	1.64	4.50	6.50	0.80
04-04-06	10.2	58	7.49	3.28	1.28	0.50	4.70	0.40

Dilution experiments

VLP counts of the 0.2 µm and 30 kDa filtrates indicated that viral particles indeed passed the 0.2 µm filters but were retained by the 30 kDa filter. A significant gradient in VLP was obtained when the whole lake water was diluted with 30 kDa filtered lake water, whereas no such gradient could be observed in the 0.2 µm dilution series (Figure 4.2). Only during the winter experiments some of the 30 kDa samples were observed to deviate from the anticipated dilution levels. The 30 kDa dilution levels (D) of these experiments were therefore corrected for the actual number of viruses present in each sample at t_0. During these

experiments also a slight retention of viral particles on the 0.2 µm filter was observed, but this loss did not result in a significant VLP gradient in the 0.2 µm dilution series. Even more careful filtration and preparation of the dilution series prevented these problems in the subsequent experiments.

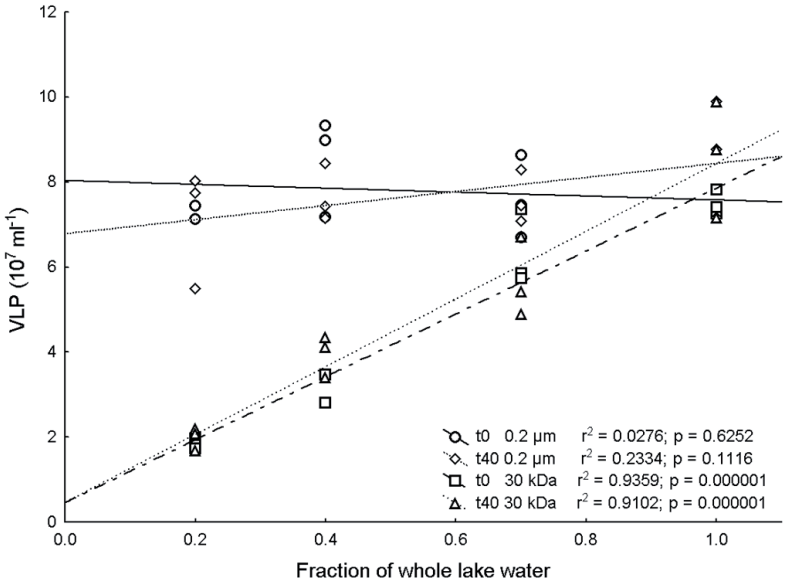


Figure 4.2 Plot of VLP concentration against fraction of whole lake water for a typical dilution experiment (04-04-2006) at t_{start} and at t_{end}.

Viral counts indicated that the relative increase in VLP in the 30 kDa series over time was slight (Figure 4.3). The desired viral dilution levels were maintained over the course of the experiments for all but 2 samples (Figure 4.2 & 4.4). These two datasets (one of the triplicate samples of the 30 kDa 0.2 dilution in both November 2005 and December 2004), were omitted from further analysis. Furthermore the regression slopes of VLP in the dilution series at the start and at the end of the experiment never differed significantly (F-test), confirming that the foreseen viral dilution levels were maintained over time.

Impact of viral lysis vs. grazing

The apparent growth of the different plankton groups in the samples that did not receive nutrient additions was within the range of the apparent growth observed in the nutrient-enriched samples during all dilution experiments (Figure 4.4a-e).

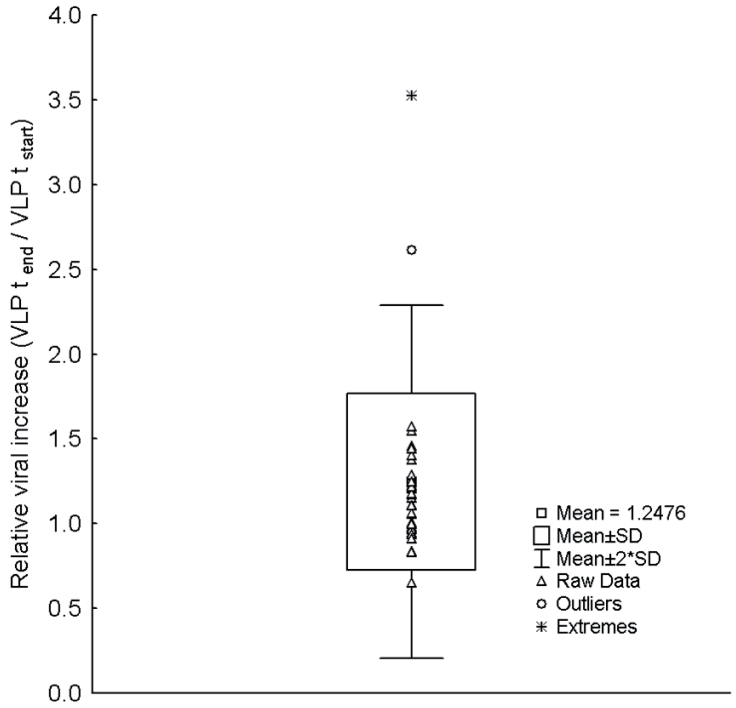


Figure 4.3 Box plot of relative increase of VLP in samples of 30 kDa dilution series over experimental time (VLP t_{end} / VLP t_{start}). Outliers and extremes correspond to 2 samples in which the relative increase in VLP was too strong (>average + 2 * SD).

These observations indicated that the growth of the different plankton groups was not phosphorus or nitrogen limited in Lake Loosdrecht at the time of the experiments. The form of the relationship of apparent growth rate vs. fraction of whole lake water and the impact of viral lysis and microzooplankton grazing was found to vary strongly between the different experiments and plankton groups (Table 4.4). This diversity in form of relationship between apparent growth rate vs. fraction of whole lake water can be roughly summarized into five different result types (Table 4.4), of which representative graphs are shown in Figure 4.4a-e. The first type of results (Figure 4.4a; experiment January 2005) shows no significant increase in apparent growth rate of the filamentous cyanobacteria upon dilution of the microzooplankton (0.2 µm series), whereas a significant increase in apparent growth was observed in the virus and microzooplankton dilution series (30 kDa series). The regression slopes of the 0.2 µm and the 30

kDa series were significantly different (Table 4. 4), indicating that the gradient in viral abundance created in the 30 kDa series had a significant impact on the filamentous cyanobacterial mortality. During this experiment the potential virally mediated mortality was therefore estimated to be 0.35 d^{-1}, representing 84% of potential filamentous cyanobacterial production (Table 4.4). Microzooplankton grazing did not have a detectable impact on the mortality of filamentous cyanobacteria during this experiment. Similar results were obtained for the bacterial population during the same experiment, where the potential virally mediated mortality was even estimated to be 0.87 d^{-1}, representing 100% of the potential bacterial production (Table 4.4). Also in December 2004 viral lysis was found to remove a significant part of the filamentous cyanobacterial population in contrast to microzooplankton grazing which did not have a detectable impact at the time (Table 4.4).

During several experiments no significant relationship was detected between apparent growth rate and fraction of whole lake water for both 0.2 µm and 30 kDa dilution series (type 2 results, Table 4.4 & Figure 4.4b). For example, the prochlorophytes during the March 2006 experiment did not show a significant increase in growth upon dilution of virioplankton and/or microzooplankton and the slopes of the two regression lines did not differ significantly either. This type of result indicates that neither microzooplankton grazing nor viral lysis had a detectable impact on plankton mortality.

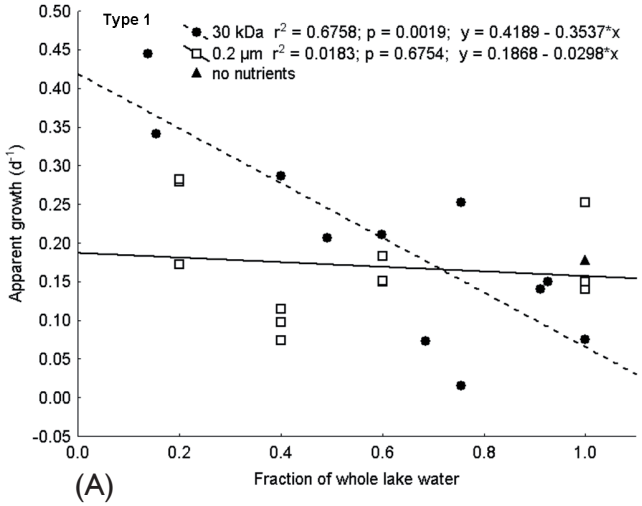
In case of type 3 results a significant increase in the apparent growth of unicellular cyanobacteria was observed when the microzooplankton community was diluted as well as when both microzooplankton and virioplankton communities were diluted (Table 4.4 & Figure 4.4c). The regression slopes of the 0.2 µm and the 30 kDa dilution series did not differ significantly from each other, indicating that the gradient in viral abundance created in the 30 kDa dilution series did not have a detectable impact on unicellular cyanobacterial mortality. The potential microzooplankton mediated mortality of unicellular cyanobacteria was estimated to be 0.45 d^{-1} in April 2006 and 1.25 d^{-1} in March 2006, corresponding to 102% and 107% of potential unicellular cyanobacterial production, respectively (Table 4.4). Grazing was also identified as an important source of bacterial mortality in April 2006, removing 46% of potential bacterial production.

Closer examination of Figure 4.4c reveals that apparent growth of the unicellular cyanobacteria only starts increasing in the highly diluted lake water

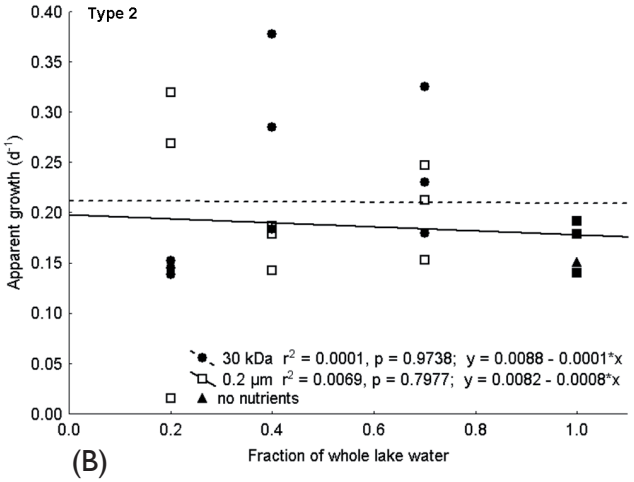
samples (D<0.6), suggesting nonlinear microzooplankton feeding kinetics. This nonlinearity may have been due to saturated microzooplankton grazing in the high whole lake water fractions (Gallegos 1989). Saturated microzooplankton grazing of unicellular cyanobacteria was also detected during the March 2006 experiment. Using the approach of Gallegos (Gallegos 1989) to correct for saturated microzooplankton grazing, the potential grazing rate was found to be 0.52 d^{-1} in the April and 2.29 d^{-1} in March 2006 with extrapolated µ-values of 0.58 d^{-1} and 2.32 d^{-1}, respectively. The tendency of saturated microzooplankton grazing was also observed for the bacterioplankton community in March 2006, but no significant relationship could be detected (Table 4.4).

From the experiments of November 2004 and April 2006 type 4 results were obtained (Table 4.4 & Figure 4.4d), exhibiting significant decrease of the apparent growth rate of filamentous cyanobacteria and prochlorophytes upon dilution of virioplankton and/or microzooplankton. The regression slopes of the 0.2 µm and the 30 kDa dilution series do not differ significantly from each other, indicating that the gradient in viral abundance created in the 30 kDa dilution series did not have a detectable impact.

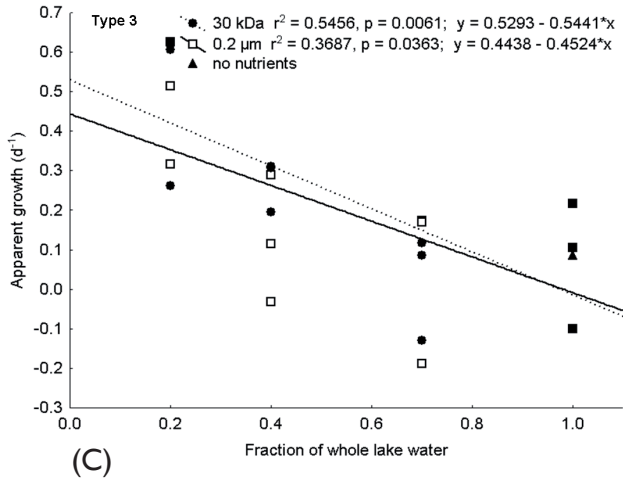
In the April 2006 experiment, the algae showed a significant decrease in apparent growth rate upon dilution of the microzooplankton community, whereas no significant change in apparent growth rate could be detected upon dilution of the microzooplankton and the virioplankton community (type 5 result, Table 4.4 and Figure 4.4e). The regression slopes of both dilution series were significantly different, indicating that viral lysis did have an impact on algal mortality. Post hoc analysis (Fisher LSD; factorial ANOVA) indicated that apparent algal growth rate in both the 0.2 and the 0.4 fractions of whole lake water of the 30 kDa diluent was significantly higher than in the 0.2 µm diluent (respectively $p = 3 \times 10^{-6}$ and $p = 5 \times 10^{-5}$), indicating a significant impact of viral lysis on algal mortality. In March 2005 the algal growth also showed the tendency of a type 5 result. The apparent algal growth in the 0.2 fraction of whole lake water of the 30 kDa diluent was significantly higher than in the 0.2 µm diluent ($p = 0.015$) indicating an impact of viral lysis on algal mortality. On the other hand no significant relationships were observed between the apparent growth rate and the fraction of whole lake water for both dilution series. Also the regression slopes of both dilution series did not differ significantly from each other during this experiment.


