REVIEW

Complex interactions in the microbial world: underexplored key links between viruses, bacteria and protozoan grazers in aquatic environments

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ABSTRACT: Viruses are the most abundant biological entities in aquatic ecosystems, and heterotrophic bacteria form the major host reservoir for these viruses. Together with small protozoan grazers, these biological compartments are now recognized as the most abundant microorganisms on earth. Bacterial loss, which constitutes a key process in pelagic ecosystems, is mainly a result of lysis by viruses and grazing by protists, which may be comparable at times. Both viruses and protozoan grazers are known to be driving forces in shaping bacterial diversity. A significant correlation between viruses and protozoan grazers has been reported in various ecosystems. Some studies have also shown that grazers can feed directly on viruses, although the transfer of carbon to the higher trophic levels via this process seems to be of minor importance. Thus, there are many routes of interaction between grazers, bacteria and viruses in pelagic systems, but few studies have attempted to characterize them. Interestingly, experimental studies have recently revealed both direct and indirect control by viruses and protozoan grazers on bacteria. Interactions between bacteria, between viruses and between heterotrophic flagellates are also likely to occur. From a mathematical point of view, we can argue that there are 9 different possible pathways. Although some of these have been well documented, others have barely been mentioned. This review attempts to cover advances in the field in recent years and concentrates on addressing the underexplored interactions among viruses, bacteria and heterotrophic nanoflagellates.

KEY WORDS: Viruses · Bacteria · Protists · Interactions · Aquatic ecosystems

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INTRODUCTION

Over the last 20 yr, extensive studies have revealed the crucial roles of microbes in marine and freshwater ecosystems. It has been shown that bacteria, viruses and protozoan grazers are dominant in terms of abundance and biomass (Suttle 2007). Bacterial abundance in the ocean is on the order of 10^8 to 10^9 cells l^{-1}, and the typical relative abundance for viruses, bacteria and heterotrophic nanoflagellates (HNF) is 10^{-1} to 10^{-3} (Noble & Fuhrman 1998, Tanaka & Rassoulzadegan 2002). This implies that interactions among organic and inorganic substrates, bacteria, viruses and protozoan grazers are often likely to occur. The frequent interactions among these microbiological compartments are responsible for strong trophic links from dissolved organic matter (DOM) to higher trophic levels via the heterotrophic bacteria, which form the basis for the important biogeochemical roles of microbial food webs in aquatic ecosystems (referred to as the 'microbial loop' by Azam et al. 1983, and the 'viral loop' by Bratbak et al. 1992). Moreover, such interactions exert strong selection pressures on each biotic agent, contributing to the evolution and maintenance of the high

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phylogenetic diversity of microbes (Breitbart et al. 2002, Venter et al. 2004) and the diversified nature of these interactions, which may also contribute to maintaining high biodiversity.

As a result of these diversified interactions, each component and functional group (heterotrophic bacteria, viruses and protistan grazers) is likely to have a major impact on the community structure and biogeochemical functions of the other groups. In this review, our aim was to focus on the interactions between 3 major players: heterotrophic bacteria, viruses and HNF. From a mathematical point of view, there are potentially 9 (3 × 3) pathways or processes by which each component affects the other components as well as itself (Fig. 1). Evidence has already accumulated for several of these processes, which have been extensively reviewed. For the other processes, however, we do not yet have sufficient evidence, and the time may have come to review what information is available and what is still missing.

We first briefly summarize the important concepts involved in several processes that have emerged from the accumulated evidence. These concepts include the effects of HNF grazing (and viruses) on bacteria, and the responses of bacteria to HNF grazing and viruses. We then review the less-explored processes in detail to obtain an overall picture of current understanding of these interactions. This includes the underexplored area of mortality and the processes by which viruses and HNF are removed, and the indirect interactions between HNF and viruses. We also discuss other aspects of the other 9 pathways that could form the basis of future studies. These include the interactions

between different groups within bacteria, viruses and HNF, and the combined effects of viruses and HNF on bacteria. We hope that this review will allow the reader to realize that interactions among the 'black boxes' (bacterioplankton, viruses and grazers) are much more complex than generally assumed.

WELL-DOCUMENTED PATHWAYS AND PROCESSES

The interactions between bacteria, viruses and HNF that have been studied most extensively are the trophic interactions between bacteria and HNF (Pathways 1 and 2, Fig. 1) and between bacteria and viruses (Pathways 3 and 4, Fig. 1). HNF bacterivory and viral lysis are now known to be 2 major causes of bacterial mortality (Concept 1, Table 1). Dozens of papers have been published on these processes. HNF grazing is responsible for 5 to 250% of daily bacterial mortality (Andersen & Fenchel 1985, Jacquet et al. 2005). Numerous studies suggest that grazing loss roughly counterbalances bacterial production in oligotrophic systems, whereas grazing alone cannot explain the balance between bacterial production and mortality in more productive systems (Strom 2000). On the other hand, viral lysis is responsible for 5 to 50% of daily bacterial mortality (Fuhrman 1999, Wommack & Colwell 2000). It is easy to speculate that both grazing and viral lysis make major contributions to bacterial mortality in more productive systems. A few studies have simultaneously compared virus-induced bacterial mortality (VIBM) and grazing-mediated bacterial mortality (for pioneer studies, see Bratbak et al. 1992, Fuhrman & Noble 1995, Weinbauer & Peduzzi 1995) and shown that viral lysis can be as important as grazing pressure in daily bacterial removal (Fuhrman & Noble 1995, Jacquet et al. 2005).

