I - EXECUTIVE SUMMARY

This synthesis, initiated during the meeting, was consolidated thereafter by inputs received from the participants.

1. INTRODUCTION

The workshop was held at the historical Laboratoire Arago, Observatoire Océanologique de Banyuls-sur-mer, from 19 to 22 March 2003. Twelve scientists from nine different countries (see list at the end of volume) attended the meeting at the invitation of CIESM.

After welcoming remarks from Gilles Boeuf, Director of the Observatory, the meeting was opened by Frédéric Briand, Director General of CIESM, and Gerhard Herndl, Chair of CIESM Committee on Marine Microbiology and coordinator of the workshop, who briefly presented the context, background and objectives of this event.

1.1. Background and objectives

The initial aim of the workshop was to stimulate research on this fast-emerging subject in the Mediterranean Sea. The discovery that viruses are far more abundant – by three orders of magnitude (!) – in the marine environment than previously assumed is only ten years old. By now it is also well established that viruses are an active component of the microbial food web. As viruses may influence microbial species composition and regulate the abundance of specific species, they probably represent one of the driving forces of microbial successions. Therefore they directly influence nutrient regeneration and carbon cycling through the microbial communities. Moreover, viruses may be responsible for the occurrence of specific diseases in marine plants and animals, particularly if specific species are present in high densities such as in mariculture.

The Mediterranean Sea, with its large range of different subsystems ranging from dystrophic lagoons to oligotrophic deep waters, provides a unique opportunity to explore the function and significance of viruses under contrasting environmental conditions. Through a series of presentations and in-depth discussions, the workshop reviewed existing knowledge on marine viruses and explored promising paths for future investigations, taking the specific conditions of the Mediterranean Sea into account. After the first two days of presentations, two parallel sessions were organized to facilitate exchanges and brainstorming. The first group focused on viral abundance and virus-mediated mortality, the second on viral diversity. The final session included a general discussion where both groups presented their findings. Their main conclusions are reported in this executive summary.

2. VIRAL ABUNDANCE, PRODUCTION AND VIRUS-MEDIATED MORTALITY.

Section prepared by R. Noble, C. Brussaard, R. Danovaro, P. Lebaron, D. Mestivier, and T.F. Thingstad

Viruses are now known to be ubiquitous, numerically dominant members of aquatic microbial communities. Because of the nature of viruses as obligate parasites, viral infection commandeers an important role in structuring marine microbial communities, making them drivers of nutrient flux, elements in the global change puzzlework, and ferries of genetic information. The consequences of viral infection can be studied on a wide range of scales, from global to micron-size scales of microbial interaction, and from time scales ranging from thousands of years to a few seconds. Viral infection affects the dynamics of “microbial loops”, and has demonstrated impacts (sometimes dramatic) on both bacterial and phytoplankton populations, yet our understanding of

the consequence and complexity of these interactions is still limited. In particular, development of an understanding of the importance of viruses to issues such as nutrient flux will require improvement of current methods to assess viral abundance, rates of virus production, and virus-mediated microbial mortality. All of these developments will necessitate further understanding of more "static" virus parameters such as viral diversity, host specificity, lysogeny *vs*. lytic virus types, and host range.

Because the field of viral ecology is multi-scalar, we choose to present issues in viral ecology, and needs for future advancement of the field from a global - ecosystem - community - population - genotype perspective. We consider the Mediterranean Sea as a potential example for an excellent study system. With its anti-estuarine circulation, hydrographic mesoscale features with permanent cyclonic and anti-cyclonic gyres, general west-east trophic gradient (increasingly oligotrophic moving east), deep light penetration, seasonal and spatial shifts in pelagic food web structure, postulated phosphorus limitation, and winter deep-water formation, the Mediterranean is a perfect natural laboratory for the study of the interactions between microbial ecology and ocean biogeochemistry. Viruses are thought to influence microbial diversity, food web structure, and the partitioning of material fluxes between dissolved and particulate forms, processes believed to be modified by environmental factors such as UV light, temperature, and nutrient availability. Studies of these processes in the Mediterranean thus have the potential, not only to improve our understanding of the Mediterranean ecosystem *per se*, but to provide new generic knowledge on these processes in general.

2.1. Importance of understanding large scale issues

The theme of temporal and spatial dynamics of viral communities is one that contains both a descriptive and an analytical aspect. There is still a lack of knowledge as to how abundance, diversity and activity of viruses vary over long-time and large-spatial scales in different aquatic environments, the Mediterranean Sea included. Over long time scales, the aim of such studies should be to understand the underlying mechanisms that control abundance, diversity and activity of viruses in natural ecosystems, especially in the light of the possible climate change scenarios. At present, no commonly accepted theory exists for this. Analytically, an understanding of temporal and spatial dynamics will improve our ability to accurately place viruses into the context of microbial food webs, and will allow to create models of their effects on nutrient cycling, food web dynamics and biodiversity.

There are several environmental factors: UV-light, particulate organic matter (POM), dissolved organic matter (DOM), turbulence, and temperature, for example, that have known or suspected direct effects on viral processes such as decay, latent period, host encounter, and burst size. These direct effects are important subjects of study, and specific mechanisms such as UV-effects on viral decay may be particularly relevant in the high intensity solar radiation common to the Mediterranean Sea environment.

It is generally believed that viruses interfere and interact with the rest of the ecosystem in a number of ways: increasing the diversity of host populations by preventing permanent dominance of otherwise successful species and toxic species, diverting the flux of energy and matter from the particulate part of the food web into the DOM-pool, and by increasing the rate of horizontal gene transfer between hosts.

However, there is a difficult but crucial step in translating knowledge of direct effects into an understanding of indirect effects; or how the direct effects spread via trophic interactions to other levels in the food web. In other words, there is an actual need for field studies aimed at verifying how isolated processes observed in laboratory experiments behave in the context of natural ecosystems. The lack of any general theory of viral ecology makes this translation from direct to indirect effects particularly difficult.

To understand these relationships, data and descriptions of whole systems are needed and comparisons of systems should be encouraged. The ultimate goal will be to explain the properties of different systems from common generic principles. Mathematical ecosystem models will be a useful tool in this regard.

2.2. Viral induced mortality and its impact on biogeochemical cycling

The main biogeochemical function of viruses is thought to be their function as catalysers for the transformation of particulate (POM) into dissolved (DOM) organic matter as the content of the host organisms is released during cell lysis.

Whether microorganisms are grazed upon, or die due to viral-induced cell lysis has major implications for the flow of material and energy cycling in marine pelagic food webs. DOM-release will force the photic zone food web towards a more regenerative system. With more of the respiration occurring at the level of small organisms, the expectation on an intermediate time scale (see below) would be a reduction in the yield of larger organisms in the ecosystem.

To comprehend the microbial compartment of the Mediterranean pelagic ecosystem, an understanding of the processes retaining bioavailable forms of nitrogen (N) and phosphorus (P) within the photic zone as the surface water is moved eastwards from Gibraltar is essential. Again, the lack of a general food web theory makes it difficult to analyze the effects that viruses may have. Based on the steady state argument that viral lysis increases loss rates of the organisms present, these organisms also have to grow faster in the presence of viruses. Faster growth requires more food, with the consequence that more of the available total nutrients would have to be distributed into the smaller-sized organisms serving as food for larger predators. If such reasoning is correct, viral lysis should promote food chains dominated by small-celled organisms, producing little export and thus retaining more nutrients in the photic zone. With this expected improved nutrient retention, the long term net effect of viral lysis on ecosystem yield at higher trophic levels is not trivial. A higher viral abundance in eutrophic systems (typically characterised by large-celled organisms) has been demonstrated, but adequate information on viral infections and production in oligotrophic (long food chain) *versus* eutrophic (short food chain) systems is still lacking. As a result the differences in the role of viruses in aquatic trophodynamics and biogeochemical processes remain largely unknown.

The composition and fate of released DOM need to be examined in more detail. Viral lysis does not only produce a selective top-down mechanism thought to affect microbial diversity, but also potentially a bottom-up effect by alteration of the substrate spectrum for the osmotrophs (phytoplankton and heterotrophic bacteria). Comparison of the impact of the released carbon and nutrients on the biodiversity and function of pelagic food webs with different trophic status is still lacking.

Diversion of the flux of energy and material from particles to DOM also implies a diversion from potentially sinking into non-sinking forms. However, bacteria attached to sinking particles are also believed to dissolve the particles through enzymatic activities. Lysis of these bacteria may thus also have a reducing effect on particle dissolution. The biogeochemical distribution of elements and the efficiency of the biological carbon pump depend upon the depths at which carbon C, N, and P are transferred from sinking to non-sinking forms. In the Mediterranean circulation pattern – with the west-flowing deeper currents of the Levantine Intermediate Water and Mediterranean Deep Water – the biogeochemical distribution of C, N and P in the water column is presumably sensitive to the depths at which these elements are released into non-sinking forms. Since the depth of release is a combination of release rate and sinking velocity of the particles, the net result of these processes on Mediterranean biogeochemistry is difficult to predict (Fig.1).

Viral lysis of coccolithophoride phytoplankton species has previously been described. One may speculate that viral lysis, presumably releasing the slow-sinking coccoliths within the photic zone, versus grazing by macrozooplankton that may pack the coccoliths into rapidly sinking fecal pellets, may have very different consequences for the Ca-distribution in the sea, and thus for the so-called alkalinity pump. Dissolution of the coccolith $CaCO_3$ increases the alkalinity. If dissolution occurs in surface waters, rapid re-sequestration into the ocean of the CO_2 released when the $CaCO_3$ was formed is thus possible, and the potential large negative effect of coccolithophoride blooms on the ocean's ability to sequester atmospheric CO_2 is neutralized. The consequences for our ability to understand the feedback between global change and ocean biogeochemistry are large.

Several studies showed that viral-induced cell lysis of phytoplankton represents a mechanism of DMSP / DMS release in the marine environment, suggesting that viruses may play a possible role

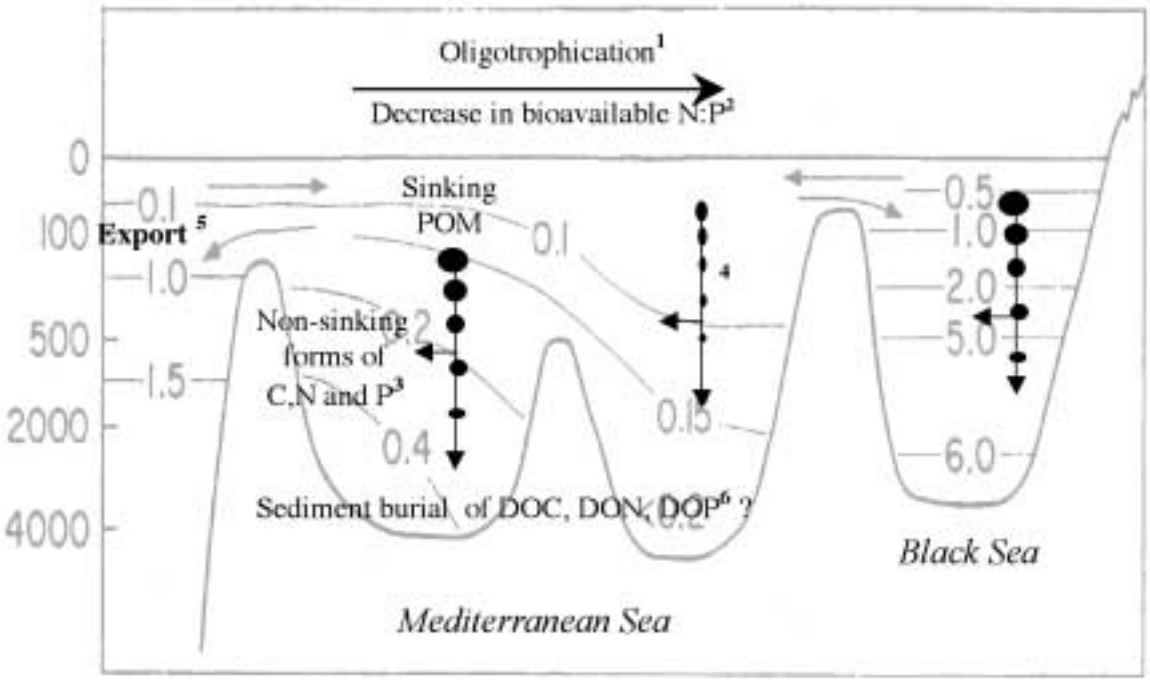

Fig. 1. Potential effects of viruses on Mediterranean biogeochemistry. Background figure shows isopleths of phosphate concentration (µM-P) in the Mediterranean and Black Seas (modified from Redfield *et al.,* 1963). If transfer of matter from particulate to dissolved forms via viral lysis is a quantitatively important process, viral lysis may affect such biogeochemical distributions through different mechanisms:

1. The east-to-west oligotrophication of the surface waters is potentially influenced by viral lysis affecting nutrient retention in the photic zone, and thus structural changes in the pelagic food web.
2. Viruses potentially affect a differential loss of N and P to non-available forms of DON and DOP, thereby possibly playing a role in shifting the Mediterranean system towards P-limitation.
3. Viral lysis of sinking organisms or organisms attached to sinking particles will release C, N and P from sinking to non-sinking forms.
4. Sinking to non-sinking transfer of matter may differ in the different basins due to differences in the photic zone food web structures, producing quantitative as well as qualitative differences in the vertical fluxes of POM.
5. Biogeochemical distributions in the Mediterranean and export of C, N, and P through Gibraltar will depend upon whether the sinking to non-sinking transfer occurs in the Levantine Intermediate Water or in the deep water.
6. DOC, DON, DOP release from POM in sediments may promote burial of C, N and P.

in climate regulation. The release of DMS / DMSP from lysed phytoplankton cells is of significant importance during blooming events such as typically found in eutrophic coastal areas. In more oligotrophic systems, the effects of anthropogenic impacts (PCBs, eutrophication, environmental stressors and micropollutants) on the significance of viral-induced mortality (induction and lysogeny, algal bloom dynamics) may be of special interest, especially for the Mediterranean Sea with its high degree of human activities and ever increasing coastal development. For a better understanding of the quantitative importance of viral infection on the pelagic food web, there is strong need for methods able to specifically detect viral abundances and lysis rates of bacteria and of phytoplankton, separately. Beside these two obvious groups of organisms, viral lysis of other members of the microbial food web, such as pico-eukaryotes and protozoans, can also be expected to contribute significantly to the flux of matter and energy. At this time, our understanding of the relative importance of viral lysis of specific groups is poor. The development of accurate methods will allow us to also estimate the magnitude of community specific, or possibly even species specific losses, a necessity for our research field. Virus-host systems based on key planktonic species should be used to further optimize our knowledge of actual lysis rates.

Mortality of microbial populations due to viral lysis has further implications for substrate availability and development of host infection resistance modes. Several recent studies have demonstrated the “usefulness” of viral infection products for successful growth by heterotrophic

bacterioplankton. The availability of the lysis-derived organic material for assimilation and further degradation has many biogeochemical implications. As in other areas, dissolved organic carbon (DOC) accumulates in Mediterranean surface waters in the stratified season. Does this occur because viral lysis produces recalcitrant organic molecules, because there are photochemical processes transforming degradable into recalcitrant chemical forms, or because of accumulation of degradable material as a consequence of P-limitation of heterotrophic bacteria? In any case, the accumulation will increase the C-sequestration capacity of the sea. With a large fraction of total N and P in the dissolved organic nitrogen (DON) and dissolved organic phosphorus (DOP) pools, one could also speculate whether a small difference in the loss rates of N and P to recalcitrant forms of DON and DOP may play a part in the so far unexplained shift towards P-limitation in the Mediterranean Sea. The process of viral lysis in sediments is also speculated to release host DNA into an environment with low DNA degradability. If so, viral lysis could be a process influencing P cycling in the ocean's sediment.

Pilot studies indicate that the viral lysate can affect the susceptibility of the host population for further virus infections, something that warrants more dedicated research. The issue of sensitive versus resistant host populations has just begun to be explored. Techniques that enable the researcher to examine these processes *in situ* are vital for an accurate understanding of this process. Another intriguing issue is the evolutionary implication of viral lysates and viral infection events, as it has been hypothesized that repackaging of cellular DNA by viruses contributes to wide-scale spread of specific genes and that viral lysis from dominant species yields DNA release for potential gene transfer to other microorganisms.

2.3. What are the controlling factors for microbial loop components ?

Based on traditional Lotka-Volterra formulations, one can construct simple idealised models for the microbial part of the food web. In these, size-selective grazing will allow steady states with coexistence of size groups of osmotrophs (phytoplankton and heterotrophic bacteria) even when all of these compete for a common limiting substrate such as e.g. phosphate. Including size-selective predation is thus one simple way to resolve the classical Hutchinson's paradox. Adding host specific viruses to this description will in an analogue manner allow coexistence of host-groups inside each size-group of osmotroph competitors. This theory has been argued to allow for more microbial diversity than presently suggested for bacteria by methods such as denaturing gradient gel electrophoresis (DGGE). In a sense, this reverses Hutchinson's classical paradox to the question of "*why do we seem to observe less diversity than can be explained by simple steady state models?*". The above principle of selective loss mechanisms "*killing the winner*" is, however not readily extended to viruses since selective loss mechanisms for viruses are not presently known. A theory has been proposed where viral lysis is the mechanism that compensates for differences in growth rate between coexisting (dominant) bacteria, leading to the conclusion that viral abundance is linked to the magnitude of physiological diversity in the bacterial community. Interestingly, such a theory links biodiversity directly to the pattern of biogeochemical cycling of elements. This theory seems, however, able only to explain the stable existence of one virus per "host group". With indications that there may be many (coexisting) viruses attacking the same host, Hutchinson's paradox may seem to return, but this time at the level of viruses: how can several viruses coexist on one host population?

In the above description, diversity in the sense of number of simultaneously coexisting "species" becomes a phenomenon that is "top-down" controlled. It does, however, not give any clues as to the organisms that will occupy these niches. This, to a much larger extent, is presumably a question of the relative competitive abilities among the potentially coexisting hosts. With this in mind, predation (top-down), viral lysis (side-in), and nutrient competition (bottom-up) control different aspects of host diversity. There is a large need for relevant experimental model systems that can be used to elucidate these different modes of control.

An interesting aspect of the community of coexisting hosts is whether the individual species remain at a (more or less) stable abundance, whether the individual host-virus system exhibit Lotka-Volterra type oscillations, or whether there are spontaneously occurring oscillations that collectively determine the stability of the system.

2.4. Impact of viruses at the population level

Viruses exert significant impacts on the structure of microbial populations in aquatic environments. Because of their relative specificity, viruses are capable of altering microbial community composition over short time scales. Since viral infection is thought to be driven largely by encounter rates, this process causes immediate and direct effects shifts in community composition by "killing the winner" where the success of a particular microbial species can quickly translate into death by viral infection. Direct effects of community composition shifts include the obvious removal of specific microbial species or groups, which in the case of diverse aquatic environments can be a complex issue to resolve. Indirect effects of viral infection, such as alterations in substrate availability, promotion of resistance strains of host cells, and shifts in competition among microbes for available substrates, are even more complex and thus far poorly studied. Advancements in methods to assess these effects (both direct and indirect) in natural systems are in dire need, as well as the use of relevant model virus-host systems to generate input for deterministic and probabilistic models.

There have been several important factors in virus control that have been speculated in recent publications. The first example is the development of host resistance to viral infection. It has been demonstrated in several studies that host cells are capable of developing resistance to viral infection over short time scales, thereby preventing decimation of the population. From experimental model systems, it is known that resistance in bacterial hosts often has a price in the form of compromised competitive abilities of the host. The bacteria-phage "war" is thus not only a fascinating subject of study from the perspective of the microbial food web dynamics, but also is an ideal system for the study of evolutionary processes within an ecosystem context. The processes of development of resistance and its ecosystem consequences are not well understood, and this area deserves more attention.

Viral impacts on populations have been demonstrated under bloom conditions, where viruses have the potential to cause immediate termination of bloom species. We know little about the factors and interactions of this process, the importance of host range, clonal variation within host species, morphotypes within species, and relation to other parameters such as nutrients and grazing impacts. It has been speculated that the range of clonal variation within host populations is important to the success of viral infection, therefore clonal variation may play an important ecological role in biodiversity. However, clonal variation within the virus populations infecting the same host species, or even the same strain, suggests that the effect of viruses on biodiversity is more complex than thought so far.

Although it goes almost without saying that it is important to study virus control of populations in natural systems, we would like to stress that model host-virus systems are vital for an optimal understanding of the mechanisms behind virus control of host populations. Relevant model systems can be used to study decay, infection rates, latent periods, lysis rates, clonal variation in host populations, and variables that control success of infection, microscale studies on surface charge changes of the host cells, interactions of receptor sites and nutrients, as well as the adsorption mechanisms. *Prochlorococcus*, with its synchronized life cycle and occurrence almost as a monoculture in the stratified season in the eastern Mediterranean Sea, is an intriguing model system for the study of virus ecology.

There are specific issues related to population level processes that deserve mention, mostly because of our poor general understanding. For example, because of the sheer number of virus host encounters in marine environments, and the known processes related to gene transfer (repackaging of cellular DNA by viruses, transduction, conversion) by viral infection, it is thought that viruses may be responsible in large part for microbial evolution. With an understanding of the rates of gene transfer in natural environments, it may be possible for us to assess relative time scales of evolution, and thereby determine if they are shorter than time scales of global change. We suggest that by studying the process of gene transfer from a rate perspective, and by direct linkage to a better descriptive understanding of viral diversity, it will be possible to assess the importance of gene transfer and its role in diversity.

Box 1 COMMON METHODS IN VIRAL ECOLOGY

For the benefit of the reader, we have compiled a list of viral ecology methods that are commonly employed in the field of aquatic viral ecology. Each method is listed with one or two relevant bibliographic references (among others), so that the reader can access further details of the methods. This list is not intended to be exhaustive, but merely a summary of some of the most routinely methods currently used.

Virus enumeration

- Transmission electron microscopy counts (Børsheim *et al*., 1990, AEM)
- Epifluorescence microscopy (Yo-Pro 1: Hennes and Suttle, 1995; SYBR Green I: Noble and Fuhrman, 1998)
- Flow cytometry (Marie *et al*., 1999; Brussaard *et al*., 2000)
- Most probably number assay of infectious units (Suttle, 1993)
- Plaque assays of infectious units (Suttle, 1993)

Viral lysis rates

- Frequency of visibly infected cells (Hennes and Simon, 1995; Weinbauer *et al*., 1993)
- Production of radiolabeled, virioplankton sized biomass (Steward *et al*., 1992)
- Use of fluorescently labelled viruses as tracers (Noble and Fuhrman, 2001).

For detailed protocols for the above approaches, see Noble and Steward, 2001.

- Dilution approach (Wilhelm *et al*., 2002)
- L&H method (Evans *et al*,. 2003)

Viral diversity

- Pulsed Field Gel Electrophoresis (PFGE, Steward, 2001)
- Denaturing Gradient Gel Electrophoresis (DGGE, Short and Suttle, 2002; Schroeder *et al*., 2003
- Nucleic acid sequencing of viral genotypes (Lu *et al*., 2001)

Virus-host interactions, host range, viral typing

- DNA-DNA hybridization (Wichels *et al*., 2002)
- Flow cytometry (Brussaard *et al*., 2002)
- Use of fluorescently labelled viruses as probes (Hennes *et al*., 1995)

2.5. Persistence of virus populations

There have been several studies conducted to assess viral decay and/or loss of infectivity in aquatic environments. However, these experiments have largely been conducted either on specific virus-host systems, or by using a whole system approach over very short time- scales. Most of these studies have been executed only in laboratory microcosms, examining single alterations in a single environmental variables. Although these experiments have yielded some useful information, they are far too limited to provide an understanding of viral decay and inactivation in real-world scenarios. Field studies have provided conflicting results related to our current concepts of viral decay, as they provide evidence for viral turnover rates either much higher or in some cases much lower than those seen in laboratory studies. This suggests that there are complex interactions with several environmental parameters that can strongly influence rates of viral decay. If we focus only on single variables, we are excluding these types of interactions from our assessments of viral decay. Additionally, it is widely thought that mechanisms of loss of viral infectivity are completely uncoupled from those of viral degradation and this has not been addressed in aquatic environments.