Type 1
30 kDa r² = 0.6758; p = 0.0019; y = 0.4189 - 0.3537*x
0.2 µm r² = 0.0183; p = 0.6754; y = 0.1868 - 0.0298*x
no nutrients
Apparent growth (d⁻¹)
Fraction of whole lake water

(A)


Type 2
30 kDa r² = 0.0001, p = 0.9738; y = 0.0088 - 0.0001*x
0.2 µm r² = 0.0069, p = 0.7977; y = 0.0082 - 0.0008*x
no nutrients
Apparent growth (d⁻¹)
Fraction of whole lake water

(B)


Type 3
30 kDa r² = 0.5456, p = 0.0061; y = 0.5293 - 0.5441*x
0.2 µm r² = 0.3687, p = 0.0363; y = 0.4438 - 0.4524*x
no nutrients
Apparent growth (d⁻¹)
Fraction of whole lake water

(C)

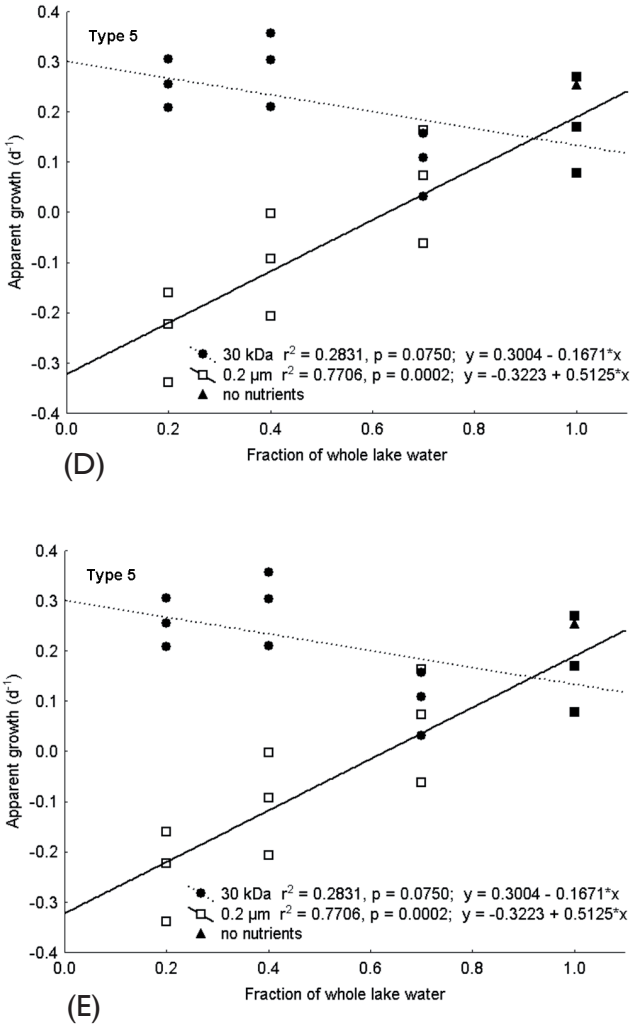


Figure 4.4 Plots of apparent growth rate versus fraction of whole lake water for different dilution experiments. A) Plot derived from apparent growth of filamentous cyanobacteria during 11-01-2005 experiment showing a type 1 result. B) Plot derived from apparent growth of prochlorophytes during 23-03-2006 experiment showing a type 2 result. C) Plot derived from apparent growth of unicellular cyanobacteria during 04-04-2006 experiments showing a type 3 result. D) Plot derived from apparent growth of filamentous cyanobacteria during 04-04-2006 experiment showing a type 4 result. E) Plot derived from apparent growth of eukaryotic algae during 04-04-2006 experiment showing a type 5 result. Dark triangles represent samples which did not obtain nutrient additions.

Date	Prey	Diluent	Regression equation	Linear fit		Regression slopes	Viral mediated mortality		Grazer mediated mortality		Type
(dd-mm-yy)				R^2	p	p	d^{-1}	% of potential production	d^{-1}	% of potential production	
02-11-04	Filamentous cyanobacteria	0.2 µm	y = -0.224 + 0.646 x	0.804	<0.01	NS	0	0	NA	NA	4 / 2
		30 kDa	y = 0.242 + 0.238 x	0.186	NS						
07-12-04	Filamentous cyanobacteria	0.2 µm	y = 0.292 + 0.037 x	0.039	NS	<0.01	0.284	96.6	0	0	1
		30 kDa	y = 0.294 – 0.284 x	0.732	<0.05						
	Bacteria	0.2 µm	y = 0.572 – 0.600 x	0.133	NS	NS	0	0	0	0	2
		30 kDa	y = 0.273 – 0.247 x	0.281	NS						
11-01-05	Filamentous cyanobacteria	0.2 µm	y = 0.187 – 0.030 x	0.018	NS	<0.05	0.354	84.4	0	0	1*
		30 kDa	y = 0.419 – 0.354 x	0.676	<0.01						
	Bacteria	0.2 µm	y = 0.030 + 0.145 x	0.042	NS	<0.01	0.868	100.5	0	0	1
		30 kDa	y = 0.864 – 0.868 x	0.769	<0.001						
23-03-06	Filamentous cyanobacteria	0.2 µm	y = -0.278 + 0.288 x	0.288	NS	NS	0	0	0	0	2
		30 kDa	y = -0.125 + 0.185 x	0.194	NS						
	Prochlorophytes	0.2 µm	y = 0.197 – 0.019 x	0.007	NS	NS	0	0	0	0	2*
		30 kDa	y = 0.211 – 0.002 x	0.000	NS						
	Unicellular cyanobacteria	0.2 µm	y = 1.163 – 1.247 x	0.530	<0.01	NS	0	0	1.247	107.2	3
		30 kDa	y = 1.103 – 1.177 x	0.482	<0.05						
	Algae	0.2 µm	y = 0.154 + 0.197 x	0.161	NS	NS	NA	NA	0	0	2 (5)
		30 kDa	y = 0.468 – 0.137 x	0.163	NS						
	Bacteria	0.2 µm	y = 0.300 – 0.192 x	0.154	NS	NS	0	0	0	0	2 (3)
		30 kDa	y = 0.302 – 0.190 x	0.208	NS						
04-04-06	Filamentous cyanobacteria	0.2 µm	y = -0.121 + 0.483 x	0.847	< 0.001	NS	NA	NA	NA	NA	4*
		30 kDa	y = 0.011 + 0.334 x	0.663	<0.01						
	Prochlorophytes	0.2 µm	y = -0.180 + 0.406 x	0.600	<0.01	NS	NA	NA	NA	NA	4
		30 kDa	y = -0.036 + 0.324 x	0.536	<0.01						
	Unicellular cyanobacteria	0.2 µm	y = 0.444 – 0.452 x	0.369	<0.05	NS	0	0	0.452	101.8	3*
		30 kDa	y = 0.530 – 0.544 x	0.546	<0.01						
	Algae	0.2 µm	y = -0.322 + 0.514 x	0.771	<0.001	<0.01	NA	NA	NA	NA	5*
		30 kDa	y = 0.300 – 0.168 x	0.283	NS						
	Bacteria	0.2 µm	y = 1.703 – 0.781 x	0.630	<0.01	NS	0	0	0.781	45.9	3
		30 kDa	y = 1.260 – 0.298 x	0.212	NS						

Table 4.4 Results of all 5 dilution experiments. Equations describing linear regression of apparent growth of different plankton groups (prey) versus fraction of whole lake water are given. The linear fit of regression analysis is given by R2 and p (ANOVA). The significance between the slopes of the regressions of the 0.2 µm and 30 kDa dilution series is given as p (F-test). Viral mediated mortality is expressed as viral mortality rate (Mv d-1) and the % of potential phytoplankton production which is removed by viral lysis. Grazer mediated mortality is expressed as grazing mortality rate (Mg d-1) and the % of potential phytoplankton production which is removed by microzooplankton grazing. The results of each experiment correspond to type 1 – 5 results, of which representatives are shown in figure 4 (*). NS = not significant; NA = not applicable.

Chapter 4

Discussion

To estimate viral lysis and microzooplankton grazing rates on different plankton groups simultaneously, an extended version of the dilution technique was employed (Landry & Hassett 1982, Evans et al. 2003). The advantage of the dilution method compared to other methods to estimate viral impact is that it gives an essential insight into the quantitative significance of both viral lysis and zooplankton grazing without the need of conversion factors. The dilution technique is essentially based on two critical assumptions. First, the plankton mortality rates due to predation are assumed to be proportional to the dilution effect on predator abundance (Landry et al. 1995). This implies that the abundances of predators should not change during the experiment, leaving the initial predator dilution gradient intact (Gallegos 1989, Landry 1993). VLP counts at the start and end of each experiment indicated that this assumption indeed holds for viral abundances. The initial viral dilution series were maintained over time, despite the long incubation times applied during the present experiments. Only two samples out of 36 had to be omitted from further analysis due to a relatively strong increase in viral numbers.

The first assumption also implies that the per capita predation rate by the predators remains constant at all dilutions (Gallegos 1989, Redden et al. 2002). Viral infection rates are indeed mainly density dependent (Murray & Jackson 1992, Fuhrman 1999), but microzooplankton clearance rates have been reported to alter in response to phytoplankton density in some studies (Evans & Paranjape 1992, Gallegos & Jordan 1997, Strom et al. 2001). Especially in environments with high plankton concentrations, ingestion rates of consumers may be saturated at natural food concentrations, resulting in a nonlinear relationship between net plankton growth and grazer density (Landry 1993). In the present study saturated microzooplankton grazing on unicellular cyanobacteria was observed during the spring experiments (type 3 results). Therefore the approach of Gallegos (1989) was applied to estimate grazing induced mortality, revealing that >90 % of the potential unicellular cyanobacterial production was removed by microzooplankton grazing in the spring experiments. When linear feeding kinetics are assumed and traditional linear regression is applied to these data sets, the microzooplankton mortality impact is overestimated, resulting in a grazing induced mortality of about 100% of potential unicellular cyanobacterial

production for the spring experiments (Table 4.4).

The second assumption of the dilution method implies that plankton growth is exponential and independent of the dilution factor (Landry 1993). Plankton growth should therefore not be limited by regenerated nutrients. In the present experiment it was tried to prevent phytoplankton growth limitation by nutrient additions (N and P) and incubation at growth saturating light. An additional advantage of nutrient enrichment is that nutrient-limited growth can be detected by including undiluted lake water samples which did not receive nutrient additions and comparing apparent growth rates (Andersen et al. 1991). Plankton growth in Lake Loosdrecht did not appear to be limited by N and P during any of the experimental periods. This is not surprising however, since nutrients generally become limiting later in the year in favourable conditions of light and temperature (Hecky & Kilham 1988, Wetzel 2001).

Worden and Binder (2003) reported that the addition of nutrients stimulated zooplankton induced mortality rates of unicellular cyanobacteria in oligotrophic environments, leading to an overestimation of microzooplankton grazing rates. This is probably due to improvement of food quality of the cyanobacterial cells in nutrient-replete versus nutrient-limited conditions. Addition of nutrients has also been reported to lead to an increase in viral burst size, thereby increasing viral production (Wilson et al. 1998, Weinbauer et al. 2003, Gons et al. 2006), which could lead to an overestimation of virally induced mortality in the present experiment. The effect of nutrient addition is thought to be of less importance for both microzooplankton grazing and viral burst size in the eutrophic Lake Loosdrecht, since these nutrients did not appear to be limiting during the experimental periods.

When applying the dilution technique, the slope of the regression line of apparent growth versus fraction of whole lake water is expected to be negative in case of significant mortality impact of microzooplankton and/or virioplankton (type 1 results), or neutral when grazing and/or viral lysis are not influencing phytoplankton mortality (type 2 results). Contrary to expectations, the apparent growth rate of filamentous cyanobacteria and prochlorophytes decreased significantly upon dilution of virioplankton and/or microzooplankton during the experiments of November 2004 and April 2006, resulting in a positive slope of both 0.2 µm and 30 kDa regression lines (type 4 results). The decrease of apparent growth rate in the highly diluted lake water was probably due to the

removal of particles >0.2 µm, since the dilution gradient in the 30 kDa series did not have an additional effect on apparent growth rate.

Since apparent growth rate is a function of mortality rate and instantaneous growth rate, either the grazing induced mortality rate increased or the instantaneous growth rate decreased upon dilution of the whole lake water with 0.2 µm filtered lake water. Both possibilities are not in agreement with the critical assumptions of the dilution technique. An increase in grazing induced mortality upon the dilution of the microzooplankton community is difficult to explain. A decrease in instantaneous growth rate upon dilution could occur if the filtrated water was contaminated with substances that negatively affected phytoplankton growth (Landry 1993). Since type 4 results were not observed in most other experiments using the same methodology, this potential explanation is also considered to be unlikely. Another explanation could be that growth of phytoplankton was actually stimulated by zooplankton activity, which could fuel the microbial loop trough fecal pellets and mechanisms such as sloppy feeding (Peduzzi & Herndl 1992, Strom et al. 1997). It is most plausible however that through the dilution of bacteria in the 0.2 µm series, nutrients other than N and P became limiting due to a reduction in nutrient remineralization (Hagstroem et al. 1984, Lancelot & Billen 1984), resulting in a decrease in phytoplankton instantaneous growth rate.

The same explanations could also be applicable to the significant decrease in apparent algal growth upon dilution of the microzooplankton community during the April experiment (type 5 result). In this case however, apparent algal growth was significantly higher in the samples where the viral population was reduced, suggesting an impact of viral lysis on algal mortality. The instantaneous growth rate may also have decreased in the 30 kDa dilution series due to the dilution of bacteria and therefore of nutrient regeneration, which was countered by the reduction in virally induced mortality upon dilution, resulting in a net neutral relationship between apparent algal growth rate and fraction of whole lake water. Interestingly, type 4 and 5 results were only obtained for filamentous cyanobacteria and algae during spring and autumn, but never during winter. It might be that growth substrates other than N and P became limiting for phytoplankton growth only during spring and autumn.