It has also been shown that HNF grazing and viral lysis not only cause bacterial mortality, but also affect the activity of bacteria (growth and production), and induce changes in the phenotype and composition of the bacterial community. On the one hand, HNF grazing affects the size distribution of the bacterial community through 'size-selective mortality' (Concept 2, Table 1). HNF preferentially graze on medium-sized bacterial cells, resulting in a bimodal size distribution within the bacterial community (Pernthaler et al. 2001, Hahn et al. 2003). In addition, HNF grazing enhances the activities of bacteria at the (individual) cell level by reducing the competition for resources, and improving growth conditions by reducing the total abundance of bacteria and regenerating organic and inorganic nutrients (Rothhaupt 1997, Salcher et al. 2007). HNF not only act as a 'node' that transfers dis-
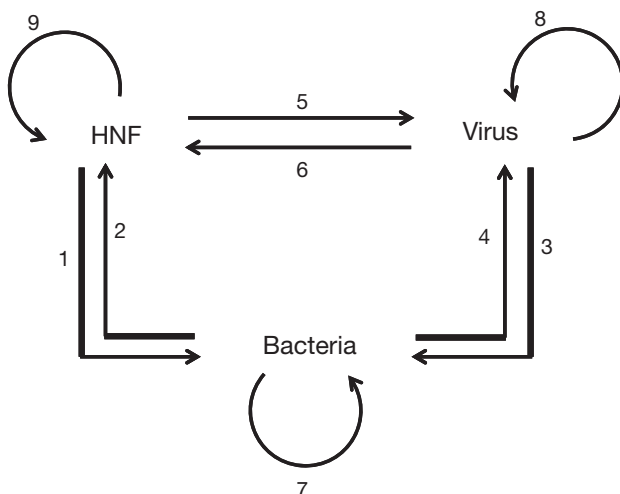

Fig. 1. Possible interactions and pathways (numbered; see text for explanation) among bacteria, viruses and heterotrophic nanoflagellates (HNF)

Table 1. Well-documented pathways and emergent concepts

Pathway	Concept	Source (Reviews unless otherwise indicated)
1. HNF → Bacteria	1. Major source of bacterial mortality 2. Size-selective mortality 3. Recycler of nutrients	Strom (2000) Hahn & Höfle (2001), Pernthaler (2005) No reviews. Nagata & Kirchman (1992), Dolan (1997), Nagata (2000)
2. Bacteria → HNF	4. Induced phenotypic changes in bacterial community	Pernthaler (2005)
3. Viruses → Bacteria	1. Major source of bacterial mortality 5. Phylogenetically selective mortality	Fuhrman (1999, 2000) No reviews. Important mathematical (Thingstad & Lignell 1997, Thingstad 2000) and conceptual (Wommack et al. 1999, Wommack & Colwell 2000) models of 'kill the winner' hypothesis. Hewson et al. (2001, 2003) supported it. Wichels et al. (1998) and Sullivan et al. (2003) did not support high host-specificity of bacteriophages and cyanophages.
	6. Mediator of evolution of bacterial genome by horizontal gene transfer 7. Virus shunt 8. Diversity of viral life cycles	Fuhrman (1999), Wommack & Colwell (2000), Suttle (2005) Fuhrman (1999, 2000), Suttle (2007) Fuhrman (2000), Weinbauer (2004)
4. Bacteria → Viruses	4. Induced phenotypic changes in bacterial community	Weinbauer (2004)
3–4. Bacteria ↔ Viruses	9. Co-evolution between host and viruses	Lenski (1988), Bohannan & Lenski (2000), Weinbauer (2004)

solved organic carbon (DOC) assimilated by heterotrophic bacteria to higher trophic levels (e.g. large zooplankton), they also recycle organic and inorganic nutrients (Concept 3, Table 1). In addition to passive changes in size distribution as a result of size-selective mortality, the bacterial community in turn reacts actively to HNF grazing as the result of either a direct response or a response to improved growth conditions (Hahn et al. 1999, Šimek et al. 2007). As a result of both the phenotypic plasticity at the individual level and shifts in community composition, the bacterial community induces various types of phenotypic changes (Concept 4, Table 1), which can act as a resistance mechanism against HNF grazing (Šimek et al. 1999, Pernthaler 2005).

On the other hand, viral lysis is also known to have a major impact on bacterial community composition (BCC) through selective mortality with high 'host specificity' (Winter et al. 2004, Bouvier & del Giorgio 2007). By analogy to 'size-selective' mortality by HNF grazing, we can interpret this as 'phylogenetically selective' or 'taxonomy-selective' mortality (Concept 5, Table 1) resulting from viral lysis. Based on these concepts, it has been suggested that viral lysis may contribute to the coexistence of bacterial species competing for a small number of limiting nutrients (known as the 'kill the winner' hypothesis; Thingstad & Lignell 1997, Thingstad 2000). Viral infection also induces

phenotypic changes in the bacterial community (Concept 4, Table 1) and contributes to the evolution of the bacterial genome by acting as a vector for horizontal gene transfer (Concept 6, Table 1; Jiang & Paul 1998). Virus-induced bacterial cell lysis also results in the release of DOM, particulate organic matter (POM) and inorganic nutrients (Riemann & Middelboe 2002). Although this resource pool is subsequently utilized by heterotrophic bacteria, and temporarily enhances bacterial growth and production (Noble et al. 1999), part of it is respired as CO_2. This 'virus shunt' (Concept 7, Table 1) is therefore likely to have a negative impact on the transfer of bacterial production to higher trophic levels (Fuhrman 1999), although short-term experiments do not always appear to support this expectation (Weinbauer et al. 2007). It is also argued that the release of POM into the surface waters could influence the vertical flux of organic matter, and may reduce the efficiency of the biological pump (Suttle 2007). In addition, viruses have diverse strategies for reproduction, i.e. lytic and chronic infections, and lysogeny ('diversity of viral life cycle': Concept 8, Table 1), which may have different impacts on bacterial mortality and physiologies. Another important aspect of interactions between viruses and bacteria is the co-evolution between virus and host (e.g. resistance and counter-resistance), which has been reviewed by several authors (Concept 9, Table 1).