In addition to environmental variables such as UV light, enzymes (nucleases/proteases), it is important to consider the relevance of the mechanical damage, and the process of viral adsorption and their relations with removal of viruses from the system. Still very little is known on the

preservation of free viruses that are attached or entrapped into an aggregate matrix. There are several mechanisms by which this is hypothesized to occur: viruses may be associated with exopolymeric substances, aggregates, and marine snow, and subsequently removed from the system, or can be incorporated into the aggregate and subsequently released into the environment during aggregate degradation. It has been also suggested, based on preliminary experiments, that successful viral infection of *Heterosigma akashiwo* increases the sinking rates of the infected cells, causing removal of the specific, potentially damaging virus strains and nutrients from the water column. Furthermore, the importance of viruses as actual food source is largely unexplored, but it can be hypothesized as potentially important in P-limited environments.

On larger spatial scales, it is important for us to understand rates of viral decay and inactivation in relation to the potential of viral transport throughout the world's oceans. Distribution of virus types in a global context is not only dependent upon the presence of viable hosts in sufficient abundance, transport by mixing, and encounter rates, but is also directly related to rates of viral persistence. Understanding viral decay rates in specific systems may permit us to model the transport of "globetrotter" viral types, extending the theory of supply-site ecology in aquatic systems.

2.6. Reservoirs of viruses

Deep water masses and sediments can be thought of as reservoirs of viruses, able to segregate or exchange virus assemblages with the overlying waters. Although investigations of benthic viruses are still at the scientific frontier's edge, there is increasing evidence that surface sediments, down to abyssal depths, can host very large virus numbers. Viruses also display a sharp decrease with depth in the sediment, but large numbers still are present in deeper sediment layers. This would suggest that viruses can be preserved in the sediment matrix and particularly in anaerobic sediments. Practically no information is available on decay rates of viruses in the sediments. If slow decay rates of benthic viruses are confirmed, this would provide a potential role of sediments as reservoirs for viruses. The overall importance of studying sediment viral and microbial communities is also related to theoretical questions. For instance, the abundance of specific groups of bacteria in marine sediments seems to be similar to those found in the water column. However, total abundances of bacteria are very different between sediments and water column, with more coexisting bacteria in the sediment. Why does this difference exist, and could this difference be explained by the difference in control of microbial communities exerted by protozoan grazing and viral lysis?

What is still unclear is whether viral assemblages in the water column and sediments are similar or not. If one assumes that benthic bacteria are different from pelagic bacteria, then their respective viruses should also be different. Preliminary electron microscopy evidence would suggest rather different morphological features. Is virioplankton able to infect benthic bacteria, and/or is viriobenthos able to infect virioplankton? If so the role of sediments as reservoir of viruses would have major implications for bacterioplankton dynamics and would suggest that the benthic-pelagic coupling of viral infections should be taken into account for modelling pelagic processes. This would increase the importance of physical processes in promoting viral exchange between different marine compartments. Sediment resuspension, slope currents, and upwelling could have a major relevance from epidemiologic perspectives, and could contribute to the exchange of viral diversity between sediments and the water column. Finally, as sediments present completely different environmental (physical, chemical, trophic) conditions from the water column, does this implies that virioplankton and viriobenthos have different viral life strategies?

2.7. Role of viral ecology in cross disciplinary studies

Viral ecology is an important, yet often unaddressed component of interdisciplinary aquatic studies. Not only could the field of viral ecology benefit from cross-disciplinary interaction, but other fields could benefit from understanding the wide array of processes that viruses impact in aquatic systems. There are several specific examples of the need for intertwining studies in the field of viral ecology with other disciplines. There is a need for incorporating specialized studies of phage and viral function, including assessment of receptor protein structure, attachment mechanisms, induction of lysogeny in aquatic species. On a wider scale, there is also a need for involvement of physical oceanography expertise in the field of viral ecology, for example to help elucidate

Box 2. PRIORITY RESEARCH QUESTIONS

- How do abundance, diversity and activity of viruses vary over time and space in different aquatic environments? At present, no commonly accepted theory exists explaining the underlying mechanisms that control abundance, diversity and activity of viruses in natural ecosystems. It is important to understand both the direct and indirect controlling factors.
- To what extent does viral lysis contribute to and maintain observed nutrient gradients ?
- Does viral lysis significantly influence biogeochemical distribution, especially in comparison to large scale processes like physical mixing of water masses ?
- What is the impact of climate change on viral infection and related biogeochemical processes ?
- What is the fate of viruses (decay, removal rate from pelagic to sediment, inactivation), specifically in relation to temperature, UV light, salinity, and pollutants ?
- What are the effects of anthropogenic impacts (PCBs, eutrophication, environmental stressors and micropollutants) on viral-induced mortality of microorganisms ?
- How well-preserved are viruses that are trapped in aggregates or sediments ? Are the free viral communities similar to the trapped viral communities ?
- Is viral infection the agent of control that is responsible for maintaining existing microbial community structures ?
- Does viral lysis promote food chains dominated by small-celled organisms, producing little export and thus retaining more nutrients in the photic zone ?
- What is the impact of lysogeny in aquatic systems ?
- Are viruses a significant food source, and if so, under what conditions ?
- Considering that each host species and likely each host strain can be infected by more virus types, how does viral infection regulate host diversity ?
- How can several viruses coexist on one host population ?
- What factors mediate and control resistance of the host cell to virus infection ?
- What are the evolutionary implications of viral infection events and viral lysates (repackaging of cellular DNA by viruses contributes to wide scale spread of specific genes. Viral lysis from dominant species yields DNA release for potential gene transfer to other microorganisms).
- How important is viral infection to the process of gene transfer? At what rate does gene transfer take place in natural environments ?
- Can we use mathematical ecosystem models that include a thoroughly tested virus module, and are validated against actual data, to model interactions of viruses in natural systems ?
- What is the ecological relevance of novel viruses (e.g. picophytoplankton)?
- What controls stability in virus-host interactions, e.g. why can some viruses coexist stably with their hosts ?

processes of water parcel mixing and its role in transport of viruses. There is need for involvement of chemical oceanography to help elucidate the composition of viral lysis induced released cellular organic matter and how this affects the functioning and structure of the microbial pelagic food web.

In applied microbiology, there is a need for applying currently used methods in the field of viral ecology to the study of eutrophication and water quality and vice versa. For example, current studies of eutrophication and bloom formation tend to focus on nutrient inputs and "top-down" controls such as grazing, but few of them examine the virus-related control of communities. Viruses are capable of exerting both direct and indirect impacts in these systems and these controls need to be further evaluated. Developments in the field of viral ecology, and more specifically related to the impact of viral infection in controlling bloom-forming species, can be expected to help understanding how to mitigate harmful algal bloom (HAB) species. Furthermore, there is a need for linking the fields of phytoplankton ecology, viral ecology, nutrient cycling and physical oceanography to better understand whole ecosystems. In a time of increased development along the coastlines of the world, there is a recent wave of efforts to develop whole ecosystem models. In a specific sense, there is an immediate need for understanding the linkages between the microbial loop, the process of eutrophication (nutrient uptake by phytoplankton, hypoxia/anoxia, fish kills) and the increasing occurrence of HABs, something that is currently omitted from most coastal and estuarine modelling approaches.

3. VIRAL DIVERSITY

Section prepared by K. E. Wommack, C. A. Suttle, W. H. Wilson, M. Weinbauer, C. Pedrós-Alió, and C. Schuett

Appreciation of biological diversity is key to many investigations of ecosystem processes. Diversity exists on several levels, communities, populations, organisms and ultimately genes, and can be defined with increasing levels of resolution. Because viruses are incapable of replication outside of host infection, virus diversity is inextricably linked to host diversity. Thus, deterministic studies of marine viral diversity at increasing levels of resolution (community, population, and strain) are critical to a better appreciation of the role of viral infection and lysis in the population biology and ecology of marine microbial host communities. In this sense, diverse communities of marine viruses may represent a unique paradigm within ecology.

3.1. Scales of viral diversity

The past decade of research in marine viral ecology has revealed that characterization of viral diversity is critical to constraining the ecological role of viruses within marine microbial communities. Diversity of viral communities can be defined and measured on several levels which have implications for the evolutionary and ecological impact of viruses within marine microbial communities. Perhaps the earliest indication that viral diversity probably influences co-occurring host diversity came through the general realization that viral strains can be very restricted in the range of hosts they infect. The routine observation of host specificity has important implications for understanding the interaction of viral diversity on host community diversity. Unlike other predators of bacteria and phytoplankton, the specificity of viral infection indicates that viruses may selectively affect the occurrence and distribution of host strains within communities of plankton (i.e., killing the winner hypothesis) (Thingstad, 2000). Conversely, some viruses infect a diverse range of hosts. Thus, it is easily imagined that the diversity of host ranges within viral communities, from highly specific (strain) to broad (intergenic), is a key parameter to understanding the ecological impact of viral infection within marine microbial communities.

While host range is a critical, albeit poorly constrained, ecological parameter for marine viruses, it is exceedingly difficult to determine for a large suite of viruses. Moreover, host range is only informative to ecological studies if this parameter is known for the viral strains within a natural community. Thus, characterizing viral diversity at a genetic level is a more tractable approach for investigating the effect of viral infection on host community diversity. For uncultivable microorganisms, small sub-unit ribosomal RNA has been a critical marker for phyletic studies of microbial communities and has revealed that the biosphere contains an amazing diversity of

prokaryotes and picoeukaryotes. However, because of their reduced genomes and varied life strategies, no universal genetic marker exists with which to compare the diversity among all viruses. Nevertheless, through molecular genetic tools, it is possible to characterize viral diversity, from an evolutionary standpoint, using marker genes specific to broad groups of viruses. Moreover, sequence analysis has revealed the mosaic nature of viral genomes and the complex nature of their evolutionary history. On both these levels, single gene to whole genome, we can begin to appreciate how the diversity of viruses affects the structure and activity of planktonic communities.

A conceptual diagram illustrates the interconnected relationships that define how viral diversity within marine ecosystems is characterized (Fig. 2). We define the smallest unit of viral diversity as the genogroup which is a group of related viruses that is clearly monophyletic based on a specific marker gene. The difficulty of cultivating viruses and their hosts, as well as the modular nature of viral evolution, makes it necessary to focus on single genes as defining the basic units

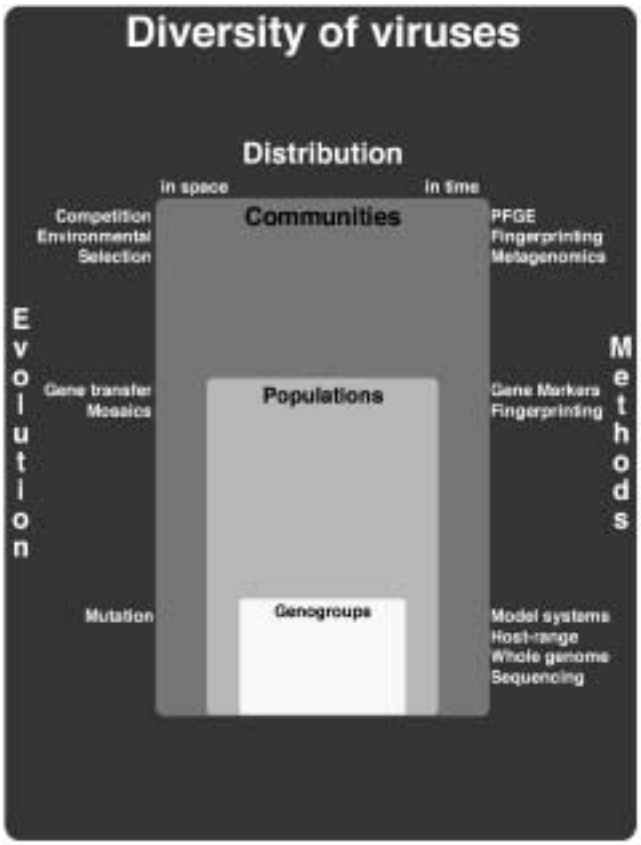

Fig. 2. Scales of viral diversity

of viral populations. In this regard it is imperative to focus on viral genes with a well-defined evolutionary history. At present examples of such viral genes are few, making the search for viral gene markers essential for more sophisticated examinations of marine viral diversity. Ultimately it is desirable to include genes in our molecular toolbox, which confer viral phenotypes of ecological consequence, (e.g., host range, burst size, and latent period.)

At the level of the genogroup, evolutionary processes can be rapid, principally deriving from point mutations in key genes that alter host range, speed of infection (latent period), life cycle (temperate, virulent, chronic) and burst size. Each of these alterations in viral phenotype has significant implications for host population biology. Understanding how these phenotypes (key genes) change on environmentally relevant time scales will be critical to the accurate estimation of the role of viral infection and lysis within marine microbial communities. At present, each of these specific characteristics of host-viral interactions is poorly constrained for natural populations. Thus, isolation of appropriate viral-host model systems, as well as more encompassing data sets of whole viral genome sequences are critical needs.

The classic ecological definition of a population is a collection of strain (sub-species) groups that can exchange genetic information. While the modular theory of bacteriophage evolution was predicted before the wider availability of genome sequence, it appears that the genomes of tailed bacteriophages are comprised of genetic modules which are disseminated among apparently dissimilar strain groups (Botstein, 1980; Hendrix *et al.*, 1999). In effect, the genomes of tailed phages are mosaics of connected modules that mix and match over evolutionary time scales. At present, it is not known whether viral groups outside of the tailed bacteriophages display similar genetic promiscuity over evolutionary time. Thus, whole genome sequencing of viruses infecting marine eukaryotes is warranted to determine the underlying mechanisms driving evolution of these viruses. The mosaic nature of tailed bacteriophage genomes confounds easy definition of a viral species; nonetheless, empirical evidence demonstrates that key marker genes can define monophyletic groups of related marine viruses. These monophyletic groups infect a defined, albeit broad, range of hosts. Examples are the use of *pol* (the gene for DNA polymerase I) to characterize viruses infecting eukaryotic microalgae (Chen and Suttle, 1996). It is possible that for a given functional or genetic group of marine microorganisms there are key genes that define the groups of viral genotypes to which they are susceptible. In the case of the Phycodnaviridae the genetic sequence of pol has been critical for examining the distribution and characterization of these viruses and for making inferences on their ecological impact (Short and Suttle, 2002). In essence, more genetic markers are needed to define the population ecology of specific viral groups. Thus far, faithful genetic markers have only been identified for viruses infecting photoautotrophs. In depth understanding of the impact of viral infection on the community ecology of hydrotropic bacteria is now limited by a lack of key, monophyletic markers for this disparate group of marine microorganisms.

At the broadest scale of diversity, collections of viral populations form a community known as the virioplankton (Sieburth, 1979). Virioplankton composition is influenced by a number of factors, the first of which may be the selective forces determining the assortment of co-occurring host species. Determinants of virioplankton community composition are complex however, due to the negative feedback between host lysis and viral production. Thus, selective viral infection may shape host community composition on short time scales; ultimately virioplankton communities are an assortment of individual genogroups infecting a diverse range of hosts. Examination of viral community composition has been aided through the use of molecular fingerprinting tools such as pulsed-field gel electrophoresis (PFGE). This molecular fingerprinting technology is beginning to show that estuarine environments exhibit rapid (i.e. less than a month) shifts in virioplankton composition (Wommack *et al.*,. 1999) while near coastal oceanic environments have relatively stable viral communities (Steward *et al.*,. 2000; Riemann and Middelboe, 2002).

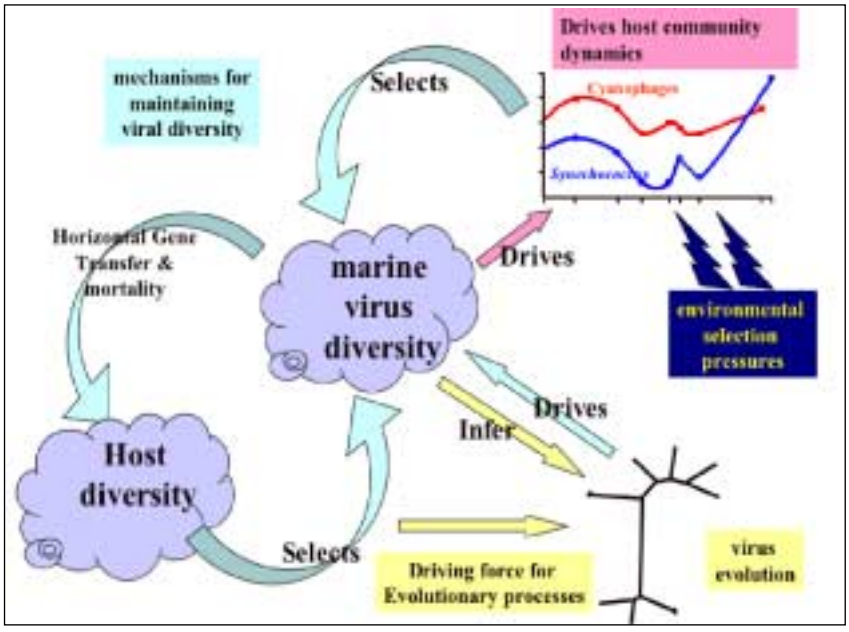

Fig 3. Conceptual diagram of the relationships between viral and host diversity.

Box 3. RESEARCH PRIORITIES IN MARINE VIRAL DIVERSITY

Genomics

- Broaden the search for genetic markers of viral evolution and diversity.
- Identify specific genes or gene cassettes that confer ecologically relevant phenotypes (e.g. burst size, latent period).
- Determine the extent of mosaicism and other genetic mechanisms in viral evolution.
- Examine the evolutionary interplay between diversity of specific host populations and their viruses.
- Work towards increasing levels of resolution and information with regards to viral genomics by moving from comparative to functional and ultimately to ecological studies.
- Examine the extent and impact of viral-mediated gene transfer on host diversity and evolution.
- Better connect primary genetic data with actual viral phenotypes.

Scales

- Determine whether viral genogroups have specific bio-geographic distributions.
- Investigate the temporal and spatial environmental gradients constraining viral diversity.
- Constrain the extent of co-variation/co-evolution of host and viruses.
- Explore the extent to which viral genotypic diversity drives the population genetics of host species over ecologically relevant time.
-Examine the role of viral infection in the dynamics of microbial blooms.
-Place viral activity within the larger context of annual biological cycles in marine ecosystems.
- Identify of ecologically relevant virus/host systems.

Observations of virioplankton populations over environmental gradients (e.g., the photic to euphotic, or oxic to hypoxic) have demonstrated significant shifts in composition. Thus, even at the relatively poor resolution of PFGE we now appreciate that viruses are dynamic members of microbial communities. The question now is what factors and mechanisms contribute to the relative diversity and dynamism of a given virioplankton community ?

3.2. A unifying theory of viral and host diversity

A conceptual diagram of the complex ecological relationship between virus and host diversity is shown in Fig. 3. Examinations of viral diversity provide data to infer the course of viral evolution and can suggest possible genetic mechanisms at work (e.g. the mosaic nature of tailed phage genomes). In turn, these molecular genetic mechanisms drive, over evolutionary time, changes in the diversity of marine viral communities. On shorter, seasonal, timescales, changes in virioplankton diversity (i.e., community composition) as well as external selective pressures likely influence the diversity of specific host populations (e.g. marine *Synechococcus*). However, due to the negative relationship between viral lysis and host productivity, ultimately the assortment of host species within a community shapes overall virioplankton diversity and composition. Finally, because viruses are both potent killers and vectors for genetic exchange between dissimilar host species (i.e., horizontal gene transfer), viral activity can influence co-occurring host diversity. The challenge to marine microbial ecologists is to determine the exact nature of these relationships, the conditions under which they occur and the relevant timescales for each process.

In this regard, integrative studies incorporating viral diversity measures along with estimates of microbial activity (i.e. primary and secondary production) and environmental conditions will provide insights on the changing role of virus infection within microbial communities. It is quite possible that under certain conditions viral lysis plays a key role in restricting bacterial and phytoplankton production; while under other conditions, viral lysis principally effects host community composition with only modest effects on overall production.

Virioplankton and the biological paradigms of the sea

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The discovery that viruses are the most abundant class of microorganisms in aquatic environments, typically 10 times more abundant than bacteria, is perhaps the best example of our ignorance as to the true nature of microbial communities. With this seminal discovery came the realization that a new and unaccounted for process was involved in the flow of energy and organic matter through aquatic microbial communities. In the years since 1989, investigations of virioplankton ecology have been driven by two over-reaching questions. First, to what extent does viral infection and lysis impact the flow of carbon from microbial biomass into the dissolved organic matter pool ? Second, what is the impact of specific viral infection on the diversity and composition (i.e., structure) of co-occurring host communities? Answers to these questions are complementary as loss of hosts to viral lysis directly impacts both C flow and community structure. Indirectly, infection and lysis-induced changes in microbial community structure may affect both the suite of microbially-mediated biochemical transformations and the rate at which these processes occur in the water column.

The concept of bacterioplankton as a central component of the aquatic food web was established by Pomeroy over 25 years ago (Pomeroy, 1974; Hobbie, 1994). However, quantification of bacterioplankton involvement in the cycling of organic matter and nutrients through the aquatic environment was initially restricted by a lack of suitable *in situ* methods. In an analogous way, our ability to examine the diversity, activity and community dynamics of aquatic virus populations was, until recently, inhibited by a lack of methodological approaches. Soon after direct counting techniques revealed the true abundance and thus biomass of bacteria in aquatic environments (Zimmerman and Meyer-Reil, 1974; Hobbie *et al.*, 1977), came the realization that a significant proportion (10 to 50%) of primary production is incorporated by bacterioplankton. By utilizing dissolved products of primary production, aquatic bacterial populations recycle the primary production lost to higher order consumers back into biomass. This process, known as the "microbial loop" (Azam *et al.*, 1983), effectively makes a larger proportion of primary production available to the marine food web.

The discovery of abundant virus populations in aquatic ecosystems had an immediate impact on the accepted dogma of the microbial loop. A conceptual model of viruses and viral lysis introduced into the aquatic food web, as shown in Figure 1, demonstrates that viral lysis enhances the flux of bacterial biomass into the dissolved organic matter (DOM) pool. Viral lysis of algae and cyanobacteria likely augments the flux of photosynthetically fixed carbon from phytoplankton biomass into the DOM pool; however, currently this flux is poorly constrained. Indeed, all of the products of viral cell lysis (e.g., macromolecules, cell organelles, and virus particles) contribute to the DOM and particulate organic matter pools (Fuhrman, 1992). The theoretical effect of viral

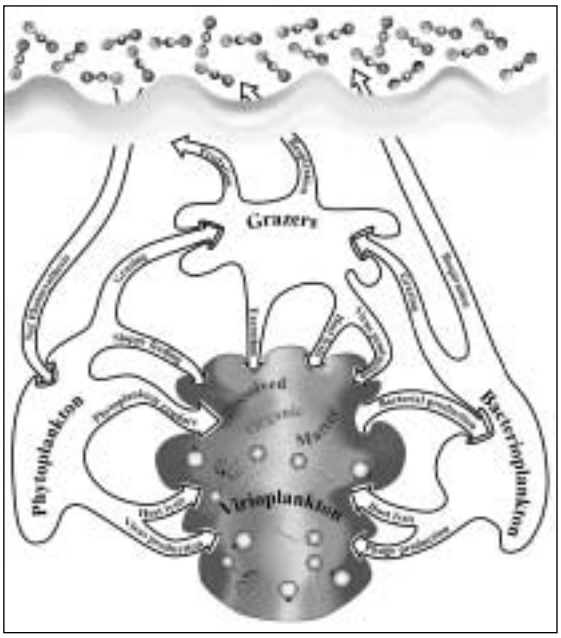

Fig. 1. Viruses and the microbial loop. This schematic drawing highlights the potential role of viral infection and lysis in the production of DOM in aquatic ecosystems.

lysis is to divert carbon, fixed as phytoplankton and bacterial biomass, away from mesozooplankton consumers into the DOM pool (Fuhrman, 1992; Thingstad *et al.*, 1993; Bratbak *et al.*, 1994; Murray and Eldridge, 1994; Thingstad and Lignell, 1997).