The type 4 and type 5 results confirm the notion that the dilution approach is only a simple manipulation of a complex food web (Gallegos 1989), and

thus caution should be taken when interpreting the results. Processes such as remineralization of nutrients due to viral lysis (Bratbak et al. 1992, Fuhrman 1999, Suttle 2000b) zooplankton fecal pellets and sloppy feeding (Strom et al. 1997) and chemotrophic bacterial activity (Fuhrman 1992) can potentially complicate the results of the dilution technique. Interactions between zooplankton and virioplankton, such as grazing of VLP by nanoflagellates (Gonzalez & Suttle 1993, Bettarel et al. 2005) or stimulation of viral production by grazing (Weinbauer et al. 2003) could also influence the outcome of the dilution technique. The exact influence of these processes on the results of the dilution technique most likely depend on the trophic state, abundances of prey and predators and the type of plankton of the environment under study. In previous research the dilution technique was shown to give reliable virally induced mortality estimates (Evans et al. 2003, Baudoux et al. 2006) when tested with laboratory cultures and against actual mortality rates in the field, respectively. More testing of the dilution technique is needed however to obtain the optimal incubation times, and experimental procedures (such as nutrient additions) to asses accurate viral lysis rates for different plankton groups and environments. The adapted dilution technique of Andersen (1991) could be applied in future experiments to identify and quantify nutrient sources, such as remineralization.

During the January experiment viral lysis was found to have a significant impact on bacterial mortality, potentially removing 87 % of the standing stock of bacteria, indicating that 100 % of the bacterial production in Lake Loosdrecht at that time could be removed by viral lysis. Together with estimates reported for other eutrophic lakes, this viral mortality rate of bacterioplankton in lake Loosdrecht is at the high end of viral mortality estimates published for a variety of freshwater environments, which are mostly based on transmission microscopic estimates and range from 0 to 130 % (Weinbauer et al. 2002, Jacquet et al. 2005). Using the dilution approach in the mesotrophic lake Bourget, Jacquet et al. (2005) observed that viral lysis could remove between 29 and 114 % of the bacterial standing stock and between 35 and 60 % of potential daily bacterial production.

To obtain a correct estimate of virally induced mortality with the dilution approach, the incubation time should preferably correspond to the length of one lytic cycle, which is often considered to correspond to the growth rate (μ) of the host (Jacquet et al. 2005). Since in the present study the effect of

viral lysis on different plankton groups was studied during single experiments, the applied incubation time might not have been optimal for all plankton groups. The estimated instantaneous growth rate of bacteria during the January experiment and of unicellular cyanobacteria during the March experiment was > 1.0 d^{-1}, indicating that an experimental period of 24 hrs would have been more accurate to estimate viral mortality rates. The estimated viral mortality rate of bacterioplankton during the January experiment might therefore have been an overestimation. Instantaneous growth rates estimated for the filamentous cyanobacteria, prochlorophytes and algae, never exceeded 0.5 d^{-1}, indicating that the applied incubation times might have been too short for these plankton groups, possibly leading to an underestimation of actual viral activity. This problem could be solved in the future by applying different sampling times for each plankton group during the same dilution experiment.

During the winter experiments viral lysis potentially removed between 28 and 35% of the standing stock of filamentous cyanobacteria per day, corresponding to 97 and 84% of potential filamentous cyanobacterial production. These rates are comparable to the viral mortality rates obtained for the haptophyte *Phaeocystis globosa* (Baudoux et al. 2006) and the chlorophyte *Micromonas pusilla* (Evans et al. 2003) when applying the dilution approach in marine environments, but at the high end of viral mortality rates reported for marine cyanobacteria (Proctor & Fuhrman 1990, Suttle 1994, Ortmann et al. 2002, Mann 2003, Brussaard 2004, Hewson et al. 2004). Contrary to viral lysis, grazing did not seem to influence plankton mortality during winter. Even though lower growth and grazing rates of zooplankton may be expected in winter (Gulati et al. 1992, Wetzel 2001), it is surprising that grazing did not even seem to have an impact on bacterial mortality. These results indicate that viral lysis was the main mortality cause in winter, and also imply that the nutrients released upon viral lysis of the filamentous cyanobacteria and bacteria were recycled within the microbial loop (Gobler et al. 1997, Noble & Fuhrman 2000).

In spring, however, grazing by microzooplankton was identified as a significant mortality source of unicellular cyanobacteria and bacteria, whereas little viral activity could be detected during this period. Jacquet et al (2005) also reported lower viral lysis rates in comparison to microzooplankton grazing rates during spring. Interestingly microzooplankton grazing was especially found to impact the mortality of small, edible plankton groups, whereas viral lysis was identified as a

significant mortality source of the large, poorly edible filamentous cyanobacteria in Lake Loosdrecht (Gulati et al. 1992, DeMott et al. 2001, Pel et al. 2003). Pel et al. (2003) identified algae as the main food source of zooplankton in Lake Loosdrecht using stable isotope analysis, whereas during the present study no impact of zooplankton grazing on algal growth could be detected. Only viral lysis seemed to influence algal growth during the spring experiments. These contrasting results might be due to the fact that Pel et al. (2003)mainly studied the diet composition of larger bodied zooplankton whereas we studied the mortality impact of microzooplankton.

In summary, the present study revealed viral lysis as a potential important mortality source of bacterial and filamentous cyanobacterial populations in a shallow eutrophic lake during winter time, when microzooplankton grazing did not have a detectable impact on plankton mortality. Microzooplankton grazing was identified as the major mortality source of unicellular cyanobacterial and bacterial mortality during spring, when viral lysis only seemed to influence algal mortality. The shape of the relationship of apparent growth rate vs. fraction of whole lake water and the impact of viral lysis and microzooplankton grazing was found to vary strongly between different experimental periods and plankton groups however. The dilution approach gave a valuable insight into the relative importance of both viral lysis and zooplankton grazing on different plankton groups in Lake Loosdrecht, but care should be taken when interpreting the results and to optimize the application of this technique to different environments and plankton groups.

To our knowledge, this study is the first to assess the mortality impact of both viral lysis and zooplankton grazing on cyanobacteria and algae in a freshwater environment.

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Chapter 5

Mechanisms and rates of viral decay in a shallow eutrophic lake

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Summary

As viruses are identified as a significant mortality source of both bacteria and algae in aquatic environments, it is important to understand the processes regulating viral abundance and infectivity. Loss mechanisms and rates of viruses in eutrophic lake water were estimated by adding trace amounts of cyanophage particles (LPP-1) to Lake Loosdrecht water and by following the loss in plaque forming units (PFU) on a lawn of the filamentous cyanobacterial host (*Leptolyngbya boryana*) over time. Viral decay rates in the lake ranged between 0.85 and 0.98 d^{-1}, corresponding to turnover times of 1.0 and 1.2 days. In addition the lake water received a variety of treatments to determine the impacts of different environmental factors on the viral decay rate. Environmental factors tested included total suspended matter, high molecular weight – dissolved organic matter (HMW-DOM), enzymes and other heat-labile compounds, UV radiation, bacteria and nanoflagellates. Contrary to other studies, UV radiation did not have a negative impact on viral infectivity, probably due to the high attenuation of UV light in the lake water. Bacteria were also not observed to affect viral decay rates. The addition of HMW-DOM resulted in a significant decrease in viral decay rate (0.023 h^{-1}, $p < 0.01$), suggesting that the abundant HMW-DOM in the lake prolongs viral infectivity. Eukaryotic organisms smaller than 10 µm were found to be responsible for 37 to 55 % of the daily viral decay, inactivating approximately 2.4 - 3.5 10^7 viruses per day, thus implying that grazing by heterotrophic nanoflagellates was the major viral decay source in Lake Loosdrecht.

Introduction

The mechanisms and rates of viral decay in aquatic environments have been of interest for almost half a century. Traditionally the interest mainly concerned the survival of enteric pathogenic viruses, the processes controlling their survival and the implications for waste- and drinking water treatment (e.g. Mitchell & Jannasch 1969, Kapuscinski & Mitchell 1980, Ward et al. 1986). The discovery of high numbers of viruses in aquatic environments (Bergh et al. 1989) not only fuelled research into the impact of viral infection on aquatic food webs, but also revived the interest in the mechanisms regulating viral decay. Viral lysis has since been identified as an important mortality factor of bacteria and algae (Proctor & Fuhrman 1990, Nagasaki et al. 1994), resulting in a recycling of nutrients within the microbial loop (e.g. Middelboe et al. 1996, Fuhrman 1999). It is therefore of ecological importance to understand the processes regulating viral abundance and infectivity.

So far, a large variety of mechanisms have been reported to control viral decay rate. Especially UV radiation, in particular UV-B, is considered to have a strong damaging effect on viral particles (Kapuscinski & Mitchell 1983, Suttle & Chen 1992, Wommack et al. 1996, Noble & Fuhrman 1997, Wilhelm et al. 1998). In the absence of sunlight, adsorption of viral particles onto particulate material and high molecular weight - dissolved organic matter (HMW-DOM) has been reported as an important source of viral infectivity loss (Suttle & Chen 1992, Schneider et al. 1996, Noble & Fuhrman 1997, Lu et al. 2002, Anesio et al. 2004). Other factors reported to enhance viral decay are enzymatic degradation, adsorption onto and degradation by bacterial cells, temperature, as well as grazing by nanoflagellates (Kapuscinski & Mitchell 1980, Ward et al. 1986, Moebus 1992, Gonzalez & Suttle 1993, Noble & Fuhrman 1997, Bettarel et al. 2005).

Between the viral decay studies, there is considerable variation in reported rates of viral decay and responsible mechanisms, however. The anti-viral effect of bacteria could not be detected in all studies (Suttle & Chen 1992), and results have been contradictory in case of the impact of nanoflagellate grazing and adsorption onto particulate material and DOM, with some studies actually reporting a positive impact of these factors on viral survival and productivity in aquatic environments (Mitchell & Jannasch 1969, Bitton & Mitchell 1974, Kapuscinski & Mitchell 1980, Sime-Ngando & Ram 2005, Weinbauer et al.

2007). These variations might be largely due to differences in the biological and environmental conditions of the environments studied (Gerba 1984, Weinbauer 2004).

In the present study the mechanisms and rates of loss of viral infectivity were studied in Lake Loosdrecht (The Netherlands). Lake Loosdrecht is a well mixed, shallow, eutrophic lake originating from peat excavation. The lake has a low transparency which is mainly due to particulate detritus and the predominating filamentous cyanobacterial community in the lake. Previous research indicated the presence of virally infected filamentous cyanobacteria, suggesting that viral infection might have a significant impact on cyanobacterial mortality in the lake (M. Tijdens, unpubl. data). Lake water enclosure experiments indeed repeatedly showed a dramatic collapse of the cyanobacterial community, which could be associated with viral activity (Van Hannen et al. 1999, Gons et al. 2002, Simis et al. 2005). For Lake Loosdrecht itself no such mass lysis event has ever been reported however, indicating that in the natural environment conditions prevail which prevent such events. Possible explanations for this phenomenon might be resistance to viral infection among the cyanobacterial hosts (Barnet et al. 1981, Waterbury & Valois 1993) or a high viral decay rate in the natural environment.

The rate of viral infectivity loss was studied using cyanophage LPP-1, which infects the filamentous freshwater cyanobacterium *Leptolyngbya boryana* (Safferman & Morris 1963, 1964). Trace amounts of the virus were added to different treatments of Lake Loosdrecht water to follow the decrease in plaque forming units (PFU) over time and to determine the impacts of the factors total suspended matter, HMW-DOM, enzymes and other heat-labile compounds, UV radiation, bacteria and nanoflagellates on the viral decay rate. Due to the low transparency and the high concentrations of organic matter of Lake Loosdrecht, we hypothesized that not UV irradiation but adsorption of viral particles onto particulate material and HMW-DOM would impact viral decay rates during this study.

Materials and Methods

Study site

The highly eutrophic (Chlorophyll-*a* annual average of ca 60 mg m^{-3}; P_{total} = 40 – 60 µg l^{-1}; and N_{total} = 1.4 – 1.9 mg l^{-1}), shallow (mean depth 1.85 m) and turbid (Secchi depth approximately 0.5 m) Lake Loosdrecht is located at about 25 km southeast of Amsterdam in the Netherlands. Low water transparency is due to high concentrations of phytoplankton, particulate detrital material, and coloured dissolved organic matter. The detritus mainly consists of peat fragments and fine-sized (<15µm) particles (Gons et al. 1992), with particulate organic matter (POM) values ranging between 11.9 and 27.3 g m^{-3} and mineral fractions (MF) ranging between 1.3 and 7.8 g m^{-3}. The high DOC level - up to > 12 mg C l^{-1} (Hofstra & van Liere 1992) - is characteristic for lakes originating from peat excavation. The plankton is predominated by a group of related filamentous cyanobacteria belonging to the *Limnothrix* / *Pseudoanabaena* group (Van Tongeren et al. 1992, Zwart et al. 2004). The second most abundant population of filamentous cyanobacteria closely resembles *Prochlorothrix hollandica,* a cyanobacterium which does not contain phycobilins but does possess Chl *b* (Burger-Wiersma et al. 1986). The population of eukaryotic algae is relatively sparse and comprises mainly of diatoms and green algal species (De Kloet et al. 1990). Bacterial and viral concentrations in the lake range from 1.0 x 10^6 to 9.0 x 10^6 ml^{-1} and from 6.0 x 10^7 to 13.5 x 10^7 ml^{-1}, respectively (M. Tijdens, unpubl. data). Nanoflagellate concentrations in the lake range from 1.0 x 10^4 to 9.0 x 10^4 $ml^{-1}$and the population is mainly composed of flagellates smaller than 10 µm resembling *Bodo spp.* (M. Tijdens, unpubl. data). The zooplankton community in the lake is dominated by small-bodied cladocerans, mainly *Bosmina* spp., cyclopoid copepods, and small rotifers such as *Keratella, Anuraeopsis, Filinia* and *Polyarthra* spp. (Gulati et al. 1992, Ooms-Wilms et al. 1999). In summer large-bodied rotifers such as *Asplanchna* and *Euchlanis* can be prominent (Gulati et al. 1992).