UNDEREXPLORED PATHWAYS AND PROCESSES

Underexplored mortality (or removal processes) of viruses and HNF

Removal of viruses by bacteria (Pathway 4)

In a conventional sense, the relationship between bacteria and viruses is categorized as a host–parasite interaction. Since the bacterial mortality produced by viruses is very severe, it is not surprising to find that bacteria have evolved various strategies to resist viral attack (Weinbauer 2004). One of the major evolutionary strategies used by bacteria to escape viral recognition is changing the cell membrane receptor structure (Bohannan & Lenski 2000). This prevents viral particles from attaching to the cell surface of the bacterium, but because particles in the ocean are generally negatively charged (Chattopadhyay & Puls 2000), encounters between particles with no specific attractive forces may not have the intended effect. In other words, this mechanism may not actually reduce viral survival. However, there are also 2 types of more active resistance to viral attack. One is the resistance induced after viral infection (i.e. the immune response, Lenski 1988), which destroys the viral genome: the mechanisms involved include DNA injection blocking, restriction modification and abortive infections, which are summarized by Lenski (1988) and Weinbauer (2004). This is a process by which bacteria rid themselves of viruses. The other type of active resistance is the constitutive or induced production of ectoenzymes, which can destroy the capsid proteins of free-living viral particles (Noble & Fuhrman 1997). Although this may also contribute to the elimination of viruses, it is not clear whether ectoenzymes are actually produced for the purpose of resisting viruses, or whether this is just a beneficial side effect of ectoenzymes produced to make use of other organic particles in the surrounding environment. Interestingly, Motegi & Nagata (2007) showed that adding nitrogen could enhance both bacterial production and virus decay. They argued that enhanced bacterial production might be followed by increased production of enzymes and colloidal particles, which could in turn lead to increased viral loss (enzymatic degradation and absorption to colloidal substances). If resistance mechanisms, in the broad sense of the term, prevail in viral communities, it can be speculated that the 'kill the winner' process may be less effective, resulting in lower bacterial diversity than expected (Miki & Yamamura 2005).

Removal of viruses by HNF (Pathway 5)

Suttle & Cheng (1992) were the first to suggest that HNF prey not only on bacteria but also on viruses.

HNF grazing (or predation) on viruses is interpreted as intraguild predation (IGP) (Polis et al. 1989) because HNF and viruses belong to the same 'guild' or assemblage of species sharing common resources. In other words, bacteria, viruses and HNF constitute an IGP 'module' (Arim & Marquet 2004), a situation that is widespread in both terrestrial and aquatic food webs. Gonzáles & Suttle (1993) evaluated the loss of free-living fluorescent-labeled viruses caused by HNF predation, and showed that this direct predation (omnivorous IGP) accounts for 2.6 to 4.8% of bacterial grazing in a coastal system. Using artificial virus-sized particles, Bettarel et al. (2005) showed that the loss rate resulting from HNF grazing was 0.8 and 4.1% of the viral production in a eutrophic lake and an oligo-mesotrophic lake, respectively. They also showed that the clearance rate of virus-sized particles relative to that of bacteria-sized particles was higher in the oligo-mesotrophic lake, where bacteria, the main resource for HNF, were less abundant.

At the population level, the negative impact of HNF grazing on viruses has been observed over a 10 d scale (Manage et al. 2002), although this may have included not only the effect of grazing but also the effects of competition on viruses. There is another line of evidence that indirectly suggests that HNF grazing induces viral loss. We would expect size-selective grazing by HNF to influence the size distribution of viruses, unless the grazing rate is negligible. Weinbauer (2004) argued that the observed increase in the average size of viruses observed in the vertical distribution in Lake Plußsee (Demuth et al. 1993) did indeed correspond to reduced grazing pressure by HNF. It is noteworthy that HNF could also graze on eukaryotic algal viruses, which can be considerably larger than prokaryotic viruses (Wilson et al. 2005), but to the best of our knowledge, this has never previously been documented.

Indirect predation can also occur between viruses and HNF (coincidental IGP). Viruses within bacterial host cells are killed indirectly by HNF grazing of infected cells. This process has been incorporated in the model used for VIBM estimation on the assumption that infected cells are killed at the same rate as uninfected cells (Binder 1999). In addition, the preference of HNF for infected cells and the longer latent period of viruses can theoretically be expected to lead to more severe coincidental IGP, and this in turn would influence the kill-the-winner and virus-shunt processes (Miki & Yamamura 2005). It is also noteworthy that both HNF and larger predators, such as ciliates, prey on viruses and contribute to their inactivation (Pinheiro et al. 2007). A similar 'Russian doll' type strategy was also identified by Parry et al. (2006) while studying possible ciliate strategies for feeding on infected pico-cyanobacterial cells.

HNF and viruses occur together almost everywhere abiotic conditions allow them to survive, and this has meant that few microbial ecologists have paid any attention to possible antagonistic relationships between them or to the mechanisms underlying their coexistence. From a theoretical viewpoint, however, the high prevalence of their coexistence is surprising, because ecological models predict that species can only coexist in a context of IGP interaction under specific conditions (the prey has to compete better for shared resources) and in specific environments (the system has to have intermediate productivity) (Holt & Polis 1997, Miki & Yamamura 2005, Takimoto et al. 2007). In particular, these models predict that an increase in productivity of the system would both indirectly and negatively affect the abundance of 'intraguild prey' (viruses in this case) because competition for a shared resource (bacteria in this case) between the intraguild prey and the 'intraguild predator' (HNF in this case) is less severe, whereas the top-down effects of the intraguild predator on intraguild prey are enhanced (Fig. 2A). However, this prediction appears to conflict with the observed tendency of viruses to be more important factors of bacterial mortality, especially in highly productive systems (Strom 2000, Weinbauer 2004). The prevalence of coexistence in natural systems therefore suggests the existence of additional mechanisms that facilitate their coexistence in eutrophic systems. One possibility is that increased productivity may result in a greater top-down effect of larger predators on HNF, which then weakens the negative top-down effect of HNF on viruses (Carpenter et al. 2001 and references therein). Another possibility is that increased productivity would result in enhanced bacterial physiological activity, leading to an increased viral infection and/or reproduction rate (i.e. a shorter latent period and larger burst size; Middelboe 2000). This could give viruses the competitive edge over HNF in competing for bacterial prey, and undermine the indirect negative effects of eutrophication.