In essence, viral infection is a new mechanism for the flux of microbial biomass into DOM. This mechanism of DOM release is quantitatively different from other well studied mechanisms, such as "sloppy feeding" by grazers, in that all of the cell contents is moved into the DOM pool. A single virus produced in a burst event likely represents 1% or less of the cell biomass released. To continue the cycle of virus production and, thereby, the steady state condition, only one virus needs to survive to infect another host cell. In this scenario, 99% of the original host cell contents are moved into the DOM pool (Fuhrman, 1992). Thus, viral lysis represents an extremely efficient means for biomass to DOM conversion. Moreover, phage lysis of bacterioplankton represents a source of nutrient rich growth substrate for bacterial production. While quantitative measurements are few, viral infection likely results in the rapid recycling of organic material between bacterial biomass and the DOM pool (Bratbak *et al.*, 1990). Each turn of the DOM to bacterial biomass cycle results in both respiratory loss and sustained bacterial production at higher levels than would be possible without phage-mediated DOM release. Ironically, the results of active phage lysis are higher levels of bacterial production and less transfer of organic matter to higher trophic levels. A possible manifestation of the viral loop in the Chesapeake may be the contribution of viral lysis to the efficient retention and recycling of N supplied annually to the Bay from the spring freshet (Harding *et al.*, 1999).

Quantification of the impact of viral lysis on microbial loop processes requires accurate estimates of viral and host production. Until recently, accurate estimation of viral production was an enigma as these techniques relied on a suite of poorly constrained conversion factors. For example, the most widely used method, frequency of visibly infected cells (FVIC), requires both the assumption that latent period is equal to host generation time and two poorly constrained conversion factors: one for converting visibly infected cells (FVIC) to total infected cells (FIC); and a second for average burst size. Recent experiments combining FVIC with an incubation based method for estimation of viral production place the FVIC to FIC conversion factor in a range from 4.34 to 10.78 and for most samples, FVIC abundance is only 1 to 4% of total bacteria. (Weinbauer *et al.*, 2002). While techniques such as FVIC and others have been critical in establishing that viral lysis can be a significant factor in the flow of organic matter through aquatic microbial communities, few of these approaches yield direct measures of viral production sensitive enough to allow for temporal or spatial comparative studies. Recently, two incubation-based methods were introduced for direct estimation of viral production. Noble and Fuhrman (2000)

developed an approach, based on an isotope dilution paradigm (Fuhrman, 1987), to estimate new viral production by dilution of an added fluorescently-labeled viral (FLV) tracer. Just last year, Wilhelm and co-workers (Wilhelm *et al.*, 2002) reported a dilution-based approach, similar to that for estimating phytoplankton growth and grazing rates (Landry, 1993), in which viral abundance is reduced to around 10% of ambient concentration and the subsequent return of virus-like particles indicates the viral production rate.

From our recent evaluations of these incubation-based approaches it is apparent that while the FLV method is the most scientifically appealing, significant logistical problems preclude routine application of this approach. The dilution approach, as described by Wilhelm *et al.*, utilizes dead-end vacuum filtration (Vac) and virus-free seawater to reduce viral abundance. Replicate incubations of virus-reduced water samples are followed for changes in abundance of virus-like particles (VLP). The slope of the first-order regression line of VLP abundance versus time is taken as the viral production rate. However, because bacteria are lost during filtration, it is necessary to correct viral production rates to account for the lost proportion of the host community. The vacuum filtration technique for reducing viral abundance has a significant drawback as usually around 80% of bacteria are lost during filtration. In our hands, use of tangential flow diafiltration (TFD) significantly improved both bacterial cell recovery and virus dilution over that attainable with dead-end vacuum filtration (Table 1). In turn, this methodological improvement lead to more reasonable estimates of viral production. For example, in the hypoxic bottom waters of station CB 858 different recovery rates between TFD and Vac methods resulted in dramatically different adjusted estimates of viral production. Subsequent estimates of viral-mediated mortality using a burst size conversion factor of 100 demonstrate that Vac derived estimates yielded an unrealistically high viral production rate as 201% of bacterial production would have been consumed by viral lysis. Conversely, the TFD approach for the same water sample gave a high but sustainable estimate of 96% (Table 1).

Table 1. Viral and bacterial production for Chesapeake and Delaware Bay stations.

Station[a]	Date	Method[b]	Bacterial recovery	Adjusted Viral production[c] (x 10^6 VLP/ml/h)	Bacterial production (x 10^4 cells/ml/h)	Viral-mediated mortality (100)[d] (x 10^4 cells/ml/h)	Bacterial production losse
DB 14	Aug. 02	TFD	53%	1.96	3.73	1.96	53%
DB 14	Aug. 02	Vac	19%	-1.60	3.73	0	0
CB 858T	Aug. 02	TFD	67%	2.76	13	2.76	21%
CB 858T	Aug. 02	Vac	25%	2.78	13	2.78	21%
CB 858M	Aug. 02	TFD	98%	0.81	4.83	0.81	4%
CB 858B	Aug. 02	TFD	19%	3.01	3.15	3.01	96%
CB 858B	Aug. 02	Vac	6%	6.33	3.15	6.33	201%
CB 908T	Sept. 02	TFD	47%	2.83	11	2.83	26%
CB 858T	Sept. 02	TFD	83%	3.51	9.78	3.51	4%
CB 834T	Sept. 02	TFD	50%	2.61	1.32	2.61	20%
CB 818T	Sept. 02	TFD	37%	0.45	1.63	0.45	3%

a) DB Delaware bay, CB Chesapeake bay, T surface water, M mid-water, B bottom water
b) TFD tangential flow diafiltration, Vac vacuum filtration
c) viral production adjusted for loss of bacteria during filtration
d) estimate of bacterial mortality based on average burst size of 100 virus-like particles per lysed cell
e) proportion of bacterial production lost to viral-mediated mortality

Based on the viral production data presented in Table 1, it is possible to make preliminary observations on the interaction between viral activity and physico-chemical gradients within the stratified water column of the Chesapeake. Viral production data from surface, mid-water, and bottom water samples at station CB 858 indicate that the impact of viral–mediated mortality is modest in surface waters, low at the picnocline (mid-water), and high in bottom waters. This is in contrast to bacterial production rates which decline with depth. Previous studies examining grazing and lysis over environmental gradients of salinity and oxygen concentration found that

under extreme conditions of high salinity or anoxia the relative contribution of lysis to bacterial mortality increased (Guixa-Boixareu *et al.*, 1996; Weinbauer and Hofle, 1998). Thus, under conditions adverse to grazing (i.e., hypoxia or high salinity) it is possible that mortality due to viral infection compensates for a loss of grazing, that is, where grazing rates are low viral-mediated mortality is high. In the case of station CB 858 the highest rates of viral-mediated mortality occurred in the hypoxic bottom waters where bactivory is likely to be low. Over the course of the next year we will be performing intra-annual measurements of viral production, and abundance in hopes of determining whether peaks in viral production coincide or follow peaks in recycling activity (i.e. primary and secondary (bacterial) production). Based on the annual cycle of primary productivity in the Bay we expect seasonally high levels of viral production to occur in summer and short peaks to occur following major bloom events in late spring and early fall.

There is a growing appreciation that species composition may, in large part, determine the biogeochemical and metabolic characteristics of any given microbial community. Selective factors such as nutrient availability and composition, and predation are known to be key processes which can shape the genotypic make-up of microbial communities. With the discovery of abundant viruses in marine environments came the realization that, due to its highly specific nature, the process of viral infection and lysis may strongly influence the diversity and clonal composition of bacterio- and phytoplankton populations (Thingstad *et al.*, 1993; Waterbury and Valois, 1993; Bratbak *et al.*, 1994; Suttle, 1994; Steward *et al.*, 1996; Fuhrman, 1999; Van Hannen *et al.*, 1999). Additionally, viruses may influence diversity at the population genetic level, as natural virioplankton consortia within bacterioplankton populations can mediate genetic exchange among bacterial strains via transduction (Saye and Miller, 1989; Miller and Sayler, 1992; Paul, 1999).

While the idea of viruses as mediators of host community structure is tenable, direct proof of this hypothesis for communities of marine microorganisms will be difficult to obtain. One approach which should lead to good circumstantial evidence supporting or refuting this idea will be the use of molecular fingerprinting techniques to coordinately examine compositional changes in host and viral communities. At present several molecular fingerprinting techniques exist for bacteria, the most widely applied of which is denaturing gradient gel electrophoresis (DGGE) of 16s rDNA (Muyzer *et al.*, 1993). Currently only one molecular fingerprinting tool exists for analysis of total virioplankton communities. Pulsed-field gel electrophoresis (PFGE) of natural viral concentrates can yield a fingerprint of viral community composition based on genome size of viruses within the consortia (Klieve and Swain, 1993; Wommack *et al.*, 1999a; Steward *et al.*, 2000). On the PFGE gel, single bands are the intact genomic DNA of a virus or viruses which share a particular sized genome. PFGE banding patterns are essentially a fingerprint of virioplankton community structure within the sample (Fig. 2). Using molecular fingerprints it is possible to compare bacterial and viral communities within different water samples and determine, semi-quantitatively, specific relationships between populations using community ordination techniques. Just such an analysis of virioplankton community structure is presented in Figure 3. Simultaneous application of fingerprint and community ordination analysis to both viral and bacterial communities should provide a first approximation of whether communities of hosts and

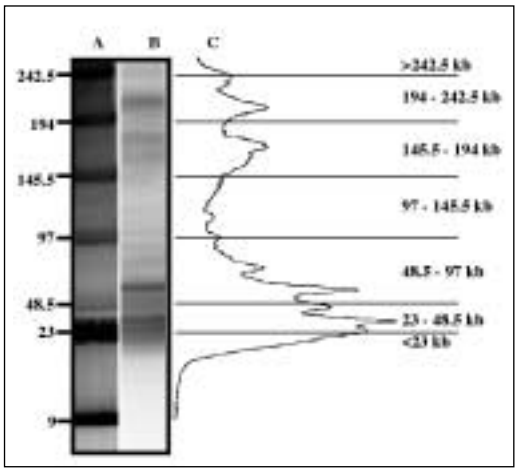

Fig. 2. PFGE profile of virioplankton genomic DNAs. **A)** Molecular weight marker (band sizes are in kilobases); **B)** PFGE fingerprint of virioplankton genomes in a single Chesapeake bay water sample; **C)** Densitometric scan of lane C. Note large bands (>150 kb) may represent phycoviruses.

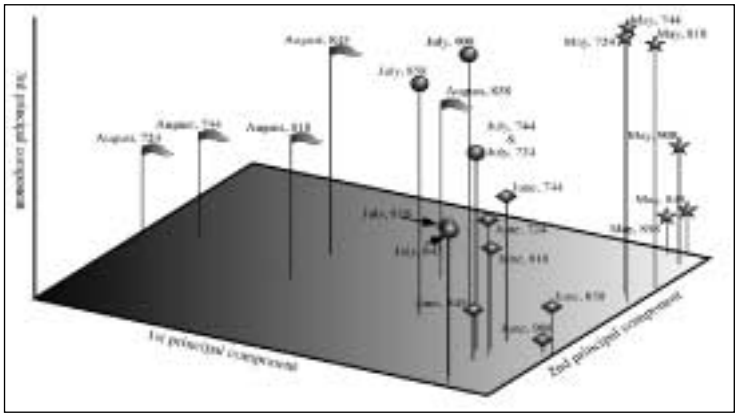

Fig. 3. Three-dimensional plot generated from principal components analysis of virioplankton PFGE fingerprints from Chesapeake bay water samples. Numbers associated with month names are station designations. Moving from north to south stations are as follows: 908, 858, 845, 818, 744, and 724.

viruses are influenced similarly by environmental changes. Riemann and Middelboe (2002a) recently utilized such an approach in Danish coastal waters and found surprisingly little change in the structure of bacterio- and virioplankton communities despite dramatic changes in bacterial productivity. This finding suggests either that PFGE and 16S rDNA DGGE fingerprinting tools are not sensitive enough to pick up population genetic changes in virus and host communities or the real possibility that a great deal of functional redundancy exists within the bacterioplankton. Nonetheless, application of molecular fingerprinting tools should provide the means to determine to what extent changes in viral and bacterial community composition co-occur or respond to environmental signals. Our annual surveys throughout the Chesapeake should yield significant data towards this objective.

Generic questions in marine microbiology addressed by analysis of simple food web models

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INTRODUCTION

Bacterial growth in Mediterranean surface waters has been suggested to be phosphate limited (Thingstad *et al.*, 1998; Zohary and Robarts, 1998; Van Wambeke *et al.*, 2002), while in deeper waters there seems, as one would expect, to be an energy/carbon limitation of bacterial growth (Van Wambeke *et al.*, 2002). As a "simplest possible" model with sufficient elements to combine bacterial growth on DOC and PO_4 as potential limiting substrates, with protozoan predation and viral lysis as potential loss mechanisms, the following scheme can be proposed (modified from Thingstad, 2000b) :

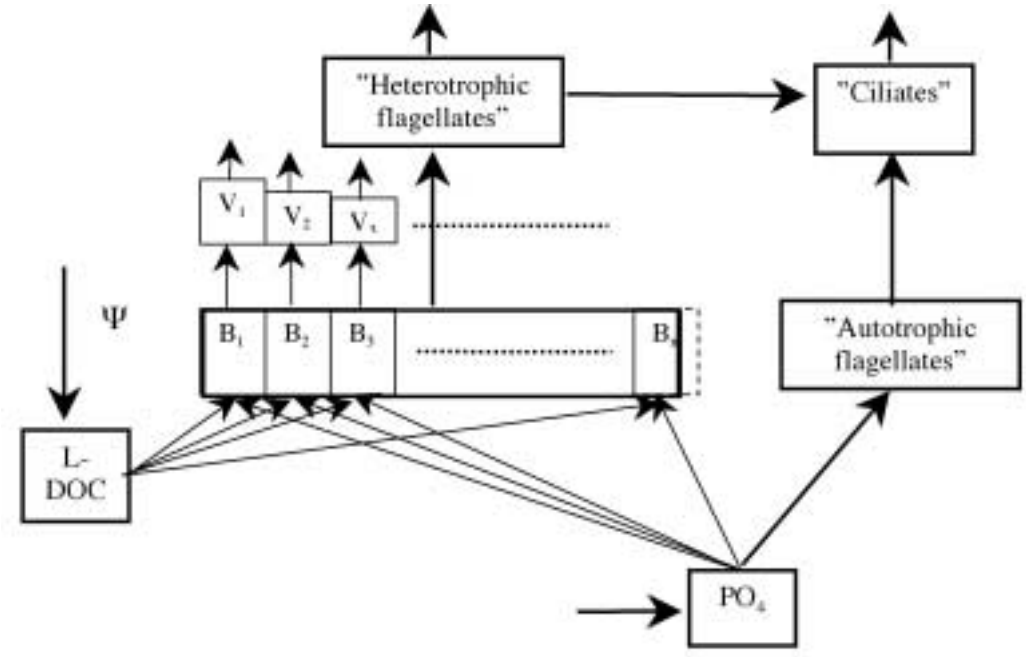

Fig. 1. Idealized model of the microbial part of the photic zone food web resolving the "black box" of heterotrophic bacteria into "host-groups" B_1, B_2, etc., each with their specific virus V_1, V_2, etc. Modified from Thingstad (2000b) by inclusion of labile DOC (L-DOC) as a 2nd potential limiting substrate for bacterial growth rate.

As illustrated in Figure 1, a framework like this can be used to address some of the basic questions in marine microbiology.

QUESTION 1: HOW MANY ?

• *Bacterial community size*. This model contains a formalisation of the widely (?) accepted hypothesis that the relatively constant community size of bacteria ($3\ 10^5 – 3\ 10^6$ bacteria ml^{-1}) is a consequence of control by protozoan predators. (For some purposes, the model could be refined with a group of bacteria avoiding predation from heterotrophic flagellates by e.g. filament formation.) Basically, the steady state argument leading to a control of bacterial community size is: there has to be enough food (bacteria) for heterotrophic flagellates to grow as fast as they die. Higher loss rates for heterophic flagellates (= more ciliates ?) thus gives a larger bacterial community.

• *Size of individual host groups*. Steady state also requires that each type of virus is produced as fast as it decays. The consequence is that a high viral decay rate, a low affinity constant, or a low number of viruses produced per lytic event, are all factors that would increase the size of an individual host group.

• *Viral abundance.* In the simplest version with protozoan predation being non-selective among bacteria, viral lysis is the only mechanism that can give the additional loss needed to compensate for high growth rate in bacterial species with high affinity for the common limiting substrate. The consequence is that viral abundance is related to the difference in growth rates between the co-existing bacteria. A wide spectrum of bacterial growth rates should therefore correlate with high viral abundance. (This untested hypothesis is, to my knowledge, the only present theory for a mechanism controlling viral abundance).

QUESTION 2: DIVERSITY ?

• *Species richness* in the sense “number of dominant co-existing bacterial species” is, in the above scheme, the size of the bacterial community divided by the average size of the individual host groups. In the ideal model world, the number of co-existing types of viruses is 1 less than the number of bacterial host groups, so viral species richness and bacterial species richness should be closely correlated and approximately equal. Since the number of bands on DGGE and PFGE gels seems to be roughly equal, observations may seem qualitatively to support the simple theory. However, if one “host group” may be a variant of another, only with a mutation that makes it resistant to (on type of?) virus, DGGE bands probably does not reveal species richness at the required microstructure level (?)

• *Numerical evenness*

Bacteria: Evenness in the sense “similarity in abundance between co-existing host-groups” is, in the simple model, determined by the differences in properties of the respective viruses. A large difference in decay rates, adsorption constants or in number of viruses produced per lytic event, would all be factors reducing evenness in the bacterial population.

Viruses: Since viruses act as growth rate compensation mechanisms (as discussed above), evenness in viral abundances is related to evenness in growth rates between the co-existing host-groups. While community size and evenness can vary independently for bacteria (one can have a large community where all host-groups have identical size), evenness and abundance are linked for viruses: If host group A grows faster than host-group B, a larger viral population is needed for A than for B. I.e., the increase in abundance is linked to a decrease in evenness.

• *Who ?* The number and size of niches for bacteria to a large extent is a top-down controlled phenomena in this scheme. Diversity in the sense of who that will occupy these niches is, however, to a large extent a bottom-up controlled matter. The system can be invaded by a bacterium that, at low abundance, has a positive net growth rate. Type of limiting substrate (here P or C limitation), and the abilities of different bacteria to grow fast at low concentrations of this substrate (high affinity constant), will thus be major factors in determining which species eventually occupy the niches.

QUESTION 3: MAGNITUDE AND PARTITIONING OF MATERIAL FLOWS ?

• *Partitioning of material and energy flows in the food web.* The larger the difference in growth rates between co-existing bacteria (low "physiological evenness") the more viral lysis is needed to produce the compensating losses needed to give a steady state, and the more material is diverted from the upwards flux in particulate food chain (by predation) to loss as DOM (by viral lysis). The partitioning of biogeochemical flows is thus directly linked to physiological evenness between co-existing bacterial host-groups.

• *Bacterial production and growth rate limitation.* Control of bacterial production in an idealised model, with bacteria as one single functional group, has been analysed by Thingstad and Lignell (1997) and Thingstad (2000a). Summarised, a simple food web of heterotrophic bacteria, autotrophic flagellates, heterotrophic flagellates, and ciliates, will have steady states characterised by phosphate- or carbon-limited bacteria according to whether a dimensionless index η (see Box 1) is larger or smaller than 1. Note that η contains bacterial C:P-ratio and respiration coefficient. A physiological flexibility at the cellular level in these parameters can thus work as kind of homeostatic mechanism at the ecosystem level, keeping the system at the border between bacterial C- and P-limitation.

Box 1

In a model such as the one in Fig.1, bacterial carbon demand is constrained upwards by two mechanisms; either the supply rate of labile organic carbon, or by the combined constraint of bacterial biomass by predation and bacterial growth rate by phosphate competition. The two situations correspond to C- and P-limitation, respectively, of bacterial growth rate. The relationship can be summarized in a dimensionless "limitation index" η defined as the ratio between the potential carbon demands under P- and C-limitation. For the model in Fig. 1, but with heterotrophic bacteria represented as one functional group only, h can be expressed as:

$$\eta = \frac{f}{(1-r)} \Psi^{-1} Y_H^{-1} \frac{\alpha_B \alpha_C^2}{\alpha_A \alpha_H} C^2 \ ,$$

with η >1 and η < 1 corresponding to steady states with C- and P-limitation, respectively, of bacterial growth rate.
f : C:P-ratio in bacterial biomass,
r : bacterial respiration coefficient,
Ψ : supply rate of labile DOC,
Y_H : biomass yield of "heterotrophic flagellates" on their bacterial prey
α_B, α_A : specific affinity constants for phosphate, bacteria and autotrophic flagellates, respectively
α_H, α_C : specific clearance rates, "heterotrophic flagellates", and "ciliates" respectively,
C : "ciliate" biomass.

Outline of the definition of a "limitation index" η defining whether a model of the type outlined in Fig.1 will have C- or P-limited bacteria. See Thingstad (2000a) for more details.

The model in Figure 1 with a multi-host bacterial community would be expected to increase such homeostasis: Assuming bacterial hosts to occupy a gradient from C-specialists (high affinity for DOC, low affinity for PO_4), via generalists (intermediate affinities), to PO_4 specialists (high PO_4, low-DOC affinities), one can get a range of steady states with P-limited C-specialists coexisting with generalists and with C-limited PO_4-specialists (Thingstad, unpublished). The potential for shifts in balance between such physiological types would add a "homeostatic control" at the population level, keeping the system at the border between C- and P-limited bacteria by allowing cells with different strategies to co-exist..

QUESTION 4: BLOOMING SPECIES OR STABLE COMMUNITY COMPOSITIONS ?

A model of the kind outlined in Fig. 1 consists of a series of nested and linked Lotka-Volterra predator-prey and parasite-prey couples. The potentially very complex dynamics of coupled oscillators with different frequencies is simplified by the stabilizing effect of nutrient recycling. Inclusion of a time-delay between viral infection and lysis seems to break the barrier of local sta-

bility and the behaviour becomes chaotic. There are interesting stabilising mechanisms such as predator-defence and virus-resistance responses in the prey/hosts that will strongly dampen such oscillations (Thingstad, unpublished). If the strength of such mechanisms differ between host-groups, one may speculate if this can result in some species being bloom-forming, while others remain present in stable virus-host consortia.

There seems not to be any principal obstacles to the application of these models also to the different size-classes of phytoplankton. The stability question thus bears relevance also to the issues of species succession and harmful algal blooms in the phytoplankton part of the microbial food web.

CONCLUSION

In a larger perspective, the goal of marine microbial ecology studies in the Mediterranean (and elsewhere) is an integrated understanding of the mechanisms that link organism properties to trophic interactions, trophic interactions and environmental forcing to food web structure, and food web structure to biogeochemical fluxes. From such a perspective, the model outlined in Fig.1 may seem (provokingly ?) simple and is at best a kind of first order approximation to the complexity of the natural system (see e.g. Riemann and Middelboe, 2002b). Many of the properties emerging from an analysis of even this simple structure are, however, non-, or even counter-intuitive. It is thus difficult to see how a relevant analysis of the real system can be obtained without at least an understanding the properties of such idealised models.

Do viral life cycles correspond to ecological strategies ?

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Viruses infecting prokaryotes ("phages") differ strongly with respect to the type of nucleic acid of their genome (DNA or RNA, double-stranded or single-stranded). However, several common steps can be distinguished in the viral development and four main types of viral life cycles have been identified: lytic, lysogenic, pseudolysogenic and chronic infections (Ackermann and DuBow, 1987).

1) Lytic (or virulent) phages hijack the host metabolisms to produce new phages, which are released as the cell lyses.

2) Temperate (or lysogenic) phages can typically insert their genome into the host genome as prophage, which multiplies along with the host, until the lytic cycle is induced.

3) Pseudolysogeny is an infection, where cell lysis occurs only in a small fraction of the infected population.

4) A chronic or steady-state infection occurs, when a cell is infected and viral progeny is constantly released from the host cell by budding or extrusion without lysing it. This classification might also reflect basic ecological strategies.

All four main types of viral life cycles have been described for a variety of isolates from aquatic systems, and ecological strategies have been formulated for such phage isolates. For example, it was suggested that lysogeny might prevail when the host abundance, i.e. the food resource, is below that necessary to sustain lytic infection (Steward and Levin, 1984), or when the destruction rate of free viruses is too high to allow for lytic replication (Lenski, 1988). Both explanations assume that the rate of successful encounters between phage and hosts is too low to sustain lytic phage production. Consequently, lysogeny should predominate at low encounter rates with specific hosts and lytic infection should predominate at high host abundance.