Cyanobacterium-cyanophage culture

The cyanobacterial host culture *Leptolyngbya boryana* and the cyanophage LPP-1 were obtained from the American Type Culture Collection (ATCC No.18200 and No.18200-B1, respectively). *L. boryana* is a filamentous freshwater

cyanobacterium, previously characterized as *Plectonema boryanum* (Safferman & Morris 1963). Host cultures were incubated on CHU No 10 medium (Stein 1973) at 80µmol quanta m^{-2} s^{-1} photosynthetically active radiation (PAR), 12 : 12 h light : dark cycle at 20°C under continuous mixing on an orbital shaker. LPP-1 is a lytic, dsDNA cyanophage with a capsid diameter of 60 nm and a short tail of 20 nm in length and 15 nm in diameter, which belongs to the Podoviridae and was isolated from a waste stabilization pond (Safferman & Morris 1963, Padan & Shilo 1973). For cultivation 0.75 ml of LPP-1 lysate was added to 25 ml of an exponentially growing host culture; the 0.2-µm filtered lysate was added to a magnesium salt solution (1:1 vol / vol; 0.2g $MgCl_2.6H_2O$, 5.85 g NaCl in1000 ml of distilled water) and stored at 4°C for optimal preservation (Safferman & Morris 1964).

Plaque counts

An exponential growing host culture was concentrated 10 times by centrifugation at 5000 g for 15 minutes. CHU No10 soft agar (0.6% w / v) was kept at 42°C in a hot water bath, of which 3 ml was mixed with 0.75 ml concentrated host solution in sterile culture tubes, before pouring onto 1.5% CHU No10 agar plates. Viral dilution series were prepared in magnesium salt solution before carefully spotting 4 µl drops of 4 different viral dilutions in tenfold onto the solidified host lawn of a single plate (Loc Carrillo et al. 2005). The viral drops were allowed to absorb into the overlay agar before incubation at 80 µmol quanta m^{-2} s^{-1} photosynthetically active radiation overhead light, 12 : 12 hrs light : dark cycle at 20°C. After 3 to 4 days separate plaques could be counted within the 10 different spots of 1 or 2 of the applied viral dilutions, yielding the number of plaque forming units (PFU) ml^{-1}. This 'plaque count by spotting' procedure allows for the simultaneous observation of a great number of replicate experiments and viral dilutions. In case of LPP-1 and *L. boryana* this procedure did not result in a significant different outcome when compared with the classical agar layer method (Adams 1959) to determine the concentration of PFU (data not shown).

Total viral counts were obtained with a Zeiss, Axiophot epifluorescence microscope, following staining of 1% formaldehyde fixed samples with SYBR Green I (Molecular Probes, Leiden, The Netherlands), as described by Noble and Fuhrman (1998).

Sampling

Surface water was collected from Lake Loosdrecht (The Netherlands) on the 18th of October and the 2nd of November in 2005. Upon sampling Secchi-disk depth, pH and water temperature were determined. Samples were transported to the laboratory in high density polyethylene-containers and processed or fixed within 3 h after sampling.

Plankton counts

Flow cytometry (FCM) was used in the first (October) experiment to distinguish different cyanobacterial groups and eukaryotic algae. Samples were fixed with 0.01% paraformaldehyde and 0.1% glutaraldehyde (final volume), and stored at 4°C in the dark for no longer than 2 days until processing. The MoFlo flow cytometer (Dako, Ft. Collins, Co., USA) was equipped with a 70 micron flowcell and running at 60 psi and 100 mW excitation; 488 nm. A multitriggerboard was used to trigger on combinations of side scatter and orange (630/30 nm) and red (670/30 nm) fluorescence. Typically, cell clusters of filamentous cyanobacteria, prochlorophytes (Turner et al. 1989), and eukaryotic algae were distinguished by their differential clustering in orange and red fluorescence. The classification of these clusters was previously confirmed for Lake Loosdrecht by light microscopy examination and fatty acids analysis after cell sorting (Pel et al. 2004). A small volume of a known concentration of 1-µm diameter fluorescent beads (Polyscience, Warrington, USA #15702) was added for exact volume determination.

Flagellates were counted after FITC staining according to the protocol of Sherr and Sherr (in Kemp et al. 1993). In brief, initial lake water sample was fixed with 1% glutaraldehyde (final volume) and stored at 4°C until further processing within a week. Between 1 and 5 ml of sample was gently filtered on a 0.8 µm black polycarbonate filter (Nuclepore, Whatman, Brentford, United Kingdom), after which the filter was flooded for 7 minutes in 0.2-µm prefiltered FITC-staining solution (0.25 ml of 0.5M sodium carbonate buffer (pH 9.5), 1.1 ml of 0.01M potassium phosphate buffer (pH 7.2), 1.1 ml of 0.85% sodium chloride and 1.0 mg of FITC (Sigma-Aldrich, Zwijndrecht, The Netherlands). After gently rinsing the filter twice in cold 0.5 M sodium carbonate buffer (pH 9.5), the filter was mounted on a glass slide with immersion oil (pH 9.5) and at least 200 flagellates were enumerated using a Zeiss Axiophot epifluorescence microscope at blue light excitation.

Solar irradiance and vertical attenuation of UV light

Solar irradiation was measured on the roof of the Centre for Limnology using a pyranometer model CM11 with Solar integrator (Kipp & zonen BV, Delft, The Netherlands). The CM11 sensor head captures a 2π sr angle, and has a spectral range of 305-2800 nm (50%) and 335-2200 (95%).

The vertical diffuse attenuation coefficient (K_d) of Lake Loosdrecht water at the time of the experiment was obtained from absorption and scattering according to Kirk (1981). A PerkinElmer Lambda 800 UV/Vis spectrophotometer was used to measure the optical properties of untreated lake water suspension and of separated fractions of particulate matter and coloured dissolved organic matter (CDOM). CDOM was measured after filtration over 0.2 µm cellulose acetate filters, in 5 cm optical grade class cuvettes in the 288-800 nm wavelength range. These results were extrapolated down to 280 nm by fitting a linear regression line to natural-log transformed data in the 288-488 nm range. Absorption by the lake water suspension was measured in a 1-cm quartz cuvette (280-800 nm) inside an integrating sphere (Labsphere Inc, North Sutton, NH, USA), while scattering was obtained from the difference of the latter measurement with a measurement of the suspension at a distance (~10 cm) from the detector in standard dual beam configuration. Particulate absorption was obtained from the difference between a sample concentrated on a Whatman GF/F filter and a blank reference filter, measured inside the integrating sphere, covering the 280-800 nm range. The absorption and scattering by pure water were neglected for the UV-A (320-400 nm) and UV-B (280-320) wavelength range.

Decay experiments

To determine the impact of different factors on the viral decay rate in Lake Loosdrecht water, the water received a series of treatments. To measure the effect of UV on viral decay rate, 800-ml lake water samples were incubated on the roof of the Centre for Limnology in 2 different types of incubation bottles: 1 l polycarbonate bottles, transmitting PAR and both short and long wavelength UV and 1 l glass bottles, transmitting only PAR (for transmission spectra of incubation bottles see Van Donk et al. 2001). The incubation temperature was maintained between 18 and 20°C, and samples were mixed 5 times a day.

For all other treatments, 75 ml lake water was incubated in the laboratory in glass Erlenmeyer flasks at 80 µmol quanta m^{-2} s^{-1} PAR, 12 : 12 h light : dark

cycle at 20°C under continuous mixing on an orbital shaker. Different filtration treatments were applied to determine the impact of particulate material on the viral decay rate. Lake water was gently filtered over 1) 10-µm mesh plankton filters; 2) 1-µm filters (Durapore, Millipore, Billerica, MA, USA), and 3) 0.2-µm filters (Durapore, Millipore, Billerica, MA, USA). To determine the impact of HMW-DOM, the 0.2-µm prefiltrated lake water was filtrated over a 30 kD PES membrane filter using the VivaFlow 200 assembly (VivaFlow, Vivasciences, Hannover, Germany) to remove the DOM. In addition, one treatment received the concentrated DOM obtained with the above procedure, resulting in approximately doubled DOM concentrations. Autoclaving of lake water was applied to determine the impact of biological activity and heat-labile compounds such as extracellular enzymes and DNA on the viral decay rate in the lake. Additionally, cycloheximide (200 mg l^{-1}), an inhibitor of protein synthesis in eukaryotes, was added to determine the impact of eukaryotic organisms on the viral decay rate. Untreated Lake Loosdrecht water served as a control. All treatments were incubated in triplicate for 74 h after addition of a trace amount of fresh 0.2-µm filtered LPP-1 lysate which did not receive the addition of the magnesium salt solution (5 10^5 PFU ml^{-1}). Samples were retrieved for plaque counts at = 0, 7, 24, 46 and 74 h after the start of the experiment and processed as described above. The October experiment (see Sampling) included all of the above mentioned treatments, whereas the November experiment only included the 10, 1.0 and 0.2-µm filtration and cycloheximide addition treatments. During the November experiment a treatment receiving both the 1.0-µm filtration and the cycloheximide addition was also included, to check if this would result in an additional decrease in viral decay rate. Negative controls were performed during each experiment by spotting the original lake water onto the cyanobacterial host lawn; no plaques were found.

Data analysis

Specific decay rate (k, h^{-1}) was determined by plotting log transformed PFU ml^{-1} against time, with the slope of the regression line representing the decay rate (Heldal & Bratbak 1991). For all treatments the fit of these regression lines was highly significant (data not shown). To test if treatments had a significant impact on viral decay rate an F-test (one-way ANOVA) in combination with the Tukey HSD post hoc test was performed using the Statistica software package

(StatSoft Inc., Tulsa, OK, USA) after checking the dataset for normal distribution (Kolmogorov-Smirnov test) and homogeneity of variance (Bartlett, Hartley and Cochran's test).

Results

Starting conditions in Lake Loosdrecht

Physical and biological characteristics of Lake Loosdrecht at the start of the two experimental periods are shown in Table 5.1. Filamentous cyanobacteria were the most abundant phytoplankton group in the lake, but their abundances were lower as normally observed in October (chapter 2). Viral, bacterial and flagellate abundances were lower at the start of the November experiment than in the previous month.

Table 5.1 Physical and biological characteristics of Lake Loosdrecht at the onset of the experiments. NA is not available.

		18 October 2005	02 November 2005
pH		7.9	7.8
T	°C	10.0	9.5
Secchi	cm	56	49
Cyanobacteria (trichomes)	ml^{-1}	$9.85\ 10^4$	NA
Prochlorophyte (trichomes)	ml^{-1}	$2.57\ 10^4$	NA
Eukaryotic algae	ml^{-1}	$2.89\ 10^4$	NA
Flagellates	ml^{-1}	$7.33\ 10^3$	$7.01\ 10^3$
VLP	ml^{-1}	$8.62\ 10^7$	$7.52\ 10^7$
Bacteria	ml^{-1}	$7.34\ 10^6$	$6.28\ 10^6$

Decay experiment October

The decay rate of infectious LPP-1 particles was 0.0355 h^{-1} in the untreated Lake Loosdrecht water during the October experiment (Figure 5.1, control treatment). The removal of particulate material larger than 10 µm did not lead to a significant reduction in decay rate (Figure 5.1, 10 treatment), whereas the removal of particulate material larger than 1 µm did lead to a significantly longer survival of viral particles ($p < 0.001$), resulting in a 50% reduction in decay rate

(0.0179 h^{-1}). Viral infectivity was also preserved better when Lake Loosdrecht water was autoclaved and when cycloheximide was added, resulting in decay rates comparable as obtained for the 1-µm filtered water (0.020 and 0.0199 h^{-1}, respectively).

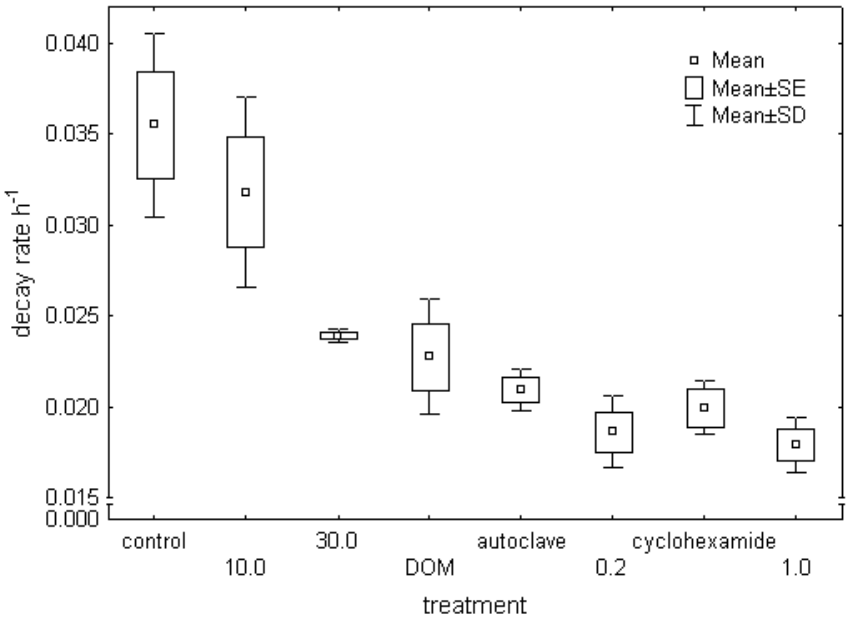

Figure 5.1 Viral decay rates during the October experiment. Box plot of the average, standard deviation (sd) and standard error (se) of the viral decay rates (n = 3) detected in the different treatments of Lake Loosdrecht water. 10 = 10-µm filtration; 1 = 1-µm filtration; 0.2 = 0.2-µm filtration; DOM = addition of HMW-DOM treatment; 30 = 30 kD filtration treatment.