Virus-induced mortality of HNF (Pathway 6)

Only 1 study has so far reported the isolation and characterization of a double-stranded DNA (dsDNA) virus infecting an HNF (*Bodo* sp.) from a natural marine system, and that was over 10 yr ago (Garza & Suttle 1995). Virus-like particles very similar to this dsDNA virus were recently observed in the cell of an HNF species (*Cafeteria roenbergensis*) but not in co-occurring species (*Caecitellus paraparvulus*) (Massana et al. 2007). It has also been suggested that the population dynamics of *C. roenbergensis* may be controlled by this virus-like particle. The mechanisms of infection by these viruses are unknown, but it is interesting to surmise to what extent HNF is infected as a result of ingesting viruses. It has been suggested that grazing on viruses is a kind of active foraging behavior engaged in by HNF (Bettarel et al. 2005). If so, we can speculate that, depending on resource abundance, active grazing behavior could increase the risk of viral infection. In another resource–host–parasite system, the foraging behavior of *Daphnia* spp. on algae is enhanced by the decrease in algal density, and that in turn enhances the rate of encounters between parasitic fungal zoospores and *Daphnia* spp., thus resulting in the increased infection rate (Hall et al. 2007).

There are many prey–parasite–predator systems in which the parasite changes the host from being a prey into being a predator ('trophically transmitted parasite'), for instance in some terrestrial insect, parasite and predatory bird systems (see review by Thomas et al. 2005). Although there are no data to suggest that bacteriophages can also parasitize protozoan grazers, this could also be an interesting topic to investigate within microbial food webs. A mathematical model for this type of interaction reveals that if a parasite
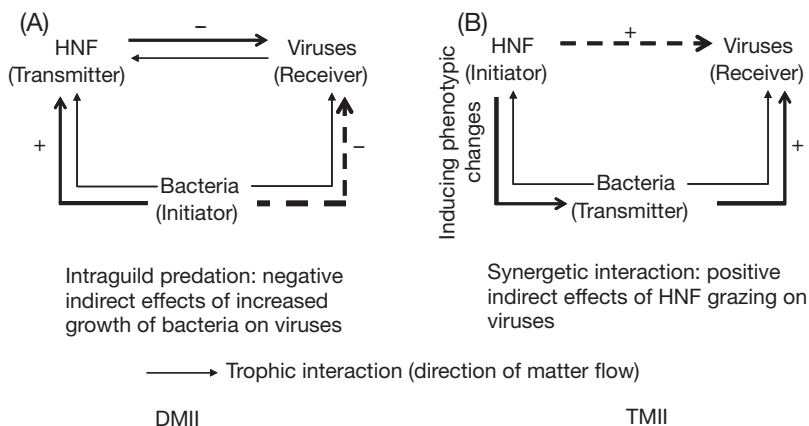

Fig. 2. Indirect effects among bacteria, viruses and heterotrophic nanoflagellates (HNF). (A) Intraguild predation (IGP) module that consists of bacteria, viruses and HNF. Indirect negative effects of increased productivity on viruses are predicted by IGP. This is a typical density-mediated indirect interaction (DMII); changes in the abundance of the initiator (bacteria) indirectly affect the abundance of the receiver (viruses) through the changes in the abundance of the transmitter (HNF). Another well-known DMII is the top-down trophic cascade. (B) Observed indirect positive effects of HNF grazing on viruses. This is a typical trait-mediated indirect interaction (TMII); the initiator (HNF) induces certain phenotypic changes in the transmitter (bacteria), which in turn affects the abundance or activities of the receiver (viruses)

changes host across trophic levels, this can have a major impact on the stability of the population dynamics of the host and predator (Fenton & Rands 2006).

Indirect interactions between HNF and viruses

Indirect effects of HNF grazing on viruses (Pathway 5)

In general, exploitative competition between HNF and viruses is to be expected, since they consume the same prey. In other words, an increase in the activity of one type of consumer of bacteria could lead to reduction in common resources, thus resulting in reduced activity of the other predator. In addition to this competitive interaction, grazing on infected cells (coincidental IGP) and free-living virus particles (omnivorous IGP) would also reduce the abundance and production of viruses. The removal of HNF could therefore always be expected to result in increased viral abundance. Although theoretical models (Thingstad 2000, Miki & Yamamura 2005) have confirmed these negative interactions between HNF and viruses, the situation is not straightforward in naturally occurring microbial communities. Several studies have clearly shown that HNF grazing actually increases viral activity (Table 2); this has been termed synergism between HNF grazing and viral lysis (see Weinbauer et al. 2007). Šimek et al. (2001) were the first to report that protozoan grazing has some beneficial effects on viral activity. By conducting size-fractionating experiments on the microbial community obtained from a meso-eutrophic dam reservoir, they showed that viral abundance, the frequency of visibly infected cells (FVIC) of bacteria and VIBM were all higher in the 'grazing-enhanced' community (subjected to a 5.0 µm filtered treatment, which reduced the top-down regulation of larger predators on HNF) than in the 'grazer-free' community (0.8 µm filtered treatment). One interesting point is that a shift in BCC had occurred and specific groups belonging to the *Flectobacillus* lineage dominated the filamentous bacteria during the incubation period in the grazer-enhanced incubation. Dominance of the *Flectobacillus* lineage was also observed in the same kind of experiments conducted a few years later (Šimek et al. 2007). Similar beneficial effects of protozoan grazing on viruses have been reported from the same reservoir (Weinbauer et al. 2003, 2007), another oligotrophic reservoir (Sime-Ngando & Pradeep Ram 2005) and peri-alpine lakes (Jacquet et al. 2007) (Table 2).