About 15 years ago, it was shown for aquatic systems that viral numbers exceed those of prokaryotes and that viral infection frequencies of bacterioplankton are high (Fuhrman, 1999). Due to methodological progress we can now investigate lysogeny and lytic-stage infection at the community level. This has shed some light on the distribution of lysogeny and lytic infection in systems varying with respect to productivity. Several studies have shown that viral infection increases along with bacterial or system productivity (Steward et al., 1992; Weinbauer *et al.*, 1999). Moreover, estimates of the frequency of infected cells (FIC) range from 1-7% in mesoplagic and deep marine waters, from 1 to 26% in oceanic waters and up to 43% in shelf/coastal waters. This suggests that the higher bacterial numbers and their higher activity in more produc-

tive systems also support higher viral infection frequencies. The relationship between lysogeny and bacterial or system productivity is less clear. Although there is evidence from some studies that lysogeny increases towards more oligotrophic offshore environments, others have suggested that there is no clear relationship between lysogeny and system trophy (Jiang and Paul, 1996; Weinbauer and Suttle, 1999). In a study investigating lysogeny along system gradients from productive estuarine to less productive deep water systems, lysogeny increased only slightly from estuarine to offshore systems, but dramatically in deep waters with more than 50% of the bacterioplankton cells being lysogens (Weinbauer *et al.*, 1999). This suggests that lysogeny predominates in low productivity/ low abundance environments. These data provide some support to the lysogeny *vs.* lytic infection hypothesis at the community level and indicate that environments exist where one of the two dominates.

While lysogeny makes evolutionary sense at low host abundance or activity, it is less intuitive to understand why lysogens should have a disadvantage in productive environments. The number of genomes of a prophage population depends on the multiplication rate of the host and results in one additional viral genome per cell division. Lysis of cells produces multiple genome copies determined by the number of viruses released (burst size). This may confer a competitive advantage to lytic phages, which could out-compete lysogenic phages by higher replication rates. Some support for this has been provided at the community level.

It has been suggested that the length of the latent period might be a strategy of phages to increase reproduction success and maximize resource exploitation (Abedon, 1989), i.e. an increased latent period would allow for the formation of more viral progeny due to a higher burst size (Table 1). This extension of the latent period might be an ecological strategy of a lytic phage to survive in a slow or non-growing host population, and lysis might occur when conditions are more favorable for host growth and thus infection. A short latent period and hence low burst sizes would then be sufficient to sustain high infection rates at high host abundance. Thus, a short latent period and consequently a low burst size should prevail in productive, high-host-density environments. Such a mechanism might occur in single phage species conferring some ecological plasticity, however, it might have also evolved along productivity gradients. Indeed, prophages might be considered as very long latent period phages at very low host density (Abedon, 1989). Also, pseudolysogeny might be a strategy to increase the latent period. This model is in agreement with the suggestion that lysogeny is a survival strategy at low host densities (Steward and Levin, 1984). Moreover, the physiological status of the host cells seems to be important for resource exploitation.

Table 1. Viral life cycles in potential relation to types of interaction with host, evolutionary trade-offs and some life strategy traits. NA, not applicable.

Life cycle	Type of interaction with host	Trade-off by resource	Life strategy traits			
			Resource abundance	Production rate	Latent period	Burst size
Lytic	Predator	Abundance	High	High	Short	Low
Lysogenic	Parasite/Mutualist	Scarcity	Low	Low	Long	High
Pseudolysogenic	Parasite	Scarcity?	Low?	Low?	Long	Low?
Chronic	Parasite	Scarcity	Low?	Low?	NA	NA

Ecological strategies of viruses can be evaluated by their interaction with host cells (Table 1). Thus, lytic phages should be described as predators and some lysogenic interactions seem to resemble mutualism, whereas pseudolysogeny and chronic infection represent true parasitism. Adopting a different view, viral life strategy concepts might be the result of an evolutionary trade-off between resource abundance and scarcity (Velicer *et al.*, 1999). Thus, lytic infection can be seen as adaptation to resource abundance, whereas lysogeny, pseudolysogenic or chronic infection might be adaptation to resource scarcity (Table 1). Pseudolysogeny could be an adap-

tation to the physiological status of the host cells, since this life strategy might be due to lytic or temperate phages, which cannot continue their life cycle because of energy limitation in the cell (Ripp and Miller, 1997). It has also been suggested that pseudolysogeny might be a life strategy allowing phages to quickly react to changes in the environment and that it might be an evolutionary transition step towards lysogeny (Wommack and Colwell, 2000).

A compilation of life strategy traits suggests similarities to concepts such the r/K continuum with lytic phages being rather r-strategists and lysogenic phages rather K-strategists. The concept of evolutionary trade-offs should be extended to “resource-independent” survival mechanisms such as resistance against destruction (such as sunlight-induced nucleic acid damage), repair capacity, and temperature and salinity tolerance, which differ between phage species. To further tackle the question, whether or not different types of life cycles have evolved as distinct ecological strategies to optimize resource allocation and survival, more information on parameters such as infection efficiency, carrying capacity, burst size, intrinsic rate of increase, and viral survival must be obtained from in situ studies or in experiments relevant for in situ conditions. Hypotheses on life strategy traits might also be inferred from sequencing viral genomes (Rohwer *et al.*, 2000).

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Viruses in aquatic systems : agents of disease and ecosystem impacts

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INTRODUCTION

Estuaries and coastal areas are among the most productive, biologically diverse, and economically important aquatic ecosystems. Coastal regions world-wide are increasingly impacted by anthropogenic activities, with the majority of the global human population residing within 100 km of a coastline. Worldwide, coastal development is twice that of inland sites, with approximately 90% of the generated wastewater being released untreated into marine waters. (Henrickson *et al.*, 2001). At least two-thirds of the world's fish stocks depend on estuaries as nurseries. These systems support recreational, residential, commercial, and aquaculture activities. Ultimately, estuaries, being on the downstream end of the terrestrial watersheds, receive and process urban, industrial, municipal, and agricultural nutrient and pollutant wastes. Estuarine and coastal watersheds support more than 70% of the USA population, and the migration to the coast continues (Vitousek *et al.*, 1997). Water quality can be compromised due to fecal contamination from a variety of sources, including agriculture (poultry litter, swine waste), forestry, and human sources (failing septic systems, leaking sewer systems) and industry. Fecal contamination, whether from animals or humans, contains known agents of disease, bacteria, viruses, and protozoa. Consequently, the public health of those using the waters for recreation and food can be compromised, with common resulting illness such as gastrointestinal disease, myocarditis, ear and skin infections, hepatitis, and meningitis. Inorganic and organic nutrient enrichment from runoff and fecal contamination are responsible for biogeochemical and ecological perturbations in estuarine and coastal waters, including increases in primary productivity (eutrophication), harmful algal blooms (HABs) and food web alterations, hypoxia, anoxia, and increases in finfish and shellfish disease and mortality (Nixon, 1995; Bricker *et al.*, 1999; Paerl *et al.*, 1998, 2001, 2002; Rabalais and Turner, 2001).

Waterborne disease due to pathogenic bacteria and viruses is a serious issue. Enteric viral disease outbreaks continue to be associated with both drinking water and recreational water (CDC, 2002). Shellfish, especially bivalve mollusks, continue to be an important source of foodborne outbreaks (Rippey, 1994; CDC, 2000). Disease outbreaks have been associated with viruses, with Norwalk-like viruses (NLV) being the leading cause of illness associated with shellfish in the United States (CDC, 2002). When risk assessments have been done in conjunction with environmental studies, risks associated with consumption of shellfish have ranged from 1 to 17 cases per 100 consumers for oysters and 1.7 to 6.8 per 100 consumers for clams. Surveys conducted in southern California indicate that viral infections resulting in mild symptoms such as gastroen-

teritis, and more serious viral infections from Hepatitis A virus (HAV), are quite common among surfers. Tests in Europe (Pina *et al.*, 1998), Florida (Rose *et al.*, 1997; Griffin *et al.*, 1999), and Southern California (Tsai *et al.*, 1993; Tsai *et al.*, 1994; Noble and Fuhrman, 2001; Jiang *et al.*, 2001) indicated positive detection of enterovirus, HAV, rotavirus, and adenovirus in coastal environments. NLV are now recognized as a major cause of human food and waterborne disease worldwide, with an estimated 23 million cases occurring annually in the USA (Mead *et al.*, 1999). Over the past five years in the USA at least five waterborne outbreaks of gastroenteritis, for an estimated 800 cases, have been attributed to NLVs (Beller *et al.*, 1997). There have been recent reports of high levels of NLV in river water (Gilgen *et al.*, 1997; Wyn-Jones *et al.*, 2000) and documented outbreaks from recreational water. As a tool for understanding viral fate and transport, male-specific coliphage have been indicated as possible proxies for a variety of different types of viral pathogens (Hsu *et al.*, 1995). In North Carolina, USA, the risk of disease is through ingestion of contaminated fish and shellfish, and the relationship between ingestion and the incidence of disease can be modeled for a variety of pathogens (Latimer *et al.*, 2001; Latimer *et al.*, 2002).

APPLICATIONS AND METHODS

Both the expansion of potentially harmful, coastal microbial populations and increased environmental impacts from anthropogenic activities, highlight the need for improved protection of coastal and estuarine waterbodies. For protecting public health of those using the waters for food, recreation, or as a drinking water source, levels of fecal coliforms or fecal streptococci (enterococci) have typically been measured as indicators of pollution. The methods currently employed to accomplish this – membrane filtration and multiple tube fermentation – are decades old, and these measurements provide little information on the sources of fecal pollution. In addition, several groups have demonstrated a weak relationship between bacterial indicators and human pathogens, such as adenoviruses and enteroviruses (Noble and Fuhrman, 2001; Jiang *et al.*, 2001), indicating the need for development of molecular methods for virus detection and quantification. In waters with multiple types of fecal contamination, it is extremely difficult to partition the contamination by source. Therefore, the source(s) of fecal contamination are often unknown, making mitigation of pollution difficult. Fortunately, the advance of molecular methods in the last decade enables molecular assessment of aquatic systems. There are a few specific advancements that hold promise for near real-time and quantitative detection of microbiological components of fecal contamination in environmental samples, especially useful for the field of water quality. DNA microarrays and Quantitative Polymerase Chain Reaction (Q-PCR) are two examples of the many types of new molecular approaches. DNA microarrays are essentially slides to which complementary sequences of DNA are attached in an orderly arrangement. Hybridization of fluorescently labeled mRNAs or DNA from the environmental samples to microarrays indicate the presence of genes or cell types of interest, with the level of fluorescence indicating the quantity of the genes or cells. The absence of hybridization indicates that the cell, indicator, or pathogen of interest is not present. One of the advantages of microarrays is that researchers can probe for hundreds of different genes, as opposed to older techniques (such as PCR) that allowed the researcher to only detect one or a few types of genes at a time. Microarrays have been used primarily for research in the pharmaceutical and biotechnology sectors, but show promise with environmental samples. Q-PCR will be discussed in more detail later in this document.

Currently, source tracking efforts are being conducted because fecal coliform standards for shellfish harvesting are often exceeded when no source of contamination is readily apparent (Kator and Rhodes, 1994). This causes a disjoint between management action and potential public health risk. Peak bacterial densities in surface waters have been correlated with rising, falling or peak portions of the hydrograph, in both summer and winter, and found to be higher when closer to fecal sources (Elder, 1987; Hunter and McDonald, 1991; Auer and Niehaus, 1993; NCDEM, 1994). In non-urbanized areas, a significant portion of the fecal coliforms contributed to a system can come from wildlife, birds, and dogs. Due to our poor understanding of the relationship between sources of fecal contamination (and therefore inherent public health risk and levels of bacterial indicators), management action is inherently inaccurate. Shellfish harvesting waters

are often closed due to failure of fecal coliform standards, even though the contamination may not represent a public health risk. Conversely, waters are sometimes not managed properly, due to the fact that low fecal coliform levels can occur simultaneously with presence of human pathogens (Beril *et al.*, 1996). These findings do not appear to be restricted to shellfish harvesting waters. In coastal bathing waters, the presence of human pathogenic viruses cannot be predicted by measuring fecal indicator bacteria (Noble and Fuhrman, 2001; Jiang *et al.*, 2001).

With the needs for improved quantitative methods for virus detection in mind, we have been developing Q-PCR methods for the detection and quantification of human pathogenic viruses in coastal waters. Traditional PCR methods were previously developed in the laboratory (Noble and Fuhrman, 2001) for the detection and semi-quantitation of enteroviruses from coastal waters in southern California. Q-PCR is a novel primer-based molecular technique that combines the specificity of traditional PCR with the quantitative measurement of fluorescence for determination of presence of specific types of nucleic acid in environmental samples. As opposed to traditional PCR, which is limited to a presence/absence result, Quantitative PCR provides for quantification of a wide range, from single viruses to tens of thousands of viruses. One of the Q-PCR approaches is the TaqManTM, or 5'-nuclease assay (Heid *et al.*, 1996). This technique employs a dual-fluorescent labeled oligonucleotide probe to quantitatively measure the accumulation of target molecules during each cycle of PCR. The oligonucleotide probe bears a "reporter" fluorescent dye at the 5'-end and a second "quencher" fluorescent dye at the 3'-end. The 3'-dye is chosen to absorb light at a longer wavelength than the emission spectra of the 5'-reporter dye. This configuration allows for fluorescence resonance energy transfer (FRET) to occur and fluorescence of the 5'-dye is "quenched" by the 3'-dye. The most common configuration is 5'-Fluorescein and 3'-Tamra. In addition, the probe is 3'-end blocked to prevent it from also functioning as a primer in PCR. The sequence of the oligonucleotide probe is chosen to be complementary to a site within the nucleic acid sequence of interest. During the course of the PCR reaction, the probe anneals to its target sequence and is subsequently degraded by the 5'-nuclease activity of Taq DNA polymerase. This physically separates the reporter dye from the quencher dye and releases the reporter dye from FRET quenching, allowing fluorescence emission of the reporter to be detected and quantitatively measured (Fig. 1).

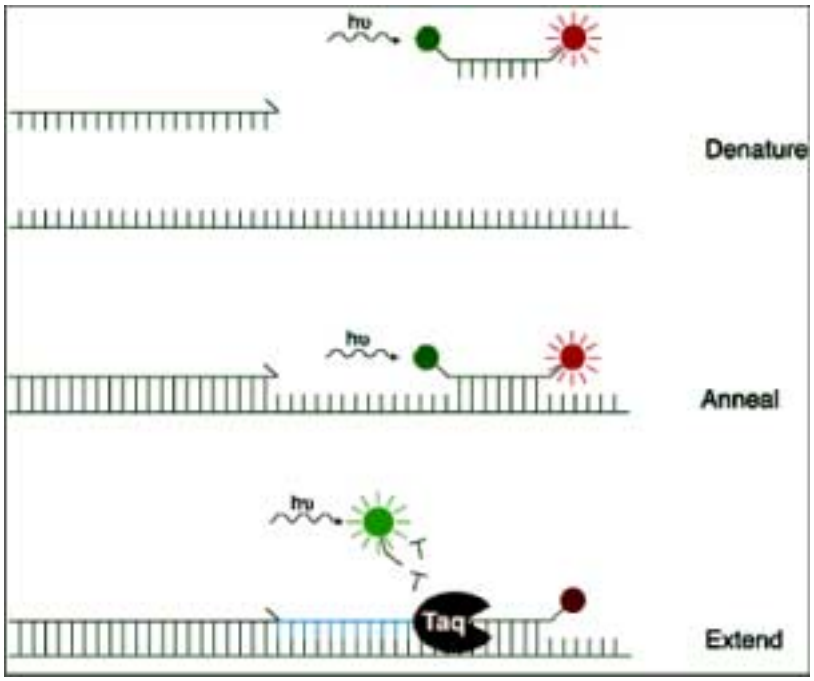

Fig. 1. Schematic representation of Q-PCR with TaqMan primers. In the intact TaqMan probe, energy is transferred from the short-wavelength fluorophore on one end (circle on left) to the long-wavelength fluorophore on the other end (circle on right), quenching the short-wavelength fluorescence. After hybridization, the probe is degraded by the endonuclease activity of Taq polymerase. Upon degradation, FRET is interrupted, increasing the fluorescence from the short-wavelength fluorophore and decreasing it from the long-wavelength fluorophore.

In the 1990s, research in the field of water quality focused on the use of traditional PCR for the detection of specific types of viral and bacterial pathogens in seawater and shellfish harvesting waters(Jiang *et al.*, 2001; Le Guyader *et al.*, 1998; Noble and Fuhrman, 2001). The prerequisite sampling required for traditional PCR is the same as that required for Q-PCR The new technology offers speed and "real-time" quantification, allowing for detection of particular DNA or RNA sequences with specific probes that fluoresce only when the target sequence are present, thus removing the need to run a gel (Heid *et al.*, 1996). Samples are prepared with reagents in a single tube, similar to standard PCR as mentioned above (Monpoeho *et al.*, 2000). To make these approaches fully quantitative, an internal standard is used, which is necessary for determining the efficiency of amplification by PCR. Internal standard can be created for RNA viruses using an SP6, T6, or T7 promoter to create RNA transcipts of the same gene amplified with our PCR primers (by RT-PCR from cultures obtained from culture collections). For DNA viruses, plasmids can be created from a suitable cloning vector. The desired DNA is excised from the plasmids, purified and quantified for use with Q-PCR. With knowledge of the molecular weight of the RNA/DNA products and the concentration, a known amount of product can be added to each Q-PCR reaction along with fluorophore-labeled primer/probe combination (of a different color than for the analysis reaction itself) for amplification. A standard graph of the threshold amplification values obtained from the serially diluted standards is constructed, plotted on a standard curve, and the copy number calculated automatically by a software package for data analysis.

Q-PCR has the potential to provide quantitative information on the presence of viral pathogens. Rather than just a qualitative description, this has the potential to provide an important immediate measurement of fecal contamination. Given quantification of viral pathogens, determination of waste load allocations and public health risk are possible. Viral pathogens are not susceptible to growth and reproduction during transport through a watershed, because viruses are not living organisms and are dependent upon their hosts for replication. Even though the focus of our work has been upon viral pathogens, detection of bacterial pathogens (such as *Salmonella*, *Shigella*, and *Enterococci* spp., etc.) has also been pursued to provide additional, important information. We have used a combination of standard methods and novel molecular techniques to measure different components of microbial contamination for development of decision matrices, taking a tiered approach, which may prove more useful than use of a single method for management of water quality resources.

The first step in ensuring the use of Q-PCR for quantitative assessments is optimization of sample concentration, nucleic acid extraction and Q-PCR methods. Our original focus has been on measuring levels of the known human pathogens, enteroviruses. The use of viral pathogens as tools for determining sources of fecal contamination provides vital, albeit different information from the currently globally conducted routine bacterial indicator monitoring. Viral pathogens are species-specific, making it possible to determine the presence of a specific type of fecal contamination (e.g. human *versus* animal) based upon the direct detection of viral pathogens specific to each. Our work has focused on enteroviruses, as they are an important causative agent of waterborne disease, they are prevalent in human populations, and they are RNA viruses (making their presence likely to be indicative of recent fecal contamination as RNA degrades in aquatic systems more rapidly than DNA). To start we have focused upon optimization of filtration and concentration techniques, extraction of nucleic acids, and optimization of Q-PCR methods. Our approaches currently involve concentration of 1 to 5 liters of seawater sample, with concentrated material extracted using nucleic acid extraction methods. For RNA we have tested the use of Qiagen RNeasy kits, Trizol® based extraction methods, with and without PVP and DNase treatments. For DNA, we have tested the use of Mol Bio Fecal DNA extraction kits, bead milling methods, and direct boiling of the concentrates. For environmental samples we have found that it is important to characterize the components of the samples before extraction, and that no one extraction method always works best. We are interested in using viruses as potential tracers of specific types of fecal contamination. For example, we are pursuing the use of canine parvovirus (CPV) as an indicator of the presence of dog fecal contamination. CPV is routinely found in the intestinal tracts of dogs, and is a DNA virus that can be used as an indicator of the presence of canine fecal contamination. In several sets of preliminary experiments, we have detected CPV multiple sets of dog fecal samples, and have optimized our quantitive PCR methods for low detection limits for CPV.

One of our main priorities in quantifying human pathogens in aquatic samples has been to assess whether or not there is a quantitative relationship between pathogens and current measures of water quality like fecal coliforms, *E. coli*, and enterococci (fecal streptococci). To date there has been no significant relationship identified between the two, in a variety of environments, even with the use of quantitative methods (t-test, $p < 0.05$). In addition, we have been using molecular methods to assess the presence of specific species of enterococci (fecal streptococci, recommended for routine monitoring of marine recreational waters) and human pathogens such as adenoviruses, enteroviruses, and HAV. Enterococci, or fecal streptococci, are important components of many marine monitoring programs. However, it has been suggested recently that some enterococci species may be typically associated with specific types of fecal contamination (i.e. *Enterococci faecalis* may be commonly associated with fecal contamination from waterfowl (Hartel, pers. comm.). Therefore, it may be useful to speciate members of the *Enterococcus* group and attempt to relate subsets of this group to known human pathogens, for better assessment of public health risk.

FUTURE CONCERNS AND APPLICATIONS

There are several other interesting issues related to viruses in aquatic systems. Other important areas where viruses have potential impacts on disease and ecosystem change are: their roles in harmful algal bloom formation and termination, viral proliferation in aquaculture effluents, impacts on specific aquatic populations, like coral reefs, and roles in the processes of eutrophication. An example of other future virus-related concerns and implementation of molecular tools to address these concerns follows. With increasing development along our coasts, the coastal oceans are experiencing an increasing frequency of harmful algal blooms (HABs), as well as increased loads of nitrogen, phosphorus, pathogenic bacteria, and viruses. The increasing frequency of HAB events appears related to progressively greater nutrient loads reaching coastal waters (Paerl *et al.*, 2001). HABs are often associated with a complex community of bacteria and viruses, that have a potentially significant role of this associated microbial community in initiating and controlling these blooms. It may be possible to utilize new molecular methods, and our newly developed (but still minimal) understanding of viral diversity in marine systems, to quantify the relative importance of viral populations in bloom formation and termination. Molecular approaches can be used to characterize the bacterial and viral communities associated with bloom formation, assess the numbers of specific types of viruses, and detect HAB species even at very low abundances.

Relationships between viral abundance and environmental factors across diverse aquatic environments

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INTRODUCTION

The main objective of the present work was to identify the factors that control and covary with viral abundance in nature. For this purpose we sampled a diverse set of environments including both marine and freshwater ecosystems. Among the first we included two solar salterns from the Spanish coast (Guixa-Boixereu *et al.*, 1996), and transects in the Mediterranean Sea (Guixa-Boixereu *et al.*, 1999a) and in Antarctic waters (Guixa-Boixereu *et al.*, 2002). The fresh water systems sampled included several karstic lakes with anoxic hypolimnia. In these environments we measured salinity, temperature, oxygen concentration, VLP abundance, bacterial abundance and cell volume, chlorophyll a, bacterial heterotrophic production and growth rate, and bacterivory. We also collected VLP abundance data in natural environments reported in the literature to extend the study of Maranger and Bird (1995). We then used this data base to determine the empirical relationships between VLP abundance and the other variables.

RESULTS AND DISCUSSION

Bacterial abundance

As expected, a strong relationship was found between viral and bacterial abundance (Fig. 1). An analysis of covariance did not find significant differences in the slopes of the viral-bacterial relationships in freshwater and in marine systems. According to the general relationship bacterial abundance explained 66% of the variability in VLP abundance. The relationship was significant for each of the marine environments separately. Some of the freshwater environments, however, did not show a significant relationship (Canadian lakes and Danube river). A significant relationship between these two variables has been found in several studies from different specific environments suggesting, indirectly, that bacteria are the most abundant hosts for aquatic viruses. The fact that the strength of this relationship is maintained over the wide range of ecosystems included in the present study seems to confirm this suggestion.