The decay rate of the 0.2-µm filtration treatment did not differ significantly from the 1-µm filtration treatment. The 30-kD filtration treatment did not result in a further decrease in decay rate, but actually led to a slight, but non-significant, increase in decay rate (0.0239 h^{-1}; Figure 5.1, 30 treatment). A positive effect of HMW-DOM on the preservation of viral infectivity was also suggested by a significant reduction in decay rate after addition of HMW-DOM (0.0228 h^{-1}, $p < 0.01$), when compared to the control treatment.

The treatment receiving both PAR and UV irradiation (0.0222 h^{-1}) did not result in an increase in decay rate but to a significant decrease in decay rate compared to the treatment which only received PAR (0.0293 h^{-1}, $p < 0.05$; Figure 5.2). Despite differences in incubation circumstances, the viral decay rate in the treatment

receiving PAR did not differ significantly from the control Lake Loosdrecht water, which was incubated in the laboratory. Measured solar radiation was 2505, 716, 577, and 925 Wh m^{-2} on 18, 19, 20 and 21 October, respectively. The vertical diffuse attenuation coefficient (Kd) obtained from spectrophotometry indicated that UV-A would be attenuated to a level of 10% of the incident UV-A light at 0.15 m depth, while for UV-B light the 10% level occurred already at 0.07 m; with 99% attenuation occurring at twice these depths.

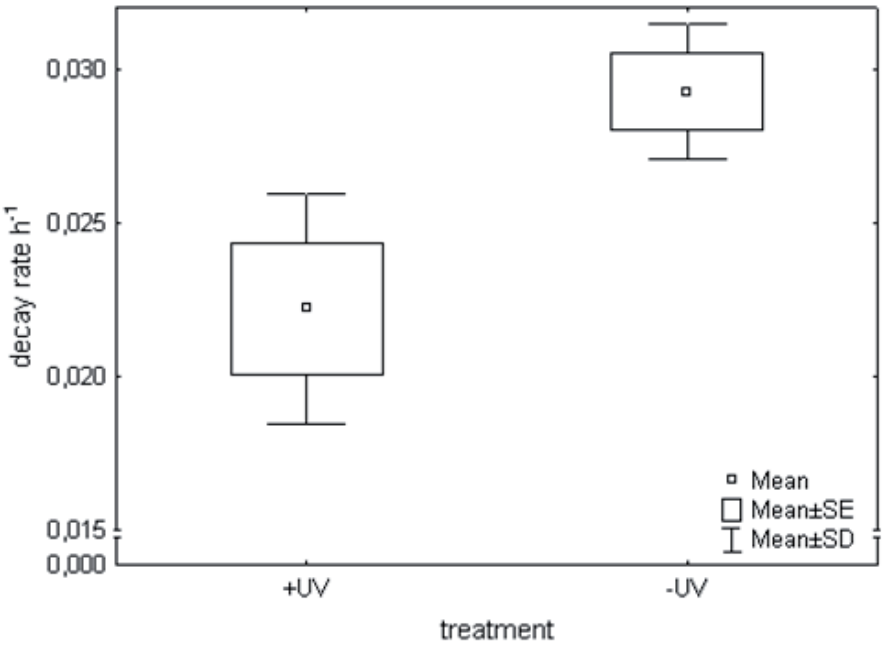

Figure 5.2 Viral decay rates with and without UV radiation. Box plot of the average, standard deviation (sd) and standard error (se) of the viral decay rates (n = 3) detected in the two different treatments of Lake Loosdrecht water. + UV = treatment receiving UV and PAR radiation; - UV = treatment receiving only PAR radiation.

Decay experiment November

The decay experiment was repeated with particular reference to the effects of the cycloheximide treatment and particles larger than 1 µm. The viral decay rate obtained for the untreated Lake Loosdrecht water (0.0410 h^{-1}; Figure 5.3) during the November experiment was comparable with the rate obtained for the same treatment during the October experiment. Again a sig-

nificant decrease in viral decay rate was observed when particles larger than 1 µm were removed (0.0233 h^{-1}, $p < 0.001$; Figure 5.3). Also, the 0.2-µm filtration did not lead to a significant additional change in decay rate (0.0180 h^{-1}, $p = 0.15$). Viral decay rate in the cycloheximide treatment (0.0180 h^{-1}) was similar to the 1-µm and 0.2-µm filtration treatments (Figure 5.3). The treatment receiving both 1-µm filtration and cycloheximide addition (0.0162 h^{-1}) did not have a lower viral decay rate than the cycloheximide treatment, but exhibited an additional decrease in decay rate when compared to the 1-µm filtration treatment ($p < 0.041$). The decay rates obtained for both the 0.2 µm filtration and cycloheximide addition treatments did not differ significantly from the values in October, whereas the decay rate in the 1-µm filtration treatment was significantly higher in November than in October ($p < 0.05$).

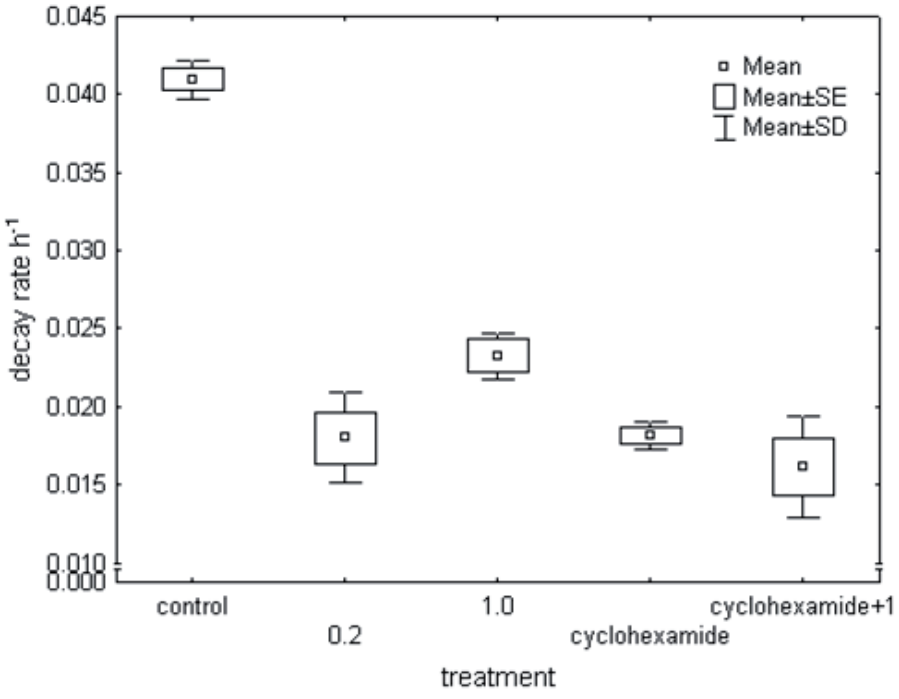

Figure 5.3 Viral decay rates during the November experiment. Box plot of the average, standard deviation (sd) and standard error (se) of the viral decay rates (n = 3) detected in the different treatments of Lake Loosdrecht water. 1 = 1 µm filtration; 0.2 = 0.2 µm filtration; cycloheximide + 1 = treatment receiving both 1 µm filtration and cycloheximide addition.

Discussion

Loss rates of viral infectivity (LRVI) were estimated to range between 0.85 and 0.98 d^{-1}, when using the cyanophage LPP-1 as a tracer in the lake water and following the loss in PFU over time. These rates correspond to turnover times ranging between 1.0 and 1.2 days. Previously published turnover times for aquatic viral communities range between 0.036 and 23.9 days (Wommack & Colwell 2000, Weinbauer 2004). These values have been obtained with various methods, such as measuring tracer dilution rates using fluorescently labelled viruses (Noble & Fuhrman 2000), estimating viral production rates (Proctor & Fuhrman 1990, Weinbauer et al. 2002, Wilhelm et al. 2002) or by addition of cyanide to stop viral production and estimate the loss rate of viral particles (LRVP) (Heldal & Bratbak 1991).

The viral turnover times obtained in the present study are comparable to turnover times reported so far for freshwater environments (Wommack & Colwell 2000, Weinbauer 2004). Applying the same method (LRVI) with enteric viruses in freshwater environments, turnover times between 0.8 and 1.2 days were reported (Ward et al. 1986, Gersberg et al. 1987). Turnover times of freshwater viral communities obtained with the LRVP approach, range between 0.09 and 2.2 days and are mostly lower than the turnover times established in the present study. This finding is surprising since the LRVP method is generally thought to yield lower estimates of viral decay rates, since it detects the decay of viral particles instead of infectivity, and excludes potential impacts of biological factors on viral decay due to the addition of cyanide (Heldal & Bratbak 1991, Noble & Fuhrman 1997).

The obtained results should be interpreted with caution, however, since they have been obtained testing only one type of phage during one season, and decay rates have been reported to vary considerably from between different phages and seasons (Gerba 1984, Suttle & Chen 1992, Noble & Fuhrman 1997). Noble (1997) reported higher losses of infectivity for non-native phages than native phages. The LPP-1 phage has been isolated from a hyper-eutrophic freshwater environment where it infects a filamentous cyanobacterium related to the dominating cyanobacteria observed in Lake Loosdrecht. Furthermore, the LPP-1 phage belongs to the podoviruses, which together with the non-tailed phages have been reported to be most abundant in natural environments (Suttle 2005)

and it is representative of the average size range observed for Lake Loosdrecht viruses (M. Tijdens, unpubl. data) and other aquatic viruses (Safferman & Morris 1963, Weinbauer 2004). The LPP-1 virus might therefore be a good representative of the viral community in Lake Loosdrecht. We would also like to point out the remarkably similar decay rates and impact of the cycloheximide and 0.2 µm filtration treatments obtained during the two experiments, even though performed two weeks apart.

UV irradiation

Viral decay rates did not increase when the lake water was exposed to both UV and PAR irradiation, compared to water which only received PAR. At first glance, this result is surprising since UV irradiation has been reported as the most important cause of viral decay in aquatic environments (Suttle & Chen 1992, Wommack et al. 1996, Noble & Fuhrman 1997), accounting for 25 to 66 % of the total viral decay. UV irradiation is a potential mechanism causing viral DNA and capsid or tail protein damage (Hartman & Einsenstark 1982, Ronto et al. 1992). Sensitivity to UV irradiance has been reported to vary between different phages due to the formation of resistance mechanisms against DNA damage (Noble & Fuhrman 1997, Garza & Suttle 1998, Kellogg & Paul 2002) and the ability to repair DNA damage within the host (Bernstein 1981). The absence of UV impact on viral decay during the present study is, however, most likely due to the high absorption of both UV-A and UV-B by the presence of dissolved organic matter in humic lake water such as Lake Loosdrecht (De Haan 1993, Simis et al. 2005). The high attenuation coefficient in Lake Loosdrecht indicates that the 10% levels of incident UV-A and UV-B light will be as close as 15 and 7 cm to the surface, respectively. Thus, at any given point in time only a small fraction of the viral particles in Lake Loosdrecht is exposed to damaging levels of UV radiation.

Nanoflagellates

The removal of particles between 10 and 1 µm, heat-labile compounds (autoclave treatment), and inactivation of eukaryotic organisms (cycloheximide treatment) resulted in a significant decrease of the viral decay rate of up to 57 %. Since the same reduction in decay rate was obtained in the cycloheximide treatment, even though particles between 10 and 1 µm and heat labile compounds were

not removed from this treatment, it can be deduced that eukaryotic organisms ranging between 10 and 1 µm were responsible for most of the viral decay in Lake Loosdrecht during the experimental period. This was confirmed during the November experiment, when the combined treatment of both 1-µm filtration and cycloheximide addition did not lead to an additional decrease in viral decay rate as compared to the cycloheximide treatment. The significantly higher decay rate detected for the 1-µm November filtration treatment as compared to its October counterpart and the combined 1-µm and cycloheximide treatment, suggests that part of the small eukaryotic population causing viral decay passed the 1-µm filter during the November experiment.

The nanoflagellates are eukaryotic organisms which fit this 1 to 10 µm size range. They have been reported to impact viral decay rates in both marine and freshwater environments (Suttle & Chen 1992, Gonzalez & Suttle 1993, Manage et al. 2002, Bettarel et al. 2005). When comparing the October treatments containing active flagellates with the treatments which did not, a highly significant difference in viral decay rate could indeed be detected (Figure 5.4; $p < 0.001$). Gonzalez & Suttle (1993) demonstrated for natural population densities that marine viral particles can be ingested and digested by nanoflagellates at ingestion rates about 10 times lower than for bacteria. Bettarel et al. (2005) estimated that 4.1 and 0.8 % of viral production was removed by grazing of nanoflagellates in an oligo-mesotrophic and eutrophic lake, respectively. However, whereas in the above studies the grazing loss was only a small fraction of the viral decay, the present results suggest that HNF grazing is the major viral population loss factor. Our estimates imply that HNF grazing represents 37 to 55 % of the daily viral decay, inactivating approximately $2.4 – 3.5\ 10^7$ viruses per day. This would imply that nanoflagellate grazing also remove a significant portion of average-sized viral particles and not only the larger viral particles as previously suggested (Murray 1995, Weinbauer 2004). Interestingly, Manage et al (2002) reported a comparably strong influence of nanoflagellates on viral decay in a hyper-eutrophic pond dominated by cyanobacteria, when studying the decay in total viral particles (LRVP). Since protist grazers are likely to meet a C-surplus in turbid, high DOC and POM lakes such as Lake Loosdrecht, it might be advantageous to consume particles which have a lower C:N:P ratio (Hessen 1992). Viral particles contain 1.6 times as much nitrogen and 3.2 times as much phosporus per unit of carbon as compared to bacterial cells (Gonzalez & Suttle

1993) and might therefore be a preferable food source of the small protists in Lake Loosdrecht.