Various mechanisms for the beneficial effects of grazing on viruses have been proposed. As we mentioned in 'Well-documented pathways and processes', HNF grazing can have various influences on the physiology of bacteria and on BCC (Concept 4, Table 1). This would in turn indirectly affect the activities and abundance of viruses. In general, HNF grazing enhances the cell-specific activity of bacteria by reducing competitive pressure for inorganic nutrients and DOM as a result of reducing the total abundance of

Table 2. Effects of HNF grazing on viral parameters in size-fractionation experiments. VA: virus abundance; FVIC: frequency of visibly infected cells, which can also be used for the estimation of VIBM (virus-induced bacterial mortality); BA: bacterial abundance; BP: bacterial production; BCC: bacterial community composition; BGR: bacterial growth rate; PA: prokaryotic abundance, including both bacteria and archaea; PP: prokaryotic production; PCC: prokaryotic community composition; VBR: virus-to-bacteria ratio; –: antagonistic relationship; +: synergistic relationship; ns: no significant relationship

Location	Trophic status	Time scale	Viral parameters (Effects of grazing)	Related parameters (Effects of grazing)	Source
Furuike Pond, Japan	Hypereutrophic	16 d	VA(–)	BA(–)	Manage et al. (2002)
Rimov Reservoir, Czech Republic	Mesotrophic	4 d	VA(+), FVIC(+)	BA(–), BP(–), BCC shifts	Šimek et al. (2001)
Rimov Reservoir, Czech Republic	Mesotrophic	4 d	VA(+), FVIC(+)	BA(–), BGR(+)	Weinbauer et al. (2003)
Rimov Reservoir, Czech Republic	Mesotrophic	4 d	VP(+), FIC(+)	BA(–), BP(–), BCC shifts	Weinbauer et al. (2007)
Sep Reservoir, France	Oligotrophic	2 d	VA(+), FVIC(+)	PA(+), PP(+), PCC shifts	Sime-Ngando & Pradeep Ram (2005)
Lake Bourget, France	Mesotrophic	1 d	VA(–/+)[a], FVIC(+)	BA(+)	Jacquet et al. (2007)
Lake Geneva, France	Mesotrophic	4 d	VA(+), FVIC(+)	BA(+), BCC shifts	Jacquet et al. (2007)
Rimov Reservoir, Czech Republic	Mesotrophic	4 d	VA(ns), FIVC(ns)	BA(–), BP(?), BCC shifts	Horňák et al. (2005)
Lac Cromwell, Canada	Dystrophic	5 d	VA(ns), VBR(–), VIBM(–)		Maranger et al. (2002)
Lake Pavin, France	Oligo-meso	4 d	VA(ns), FVIC(ns)	PA(–), PP(–)	Jardillier et al. (2005)
Adriatic Basin	Oligotrophic	24 h	VA(ns)	BA(ns), BP(ns)	Bongiorni et al. (2005)
Adriatic Basin	Eutrophic	24 h	VA(–)	BA(–), BP(–)	Bongiorni et al. (2005)

[a]Depends on the season

bacteria and so allowing these resources to regenerate (Concept 3, Table 1). Enhanced cell-specific activity may result in an increase in both viral production via cell lysis and in infection rate. First, enhanced cell-specific activity may lead to a shorter latent period (the period of infection prior to cell lysis) and an increase in the burst size of lytic viruses (Middelboe 2000, Weinbauer 2004). In addition, shifts from the lysogenic mode to the lytic mode could be induced by an enhanced growth rate of the host cells (Williamson et al. 2002). HNF grazing would therefore enhance viral production via both these mechanisms. Second, enhanced cell-specific activity also leads to an increase in the number of outer membrane nutrient transporters, which could be recognized as receptors for viruses. It is argued that an increase in the number of receptors would enhance the attachment and infection rate of viruses (Lenski 1988 and references therein). This is based on evidence obtained from *Escherichia coli* and coliphage systems, although there is no evidence from natural aquatic systems. The hypotheses that growth enhancement of bacteria by HNF grazing favors viruses are indirectly supported by the experiments in which adding nutrients enhances viral production in various environments (Hewson et al. 2001, Williamson et al. 2002, Weinbauer et al. 2003, Motegi & Nagata 2007). In addition, there is another way protozoan grazing could directly affect viral activity. It is suggested that a prophage within ingested bacterial cells in the food vacuole of ciliates may enter the lytic cycle, and that some of the resulting viral particles are not digested, but released into aquatic habitats as free-living particles (Clarke 1998, Parry et al. 2006). Changes in bacteria at the community level may also explain the beneficial effects of HNF on viruses. First, it is suggested that switches in BCC toward the dominance of bacterial groups with higher growth rates and those with grazing-resistant traits can be linked to enhanced viral production (Weinbauer et al. 2003). If we assume that the growth rate is related to the number or structure of membrane receptors involved in nutrient transporting, which are also recognized by viruses (Lenski 1988), the dominance of such groups would lead to greater susceptibility to viral attack at the community level. In addition, if there are trade-offs between anti-grazing traits and anti-virus traits, enhanced viral activity can be attributed to the dominance of groups of bacteria with grazing resistance. However, filamentous bacteria (e.g. *Flectobacillus* spp.), which are grazing-resistant, may also be resistant to viral infection (Šimek et al. 2001, 2007, Sime-Ngando & Pradeep Ram 2005). This makes the connection between shifts in BCC and enhanced viral activity less straightforward. Second, it is also speculated that reduced bacterial diversity as a result of high grazing

pressure would reduce bacterial diversity, and that shifts in BCC may also be related to higher susceptibility to viral infection at the community level (Šimek et al. 2001). In general, if the diversity of the host is much greater than that of the parasite, the successful attack rate will be reduced because each type of parasite can attack only a small fraction of the host groups in the context of high host specificity (Keesing et al. 2006).