Chlorophyll a

Maranger and Bird (1995) found a better relationship between VLP abundance and chlorophyll than with bacterial abundance. In the present study the regression between VLP abundance and chlorophyll a was also significant, but the percent of variability explained was lower than in the case of bacteria (39%). We think that this implies that algal viruses were not very important

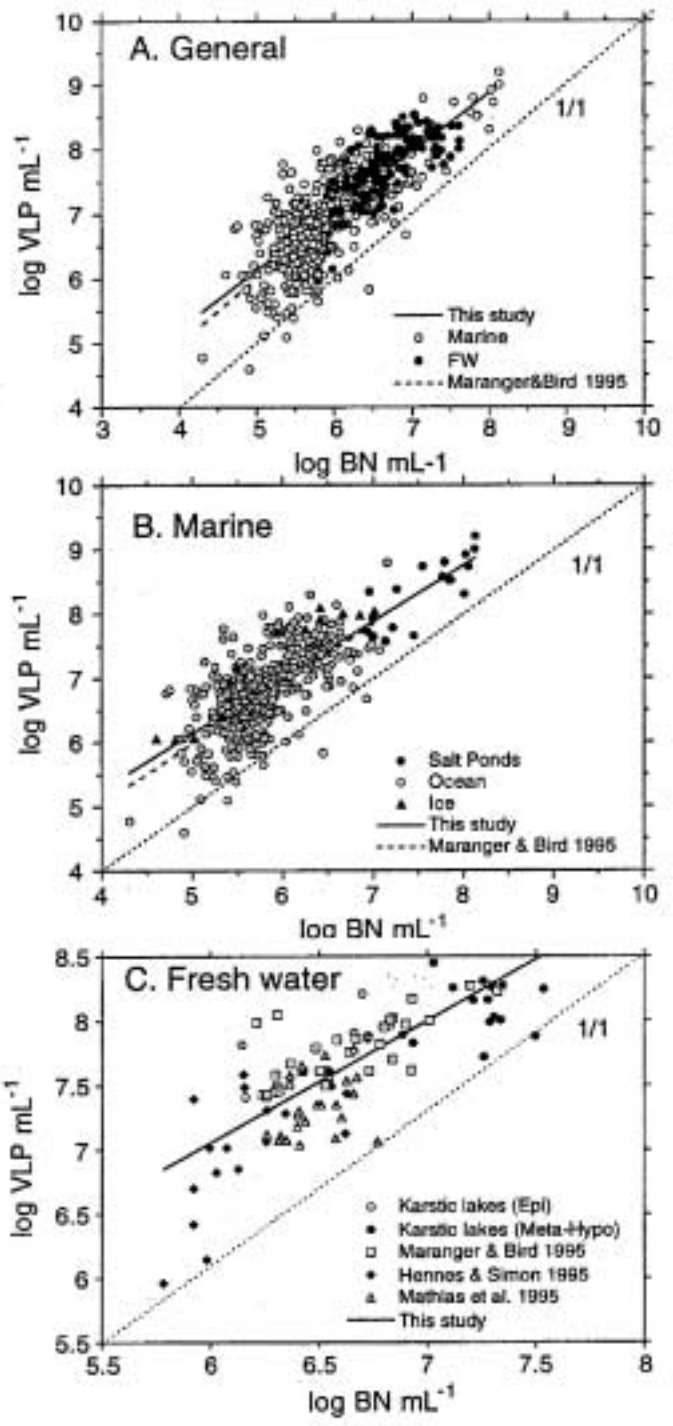

Fig. 1. Relationships between VLP and bacterial abundance. The dotted line represents a hypothetical 1:1 relationship. **A**, all systems together; **B**, marine systems; **C**, freshwater systems.

in the environments included in the data base. There were significant differences between the regressions for freshwater and marine environments. In fact, the regression for freshwater systems was very poor, explaining only 8% of the variability. Using chlorophyll a in multiple regressions did not increase the percentage of variability explained just with bacterial abundance.

Other variables

Bacterial heterotrophic production and bacterivory were also singificantly correlated with VLP abundance, but the coefficients were very low. VLP abundance was not significantly correlated with bacterial growth rate.

Visibly infected bacteria (VIB) were significantly correlated with both bacterial abundance and heterotrophic production (Fig. 2). The percentage of VIB, however, was not. This percentage did not seem to increase linearly with bacterial numbers, except in the samples from the Adriatic Sea.

The slope of the regression between VLP and bacterial abundance was significantly lower than one. This indicates that VLP abundance did not increase at the same rate as bacterial abundance. As a consequence, the VLP/BN ratio tended to decrease when bacterial abundance increased. Thus, for a bacterial abundance of 105 cells per mL, the VLP/BN ratio would be 10.8, while for a bacterial abundance of 107 cells per mL, the VLP/BN ratio would be 7.4. Before discussing the possible factors responsible for this uncoupling we should consider two points: first, although the slope of this relationship was significantly different from one, the difference was small and perhaps it will change when more data are added to the data base. Second, part of the VLP assemblage could be phytoplankton viruses. At the moment, however, there is no way of knowing what proportion of the VLP corresponds to algal viruses. We will assume that most VLP are bacteriophages. Leaving these caveats aside, several reasons could explain the uncoupling in the empirical relationship between VLP and bacteria found in the present study.

1. VLP abundance in an environment is the result of the balance between VLP production and losses. In a steady-state situation production and losses are in equilibrium, thus the rate of either process over the abundance would give the VLP turnover. The uncoupling found could be explained if VLP turnover increased with increasing bacterial abundance. In this case, the VLP/BN ratio would tend to be lower at higher bacterial abundance because VLP would remain relatively less time as free particles than at low bacterial abundance. Several of the data collected in the present study correspond to steady-state situations. Therefore, we checked if either viral

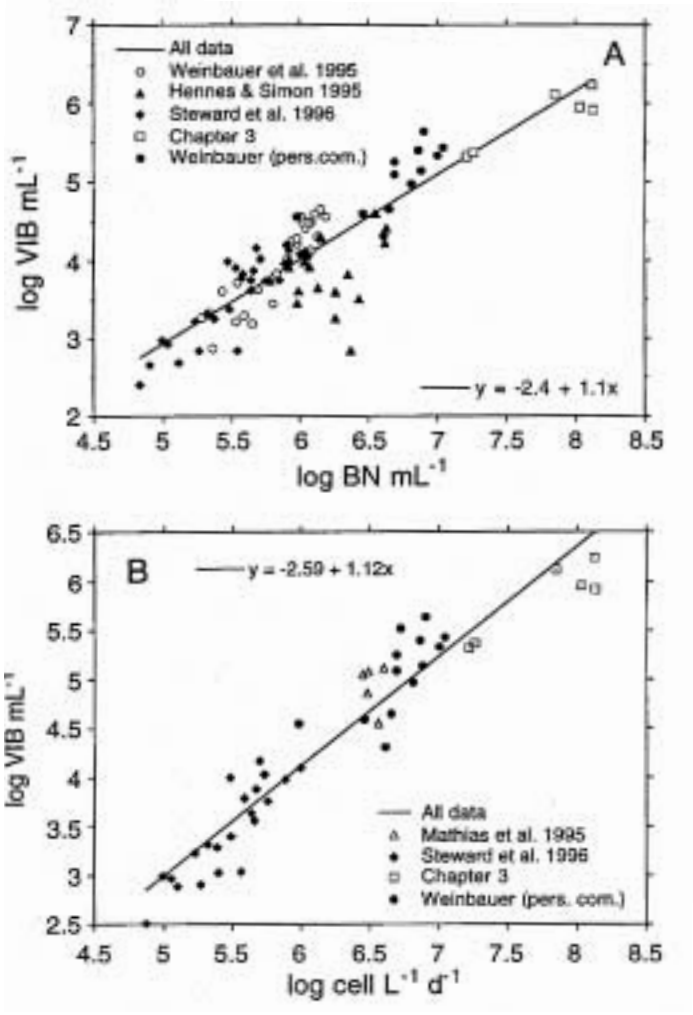

Fig. 2. Relationships between visibly infected bacteria (VIB) and bacterial abundance **(A)** and bacterial heterotrophic production **(B)**.

production or decay rates increased with increasing bacterial abundance (Fig. 3A). No significant relationship between production or decay rates and bacterial abundance was found. Thus, a difference in turnover rates with bacterial abundance must be rejected as the cause for the observed slope.

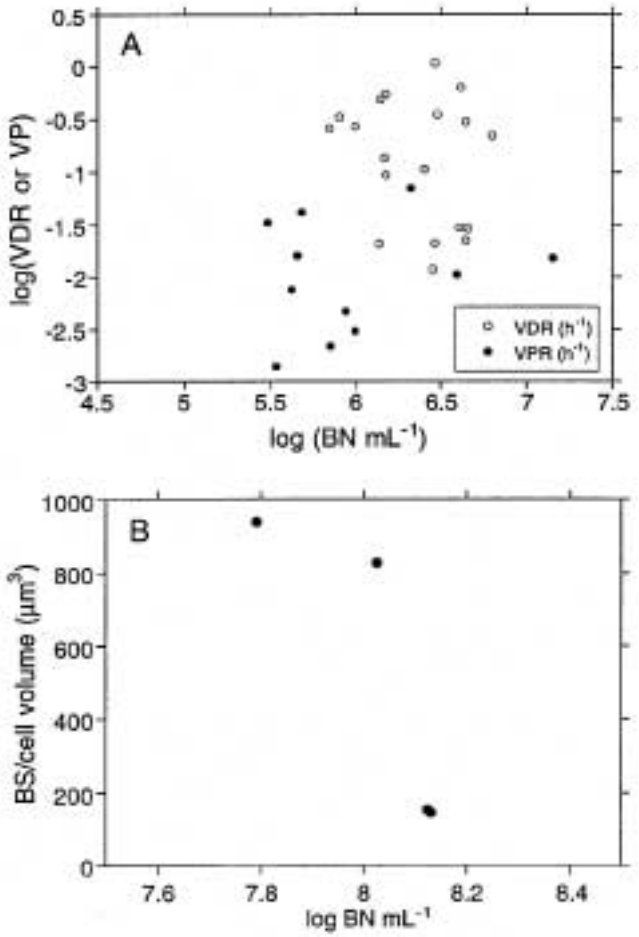

Fig. 3. **A)** relationship between viral decay rate (VDR) or viral production rate (VP) and bacterial abundance. **B)** Relationship between efficiency of viral infection as burst size per unit volume (BS/V) and bacterial abundance in the salterns.

2. In our data base, the number of infected cells increased proportionally to the total number of cells (the slope of the relationship was one). If VLP/BN ratio tends to be lower when increasing bacterial abundance, the efficiency of the infection (VLP released per cell volume) should be higher at lower bacterial abundance. If this were true, the average burst size divided by the average cellular volume (BS/V) in any environment would tend to be lower with increasing bacterial abundance. Unfortunately, the only relevant data available are those from the salterns (Fig. 3B). The data are consistent with a decreased efficiency at higher cell abundance. However, a larger data base would be needed to confirm this hypothesis.

CONCLUSION

Bacterial abundance was the variable that better explained the variability on VLP abundance across a wide range of aquatic environments with similar relationships in most of them. 66% of the variability in VLP numbers was explained by bacterial numbers. No differences were found between marine and freshwater environments. Bacterial and VLP abundance showed a slight uncoupling that could be due to a low efficiency (burst size per unit of cell volume) of the viral infection at high bacterial abundance. In particular environments such as the anaerobic layers of stratified lakes, burst size could be influenced by the physico-chemical characteristics of the medium. Thus, burst size appears to be a key variable in the dynamics of viral infection in nature. Its measurement in as wide a range of habitats as possible seems a promising source of information.

Impact of pollution on marine viruses, benthic viral production and lysogeny

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Pollutants can have an impact on marine viruses and on the virus-host systems with important ecological implications. Excess nutrients from sewage and river effluents are a primary cause of marine eutrophication and mucilage formation. Eutrophic systems can be characterised by a higher degree of viral infection (Wilhelm and Suttle, 1999; Weinbauer *et al.*, 1993). Since generally the number of viruses is greater in productive and nutrient-rich environments, the trophic state has been proposed as possible driving force controlling the spatial distribution of viruses (Weinbauer *et al.*, 1993). There are two possible explanations for the increased virus relevance in eutrophic systems: i) eutrophic environments allow a higher standing stock of bacteria (and consequently a higher number of hosts for bacteriophages) than oligotrophic systems (Weinbauer *et al.*, 1995; Weinbauer *et al.*, 1993); ii) eutrophication can directly stimulate viral development (in 22% of microcosm experiments viruses increased after inorganic P enrichment; Williamson *et al.*, 2002). However, a large-scale study on virioplankton distribution in the Adriatic Sea (the most eutrophic area of the Mediterranean basin) pointed out the lack of any dependence of viral distribution on biotic (chlorophyll-a) or abiotic (hydrology, salinity, inorganic nutrients) variables (Corinaldesi *et al.*, in press), which are commonly utilised to define the trophic state of coastal systems. Therefore, eutrophication seems only to increase viral abundance indirectly through the increase of bacterial activity and of the host-cell abundance (Corinaldesi *et al.*, 2003).

However, the presence of extremely high viral counts in eutrophic environments could also be the result of induction of prophages (i.e., lysogenic bacteria induced to lytic cycles, that deliver viruses after cell lysis). Cochran and Paul (1998) observed that there is a strong interaction between viral abundance and the presence of inducible prophages in eutrophic environments. They observed that the highest fraction of inducible prophages occurred after the phytoplankton bloom, concomitantly with a rise of water temperature (from 13 to 19°C). However, there is no evidence for a direct causality between eutrophication and prophage induction, as eutrophication can be associated to other pollutants capable of inducing lytic cycle in lysogenic bacteria. Moreover, it is possible that eutrophication acts synergistically with other pollution sources contributing to the creation of environmental conditions favouring viral development (Danovaro and Corinaldesi, 2003; Corinaldesi and Danovaro, 2002).

Recently, it has been demonstrated that polynuclear aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and pesticides determine a high percent efficiency of prophage induction in natural bacteria assemblages, from different marine environments (up to 75%; Cochran *et al.*, 1998). Jiang and Paul (1996), using aromatic and aliphatic hydrocarbons (includ-

ing Bunker C #6 fuel oil, phenanthrene, naphthalene, pyrene, and trichloroethylene) as agents inducing natural bacterial communities, found that viral direct counts increased from 129 to 345% of the non-induced control. Hydrocarbon-mediated prophage induction (up to 38% of the bacterial assemblages) was frequently found in estuarine environments. Cochran *et al.* (1998) investigating the effect of a pesticide mixture suggested that organic pollutants may induce lysogenic viral production, and that the induction of lysogenic bacteria is more efficient in coastal/estuarine systems than in off-shore environments.

Although classical pollutants (e.g., hydrocarbon, pesticides) have been recognised to induce lytic cycle in lysogenic bacteria, information on micro-pollutants is almost completely lacking. Recently, among these micro-pollutants, the effects of cosmetic sun products (sunscreen and solar oil) on viral abundance and bacterial activity were investigated (Danovaro and Corinaldesi, 2003). These studies revealed that both sunscreen and solar oil acted as pollutants, inducing viral development and leading to an increase of the virus to bacterium ratio. The impact on viral abundance was 5-10 times higher in microcosms treated with sunscreen than in those treated with solar oil. Moreover, sunscreen induced the lytic cycle in a large fraction of total bacterial abundance (13-24% of bacteria), whereas solar oil had a lower impact (6-9%). These results indicate that sun-products released in coastal waters might have a significant impact even at very low levels as observed for mixture of PCBs and pesticide (Cochran *et al.*, 1998).

In this perspective marine sediments are the optimal domain for testing the impact of pollutants on the host-phage system, as they collect and concentrate most pollutants present in marine systems. Estimates of viral production in marine sediments indicate that viral production in the sediment is one to three order of magnitude higher than in the plankton and that benthic viruses are characterised by very high turnover rates. Phage infection can be responsible for an important fraction of bacterial mortality in surface sediments (7-20%) and for 100% of bacterial mortality in deeper sediment layers. Moreover, differently from the water column, in marine sediments the lysogenic strategy seems to be rather uncommon. Comparing four different marine systems the percentage of lysogenic bacteria in surface sediments was always negligible (0 to 0.2%). The impact of pollution on viral infection and host-phage system is indirectly confirmed by studies conducted in coastal sediments subjected to a gradient of increasing impact (Fig. 1).

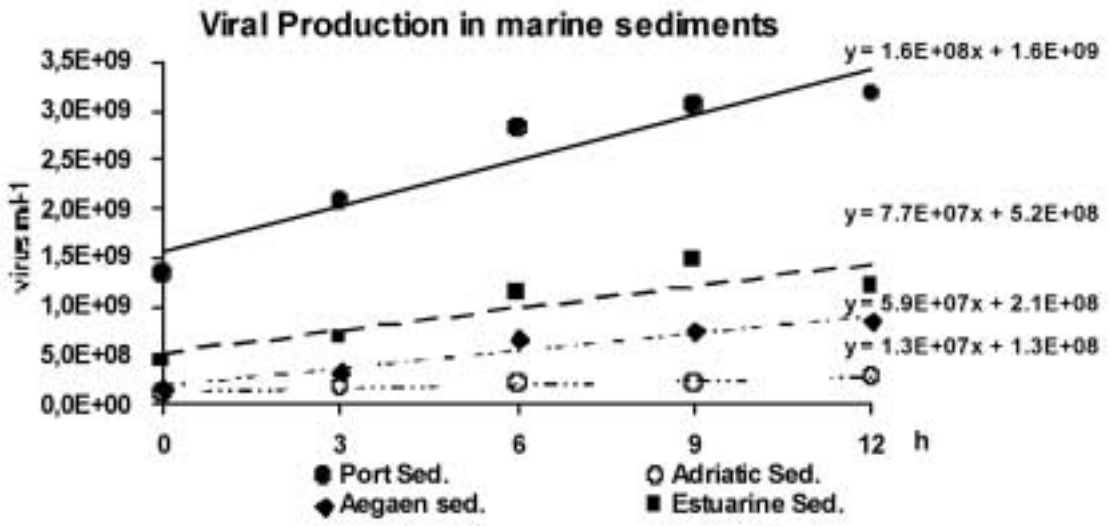

Fig. 1. Viral production (determined by dilution technique) in different marine sediments. It is evident an increasing production (see slope values) with increasing pollution from Adriatic to Aegean to estuarine to Port sediments).

These data suggest that different forms of pollution might increase prophage induction and viral abundance, and it is possible that different impacts act synergistically, but remains still to be explored which are the agents and mechanisms enhancing viral production and which pollutants might display a synergetic effect.

Bacteriophages of the North Sea – state of the art

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ABSTRACT

Bacteriophages may play ecological key roles in the complex marine bacterial communities and habitats. It is assumed that phages control bacterioplankton through their lytic activities, and that temperate phages may contribute to horizontal gene transfer. However, these statements rely on a limited set of data. Current knowledge is based on microscopic virus-like particle (VLP) counts and some observations using classical plaque forming units (PFU) providing information of bacteriophages in selected marine habitats. Molecular approaches were applied rarely. Unfortunately, the required information in order to decipher the complex puzzle(s) is presently not available. There is no technique on-hand for quick virus determination. Furthermore, the dominant bacterial hosts are often not culturable. The development of a concept on the systematic elucidation of phage ecology is overdue. It should encompass molecular and classical approaches. This paper contributes some ideas to solve these problems.

INTRODUCTION

Recent publications stress that phages play an important role in the complex marine food webs as summarized in recent reviews by Proctor (1998), Wilhelm and Suttle (1999) and Wommack and Colwell (2000). Phages are abundant in the marine environment (up to 10^8 virus-like particles ml^{-1}) (Bergh *et al.*, 1989; Wommack and Colwell, 2000). The abundances are positively correlated to the trophic level and productivity of their habitats.

Usually their numbers are determined as VLP's PFU generated by their host bacteria. Microscopic approaches require transmission electron microscopy (Bergh *et al.*, 1989) or epifluorescence microscopy (Hennes and Suttle, 1995). Phages infect and lyse planktonic bacteria. It is assumed that besides the digestion of bacteria by heterotrophic nanoflagellates, phages account for 10 to 50% of bacterial mortality (Fuhrman and Noble, 1995; Hennes and Simon, 1995; Fuhrman, 1999). Simultaneously, the release of dissolved organic and inorganic nutrients derived from lysed bacteria may enhance bacterial substrate turnover and result in higher bacterioplankton biomass. Besides their quantitative growth control, phages can influence the community structure of the planktonic bacteria also qualitatively. This may be due to the high bacterial host specificity of many marine bacteriophages (Moebus, 1983; Hennes *et al.*, 1995; Wichels, 1997; Wichels *et al.*, 1998; Wommack and Colwell, 2000) and comprises complex and diverse phage activities, like specific lysis, resistance phenomena, and/or lysogeny of host bacteria, possibly also

resulting in horizontal gene transfer by transduction (Jiang and Paul, 1998; Chiura *et al.*, 2000). If information of lysogenic bacterial hosts from the North Sea is rare (Moebus, personal communication), a convenient example for lysogeny is represented by *Sphingomonas* sp. strain B18, isolated from Lake Pluss See (Ostholstein, Germany) (Witzel *et al.*, 1994; Wolf *et al.*, 2003). Also worthy of note, the fact that a global movement of ballast water by ships creates a worldwide distribution of microorganisms including virus particles (CIESM, 2002; Ruiz *et al.*, 2002). All these phenomena have an impact on the bacterial community structure.

New biotechnological approaches focus on the utilization of phages. Phages can function as tools, agents and/or chemical compounds. For example applications make use of phages to remove phosphate from activated sludge systems (Khan *et al.*, 2002). Or phages may be used as antifoam agents (Thomas *et al.*, 2002). In another instance bacteriophage H4489A provides specific lyases to digest the hyaluronan capsule of pathogen streptococci (Baker *et al.*, 2002). Brion and Silverstein (2001) describe a successful practice using non-pathogen phages to test the efficiency of water recycling systems. Probably, in the near future new applications will be detected for marine viruses.

PHAGE RESEARCH AT MARINE STATION HELGOLAND

Extensive investigations at the Biologische Anstalt Helgoland have demonstrated a high diversity of bacteriophages in the open waters (Moebus and Nattkemper, 1981 und 1983; Moebus, 1987; Wichels *et al.*, 1998; Wichels *et al.*, 2002). All bacteriophages investigated contained double stranded DNA. Electron microscopy studies revealed that phages collected from the waters around Helgoland (German Bight) were members of the order Caudovirales with the families Myovirides, Siphoviridae and Podoviridae including different species and some conspicuous morphotypes (Fig. 1). The host specificity was striking. Seventy-three percent of a total

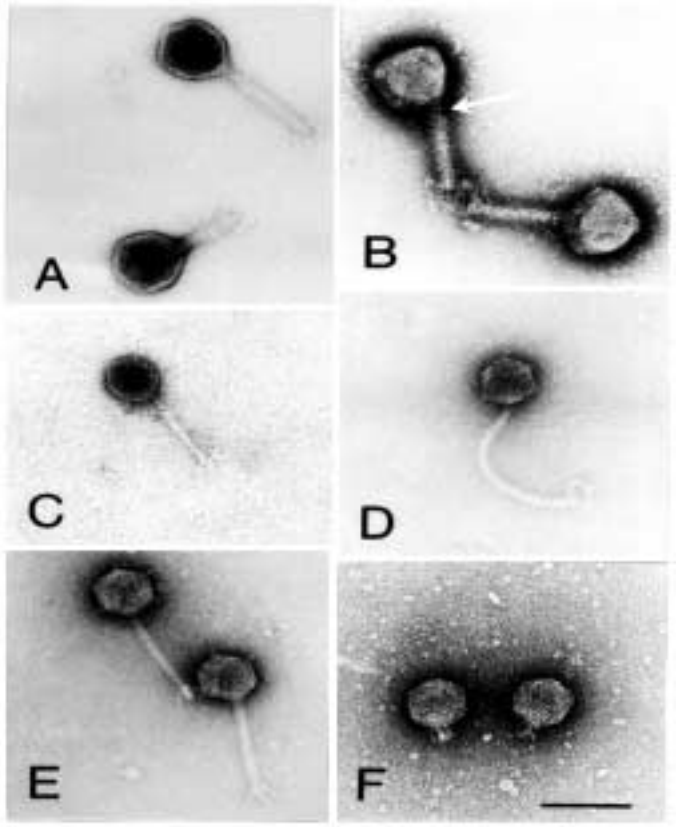

Fig. 1. Marine bacteriophages of three different virus-families, isolated from water samples near Helgoland (German Bight). **A:** Myoviridae, morphotype 1: head without antennae; short appendages on the tail; (phage H106/1); **B:** Myoviridae, morphotype 2: collar like structure between head and tail (see arrow); short appendages on the tail (phage H7/2; 15); **C:** Sipho-viridae, morphotype 1: head and tail without appendages (phage 10-77a); **D:** Siphoviridae, morphotype 2: knob-like appendages on the head, tail with a hook at the end (phage 11 68c); **E:** Siphoviridae, morphotype 3: knob-like appendages on the head, tail with short appendages (phage H105/1); **F:** Podoviridae, morphotype 1 (phage H100/1); bar: 100 nm.

of eighty-five phages investigated lysed exclusively their original bacterial host. The determination of host bacteria indicated that the bacterial isolates belong to the subgroup of proteobacteria. Phage diversity was elucidated by DNA-DNA-cross-hybridization (all phages were hybridised against each other), restriction analysis and the comparison of RAPD patterns. Cross-hybridization data unveiled a high phage diversity. Fourteen out of twenty-two phages showed no genetic relationship to the other particles tested (Fig. 2). The analysis of total phage protein patterns and restriction patterns was less helpful. Inactive restriction enzymes, and low phage protein concentrations caused major problems. The ecological approach showed that some of the phages Moebus (1983) isolated a decade earlier could still be detected in the waters of Helgoland and the German Bight. However, in using the bacterial host strains from Moebus, newly isolated lytic phages were only localized in a narrow regional area north and south of Helgoland (Fig. 3). These data suggest stable community structures over a long space of time of specific phage host systems. These findings are different from the data of Kellog *et al.* (1995). The authors detected specific *Vibrio* phages in the Atlantic Ocean which are distributed over a distance of several thousands nautical miles, whereas bacterial hosts from the German Bight were mostly members of the *Pseudoalteromonas* group (γ–Proteobacteria). The different data may not be contradictory. Presumably both the Vibrio and the *Pseudoalteromonas* group of bacterial hosts are well adapted to their marine environments. However, the German Bight with the major estuaries of the Elbe and Weser River can be regarded as an enormous melting pot, which differs nearly in every oceanographic and biological respect from the characteristics of the open Atlantic.