Contrary to other studies (Mitchell & Jannasch 1969, Ward et al. 1986, Moebus 1992), bacteria did not seem to impact viral decay rate in Lake Loosdrecht, since decay rates in 0.2-µm and 1-µm filtration treatments were comparable between each other and to the cycloheximide treated sample where bacteria were not removed or inactivated. Suttle and Chen (1992) could neither detect an impact of bacteria on viral decay rate.

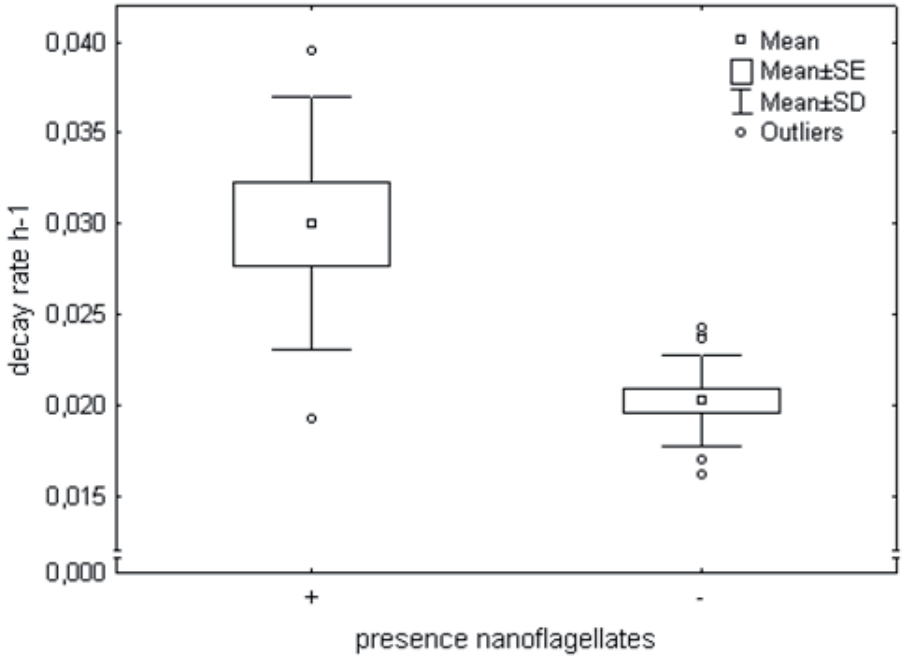

Figure 5.4 Impact of presence of nanoflagellates on viral decay rates. Box plot of the average, standard deviation (sd) and standard error (se) of the viral decay rates (n = 3) detected when nanoflagellates were present and active (+) vs. the treatments where no active nanoflagellates were present (-). The difference between the two groups was highly significant (p = 0.00009).

HMW-DOM

In the October experiment addition of HMW-DOM resulted in a significant reduction in decay rate as compared to the control treatment. Furthermore, viral decay rates increased when HMW-DOM was removed. This result is contrary to reports of a negative effect of HMW-DOM on viral infectivity due

to mechanisms such as adsorption, destruction and lower replication rates of viruses in the presence of HMW-DOM (Schneider et al. 1996, Noble & Fuhrman 1997, Lu et al. 2002, Anesio et al. 2004). Our findings do agree however with reports that presence of humic organic matter and particulate materials can protect and prolong viral survival (e.g. Bitton & Mitchell 1974, Kapuscinski & Mitchell 1980, Gerba 1984). Adsorption of viruses onto particles or colloidal organic matter is thought to offer protection from proteolytic enzymes and UV radiation which inactivate viruses, and to give increased stability of the viral capsid (Gerba 1984).

Ecological implications

When assuming a steady state in viral abundance and infectivity, viral decay will be balanced by viral production (Heldal & Bratbak 1991). The steady state condition would thus imply an average viral production rate of 0.92 d^{-1} in Lake Loosdrecht, corresponding to 6.4 10^7 viruses ml^{-1} per day. With an abundance of 1.2 10^7 cyanobacterial and bacterial cells ml^{-1} in Lake Loosdrecht (Table 5.1; with an average of 52 cells per cyanobacterial trichome), this would mean that between 0.6 and 10 % of the total bacterial community would be infected daily, when assuming viral burst sizes of 50 and 900 viruses per cell, respectively (Padan & Shilo 1973, Weinbauer 2004, Gons et al. 2006).

If the high viral decay rates due to small eukaryotic organisms are indeed the result of nanoflagellate grazing, this would indicate viral particles as a substantial nutrient source of nanoflagellates. Viruses would thus not only impact the aquatic foodweb by the release of cellular debris upon lysis of the host cell (Bratbak et al. 1994, Fuhrman 1999), they would also fuel the flow of nutrients to higher trophic levels due to nanoflagellate grazing. The release of cellular debris upon lysis is generally thought to stimulate heterotrophic bacterial production and respiration and thus to a recycling of nutrients within the microbial loop. This is thought to result in decreased nutrient availability to higher trophic levels (Bratbak et al. 1994, Fuhrman 1999). The nanoflagellate grazing of viral particles could partly compensate for this decrease in nutrient flow to higher trophic levels. To which extent this truly is the case remains to be investigated in future research. Another exciting feature for future research would be to see if nanoflagellate grazing of viral particles can indeed protect bacterial cells from viral infection, as suggested by Murray et al (1995). Murray proposed that

the exudates of healthy phytoplankton indirectly might function as a protection against viral infection as these exudates attract bacteria, which in turn would attract flagellates. This would result in an increased flagellate-induced mortality of viruses and thus to a reduction of viral infection (Murray 1995).

The present study reports on the impacts of a wide variety of different mechanisms on the decay rate of viruses in a freshwater lake. Viral turnover times in the eutrophic lake ranged between 1.0 and 1.2 days and were at the high end of turnover times previously reported for freshwater environments. This might be due to the fact that both UV irradiation and bacteria did not have a detectable impact on viral infectivity and because of the high abundances of HMW-DOM in the lake, which seemed to prolong viral infectivity in Lake Loosdrecht. Eukaryotic organisms smaller than 10 µm were found to be responsible for 37 to 55 % reduction of viral infectivity, suggesting grazing of heterotrophic nanoflagellates was the major cause of viral decay in Lake Loosdrecht.

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Chapter 6

Discussion

Discussion

The present study addressed the population dynamics, diversity, ecological impact and control of the viral community in the shallow, eutrophic Lake Loosdrecht. In **Chapter 2** the virioplankton community was identified as a dynamic component of the aquatic community in Lake Loosdrecht, with abundances ranging between 5.5×10^7 and 1.3×10^8 virus like particles ml^{-1} and viral genome sizes ranging between 30 and 200 kb. The viral community in Lake Loosdrecht was diverse with 5 to 10 different viral genome sizes (**Chapters 2 & 3**) and a multitude of different viral morphotypes (Figure 1.2).

Potential viral hosts in Lake Loosdrecht

The two most abundant plankton groups - the filamentous cyanobacteria and the heterotrophic bacteria - were expected to be potential viral hosts in Lake Loosdrecht, as the chance of viral propagation is linearly dependent on the density of susceptible hosts (Murray & Jackson 1992, Hennes et al. 1995). We indeed observed viral lysis of both the heterotrophic bacterial and filamentous cyanobacterial communities in Lake Loosdrecht, when performing experiments in the laboratory. In **Chapter 3** a series of enclosure experiments was used to study the impact of mass lysis of the cyanobacterial community on the composition of the aquatic community. High percentages of virally infected cyanobacterial cells and an increase in viral numbers during the collapse of the cyanobacterial population indicated that viral lysis was the major cause of filamentous cyanobacterial mortality. The viral lysis of the cyanobacterial bloom was accompanied by changes in both cyanobacterial and heterotrophic bacterial community composition, indicating that viral infection in Lake Loosdrecht not only has the potential to control cyanobacterial blooms, but also has a marked impact on the structure of the plankton community. Viral lysis was also identified as the main mortality cause of filamentous cyanobacteria and heterotrophic bacteria (**Chapter 4)**. Employing an adapted version of the dilution technique, viral lysis was detected to remove between 84 and 97% of the potential filamentous cyanobacterial production and up to 101% of the potential bacterial production in Lake Loosdrecht in winter.

In Lake Loosdrecht prochlorophytes are an important subgroup of the filamentous cyanobacteria. Viral abundances showed a significant positive correlation to prochlorophyte abundances (**Chapter 2**), suggesting that a substantial proportion of the viral community in Lake Loosdrecht infects prochlorophytes. During the dilution experiments performed in spring 2006 no such impact of viral lysis on the mortality of the prochlorophyte community could be detected, though (**Chapter 4**). The observed correlation between prochlorophyte and viral abundances in Chapter 2 may have been due to an interaction between viruses and heterotrophic bacteria associated with prochlorophytes. No significant correlation between viral and bacterial abundances could be detected during the same seasonal study, however. An alternative explanation might be temporal variation in the viral impact on prochlorophytes in Lake Loosdrecht, with viral infection of prochlorophytes occurring during a large part of the year except during early spring. Temporal variation in viral impact in aquatic environments has been reported in several studies (e.g. Hennes & Simon 1995, Bettarel et al. 2004, Winter et al. 2005). Furthermore, the dilution technique may not have been the most appropriate method to detect viral impact on prochlorophyte mortality, as problems with this technique were encountered when estimating mortality rates for algae, filamentous cyanobacteria and prochlorophytes during the experiments of April 2006 (**Chapter 4**).

Viral abundances also showed a positive correlation to Chl-*a* concentrations, which was initially attributed to a potential interaction between viruses and the highly abundant filamentous cyanobacteria and prochlorophytes in Lake Loosdrecht (**Chapter 2**). Alternatively this correlation could have been due to the presence of algal viruses, which are known to be widespread in freshwater environments (Van Etten et al. 1991, Van Etten et al. 2002). Viruses were indeed found to have a significant impact on algal mortality during the dilution experiment of April 2006 (**Chapter 4**). Viral infection of algae in Lake Loosdrecht was also suggested at the end of the enclosure experiment of November 2002, when the algal community started decaying rapidly on reaching high densities (**Chapter 3**). This algal peak coincided with high viral numbers with relatively large genome- and capsid sizes, which are often attributed to algal viruses (Reisser 1993). No electron microscopic data is available to confirm the presence of virally-infected algae at that time, however, so it remains to be investigated whether also the green algal collapse in the enclosures has been caused by viral lysis.

Co-variation of virus and host dynamics

In **Chapter 2** it was hypothesized that due to the selective and parasitic nature of viral infection, viral and host community dynamics would co-vary, both in abundances and community composition (Fuhrman 1999). Therefore the temporal dynamics of the total viral community were studied in relation to the most dominant plankton groups in Lake Loosdrecht, the filamentous cyanobacteria and heterotrophic bacteria. Changes in viral community composition and abundance did follow a clear seasonal pattern, reflecting the seasonality detected in heterotrophic bacterial and cyanobacterial community composition. In addition viral abundances were observed to relate well to cyanobacterial and heterotrophic bacterial community assemblage and showed a positive correlation to Chl-*a* and prochlorophytes, indirectly suggesting that a significant proportion of the viruses in Lake Loosdrecht might indeed be heterotrophic bacterial and cyanobacterial viruses. The expected co-variation between viral abundances and bacterial and filamentous cyanobacterial abundances could not be detected, however, nor could we observe a significant correlation between the assemblage of viral and bacterial or cyanobacterial communities during the overall period.

Theory thus predicts a co-variation between virus and host community dynamics, and both heterotrophic bacteria and filamentous cyanobacteria were identified as significant viral hosts in Lake Loosdrecht during laboratory experiments. However no such co-variation between viral and heterotrophic bacterial or cyanobacterial community dynamics could be detected in Lake Loosdrecht from early spring until winter. At least three potential explanations for this lack of correlation may be suggested. First, viral infection is not as host specific as generally assumed. The extent of host specificity of viral infection is indeed under discussion. Some viruses have been reported to exhibit a wide range of hosts (Mann 2003). For example, phages of *Synechococcus* can infect up to 10 different strains (Waterbury & Valois 1993). Also, some cyanomyoviridae have been observed by Sullivan et al. (2003) to cross-infect hosts of different cyanobacterial genera.

An alternative explanation for the absence of co-variation between viral and microbial community dynamics would be that viral lysis in Lake Loosdrecht only has a significant impact on heterotrophic bacterial and cyanobacterial communities during particular periods of the year; while during the remaining

periods viral infection occurs below detection levels. Subsequently one would not find a co-variation between virus and host community dynamics over a longer period of time. In Lake Loosdrecht we could indeed only detect a tight linkage between viral and microbial community composition during periods of strong fluctuations in the bacterial and cyanobacterial community during spring and summer (**Chapter 2**). A seasonal variation in viral impact was also suggested in **Chapter 4**, in which the viral community did not have a detectable impact on filamentous cyanobacterial and bacterial mortality during early spring 2006 and November 2004, whereas viral lysis was found to remove a significant part of microbial production during the winter of 2004 – 2005. All the enclosure experiments with Lake Loosdrecht water did show comparable lytic events in the cyanobacterial community, however, no matter during what period of the year they were performed (**Chapter 3**, Van Hannen et al. 1999, Gons et al. 2002, Simis et al. 2005).