Although these mechanisms could potentially contribute to indirect beneficial interactions between HNF and viruses, several studies in similar experimental settings (size-fractionation experiments) have shown that HNF grazing reduces viral activity (Maranger et al. 2002, Bongiorni et al. 2005, Horňák et al. 2005, Jardillier et al. 2005) (Table 1). Surprisingly, one of these studies (Horňák et al. 2005) was carried out using the same system in which beneficial effects had been detected (Šimek et al. 2001, Weinbauer et al. 2003, 2007). It is likely that such interactions are also dependent on a range of environmental factors so that the overall effect can vary from positive to negative. For example, Jacquet et al. (2007) have shown that the type of effect of HNF on viruses in the surface waters of some peri-alpine lakes varies depending on the season. In general, the patterns observed over large spatial or time scales, which reflect long-term effects, do not reveal beneficial relationships between HNF and viruses. Vertical distribution patterns in lakes reveal negative correlations between the abundance and activities of HNF and those of viruses (Weinbauer & Höfle 1998, Colombet et al. 2006). Short-term incubation (15 d) of the sediment systems have shown an inverse correlation between HNF and ciliate abundance and virus: bacteria ratio (VBR) and FVIC (Fischer et al. 2006), whereas the temporal dynamics at the seasonal scale does not show any significant correlation between HNF and viral activities (abundance, FVIC and burst size) (Bettarel et al. 2003, 2004).

These data could result from the combined effects of beneficial (synergistic) and harmful (antagonistic: competition and IGP) effects of relationships between HNF and viruses. Unfortunately, limiting the nutrients available to the bacterial community would affect both types of effects in similar ways: (1) Beneficial effects initiated by nutrient regeneration may have a major impact in oligotrophic systems. (2) The negative effects of competition between HNF and viruses for bacteria may also be more severe in oligotrophic systems that are bacterial growth-limited. (3) The expected longer latent period observed in a context of nutrient limitation (Middelboe 2000) would mean that the negative effects of coincidental IGP would be more marked in an oligotrophic system (Miki & Yamamura 2005). (4) Although eutrophication may enhance top-down effects on HNF because of larger protists, it would also

weaken both the positive and negative effects on viruses. This means that the trophic status cannot be 'used' as an environmental factor to change the balance between positive and negative effects of HNF on viruses. In addition, although many arguments and suppositions propose that HNF may have beneficial effects on viruses, we need much more evidence to identify all the processes involved in enhancing viral activities in response to grazing.

It is worth noting that positive relationships between HNF and viruses become apparent only in unstable, non-equilibrium situations, which tend to occur in short-term experiments (Table 2), but not in stable, equilibrium situations, which more often correspond to long-term situations or large spatial distribution patterns. Even if the net effect over long time scales is always negative, we hypothesize that positive indirect effects of HNF on viruses could offset the negative effects of competition and IGP, and contribute to the coexistence of viruses and HNF. Of course, it is difficult to test this hypothesis, because of the difficulty of separating positive and negative effects experimentally.

Indirect effects of viruses on HNF (Pathway 6)

Just as HNF affect viruses, so too can viruses be expected to have negative effects on HNF as a result of exploitative competition. In addition, viral lysis enhances carbon remineralization through the regeneration of DOM, which is then reprocessed by heterotrophic bacteria, resulting in the transfer of smaller fractions of DOM to higher trophic levels (i.e. HNF); this is confirmed by theoretical models (Fuhrman 1999, Miki et al. 2008). However, few direct comparisons have been made of microbial communities with and without viruses. It is also speculated that viruses have some beneficial effects on HNF, based on the same assumptions as those made for the beneficial effects of HNF on viruses. This would mean that enhancement of cell-specific activities of bacteria via resource regeneration and shifts in BCC might enhance their vulnerability to HNF grazing. However, results from a simplified model (Miki et al. 2008) suggest that a moderate increase in the grazing risk of virus-resistant bacteria (50% higher than virus-susceptible bacteria) does not compensate for the negative effects of viruses in reducing bacterial abundance and enhancing carbon remineralization. As far as we are aware, only a few experimental studies have examined the effects of viruses on HNF. However, no statistically significant difference was found in HNF abundance between a system with bacteria and HNF, and that with bacteria, HNF plus viruses (Weinbauer et al. 2007, Zhang et al. 2007). So

far, we can only speculate that the effects of viruses on HNF must be either harmful or negligible on the basis of the correlation patterns between them, as discussed in 'Indirect effects of HNF grazing on viruses (Pathway 5)' above (Weinbauer & Höfle 1998, Bettarel et al. 2004, Colombet et al. 2006).

Next steps for investigating indirect interactions between HNF and viruses

From a more general point of view, these indirect interactions between HNF and viruses, especially via phenotypic changes in the bacterial community, would be the first known example in aquatic systems of 'trait-mediated indirect interactions' (Ohgushi 2005) (Fig. 2B), which have been widely observed in terrestrial plant–herbivore interactions (Ohgushi 2007). In terrestrial systems, herbivory by insects and mammals often induces trait changes in plant tissues, which indirectly affects the growth or abundance of other consumers of the same individual plant. These indirect effects can be negative, as a result of induced resistance to herbivory (e.g. Faeth 1986, Karban & Myers 1989), but can also be beneficial as a result of compensatory re-growth of plant tissue (e.g. Nakamura et al. 2003), or of induced resistance that is effective against 1 consumer species, but actually benefits other consumers (Martinsen et al. 1998). When we interpret the positive effects of HNF grazing on viruses within the framework of trait-mediated indirect interactions (Ohgushi 2005), the 'initiator' of this indirect effect (HNF) induces phenotypic changes in bacteria, which in turn act as a 'transmitter'. Phenotypic changes in the 'transmitter' (bacteria) subsequently affect the 'receiver' (viruses), thus resulting in an indirect effect.