Our field experiments in the waters around Helgoland underscore the high diversity of different shapes and sizes of virus-like particles. Fig. 4 provides an example of the natural virioplankton. Seasonal phage distributions, determined by epifluorescence direct counting with YO-PRO stain (Molecular Probes), followed the seasonal bacterioplankton characteristics (Fig. 5). Low

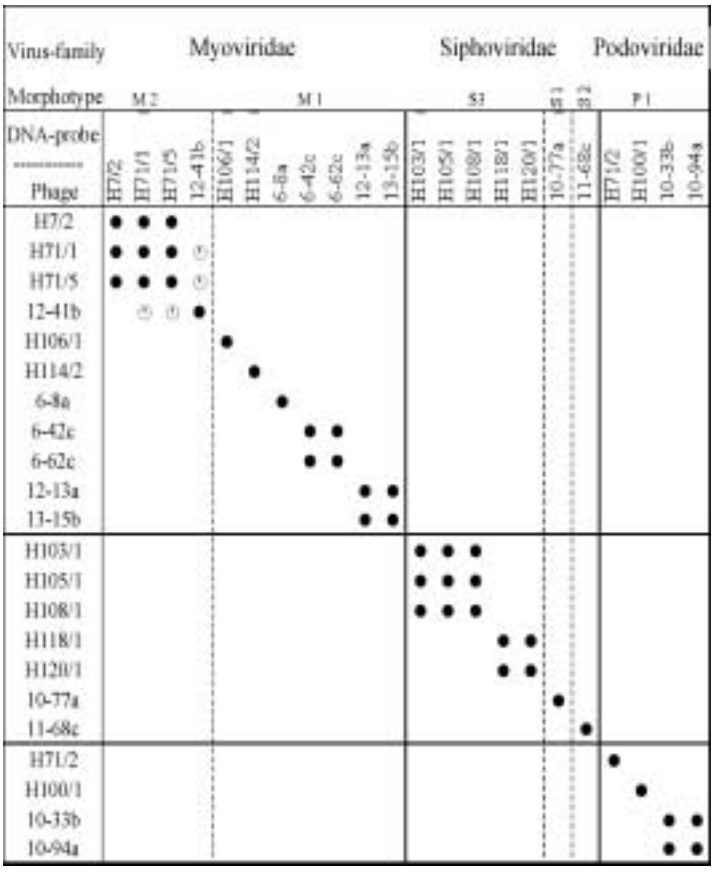

Fig. 2. DNA-DNA-cross-hybridizations of 22 marine bacteriophages collected from waters around Helgoland. l: strong hybridization signal. m: weak hybridization signal. Phages and DNA-probes were assigned to their families and morphotypes.

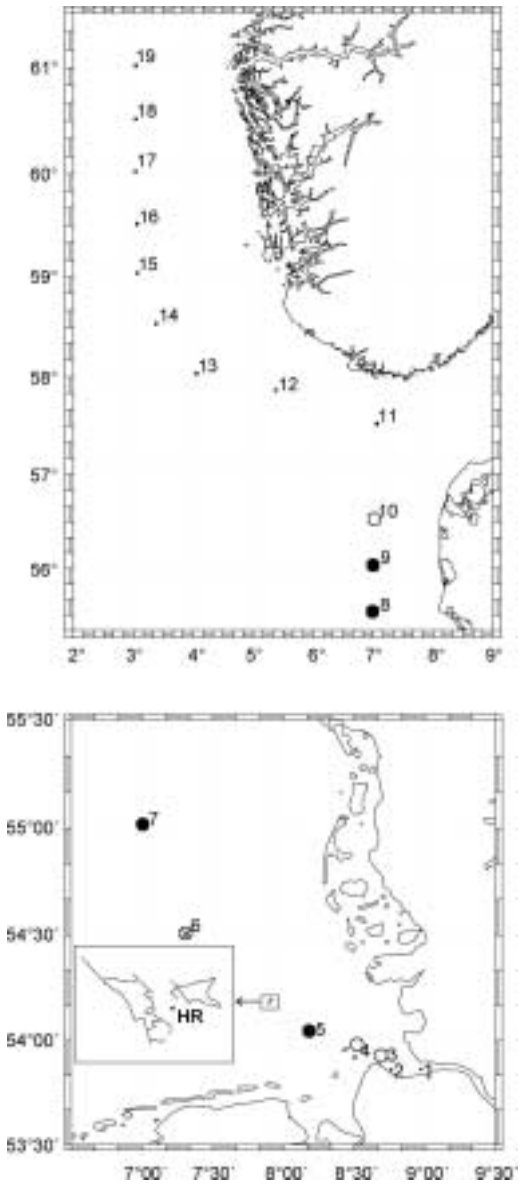

Fig. 3. Sampling sites of the RV *Heincke* cruises HE49 (September 1993), 54 and 65 (April and October 1994) in the North Sea (German Bight to Norwegian waters). Station Helgoland Roads (HR) is the origin of 70 phage host systems used in this study (Moebus 1992a,b). Circles represent the occurrence of Pseudoalteromonas phages, as determined by plaque assay, at each station (l 1993; m 1994; U both years; other stations no plaques formation).

numbers phages and bacteria were found during the cold seasons; high numbers were detected during the productive seasons from May to October.

NEEDS AND NEW APPROACHES

Presumably phages play ecological key roles in controlling marine bacterioplankton and gene transfer. However, presently there is no concept for unveiling the ecological role with lytic and temperate activities of phage host systems. The functions of the zillions of marine virus particles, which represent an exciting block of the “mare incognitum”, are still enigmatic.

Generally, there are two fundamental complexes of questions concerning the bacterial hosts and their phages. (i.) Bacterial hosts: systematic new approaches to quantify, culture and determine the dominant host bacteria in the various natural habitats are urgently needed. Since bacterial hardware is still essential in laboratory experiments, the development of new culture techniques is indispensable. In order to reach realistic answers on quality and frequencies of virus mediated natural gene transfers, thorough investigations of lysogenic bacteria and its temperate phages are required. That kind of approaches should include the development of specific primers for selected phage genes. (ii.) Phages: progress in virus research will depend on the availability of quick determination procedures for phages. In this field universal viral probes from polymerases, lyases, etc. are needed.

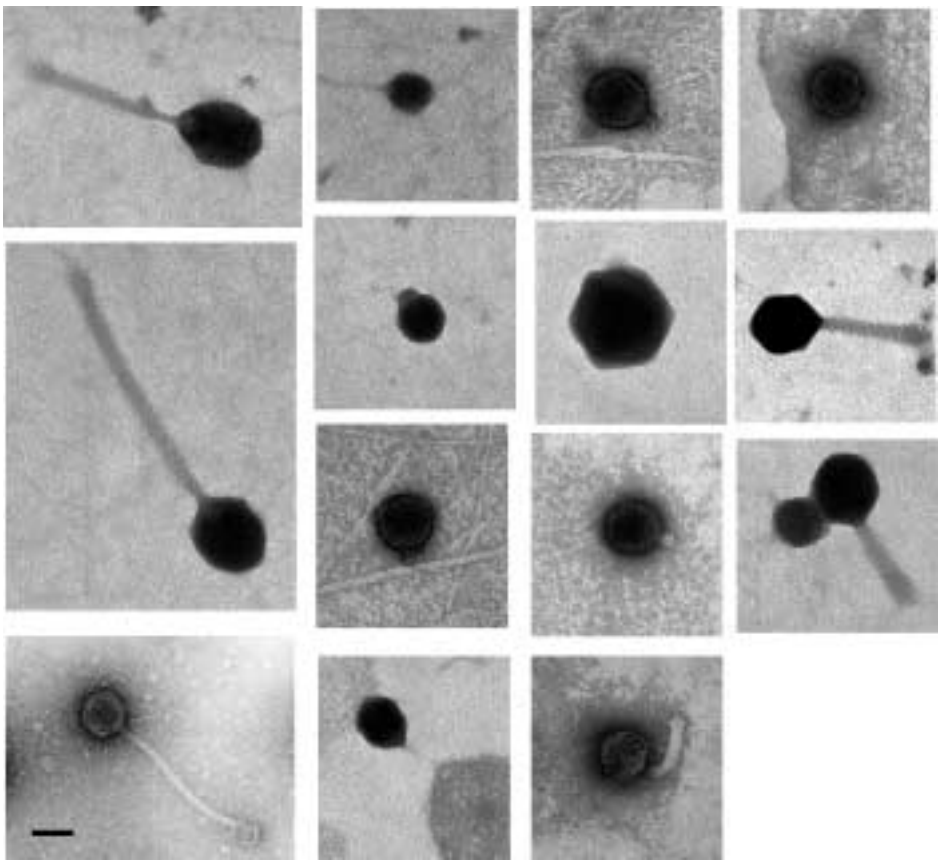

Fig. 4. Natural virioplankton in water samples from Helgoland (German Bight); sampling from April to June 2001 (bar: 80 nm).

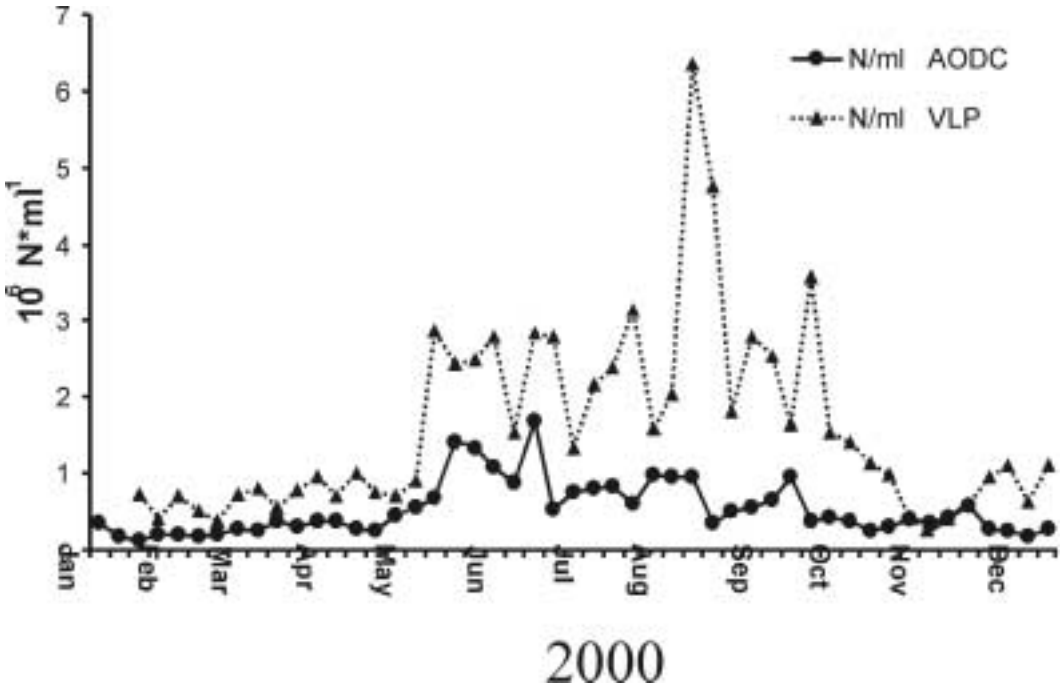

Fig. 5. Bacterioplankton (AODC) and virioplankton (VLP, YOPRO) total counts in 2000; weekly sampling near Helgoland (German Bight).

Dominant bacterial hosts: improvement of cultivation techniques

Recent studies revealed that the isolation techniques used so far (ZoBell medium, marine broth) are highly selective for the isolation of fast growing γ–Proteobacteria (*Pseudalteromonas* sp.). However, results of fluorescence in-situ hybridization (FISH) showed that these *Pseudalteromonas* species do not represent the dominant bacterial groups in the natural marine environment (Eilers *et al.*, 2000). In general, results of the standard cultivation procedures used so far do not reflect the structure of the natural bacterial composition and the majority of planktonic bacteria in the marine habitats detected by FISH, cannot be cultured by using current techniques (Giovannoni and Rappé, 2000). Among these "uncultivated" bacteria are specific phylogenetic clusters of the a Proteobacteria (e.g., SAR11, SAR116, *Roseobacter*), γ–Proteobacteria (e.g. SAR-86) and those of the *Bacteroidetes* group. There are only very few cultures available of the *Roseobacter* group, and one single isolate of the SAR11 cluster (Rappé *et al.*, 2002), which is very difficult to culture. Our culture collections do contain some strains closely related to the mentioned clusters, generated through enrichment cultures. Consequently only very few phages were isolated from these bacterial groups. One example represents the *Roseobacter*-specific podovirus, Roseophage SOI1. This phage is highly host specific. Its genome has been completely sequenced (Rohwer *et al.*, 2000).

Phage diversity: molecular approaches

It is assumed that presently uncultured but *in situ* dominant bacteria will use the same infection mechanisms as other isolates in nature, but the role and importance of lytic and/or virulent life cycles of marine phages still need to be evaluated. For a better understanding of marine phage ecology, natural dominant bacteral groups should be used in future experimental approaches. Additionally the development of new techniques is indispensable. It can be assumed that pulsed field electrophoresis (PFGE) will provide a better resolution of the diversity of marine phage communities. The genome of the total phage population, separated by PFGE, displayed significant variation in size, and showed specific band patterns due to seasonal variations (Wommack *et al.*, 1999a, 1999b; Steward and Azam, 2000). By using a combination of plaque assay and DNA-DNA-hybridization, Wichels *et al.* (2002) demonstrated the existence of a restricted distribution of specific *Pseudoalteromonas* phages for the North Sea (see above). Another promising approach of the analysis of the diversity of natural phage and virus assemblages is the denaturing gradient gel electrophoresis (DGGE) of PCR fragments. While Short and Suttle (1999) demonstrated the diversity of natural phytoplankton-viruses by DGGE from PCR-amplified viral DNA-polymerase gene fragments, there is nearly no information for marine bacteriophage diversity. Only Rohwer *et al.* (2000) showed first results of sequence homologies among DNA-polymerases genes of phages of different habitats.

Classical virology and molecular approaches

Besides the elucidation of phage diversity in marine habitats, quantification of specific groups of phages is of high interest to estimate their influence on bacterial populations in nature. Plaque tests on their own are insufficient, since this method does not differentiate between specific phage groups. Only a detailed analysis of all plaques will generate quantitative data about specific phages. An option would be the time consuming DNA-DNA-hybridization or as an excellent alternative the real time PCR. Quantitative approaches by real time PCR in detecting specific virus particles have been successfully conducted in clinical virology (Aberham *et al.*, 2001; Japs *et al.*, 2001; Najioullah *et al.*, 2001), but have not been used so far in phage ecology.

The powerful techniques (PFGE, DGGE) discussed above, including the development of specific primers and probes for dye detection of phage genes (quantitative PCR), will broaden our insight of phage ecology and of the interactions between phages and their hosts. This includes both, the quantitative assessment of phages and their hosts and additionally the qualitative impact of specific phages on the composition and dynamics of the bacterioplankton. These new technologies will provide exciting information on the potential transfer of functional genes between phages and bacteria.

Acknowledgements. We are grateful to Michael Schweickert, Univ. of Stuttgart, FRG, who provided the electrom microscopic images of virioplankton from the North Sea. The work was supported by funds of the Biologische Anstalt Helgoland.

Viral regulation of bacterial biodiversity

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Many factors contribute to regulate bacterial biodiversity. Recent experimental results support the hypothesis that viruses are involved in the regulation process (Larsen *et al.*, 2001). The control of bacterial diversity would then rely on selective destruction mechanisms, by a virus, of a bacterial population as soon as its concentration is growing, thus preventing the emergence of dominant bacterial populations (Lebaron and Nicolas, in press). In this paper, we present how mathematical models for microbial and viral populations can contribute to evaluate this hypothesis.

Data on growth parameters of bacterial and microbial populations specific to the Mediterranean sea are scarce but can be estimated. Data on bacterial and viral dynamics can also be estimated thanks to recent methods such as epifluorescence microscopy (Hennes and Suttle, 1995; Noble and Fuhrman, 1998) and flux cytometry (Marie *et al.*, 1999a). In addition, isolation of host-phage couples will allow to characterise experimentally the affinity phage/bacterium, for example the species *Vibrio splendidus*, a pathogenic agent for oysters, which is a relatively dominant species in the Bay of Banyuls. We will start our study by considering bacterial populations of *Escherichia Coli* on glucose to show how theoretical models can explore hypotheses. These models allow to isolate the influence of specific factors, and help in suggesting experimental verifications. They can also be useful to determine key parameters, such as the parameters of the chemostat.

MODELLING APPROACH

Modelling can envision large systems, taking into account bacterial growth, limiting substrates, protozoan predation, and viral-host interactions (Thingstad and Lignell, 1997). With such models, one can address the role, for instance, of viral infection on the structure of microbial populations (Thingstad, 2000b).

In this work, we adopt a bottom-up approach. Our aim is to focus on an ideal situation where viruses would be the only factor in the regulation of biodiversity. Here, we restrict ourselves to lytic viruses.

We adopt the following modelling strategy: in the first step, we consider the growth of a single bacteria population. As we want to mimic experimental conditions, we take a microbial pop-

ulation growing in a continuous culture apparatus under conditions, where a single substrate (here glucose) limits growth (Bazin, 1982). The lytic virus is then added to the model, and the analysis of this new model, compared to the reference model, allows us to define the role of this factor. In a second step, we model two bacterial populations. Because our main goal is to study coexistence between two microbial populations due to viruses, we set specific conditions where only one population survives in the absence of viruses. Then, we add virulent viruses. Due to virus regulation, conditions in which the two bacterial populations coexist can appear.

BACTERIAL GROWTH AND CHEMOSTAT PARAMETERS

The model for bacterial growth in the absence of viruses is the following:

$$dB_1/dt = \mu 1\ B_1\ S / (C_1 + S) - K\ B_1 \qquad [M1]$$

$$dS/dt = -\alpha\ \mu_1\ B_1\ S / (C_1 + S) + K\ (S_f - S)$$

where B_1 is the microbial density population (grams per liter), S the substrate concentration (grams per liter, μ_1 the maximum specific growth rate ($hour^{-1}$), a the stochiometric coefficient (grams substrate per grams cells) and C_1 a parameter describing the growth curve *versus* substrate concentration (grams per liter). K is the wash-out rate constant of chemostat (hour) and S_f is the feed substrate concentration (grams per liter). We assume that the relationship between growth rate and substrate concentration can be described by Monod kinetics ($\mu_1\ B_1\ S / (C_1 + S)$), which is more adequate than a logistic curve (Bazin, 1982; Monod 1958).

In the equilibrium state, the density of the population and that of the substrate do not change with time, so that $dB_1/dt = dS/dt = 0$. Model [M1] had two such equilibrium states. The first one is when the bacterial population disappears, which is of no practical interest. A condition necessary to avoid this situation and to stabilise the population is that $K < \mu_1\ S_f / (C_1 + S_f)$ (Bazin, 1982). This condition implies that the wash-out of the chemostat should not be too rapid compared to the growth of the microbial population. An important notion associated to an equilibrium state is its stability, that is, whether a little variation away from the equilibrium state will return to this state or not (if not, the equilibrium state is instable). Mathematically, stability can be defined by the eigenvalues of the jacobian matrix associated to the model: all the eigenvalues should be negative in order to have a stable equilibrium state.

In this situation, assuming that one can obtain experimentally growth parameters for bacterial populations (α, μ, C), stability only depends on two parameters K and S_f. We use a graphical representation to illustrate the stability conditions for K and S_f. Figure 1a shows an example of a growth curve, and Figure 1b delineates in black the stability region in the (K,S_f) space. We can choose the experimental parameters using this graphic: in the following example, we set K = 0.5, S_f = 15.

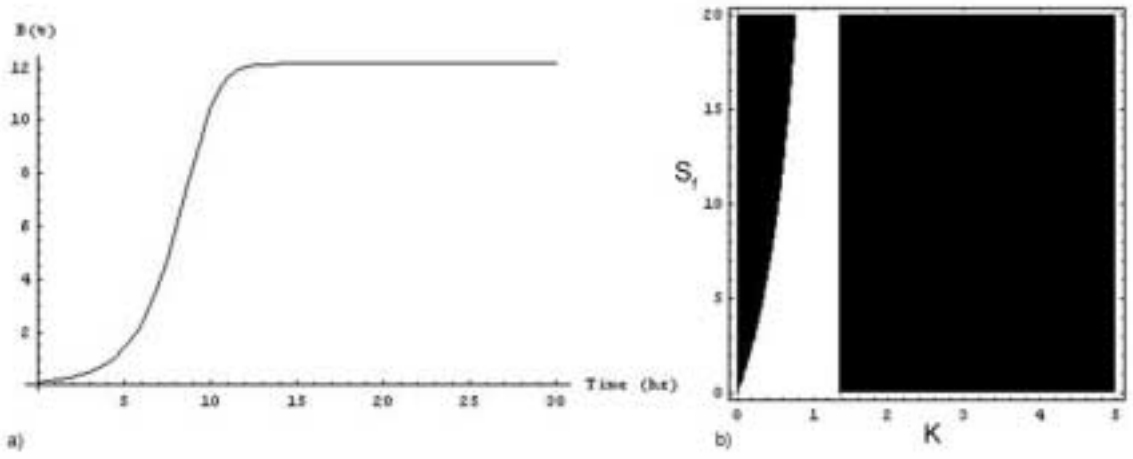

Fig. 1. **a)** Growth curve of a bacterial population (μ_1= 1.35, C_1 = 4, α = 3.39, K = 0.5; S_f = 15) for model (1)**. b)** stability region (black) of equilibrium state for K and S_f values.

BACTERIAL GROWTH WITH LYTIC VIRUSES

Following previous approaches (Campbell, 1961; Beretta *et al.*, 2000), our model of lytic phage/bacterial interaction is the following:

$$dB_1/dt = \mu_1 B_1 S / (C_1 + S) - K B_1 - \beta B_1 P \qquad [M2]$$

$$dS/dt = -\alpha \mu_1 B_1 S / (C_1 + S) + K (S_f - S)$$

$$dP/dt = n \beta B_1 P - \gamma P - K P$$

where P is the density of lytic viruses, γ the death rate of viruses, β the effective phage adsorption per bacteria. Here, bacteria-phage interaction is modelled by the term $\beta B_1 P$. We assume that each infected bacterium yields *n* phage particles (we set *n*=100 based on Ptashne, 1992). Our approach differs from Beretta *et al.* on three points: 1/ we use a Monod kinetics instead of a logistic growth curve, 2/ we do not consider delay for lytic latency, and 3/ we consider a chemostat situation when Beretta *et al.* consider a "*thermo clinic layer of sea*". The latter choice is related to our main goal: setting conditions for experimental test of our hypothesis.

For the parameters chosen previously, model [M2] has four equilibrium states: no bacteria and no viruses, bacteria and no viruses, and two equilibria where bacteria and viruses coexist. One of these two states does not have biological meaning, as S is negative. In the other, if we set β=1, γ=5, a periodic evolution for the bacteria and viruses populations appears, as shown in Figure 2b.