A third potential explanation for the lack of correlation between viral and microbial community composition would be that we were not able to detect the more subtle virus – host interactions with the applied technique to detect viral abundances and diversity, and more specific techniques with a higher resolution might be necessary to reveal virus-host interactions in natural assemblages. To describe population dynamics of cyanophages specifically, classical plaque assay, quantitative PCR (Fuller et al. 1998, Muhling et al. 2005) and denaturing gradient gel electrophoresis (DGGE) using primers developed for the *g20* gene (Short & Suttle 1999, Wilson et al. 2000, Frederickson et al. 2003, Dorigo et al. 2004) or other major capsid protein genes (Baker et al. 2006) can be applied to describe population dynamics of cyanophages specifically, and could give us more insight into this aspect of virus-host interactions in the future.

Control of viral populations

In **Chapter 2** it was noted that high nanoflagellate abundances coincided with decreasing viral numbers, suggesting that nanoflagellates could play a role in viral decay in Lake Loosdrecht. The decay experiment in **Chapter 5** also indicated nanoflagellates as the most important viral decay factor in Lake Loosdrecht, inactivating approximately 3 10^7 viruses per day. Gonzalez and Suttle (1993)

applied fluorescently labelled viral particles (FLV) to detect nanoflagellate grazing of viral particles. During the current study the FLV approach was also tested to investigate whether viral particles were ingested by the nanoflagellate community in Lake Loosdrecht. Of a wide variety of staining procedures none seemed to label LPP-1 or Lake Loosdrecht viral concentrates successfully. The tested dyes never labelled the viral particles in the first place, or the fluorescent label was lost upon the washing steps necessary to remove the unbound dye. Therefore the FLV approach could not be applied in grazing experiments with nanoflagellates from Lake Loosdrecht. An alternative approach for future research would be to perform grazing experiments by adding radiolabelled LPP-1 phage concentrate (Noble & Fuhrman 1999). An additional advantage of this approach is that it also allows detection of the actual digestion and incorporation of viral nutrients by nanoflagellates.

Virally induced cyanobacterial collapse

The reproducibility of the cyanobacterial collapse in Lake Loosdrecht enclosure experiments performed during the present (**Chapter 3**) and previous studies (Van Hannen et al. 1999, Gons et al. 2002, Simis et al. 2005) is remarkable. During each experiment the filamentous cyanobacterial community showed a period of steady growth, ultimately followed by a drastic collapse within approximately the first 2 weeks. The present work was the first, however, to actually identify viruses as the major mortality source during the cyanobacterial collapse and to give a detailed description of both viral abundances and diversity. Even though the collapse of the filamentous cyanobacterial community has been reproduced in the enclosures on several occasions, a similar event has never been observed in Lake Loosdrecht itself. It is thought that this difference between lake and enclosures is due to the ideal growth conditions in the enclosures concerning temperature, resource availability and physical mixing. These ideal conditions might select for filamentous cyanobacterial strains with high growth and metabolic rates, enabling these strains to reach high population densities. Since viruses propagate as a function of host population density (Murray & Jackson 1992, Hennes et al. 1995), the increased abundance of these strains are thought to allow rapid viral replication of their specific viruses, resulting in a collapse of the cyanobacterial bloom. Viral infection of these strains might have been further accelerated because cells with higher growth and metabolic rates generally have

higher virus yields and are more susceptible to viral infection (Lenski 1988, Middelboe 2000, Gons et al. 2006).

An additional explanation for the difference between lake and enclosures might be the presence of nanoflagellates. During the March 2003 enclosure experiments it was observed that nanoflagellate abundances decreased by 50 to 70% during the first days of the experiments and remained at low levels after that (data not shown). This decrease in nanoflagellate abundances is thought to have been due to the physical mixing with metal rods in the enclosures, which potentially damages the nanoflagellates. As nanoflagellates have been identified as a potential source of viral decay in Lake Loosdrecht (**Chapter 5**), it might have been that the decrease in nanoflagellate abundances facilitated viral spread in the enclosures.

Ecological implications

Filamentous cyanobacteria, heterotrophic bacteria and to a lesser extent eukaryotic algae were identified as important viral hosts in Lake Loosdrecht, but the impact of viral lysis on plankton mortality was observed to vary strongly between plankton groups and over time. In several experiments viral infection was found to remove a significant proportion of the standing stock of both heterotrophic bacteria and filamentous cyanobacteria, whereas during other experiments no impact of viral lysis on these plankton groups could be detected. In general viral infection was found to have a stronger impact on bacterial and especially filamentous cyanobacterial mortality than micro-zooplankton grazing, except in the case of the unicellular cyanobacteria. These results imply that a considerable part of the cellular material of filamentous cyanobacteria and heterotrophic bacteria in Lake Loosdrecht is recycled within the microbial loop due to viral lysis, instead of being transported to higher trophic levels after micro-zooplankton grazing. The release of cellular debris by viral lysis is thought to increase bacterial production and respiration, and ultimately to decrease zooplankton production, compared to a food-web where micro-zooplankton grazing has the strongest impact on plankton mortality (Fuhrman 1992). Figure 6.1 gives an overview of the interactions of the virioplankton community in the aquatic food web in Lake Loosdrecht, based on the results obtained in **Chapters 4 & 5**.

This PhD study indicated nanoflagellates as the major viral decay factor in Lake

Loosdrecht (Figure 6.1). If the high viral decay rates due to small eukaryotic organisms are indeed the result of nanoflagellate grazing, this would indicate viral particles as a substantial nutrient source of nanoflagellates. It would also imply a flow of viral nutrients towards higher trophic levels in the food-web. To what extent viral particles are indeed ingested and incorporated by nanoflagellates remains to be investigated in future research. Figure 6.1 also indicates the apparent 'protective' role that the highly abundant HMW-DOM in the lake seemed to have on viral infectivity.

In addition, viral lysis was identified as the major mortality source during the collapse of the filamentous cyanobacterial bloom in Lake Loosdrecht enclosure experiments. The lysis of the cyanobacterial bloom was accompanied by a change in community composition of both cyanobacteria and bacteria. These results imply that the viral community in Lake Loosdrecht can potentially control cyanobacterial blooms and has a marked impact on the structure of the plankton community in the lake. Viral infection is thought to influence microbial community composition due to its host-specific nature and is predicted to enhance microbial diversity (Fuhrman & Suttle 1993, Thingstad & Lignell 1997, Thingstad 2000). The impact of viral lysis on the diversity of the plankton community in Lake Loosdrecht remains unclear however, as viral abundances were negatively related to community diversity during the field work on Lake Loosdrecht, whereas during the enclosure studies a positive relationship was detected. Future experiments comparing plankton diversity in treatments with and without virioplankton community should clarify the impact of viral lysis on community diversity in Lake Loosdrecht.

This PhD study thus revealed the virioplankton as an abundant, diverse, active and dynamic component of the aquatic community in the shallow, eutrophic Lake Loosdrecht.

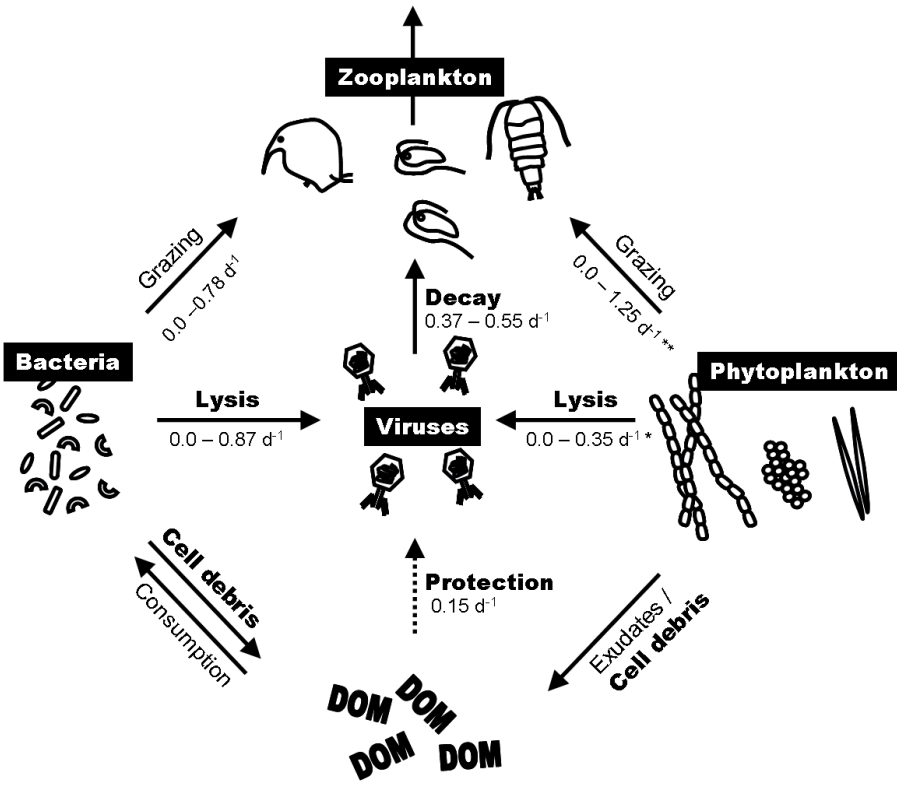

Figure 6.1 Simplified food web Lake Loosdrecht. Representation of the interactions of the viral community within the aquatic food web of Lake Loosdrecht. All interactions involving the virioplankton are indicated in bold. Viral lysis was found to be a significant mortality source of heterotrophic bacteria and the filamentous cyanobacteria. Lysis of these viral hosts results in the production of new viral particles and the release of host cellular debris. Dissolved Organic Matter (DOM) including cellular debris stimulates bacterial production. The release of cellular debris and the uptake of DOM by heterotrophic bacteria have not been quantified during the present study. Zooplankton grazing removed a significant part of heterotrophic bacterial and unicellular cyanobacterial production. Zooplankton grazing will result into the flow of nutrients towards higher trophic levels. Viral lysis and grazing rates of phytoplankton and heterotrophic bacteria are represented as estimated with the dilution technique in **Chapter 4**. *Viral lysis rates are based on estimates for the filamentous cyanobacteria, whereas **zooplankton grazing rates are based on estimates for the unicellular cyanobacteria. No impact of zooplankton grazing on filamentous cyanobacteria could be detected during the present study.
Small eukaryotic organisms were revealed as the major cause of viral decay in Lake Loosdrecht. Nanoflagellate grazing is the most likely source of this decay, which would result into a flow of viral nutrients towards higher trophic levels. The viral decay rate estimates are based on the cycloheximide treatment in **Chapter 5**. Besides stimulating heterotrophic bacterial production, DOM was also found to 'protect' viral particles in Lake Loosdrecht and prolong their survival. The protective impact of DOM on viral infectivity was estimated after doubling the concentration of DOM in the lake water. Therefore the rate represented in the figure is estimated by dividing the obtained impact of DOM addition (**Chapter 5**) by two, assuming a linear effect of DOM concentration on the longevity of the virioplankton. For simplicity viruses infecting zooplankton and zooplankton feeding on DOM are not accounted for in this food web.

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English Summary

This thesis aims to give an insight into the ecology of the viral community in a shallow eutrophic lake. To achieve this, the population dynamics, diversity and control of the viral community in Lake Loosdrecht were studied, as well as the impact of the viral community on plankton mortality and community composition.

A seasonal study of Lake Loosdrecht revealed the virioplankton community as a dynamic component of the aquatic community, with abundances ranging between 5.5×10^7 and 1.3×10^8 virus like particles ml^{-1} and viral genome sizes between 30 and 200 kb. Viral community dynamics followed a distinct seasonal cycle, and were related to Chl-*a* concentrations, prochlorophyte numbers and cyanobacterial community composition, suggesting that a significant proportion of the viruses in Lake Loosdrecht may be phytoplankton and more specific cyanobacterial viruses. Temporal changes in bacterial abundances were significantly related to viral community assemblage, and *vice versa*, also suggesting an interaction between viral and bacterial communities in Lake Loosdrecht.

During a series of enclosure experiments viruses were indeed identified as the major cyanobacterial mortality cause, when the drastic collapse of the dominant filamentous cyanobacterial population was accompanied by high percentages of virally infected cyanobacterial cells and increasing viral numbers. The viral lysis of the cyanobacterial bloom was accompanied by changes in both cyanobacterial and heterotrophic bacterial community composition, indicating that viral infection in Lake Loosdrecht not only has the potential to control cyanobacterial blooms, but also has a marked impact on the structure of the plankton community.

To simultaneously estimate the impact of both viral lysis and zooplankton grazing on the mortality of bacteria, algae and cyanobacteria, an adapted version of the dilution technique was applied on Lake Loosdrecht water. Viral lysis was identified as the main mortality source during winter experiments, removing between 84 and 97% of the potential filamentous cyanobacterial production and up to 101% of the potential bacterial production. Viral impact on plankton mortality varied strongly between different experiments however, and care should be taken when interpreting results and to optimize the dilution technique for application on a variety of plankton groups.

Loss mechanisms and rates of viruses in eutrophic lake water were estimated by following the loss in infectivity of cyanophage LPP-1 in Lake Loosdrecht water. Viral decay rates in the lake ranged between 0.85 and 0.98 d^{-1}, corresponding to turnover times of 1.0 and 1.2 days. The addition of HMW-DOM resulted in a significant decrease in viral decay rate, suggesting that the abundant HMW-DOM in the lake prolongs viral infectivity. Eukaryotic organisms smaller than 10 µm were found to be responsible for 37 to 55 % of the daily viral decay, thus implying that grazing by heterotrophic nanoflagellates was the major viral decay source in Lake Loosdrecht.