In plant–insect systems, since the generation time of the resource (plant) is generally longer (several years) than that of the consumers (insects) (a few weeks to months), and effects of the consumer community are concentrated on an individual plant and are non-lethal (in general, insects do not kill the plant but utilize part of plant tissue), the induced phenotypic changes occur only in individuals actually subjected to herbivory. Therefore, individual responses are the major process affecting the dynamics of insect populations and communities. On the other hand, in microbial systems, since the risk of HNF grazing and the positive effects on bacterial growth are shared among all the individuals in the community, individual phenotypic changes occur in all individuals in the community. In addition, these responses will persist over the generation time of the resource (bacteria), because the gap in generation time between the resource and the consumer is relatively small (bacteria generation time is about the same

as that of predators and viruses, i.e. around a day). Shifts in BCC therefore also contribute to the induced trait changes at the community level, which may occur over longer time scales than phenotypic plasticity at the individual level. Consequently, not only individual responses but also shifts in BCC affect the population or community dynamics of HNF and viruses. One distinctive feature of microbial systems is that multiple processes can contribute to phenotypic changes at the community level, mediating indirect interactions between consumers over relatively short time scales. This kind of comparison between different systems will provide fresh insights into both microbial ecology and general ecology.

One of the lessons from terrestrial systems is that the sign and strength of indirect interactions depends on the combination of initiator and receiver species, even with the common transmitter species (Ohgushi 2005). Therefore, it is necessary to pinpoint the initiator and the receiver species within the HNF and viral communities, but size-fractionation experiments are probably not up to the task. Identifying these species and their characteristics would enable us to start to answer the following questions: Why do the indirect beneficial effects of HNF on viruses only occur in some cases? When does the benefit gained outweigh the harm done? Do the positive or negative effects occur seasonally, and if so, is this attributable to the seasonal succession of HNF and viral communities or to seasonal changes in bacterial physiology? Why do apparently negative relationships between HNF and viruses appear in large spatial patterns? In addition, despite enabling us to discuss the prevalence of positive indirect effects of HNF on viruses, which are likely to be related to the increased availability of nutrients, size-fractionation experiments have methodological limitations. Physical separation of bacteria and grazers by filtration is inevitably accompanied by changes in bacterial cell physiologies and cell destruction, and furthermore the filtration process itself is likely to influence nutrient availability and bacterial growth.

It is also necessary to identify similarities and dissimilarities in different systems by comparing pelagic and benthic systems, free-living and particle-attached systems of bacteria–virus–HNF interactions, and freshwater and marine systems. There are many routes of interactions between grazers and viruses in aquatic systems, but only a few studies have attempted to characterize these interactions. This means that we should also pay attention to indirect interactions between protozoan predators and viruses, which are mediated by different players in aquatic food webs, including not only heterotrophic bacteria, but also other food web members, which constitute common resources for both protozoan predators and viruses, and can therefore mediate indirect interactions between them. Photosynthetic prokaryotes (e.g. *Synechococcus* spp. and/or *Prochlorococcus* spp.) and eukaryotic phytoplankton communities can be transmitters of indirect interactions. These phytoplankton communities are known to induce phenotypic changes in response to protozoan and metazoan grazing (Long et al. 2007), and to viral infections, although there are no reports of indirect interactions resulting from these trait changes.

Untangling these indirect interactions between HNF and viruses will also contribute to the understanding of the mechanism behind the partitioning of bacterial loss to predation and viruses. Their relative importance should be compared among various types of ecosystems and within ecosystems (i.e. at different locations and/or various spatial scales), although it has generally been studied in one type of ecosystem (whether in different seasons and/or depths or not) and more rarely in a variety of ecosystems. Recently, Lymer et al. (2008) reported such a comparison for the epi- and hypolimnion of 21 lakes differing in trophic status and humic content (see below).

Less-explored pathways and future directions

There are other underexplored pathways in the interactions among bacteria, viruses and HNF, i.e. interspecies interactions within each functional group: interactions between bacteria (Pathway 7, Fig. 1), between viruses (Pathway 8, Fig. 1) and between HNF (Pathway 9, Fig. 1) themselves. In natural aquatic systems, exploitative competition occurs between bacteria for the use of organic and inorganic resources, which is suggested by experiments (Middelboe et al. 2001, Corno 2006) and theories (Middelboe et al. 2001). Interference competition with particles is known to result from the production of antibiotic compounds by neighboring cells (Long & Azam 2001). Positive interactions (commensalism and mutualism) typically occur when bacterial density is high, and the frequency of direct interactions between cells is increased, as in biofilm or particle-attached communities (Paerl & Pinckney 1996). Dissolution of particulate organic carbon (POC) to form DOC, and the resulting increase in bacterial production by the free-living fraction (Friedrich et al. 1999, Riemann & Winding 2001), can be also interpreted as commensal interactions between particle-attached and free-living bacteria. There is also some evidence of predation by bacterivorous bacteria such as *Bdellovibrio* spp. (see review by Martin 2002, Yair et al. 2003), which have been shown to be highly diverse (Synder et al. 2002) and widely distributed in soil and aquatic environments (Yair et al. 2003).

Potential interactions between viruses (positive, negative and neutral) are summarized by Weinbauer (2004). Most viruses compete with other viruses for the same host. One virus negatively affects other viruses that share the same host not only as a result of competition for the same resources, but also via trait-mediated indirect effects, e.g. by changing the gene expression patterns of infected host cells that tend to prevent co-infection by other viruses (see Weinbauer 2004).

As for the interaction between 2 HNF species, competition for shared resources can naturally be expected. However, the prevalence of prey–predator interactions within protozoan grazers in the small size class (e.g. <5.0 µm) is unknown, whereas it is well established that larger protozoan predators (e.g. ciliates) eat both HNF and bacteria (Weisse 1990).