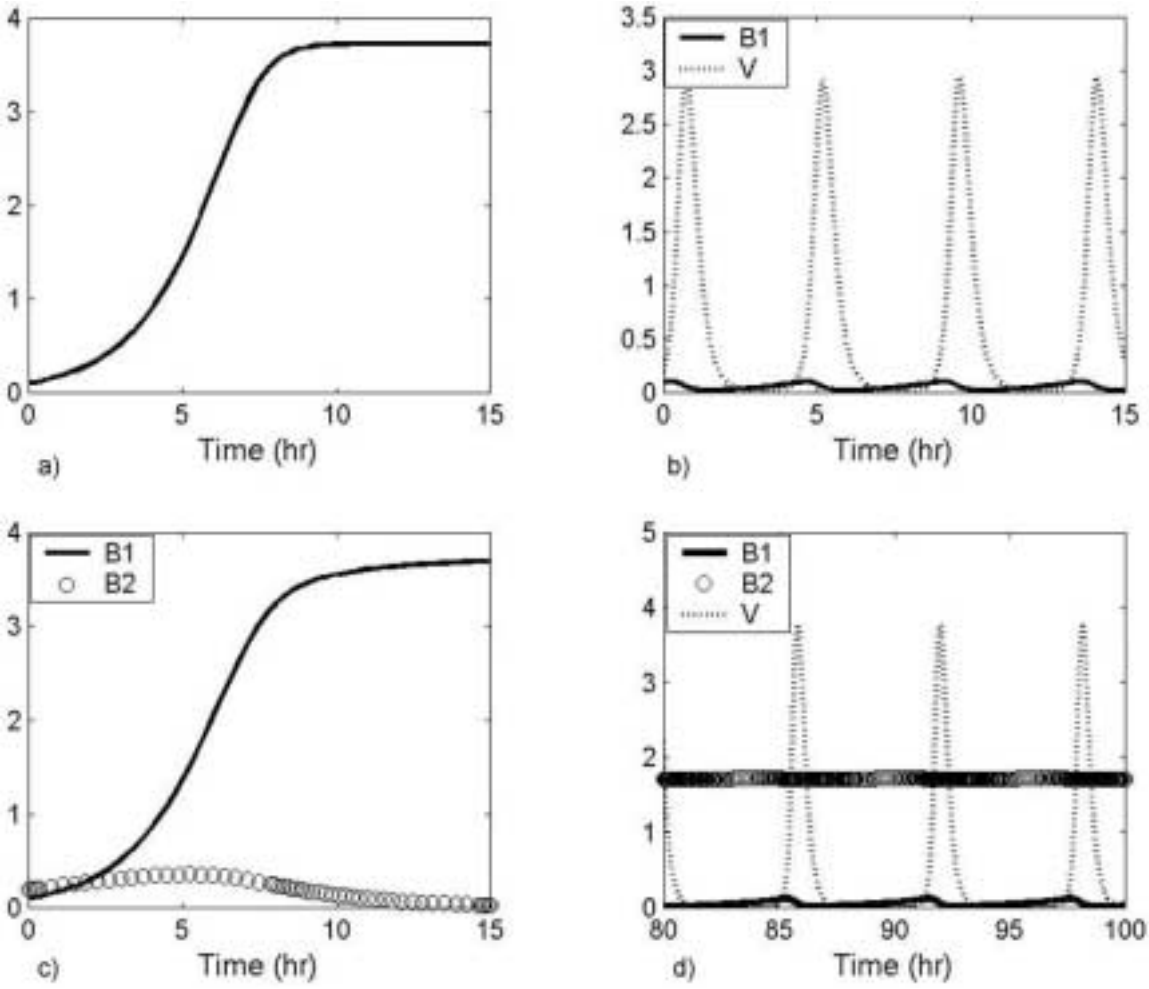

Fig. 2. Growth curve for **a)** one bacteria population (model 1); **b)** one bacteria population and lytic viruses (model 2); **c)** two bacteria populations (model 3) and **d)** two bacteria populations and lytic viruses (model 4). Parameters are : μ_1= 1.35, C_1 = 4, μ_2= 1.35, C_2 = 15, α = 3.39, K = 0.5; S_f = 15, n = 100, β = 1, γ = 5.

TWO COMPETING BACTERIAL POPULATIONS

We can now consider a bacterial population, B1, and the competing bacterial population B_2. The two populations compete for the same substrate S. In the absence of virus, the model is the following:

$$dB_1/dt = \mu_1 B_1 S / (C_1 + S) - K B_1 \qquad [M3]$$

$$dB_2/dt = \mu_2 B_2 S / (C_2 + S) - K B_2$$

$$dS/dt = -\alpha [\mu_1 B_1 S / (C_1 + S) + \mu_2 B_2 S / (C_2 + S)] + K (S_f - S)$$

We set $C_1 < C_2$, and $\mu_2 = \mu_1$. In this situation, population B_1 has a better growth on substrate S than population B_2 (see Fig. 2c). Model [M3] has three equilibrium states: only population B_1 survives, only population B_2 survives, and no population survives. We consider the first equilibrium state.

TWO COMPETING BACTERIAL POPULATIONS AND VIRULENT VIRUSES

We now turn to the following situation: two initial bacterial populations (B_1 and B_2) in the situation described above where B_2 cannot survive. Let us introduce a virulent virus. The model is the following:

$$dB_1/dt = \mu_1 B_1 S / (C_1 + S) - K B_1 \qquad [M4]$$

$$dB_2/dt = \mu_2 B_2 S / (C2 + S) - K B_2$$

$$dS/dt = -\alpha [\mu_1 B_1 S / (C_1 + S) + \mu_2 B_2 S / (C_2 + S)] + K (S_f - S)$$

$$dP/dt = n \beta B_1 P - \gamma P - K P$$

We can now compare this situation to model [M3]. As seen in Figure 2d, bacterial population B_2 can now survive. Recall that this situation was impossible in the model without lytic viruses (model [M3]). We can observe that B_1 stays at the same level as when no competing population was introduced (model [M2]). B_2 also remains at a very low level, because C_2 is set to 15. This means that without any competing bacterial population (here B_1), this population should saturate at a level of 15 instead of ~ 1.75 (see Fig. 2d).

CONCLUSIONS

Modelling allows us to isolate and study the specific contribution of the competition between population i.e. the coexistence of two bacterial populations due to lytic viruses. We can study the specific conditions in which a viral population can ensure the coexistence of the two bacterial populations.

In considering and modelling experimental situations (here, growth in a continuous culture apparatus), we would like to stress that : 1/ these simple situations can be useful to study specific hypotheses, 2/ data can be obtained for Mediterranean bacterial growth, and this can help to construct models for these specific situations, 3/ we can consider more complicated or more accurate viral live cycle and/or bacteria-phage interactions for regulating biodiversity.

Since we incorporate only one new factor a each modelling step, we can isolate the specific contribution of this specific factor and test its validity. In this paper for example, we focused on the bacteria - phage interaction.

Can we use flow cytometry to analyse the dynamic of virus-host systems ?

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INTRODUCTION

Over the last 10 years, viruses have been shown to be an abundant and dynamic component of the marine plankton and an important source of bacterial mortality (Wommack and Colwell, 2000). There is clear evidence that viruses constitute a significant loss for the bacterioplankton but factors regulating this control are far from being understood. Most studies have been devoted to the enumeration of free viral particles and visibly infected bacteria in pelagic environments. These parameters only provide indirect estimates of total viral activity and bacterial lysis in natural systems. This is probably because studies of natural viral and bacterial assemblages suffer from the lack of methods to perform direct measures of viral activity. Furthermore, these parameters suffer from large uncertainties and only provide limited information on the dynamics of single host-virus systems as well as on the regulation of these interactions in changing environments.

Despite important investigations on viral induced mortality, the complexity of the interactions between bacteria and viruses and regulation of specific virus-host systems makes it difficult to draw conclusions on the role of viruses in bacterial mortality in complex natural systems. One of the keys to better understand the role of viruses in bacterial mortality and substrate turnover may be to investigate the behavior of specific virus-host systems under controlled conditions in the laboratory in a first stage and then in the field when appropriate methods are available to follow the *in situ* dynamic of both virus and host populations.

There is clear evidence that virus-host systems should be based on key bacterial species that play a central role in biogeochemical cycles. These key species can be determined from the monitoring of bacterial diversity at fixed stations in relation with functional parameters. Once they are identified, these species should be isolated in order to be purified and then to isolate viruses.

Another important issue is to develop new tools that could be used for single cell analysis of virus-infected bacterial cells. Flow cytometry is now routinely used in aquatic microbial ecology to provide multiple information at the single cell level and parameters relative to the nucleic acid content of the cells, or to their cytoplasm density, could be useful to discriminate virus-infected cells.

This paper is related to preliminary experiments (i) to determine and to isolate key bacterial species and their specific virus from a coastal station located on the northwestern Mediterranean Sea (Banyuls-sur-mer bay) and (ii) to investigate the potential use of flow cytometry to discriminate and to quantify virus-infected cells, at least when working on a single virus-host system.

POTENTIAL USE OF FLOW CYTOMETRY TO DISCRIMINATE VIRUS-INFECTED CELLS

Application to *E. coli* cells infected by M13 or T2 bacteriophages

The first investigations were performed on *E. coli* (ATTC 23848) infected by two bacteriophages T2 or M13. The bacteriophage M13KO7 Helper is a pseudolysogenic bacteriophage (e.g., its replication does not induce the lysis of its host). This bacteriophage presents a resistance to kanamycine which is transmitted to the infected host and facilitate the discrimination between infected (kanamycine resistant) and non-infected (kanamycine sensitive) hosts. The T2 bacteriophage (ATTC 11303-B2) is a lytic bacteriophage characterized by a theorical latent phase of 20 minutes, followed by the lysis of the infected bacteria. This infection cycle lasts about 30 minutes.

Flow cytometry (FACSCalibur, Becton Dickinson) was used to analyse infected and non-infected cells stained with the nucleic acid dye SYBR I. For each individual cell we analysed the fluorescence intensity proportional to the nucleic acid content of the cell and both forward (FSC) and side scatter (SSC) signals which are respectively more or less related to the cellular volume (cell size) and to the cellular content of the cell.

Figure 1 represents the distribution of both fluorescence and SSC signals obtained with bacteriophage T2. The fluorescence intensity of infected cells is significantly increased but the two distributions (infected vs non-infected cells) are not well discriminated. The SSC signal is slightly decreased for infected cells after 35 minutes but this decrease is less clear when increasing the incubation. A similar trend was found for bacteriophage M13 (not shown).

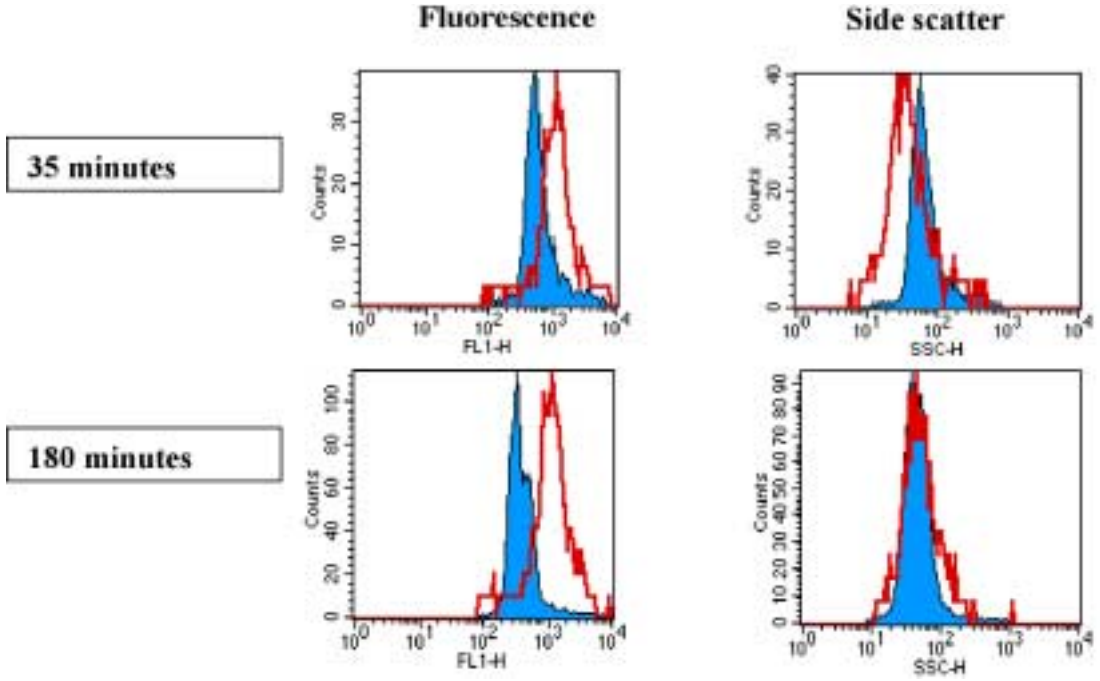

Fig. 1. Comparison of fluorescence and SSC signals of *E. coli* cells infected by bacteriophage T2 (white area) and non-infected cells (grey area) and stained with the nucleic acid dye SYBR I. The histograms were analysed after 35 and 180 minutes of contact.

From these experiments, we concluded that fluorescence and scatter signals cannot be used, also when combined together, to discriminate infected from non-infected cells.

Application to natural communities

We also investigated the cytometric properties of bacterial cells in relation with the temporal variations of virus concentrations at the SOLA station. Bacterial cells were discriminated based on their nucleic acid content since those with a high nucleic acid content (HDNA) are more active than those with a low content (LDNA cells). The relationship between HDNA cells and viruses could be explained by an increased nucleic acid content of cells and/or by an increased activity of bacterial cells which is favourable to the replication of viruses.

The mean SSC signal of HDNA cells is submitted to great variations whereas SSC values of LDNA cells are more or less constant (Fig. 2). This is congruent with the idea that HDNA cells are the reactive and dynamic fraction of bacterial communities (Gasol *et al.*, 1999; Lebaron *et al.*, 2002). When the virus concentration increased, the mean SSC signal increased. Similarly, the mean fluorescence intensity of individual cells decreased when virus concentrations increased (Fig. 3). This decrease is congruent with the trend reported for *E. coli* and could be explained by the release of viruses by infected cells. Similarly, the increase in SSC values could be explained by the release of viruses and changes in cell size and structure since a similar trend was observed with FSC values (data not shown).

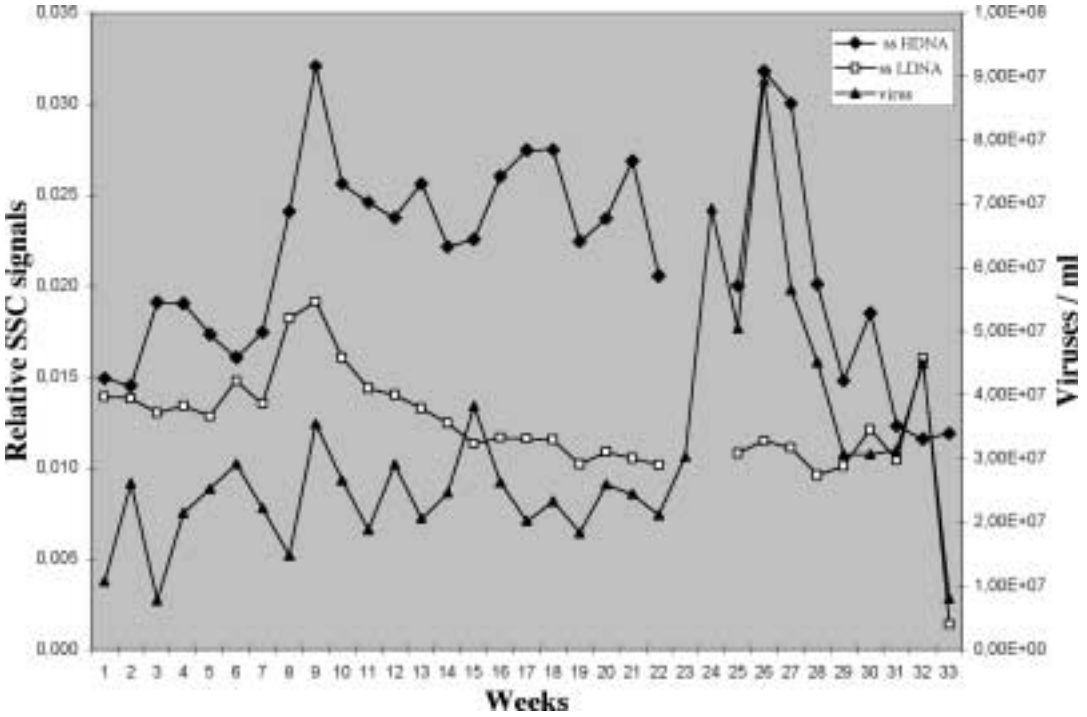

Fig. 2. Temporal evolution of virus concentrations and relative SSC signals of both bacterial cells with a high (ss HDNA) and low (ss LDNA) nucleic acid content at the SOLA station (Banyuls-sur-mer Bay, Mediterranean coast).

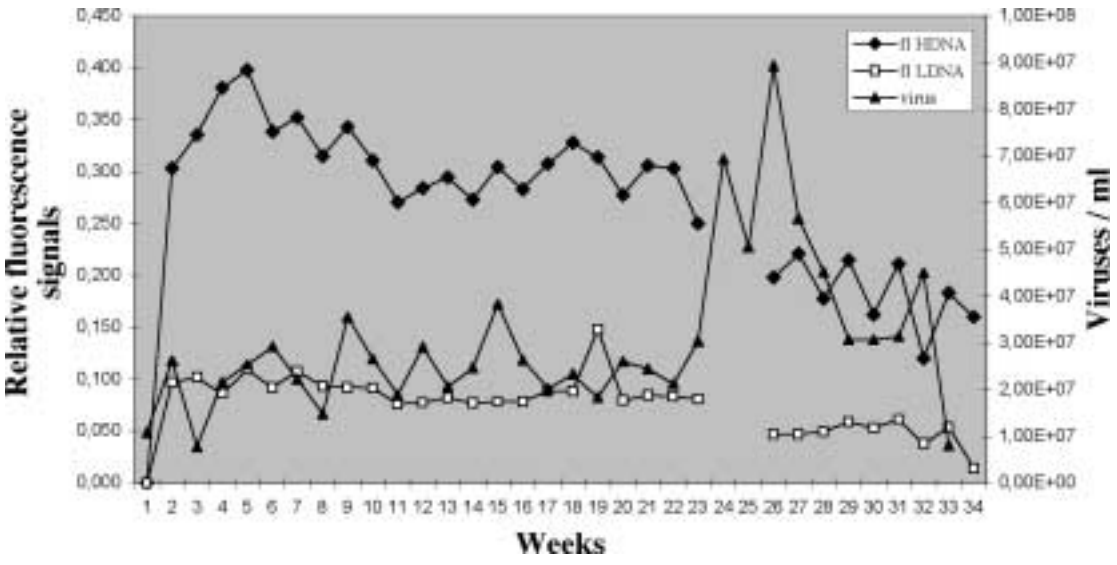

Fig. 3. Temporal evolution of virus concentrations and relative fluorescence signals of both bacterial cells with a high (ss HDNA) and low (ss LDNA) nucleic acid content at the SOLA station (Banyuls-sur-mer Bay, Mediterranean coast).

ISOLATION OF A KEY BACTERIAL SPECIES AND ITS VIRUS

Bacterial cells were isolated at the SOLA station and dominant colonies were picked, purified and identified by sequencing of the 16 rDNA gene. One of these species was identified as *Vibrio splendidus*. Viruses present at the SOLA station were concentrated each week and this bacterial species was submitted weekly to viral infection. Infection was observed in most cases and the infected cells were selected for further investigations. This species was not sensitive to mitomycin and its virus has never been purified. However, when the infected cells were starved in culture, viruses were released in late stationary phase (8-10 weeks). The cytometric properties of the cells were submitted to great variations and infected cells with low SSC signals were detected at this stage of the cell cycle (Fig. 4). After detection of infected cells, viruses could be detected as well as moribund cells, suggesting that the lytic cycle was induced by starvation.

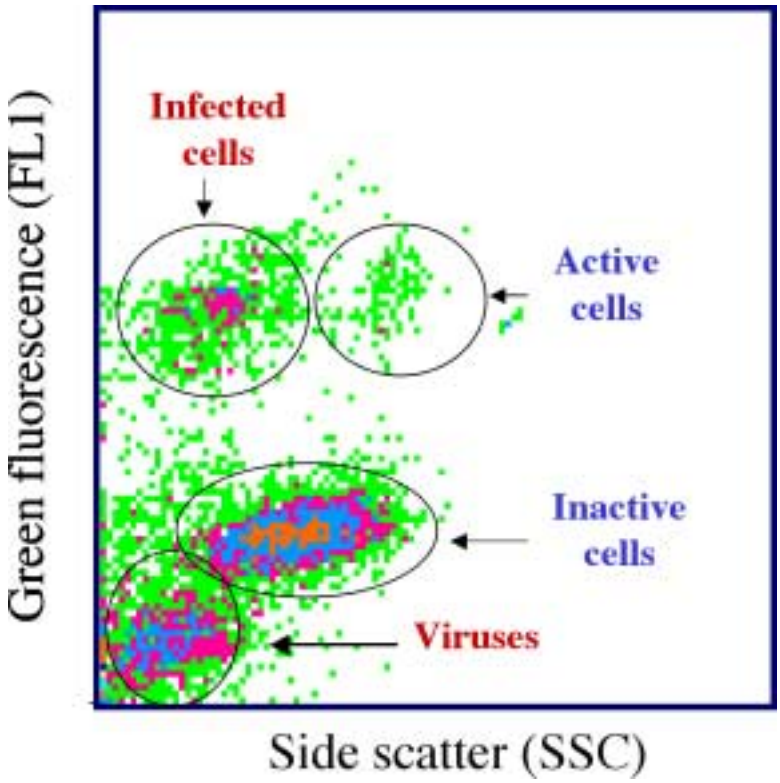

Fig. 4. Flow cytometric properties of *Vibrio splendidus* cells in late stationary phase.

Although this result should be confirmed by replicated starvation studies, it suggests that for some species flow cytometry and mainly scatter properties could be used for the detection and quantification of both viruses and infected cells.

PERSPECTIVES

The potential use of flow cytometry for the monitoring of virus-host systems in laboratory cultures should be further investigated using other bacterioplankton and phytoplankton species. Although the autofluorescence of phytoplanktonic cells is sometimes considered as an advantage for the flow cytometric detection of cells, it becomes a disadvantage when this autofluorescence signal has to be combined with the fluorescence signal induced by a nucleic acid dye. Therefore, in the case of viral infection, this approach is easier for bacteria. If this result is confirmed, it will be possible to better analyse different parameters which are of key importance for the modelling of virus effects. One important point should be to sort infected cells and to validate the presence of viruses inside the cells.

Molecular dynamics of viruses in marine ecosystems

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INTRODUCTION

You only have to look at an electron micrograph of a seawater sample, centrifuged at high speed onto an electron microscope grid, to appreciate the enormous morphological diversity of viruses in the sea. However, this only scratches the proverbial surface of the diversity that is actually out there. If you look at that same electron microscope grid you would be lucky if you see more than a dozen bacterial morphotypes. Despite this, microbiologists have been talking for years about how high marine bacteria diversity is, with estimates ranging from only approx. 1200 species (Hagstrom *et al.*, 2002) to over one million species (Curtis *et al.*, 2002), based primarily on data from phylogenetic analysis of 16s rDNA. Whatever the number is, virus diversity is likely to be at least an order of magnitude higher since every microbe in the sea must have at least one virus that infects it. If you just look at the range of viruses that infect one commonly used marine cyanobacterium, *Synechococcus* sp. WH7803 (DC2), several studies have demonstrated that a wide range of cyanophages infect this single strain (Suttle, 1994, Suttle and Chan, 1993, Waterbury and Valois, 1993, Wilson *et al.*, 1993). It is likely that each virus morphotype will be representative of a huge range of genotypes.

Such audacious speculation can only be backed up by adopting a similar molecular strategy that microbiologists have used which has been instrumental in changing our understanding of the composition of marine bacterial communities. Unfortunately it is impossible to develop a truly universal virus primer to detect all viruses (in the same way that 16S rDNA can detect all bacteria and archea) since there are so many different types of viruses. One look at an International Committee on Taxonomy of Viruses (ICTV) report on virus taxonomy (e.g. van Regenmortel *et al.*, 2000) will confirm this. Just basic taxonomic criteria such as the nucleic acid characteristics: ds and ss DNA; ds RNA; negative, ambisense, positive sense ssRNA and reverse transcribing virus taxonomic orders reveal the enormous diversity. So as virological ecologists, we must take a step back, lower our sights and focus on a more realistic aim. Rather than trying to determine overall diversity of marine virus communities we can use similar molecular approaches to investigate the diversity of specific groups of viruses and determine what effect this diversity has on the dynamics and ecological functioning of the hosts they infect.