This study thus identified the viral community as a dynamic and active component of the foodweb in shallow eutrophic lakes, which should therefore not be ignored in future ecosystem research. More specific methodology for virus detection is needed however to further unravel the virus-host interactions in these lakes.

Nederlandse Samenvatting

De zomer komt er weer aan. Nog even en we kunnen weer naar het strand of lekker zwemmen in het meer. Heerlijk van de zon genieten om vervolgens een verkoelende duik te nemen in het frisse water. Onderzoek heeft echter uitgewezen dat zich in één druppel van dat water miljoenen virussen bevinden. Kun je, nu je dit weet, nog steeds zo genieten van die verfrissende duik in het water?

In 1989 ontdekte een onderzoeksgroep uit Noorwegen dat er in het buitenwater erg veel virussen voorkomen. In meren, zeeën en oceanen kun je tussen de 5 en 150 miljoen virussen per milliliter vinden, rapporteerden zij in het vooraanstaande tijdschrift Nature. Als je met deze getallen doorrekent blijken er 10^{27} virussen in 's werelds oceanen voor te komen. Wanneer je al deze virussen in een rij naast elkaar legt, zou je een virusketting van 400.000 lichtjaren lang krijgen, wat 16 keer de doorsnede van de Melkweg is. Uit een ander rekenvoorbeeld blijkt dat de hoeveelheid organische stof in deze virussen vergeleken kan worden met 75 miljoen walvissen. Virussen kunnen dus met recht de meest voorkomende organismen in aquatische systemen genoemd worden. De logische vervolgvraag is nu wat al deze virussen in dat water doen.

Eiwitjasjes

Om deze vraag te kunnen beantwoorden, is het belangrijk om meer over virussen te weten. Virussen zijn minuscuul kleine deeltjes die opgebouwd zijn uit een stuk genetisch materiaal met een eiwitjasje daar omheen. Ze moeten andere organismen infecteren om zich voort te kunnen planten. Na infectie neemt het virus de controle van de gastheercel over, zodat deze al haar energie gebruikt om nieuwe virussen te produceren. Wanneer er genoeg nieuwe virussen zijn geproduceerd, komen deze vrij buiten de cel om zelf een gastheer te infecteren. In 's werelds meren, zeeën en oceanen drijven er dus in elke druppel water, miljoenen van deze virusdeeltjes rond. Dit is echter geen reden tot paniek, omdat verreweg de meeste virussen geen mensen kunnen infecteren.

Virussen houden dichtheden van algen en bacteriën in toom

Wanneer deze virussen onschadelijk blijken te zijn voor mensen, wat

infecteren ze dan wel? Uit onderzoek is gebleken dat vooral bacteriën en algen geïnfecteerd worden, die in hetzelfde water voorkomen. Verder blijken virussen erg gespecialiseerd te zijn. Ze kunnen vaak maar één specifiek soort gastheer infecteren. Alle verschillende soorten algen en bacteriën in het water hebben elk hun eigen specifieke virussen en de virus gemeenschap is dan ook erg divers. Bacteriën en algen sterven na een virusinfectie, doordat de cel openbarst bij het vrijkomen van de nieuwe virussen (lyse). Onderzoek in Japan heeft bijvoorbeeld aangetoond dat virussen verantwoordelijk zijn voor het afsterven van de giftige alg *Heterosigma*. Deze alg kan zomers in grote hoeveelheden voorkomen en veroorzaakt vissterfte in commerciële viskwekerijen. Virus lyse zorgt ervoor dat deze alg sterk in aantallen afneemt. De kans op een virusepidimie is erg hoog wanneer een alg zoals *Heterosigma* in de zomer in grote hoeveelheden voorkomt. De kans dat een gastheer zijn specifieke virus in het buitenwater tegenkomt en vervolgens geïnfecteerd wordt, is namelijk dichtheidsafhankelijk. Door deze gastheer-specifieke en dichtheids-regulerende eigenschappen van virusinfectie, wordt er verwacht dat virussen de soorten samenstelling van verschillende algen en bacteriën in het buitenwater beïnvloeden.

Virussen doen aan recyling

Verder verwachten onderzoekers dat virussen de stroom van nutriënten in het voedselweb beïnvloeden. Wanneer een geïnfecteerde gastheercel bij lyse openbarst, komen er behalve nieuwe virussen ook stukjes celmateriaal van de gastheer vrij. Dit celmateriaal van de afstervende gastheer kan vervolgens als voedsel dienen voor heterotrofe bacteriën. Virus lyse resulteert zo in het 'recyclen' van nutriënten binnen de bacteriële gemeenschap van het voedselweb.

Promotie onderzoek

Tijdens mijn promotieonderzoek heb ik de populatiedynamiek, invloed en interacties van de viruspopulatie in de Loosdrechtse Plassen bestudeerd. De Loosdrechtse Plassen liggen ter hoogte van Breukelen, tussen Utrecht en Amsterdam en zijn ontstaan door het afgraven van veen voor turf. De plassen zijn erg ondiep, voedselrijk (eutroof) en hebben troebel water. Er komen veel cyanobacteriën voor, in de volksmond ook wel blauwalgen genoemd. In de Loosdrechtse Plassen komen vooral veel niet-toxische, draadvormige cyanobacteriën voor.

Virus dynamiek

Allereerst heb ik de populatie dynamiek van de virussen in de Loosdrechtse Plassen bestudeerd. Tussen februari en november 2003 zijn elke twee weken de aantallen virussen, bacteriën, cyanobacteriën en algen in het Loosdrecht water bepaald. Verder is de diversiteit van de virus -, bacteriële - en cyanobacteriële populaties bepaald met behulp van DNA technieken. De virus populatie in de plassen bleekt talrijk en divers, met tussen de 50 en 200 miljoen virussen per milliliter en 13 verschillende viruspopulaties. Veranderingen in virusaantallen en populatiesamenstelling bleken seizoensgebonden en kwamen overeen met veranderingen in chlorofiel (een fotosynthese pigment in algen en cyanobacteriën), prochlorofieten (een subgroep binnen de draadvormige cyanobacteriën) en de cyanobacteriële- en bacteriële populatiesamenstelling. Deze resultaten suggereren dat bacteriën en met name cyanobacteriën belangrijke virus gastheren zouden kunnen zijn in de Loosdrechtse Plassen.

Massale cyanobacteriële sterfte veroorzaakt door virussen

Vervolgens is de invloed van virussen op de populatie dynamiek van bacteriën, cyanobacteriën en algen bestudeerd met behulp van grote kunstof-kolommen met elk 130 liter water uit de Loosdrechtse Plassen. Deze kolommen zijn speciaal ontwikkeld om de situatie in de Loosdrechtse Plassen te simuleren en in het laboratorium gecontroleerd experimenten te kunnen doen. In de kolommen kregen de cyanobacteriën voldoende licht en voedsel, waardoor deze goed groeiden en sterk in aantallen toenamen. Herhaaldelijk werd waargenomen dat de dichte populatie van cyanobacteriën na een periode van sterke groei plots afstierf en binnen enkele dagen drastisch in aantallen afnam. Tijdens het ineenstorten van de cyanobacteriën nam het aantal virussen sterk toe. Met behulp van electronenmicroscopie konden grote aantallen met virus geïnfecteerde cyanobacteriën waargenomen worden. Op basis van deze resultaten werd geconcludeerd dat virussen zeer waarschijnlijk verantwoordelijk waren voor de ineenstorting van de cyanobacteriële populatie. Met behulp van DNA technieken werd verder vastgesteld dat de populatiesamenstelling van zowel de cyanobacteriën als de bacteriën sterk veranderde tijdens de lyse van de cyanobacteriën. De viruspopulatie in de Loosdrechtse Plassen bleek dus niet alleen de dichtheden van de cyanobacteriën in toom te kunnen houden, maar ook de populatiesamenstelling in de plassen te beïnvloeden. Om daadwerkelijk

aan te kunnen tonen dat virusinfectie de samenstelling van de microbiële gemeenschap beinvloed zijn er in vervolg onderzoek controle experimenten nodig waar de virusgemeenschap door middel van filtratie actief verwijderd is.

Invloed viruslyse op microbiële sterfte

Om te bepalen wat de invloed was van de viruspopulatie op de cyanobacteriële en bacteriële sterfte in de Loosdrechtse Plassen, werden er 'verdunnings experimenten' uitgevoerd. Tijdens deze experimenten is de viruspopulatie in het water stapsgewijs verdund, waarna de netto groeisnelheid van bacteriën en cyanobacteriën bepaald is. Wanneer viruslyse veel sterfte veroorzaakt, verwacht je minder sterfte van cyanobacteriën en bacteriën in het water waarin de virussen sterk verdund zijn. Hierdoor nemen de cyanobacteriën en bacteriën in deze verdunningen in aantallen toe. Deze toename in cyanobacteriën en bacteriën is tijdens de experimenten bepaald en vervolgens als maat gebruikt voor de invloed van viruslyse op de microbiële sterfte.

Virussen bleken vooral in de winter veel sterfte van bacteriën en cyanobacteriën te veroorzaken, wanneer er tussen de 84 en 97% van de potentiële cyanobacteriële produktie en tot 101% van de potentiële bacteriële produktie door virus lyse werd verwijderd. Gedurende andere experimenten bleek virus lyse daarentegen soms geen invloed op de microbiële sterfte te hebben. Verder bleken de resultaten van de 'verdunnings techniek' vanwege de vele aannames vaak lastig te interpreteren, waardoor de techniek niet altijd even goed toepasbaar bleek.

Virus sterfte

Uit voorgaand onderzoek in zeewater bleek een aantal factoren de sterfte van virussen zelf te veroorzakten. Voorbeelden van deze voor virussen schadelijke factoren zijn zonlicht, het 'blijven plakken' van virussen aan bodemdeeltjes of het opgegeten worden door nanoflagellaten. Ik heb getest welke factoren de sterfte van virussen in de Loosdrechtse Plassen beïnvloeden. Verschillende behandelingen zijn op het water uit de Loosdrechtse Plassen toegepast om de schadelijke invloed van UV straling, adsorptie aan bodemdeeltjes en organisch materiaal, bacteriën, enzymen en nanoflagellaten te bepalen. De gemeten virussterfte in het water varieerde tussen de 0.85 en 0.98 per dag, wat inhoudt dat de totale virus populatie in ruim een dag vervangen werd. Kleine eukaryote organismen, zoals nanoflagellaten, bleken de sterkste invloed te hebben op de

afname van virus infectiviteit in de plassen. Waarschijnlijk worden de virussen gegeten door nanoflagellaten. Nanoflagellaten zijn kleine 'jagers' in het water, waarvan men voorheen dacht dat ze alleen bacteriën aten. In tegenstelling tot eerder gepubliceerd onderzoek in het zeewater bleek opgelost organisch materiaal de virus infectiviteit positief te beïnvloeden, terwijl UV-straling geen invloed bleek te hebben in de Loosdrechtse Plassen.

Ten slotte

Dit onderzoek toont aan dat virussen een dynamische, talrijke en diverse component zijn van het voedselweb in de Loosdrechtse Plassen, waar ze vooral bacteriën en cyanobacteriën infecteren. Bij uitzonderlijk sterke cyanobacteriële groei in laboratorium experimenten bleek de virusgemeenschap zelfs verantwoordelijk voor het ineenstorten van de cyanobacteriële populatie, wat samen ging met sterke veranderingen in de soorten samenstelling van de microbiële gemeenschap.

Om ook in de Loosdrechtse Plassen zelf de samenstelling van de virus en cyanobacteriële gemeenschap direct aan elkaar te kunnen relateren, zijn in de toekomst meer geavanceerde technieken nodig om specifiek virussen van cyanobacteriën te kunnen identificeren. Daarnaast is het interessant om in toekomstig onderzoek na te gaan of de virussen in de Loosdrechtse Plassen daadwerkelijk door flagellaten gegeten worden en in welke omstandigheden virussen een welkome toevoeging aan het dieet van flagellaten kunnen vormen. Dit onderzoek benadrukt het belang dat virussen als regulerende factor niet langer genegeerd dienen te worden in toekomstige ecologische studies naar het functioneren van ondiepe eutrofe meren.

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Marjolijn

Tokyo, 21 Augustus 2007

CV

Marjolijn Tijdens (1977) graduated from the University of Groningen in Microbial Ecology in 2001. As an undergraduate student she first worked in the microbial ecology group of Professor Larry Forney (RuG) under supervision of Dr. Henk Bolhuis, conducting research on the influence of stressful situations on the mutation rate of bacteria. Within this research theme she particularly looked at the influence of starvation on the mutation rate of Haloferax volcanii. For her second research project as an undergraduate student she went to Michigan State University (USA) where she worked at the department for civil and environmental engineering under supervision of Professor Syed Hashsham (MSU) and Dr. Jan Gottschal (RuG). Here she worked on the classification of environmental samples using DNA microarrays. For this project she designed and tested a DNA microarray to classify the Escherichia coli strain composition within environmental samples. After her graduation she first worked at the Dutch ministry of agriculture, fishery and nature policies after which she started in 2002 as a PhD student at the NIOO-KNAW Centre for Limnology in the Microbial Ecology group of Professor Riks Laanbroek under supervision of Dr. Herman Gons. The focus of this PhD study was the influence and interactions of viral communities in shallow eutrophic lakes, with a special interest in viruses infecting cyanobacteria. This thesis is the result of her PhD studies. After finishing her PhD work at the NIOO in 2007, Marjolijn remained interested in cyanophages and got awarded a grant from the Japanese Society for the Promotion of Science for a 5 month study into the molecular ecology of temperate phages of freshwater toxic cyanobacteria. This research was performed under supervision of Dr. Keizo Nagasaki from the harmful algae control section at the Fisheries Research Agency in Onoura, Japan.