Further exploration is needed of the combined effects of viruses and HNF on BCC, species richness and bacteria-mediated biogeochemical processes. Theoretical models predict that the balance between HNF grazing and viral lysis is determined by the degree of bacterial diversity in the competitive community (Thingstad & Lignell 1997, Thingstad 2000, Miki & Yamamura 2005). Several studies have tested the 'kill the winner' hypothesis. A mathematical analysis of pairs of hosts and lytic viruses by Thingstad (2000) showed that bacterial abundance at equilibrium increases with the decay rate of viruses, and decreases with the infection rate and the rate of transfer of nutrients from the bacterial biomass to virus. Bouvier & del Giorgio (2007) showed experimentally under *in situ* conditions that an absence of viruses resulted in the dominance of rare marine prokaryotic groups. Based on the foregoing model analysis, their findings suggest that bacterial groups with a higher competitive ability for nutrients are more susceptible to viruses (high infection rate), that the specific viruses that prey on them can more efficiently utilize the bacterial biomass for their own replication purposes (e.g. higher burst size) or that their decay rate is lower than viruses that infect bacterial groups with lower competitive ability. As discussed in Thingstad (2000), the results of Bouvier & del Giorgio (2007) suggest that there are trade-offs between susceptibility to viruses and the ability to exploit nutrients. It is also predicted that viral lysis affects the bacterial consumption rate of diverse types of carbon sources (Miki et al. 2008). It is important to clarify how HNF and viruses interactively influence the BCC, and then affect bacteria-mediated biogeochemical processes. So far, only Weinbauer et al. (2007) and Zhang et al. (2007) have demonstrated the combined effects of HNF and viruses on bacterial abundance, richness or production.

For both well-documented as well as less-explored pathways, further investigation of each concept is necessary (Table 1). For example, the relative importance of both VIBM and the potential grazer-induced mortality, which is related to Concept 1 (Table 1), has generally been studied in 1 type of ecosystem (whether in different seasons and/or depths or not) and more rarely in a variety of ecosystems. Lymer et al. (2008) reported such a comparison for epi- and hypolimnion of 21 lakes differing in trophic status and humic content. For 70% of the lakes, bacterial removal was explained more by grazing activity than by viral lysis. However, viral activity was potentially highest in humic lakes of medium trophic status and also greater in the hypolimnion than in the epilimnion. Such a synthesis has been one of the few made available. Covering various environmental conditions (e.g. deep-sea, sediments and wetlands) will be an important step for a better understanding of both the well-documented and less-explored pathways. An understanding of the very small spatio-temporal dynamics of viruses is also lacking. To date, only Seymour et al. (2006) have examined the spatial distribution of viruses at the cm scale; they reported an uncoupled relationship between bacteria and viral abundance. As far as we know, no study has focused on how viruses interact with their host and natural enemies on very short time scales (e.g. minute and hour scales). Another important step for a better understanding of well-documented pathways is to consider the links between multiple emergent concepts (Table 1), some of which have been developed relatively independently of each other. For example, co-evolution between hosts and viruses (Concept 9, Table 1) is likely to have a large impact on the degree of phylogenetic selectivity of VIBM (Concept 5) (Riemann & Middelboe 2002), and then affect the BCC and bacteria-mediated biogeochemical processes (virus shunt: Concept 7). It is also likely that the diversity of viral life cycles (Concept 8) affects VIBM (Concept 1) and the strength of the virus shunt.

The 'black boxes' within the microbial loop framework would need to be opened. For example, the focus should not only be on biodiversity at very small scales within each functional group (e.g. species level, or focusing on more minor phylogenetic differences, or even gene level; for the genetic view of ocean microbes, see Venter et al. 2004, DeLong et al. 2006), but also on reconstructing functional groups, by dividing each one in the present model of microbial loops into several functional subgroups. Prokaryotes, as a basic functional group in the microbial food web, should be divided into heterotrophic bacteria, Archaea and photosynthetic groups. We should also find a place in such a list for heterotrophic prokaryotes that have proteorhodopsin, and so are able to utilize light energy (Eiler 2006). The microzooplankton in a microbial food web actually consists of multiple

size classes of predators (HNF, ciliates, rotifers, etc.). Each functional subgroup in prokaryotes therefore must consist of both specialized viruses and specialist or generalist predators. Any increase in the number of functional groups will result in an increase in the number of potential direct and indirect interactions. This may be of particular importance with the HNF group, since recent studies have shown that generally unidentified small heterotrophic flagellates are unlikely to be bacterial grazers but are instead phytoplankton parasites or saprotrophs (Lefèvre et al. 2007, Lepère et al. 2007). Opening these 'black boxes' could add to the current complexity, but should also help explain apparently contradictory findings obtained from a coarser classification of functional groups. It would be very interesting to examine diversified and complex relationships between species, and those between functional subgroups within each functional group (and within each community). However, it should be kept in mind that studies of this kind are intended to contribute to a better understanding of the dynamics and characteristics of the entire functional group or community, and of the relationships between communities. Opening the 'black boxes' should help us understand these 'black boxes' as a whole, and the interactions between them.

CONCLUSIONS

It is surprising to find that all kinds of inter-specific interactions (competition, mutualism, predation parasitism, IGP and trait-mediated indirect interactions) are found even in the apparently simplest and oldest biological communities consisting of prokaryotes, viruses and unicellular eukaryotes. We can easily imagine that biotic interactions and their consequences for biogeochemical cycling have been very complex since the early stages of the evolution of the aquatic ecosystem on earth. However, it also makes us realize just how little is known about the interactions between bacteria, viruses and protozoan grazers, and their consequences for aquatic ecosystems. Even at the community level (at the 'black box' level), interactions between bacterioplankton, viruses and grazers are much more complex than hitherto assumed. Ecological theories on the complex interactions between macroorganisms as well as advances in molecular biology techniques and environmental genomics, if applied to microorganisms, will shed new light on the complex interactions in the aquatic microbial world.

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