Since marine virologists do not have an extensive 16S rDNA database equivalent to hand for the development of specific diagnostic virus probes, research into identifying suitable marker genes for assessing group-specific virus genetic diversity has been necessary. The approach has been to identify conserved regions within these genes for the synthesis of PCR primers. To detect algal viruses, a region in the viral DNA polymerase gene has been identified (Chen and Suttle,

1995) that has been successfully used to investigate natural marine algal virus diversity both by sequence (Chen *et al.*, 1996) and DGGE analysis (Short and Suttle, 1999; Short and Suttle, 2000; Short and Suttle, 2002). The DNA polymerase marker also provides a useful tool to determine evolutionary relationships within algal viruses (Castberg *et al.*, 2002; Chen and Suttle, 1996; Schroeder *et al.*, 2002). Furthermore, a recent provocative paper (Villarreal and DeFilippis, 2000) expanded this phylogenetic analysis and placed the algal viruses (taxonomically classified as the Phycodnaviridae) near the root of all eukaryotic DNA polymerases. Such information can date algal viruses back more than 1.2 billion years.

A similar approach, but different target gene, has been used to investigate marine cyanophage diversity and community dynamics in the marine environment. A conserved region in three cyanomyovirus isolates has been identified that is homologous to a gene encoding the T4 portal vertex protein, g20 (involved in capsid assembly) (Fuller *et al.*, 1998). Again this target gene has been used to investigate natural marine cyanophage diversity and community dynamics by sequence (Zhong *et al.*, 2002) and DGGE (Wilson *et al.*, 1999, Wilson *et al.*, 2000) analysis. This cyanophage target gene (g20) is encoded on a 10 kb module which encodes many of the cyanophage structural components and is organised similarly to other T-even phages (Hambly *et al.*, 2001). Phylogenetic analysis of the major capsid protein gene (g23), found on this same module, classifies marine cyanophages into a sub-group of phages termed the “exo T-evens” (Hambly *et al.*, 2001).

In the rest of this report I shall briefly outline some of the research that is currently being conducted as part of the Aquatic Virology Group at the Marine Biological Association and Plymouth Marine Laboratory (both Plymouth, UK). Part of our research focuses on the application of molecular techniques to investigate two groups of viruses that infect ecologically important marine primary producers, the cyanobacteria (specifically marine *Synechococcus* spp.) and *Emiliania huxleyi*. Unicellular cyanobacteria of the genus *Synechococcus* are a major component of the picophytoplankton, make a substantial contribution to primary productivity in the open oceans (Waterbury *et al.*, 1986); indeed, several studies have investigated the dynamics of *Synechococcus* in the Mediterranean ecosystem (e.g., Jacquet *et al.*, 1998). Although not a major primary producer in the Mediterranean, *E. huxleyi* is well known for forming vast mid-oceanic blooms. Its production of calcium carbonate coccoliths and its role in CO_2 cycling and dimethyl sulphide (DMS) production make *E. huxleyi* an important species with respect to past and present marine primary productivity, sediment formation and climate (Holligan *et al.*, 1993).

CYANOPHAGE DYNAMICS

Using the g20 marker (gene encoding the cyanophage portal vertex protein – involved in head assembly) we investigated the temporal and spatial dynamics of cyanophages during a south-north transect of the Atlantic Ocean (Wilson *et al.*, 1999, Wilson *et al.*, 2000). Cyanophage population structure changed dramatically in the surface (7 m) waters across the transect. It was also noted that some DNA fragments in the DGGE analysis were common throughout the transect suggesting that some genetically identical cyanophages have ubiquitous distribution in the surface ocean. DGGE analysis also revealed a high cyanophage diversity through all the depth profiles, and changes in population structure were observed with depth (Fig. 1). Maximum diversity was invariably correlated to maximum *Synechococcus* spp. abundances. Changes in cyanophage population structure reflected both nutrient and *Synechococcus* spp. concentrations at many of the stations (e.g., Fig. 1).

The next obvious step was to investigate the role of such high cyanophage diversity and determine the interactions between cyanophage and *Synechococcus* host in marine systems. We investigated the community dynamics of *Synechococcus* and the cyanophages that infect them in a station in the Red Sea (Gulf of Aqaba). The project was aimed at investigating the impact of cyanophages on the population structure of marine *Synechococcus* during an annual cycle.

The first step was to design primers to determine the diversity and dynamics of the host. PCR primers specifically amplifying rpoC1 (a gene encoding RNA polymerase) fragments from *Synechococcus* spp. were designed and used to prepare clone libraries covering an annual cycle and three sampling depths in the Red Sea. RFLP analysis of 36 clones from each of the libraries

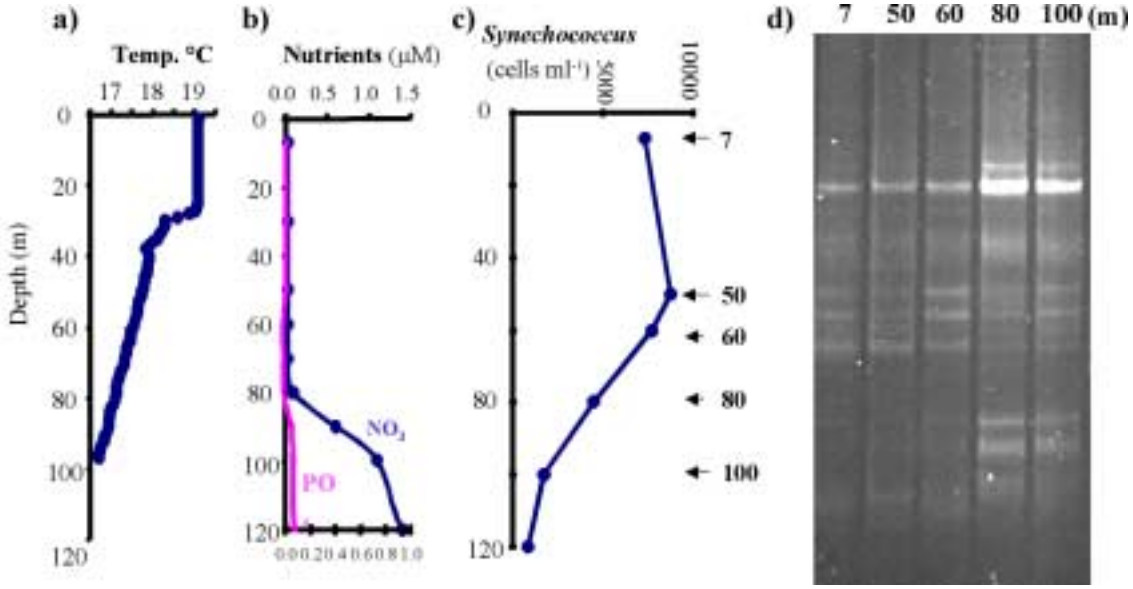

Fig. 1. DGGE profiles of cyanophages **(d)** compared to vertical profiles of temperature **(a),** nutrients (with nitrate and phosphate profiles labelled on each graph) **(b)** and *Synechococcus* spp. concentrations (actual depths where DGGE samples were collected are labelled accordingly on the right hand side of this graph) **(c)** from a station in the Atlantic Ocean (31°N 21°W) collected on 16 May 1996. Virus communities were concentrated by tangential flow filtration. For DGGE analysis, cyanophages in concentrates were amplified by PCR using primers CPS4GC and CPS5 (Cyanophage Specific) which amplifies a 205 bp fragment from g20 (Wilson *et al.*, 1999, 2000). PCR products were subject to DGGE analysis using a 15-35% urea/formamide gradient at 150 V for 5 h in 10% acrylamide and visualised by ethidium bromide staining.

suggested that during the summer the *Synechococcus* population was dominated by only a few genetically different clones, while there was great genetic diversity during the winter. This general trend was found to be independent of the depth from which the samples were taken (surface, 30 m and 100 m). Clone libraries of g20 fragments amplified with cyanomyoviridae-specific primers were prepared for the same water samples from which the *Synechococcus* (i.e host) population structure was analysed. Analysis of the g20 clone libraries by DGGE (as for the host population 36 clones were screened) showed high cyanomyoviridae diversity throughout the year and seasonal changes in cyanophage genetic diversity paralleled that of *Synechococcus* with greatest diversity in spring and winter (Fig. 2) (Martin Mühling, in prep.)

E. HUXLEYI VIRUS (EHV) DYNAMICS

Vast coastal and mid-ocean populations of *E. huxlyei*, which are readily visualised by satellite imagery due to their reflective calcium carbonate coccoliths, often disappear suddenly causing substantial fluxes of calcite to the seabed (Ziveri *et al.*, 2000) and cloud-forming dimethyl sulphide to the atmosphere (Malin, 1997). Until recently the mechanisms of *E. huxleyi* bloom disintegration were poorly understood, but it is now accepted that viruses are intrinsically linked to these sudden crashes (Jacquet *et al.*, 2002, Wilson *et al.*, 2002a, Wilson *et al.*, 2002b). Viruses that infect *E. huxleyi* can be isolated relatively easily by adding filtered seawater to exponentially growing host strains and plaque assays can also be used to obtain clonal isolates (Bratbak *et al.*, 1996, Castberg *et al.*, 2002, Schroeder *et al.*, 2002, Wilson *et al.*, 2002b). Phylogenetic analysis of DNA polymerase gene fragments in conjunction with morphological characterisation of each virus isolate revealed that they belonged to a new virus genus which we have named the *Coccolithovirus* (Schroeder *et al.*, 2002).

Recently, we reported the cloning and sequencing of amplified segments of the major capsid protein (MCP) gene from EhVs (Schroeder *et al.*, 2002). Significant sequence variation was observed between virus strains revealing the potential of using this gene as a genetic tool to differentiate viral genotypes in natural communities. We used PCR and DGGE to differentiate known EhV isolates based on the sequence variation in the MCP gene (Schroeder *et al.*, 2003).

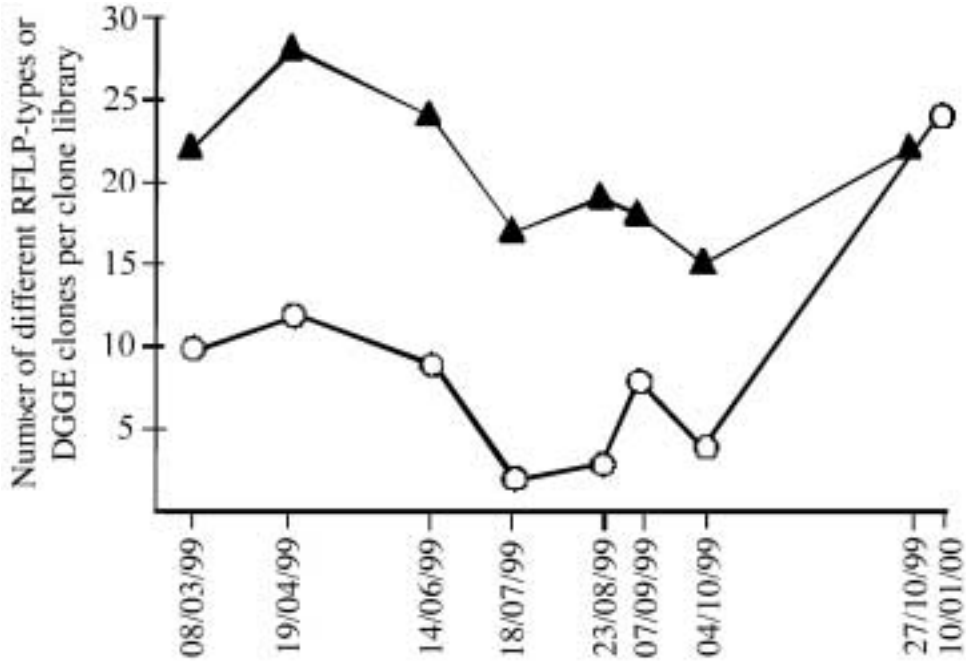

Fig. 2. The genetic diversity of *Synechococcus* (open circles) and co-occurring cyanophage (filled triangles) populations over an annual cycle (March 1999 to January 2000) are expressed as the number of different RFLP-types (*Synechococcus*) or g20 DGGE clones (cyanophages) found among the 36 clones analysed for each library (Martin Mühling, in prep.)

This technique was used to monitor the progression of the EhV community during an *E. huxleyi* induced bloom in a mesocosm experiment conducted in Norway during June 2000. Where several virus genotypes were observed at the start of an *E. huxleyi* bloom, however, only a few virus genotypes eventually go on to kill the bloom (Fig. 3) (Schroeder *et al.*, 2003). Future work will entail the use of the EhV-specific probes in conjunction with prokaryotic and eukaryotic probes to study the effects of viruses on the overall community diversity within an *E. huxleyi* bloom.

CONCLUDING COMMENTS

Virus selection pressure will undoubtedly influence the host clonal structure via infection and lysis of sensitive cells or through horizontal gene transfer events via lysogeny and/or transduction. These events will, in turn, be influenced by nutrient availability (Fig. 1), fluctuating light and changing physical conditions. Consequently, the clonal structure of the virus standing stock will be very diverse. The use of specific genetic markers has allowed us to observe changes in virus communities at the genotypic level during the progression of host blooms. These observations suggest that specific virus genotypes can influence host succession dynamics. As long as the diversity remains high, there is always going to be a virus or group of viruses that will facilitate this succession. It highlights what marine virologists had suspected for a long time - that we were seriously underestimating the level of genotypic diversity in marine viral communities (e.g. Fuhrman, 1999).

The overall picture is not likely to be quite this simple. There must be mechanisms for maintaining such a high virus diversity observed in marine ecosystems. This will start with the many environmental selection pressures which create microbial niches that allow the host to replicate productively in the first place. Observed diversity is also the key to determining evolutionary relationships between viruses. In addition, viruses are probably the key force in driving gene transfer processes in host communities. We are only just starting to understand the extent of high virus diversity in natural communities with only a few key groups currently under study. Further research is clearly required to determine the effect viruses have on a whole range of ecologically important hosts in natural marine microbial communities.

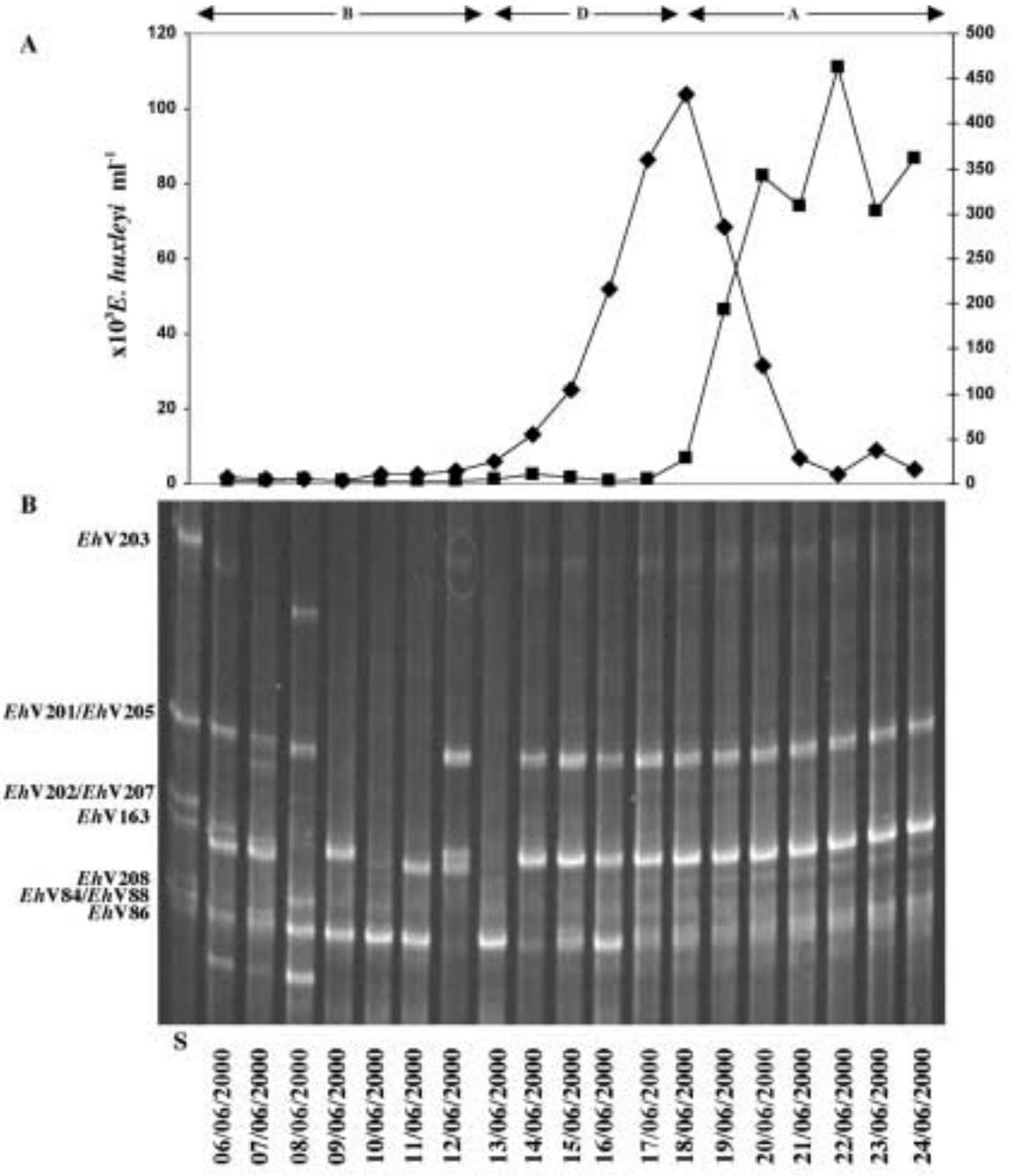

Fig. 3. (A) Time series obtained for *E. huxleyi* (◆) and EhV concentrations (■) using flow cytometry between 6 and 24 June 2000 in a mesocosm enclosure enriched with nutrients. B,D and A at the top of the graph refer to before, during and after the bloom, respectively. (B) Image of a DGGE gel of PCR fragments amplified from samples collected during the mesocosm experiment. Standards (S): all the EhV isolates (EhV84, EhV86, EhV88, EhV163, EhV201, EhV202, EhV203, EhV205, EhV207 and EhV208) in one lane (NB. virus pairs EhV201/205; EhV202/207 and EhV84/88 run at the same mobility). See Schroeder *et al.*(2003) for further sequencing analysis of DGGE bands.

Acknowledgements. Several people that have contributed to the research outlined in this report and are gratefully acknowledged. Red Sea data: Martin Mühling (University of Warwick, Plymouth Marine Laboratory, UK), Nick Fuller, Nick Mann, Dave Scanlan (University of Warwick, UK), Anton Post (The Interuniversity Institute for Marine Science Eilat, Israel). *E. huxleyi* Virus data: Declan Schroeder, Matthew Hall, Joanne Davy - Oke (Marine Biological Association, UK). The mesocosm study was supported by the Access to Research Infrastructures scheme (Improving Human Potential Program) from the European Union through Contract n° HPRI-CT-1999-00056.

Role of viruses in controlling phytoplankton blooms

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INTRODUCTION

Increased awareness of global environmental changes fostered research on the role of aquatic microorganisms in the geochemical cycling of energy. Despite the considerable knowledge on the (whole community) dynamics of microorganisms, many aspects of their specific ecology and physiology are still poorly understood. For example, very little is known about the actual *in situ* growth and loss rates of microorganisms on a species level. The problems in quantifying species-specific growth- and loss rates in a natural plankton assemblage are in part due to the multitude and complexity of processes involved.

The presence of viruses in marine environments has been acknowledged for many years, and it is by now well established that viruses are extremely abundant in aquatic ecosystems (Bergh *et al.*, 1989; Fuhrman and Suttle, 1993; Proctor, 1997; Wilhelm and Suttle, 1999; Wommack and Collwell, 2000). They are regarded as active and important members of the microbial food web (Wilhelm and Suttle, 1999; Fuhrman, 1999). Viruses are known to infect a wide spectrum of hosts, infecting not only bacteria but also eukaryotic primary producers (Suttle *et al.*, 1990; Van Etten *et al.*, 1991; Bratbak *et al.*, 1993; Peduzzi and Weinbauer, 1993; Brussaard *et al.*, 1996; Weinbauer and Höfle, 1998). Over the last decade it has been shown that viruses are important regulating factors in marine ecosystems. There is now the notion emerging that viruses influence species diversity, dynamics, population, the fluxes of organic carbon and nutrients, as well as gene transfer. Unicellular primary producers are a major group of organisms in natural aquatic communities and viruses have been shown to be an important source of mortality for phytoplankton (Van Etten *et al.*, 1991; Reisser, 1993; Proctor, 1997). Viruses or virus-like particles have been reported in all major taxa of eukaryotic algae, varying from unicellular to multicellular, swimming to non-swimming, bloom-forming to non-bloom-forming, free-living to symbionts.

VIRUS ENUMERATION

Because of the relatively short infection cycles, virus dynamics are often highly variable and rapid changes in both total abundance and diversity are reported (Suttle and Chan, 1994; Cottrell and Suttle, 1995; Castberg *et al.*, 2001; Larsen *et al.*, 2001; Wilson *et al.*, 2002a). Assays for counting viruses with high precision and fast analysis are, therefore, beneficial for the study of viral ecology.

In any field of research, the development of new methods stimulates research; either by improving speed or accuracy, or by allowing the detection or quantification of parameters not

measurable before. More traditionally, diverse culturing (plaque counts and most-probable-number assays) and transmission electronmicroscopy were used to estimate the number of viruses in natural waters. These techniques were either selective for viruses infective for a specific host, or very time-consuming. The use of very sensitive fluorescent nucleic acid-specific stains allowed faster detection of aquatic viruses, infective as well as non-infective, with epifluorescence microscopy (Hennes and Suttle, 1995; Noble and Fuhrman, 1998). With the recent introduction of flow cytometric detection and enumeration of free viruses by Marie *et al.* (1999a), speed of analysis and accuracy of counting improved largely. Brussaard *et al.* (2000) showed that a wide range of viruses, differing in morphology and genome size, could be detected flow cytometrically. Flow cytometry, applying SYBR Green I as the nucleic acid-specific fluorescent stain, has been used successfully to count viruses from laboratory experiments (Brussaard *et al.*, 1999, 2001), as well as from natural marine environments (Marie *et al.*, 1999a; Larsen *et al.*, 2001; Castberg *et al.*, 2001; Li and Dickie, 2001; Chen *et al.*, 2001; Wilson *et al.*, 2002a).

Studies using flow cytometry to count free viruses in natural samples are not uniform in the method used (Marie *et al.*, 1999b; Brussaard *et al.*, 2000; Chen *et al.*, 2001; Wilson *et al.*, 2002a). Working closely at the limits of staining methodology and instrumentation at present, however, the level of GFL is of importance for optimization of the detection of free viruses (Brussaard *et al.*, 2000). The few reports on the methodology of flow cytometric analysis of virus samples suggest that optimal detection of the free virus particles depends on various factors. A very recent evaluation of ours of the effects from a broad range of variables on the staining specifications of aquatic viruses (various specific cultured phytoplankton viruses and bacteriophages, but also natural marine samples) indicates that large variations in reactions between the different viruses were found. When dealing with unknown mixed virus communities, one specific set of variables seems to provide the best results, involving storage of fixed samples in liquid nitrogen, the use of SYBR Green I as fluorescent dye at low concentration, and heating of the sample before flow cytometric analysis. Further, comparison between epifluorescence microscopy and flow cytometry showed that the latter method to count free viruses is more sensitive and can be applied more generally (marine *vs.* freshwater, eutrophe *vs.* oligotrophe, shallow *vs.* deep water layers) than the epifluorescence microscopy method.

In general, many of the phytoplankton viruses exibit a relative high green fluorescent signals after staining (Fig. 1), allowing a convenient discrimination of these viruses from the total virus fraction (mainly bacteriophages). As long as there are no specific probes for the viruses of interest, such can be very useful for research on phytoplankton virus ecology under natural conditions.

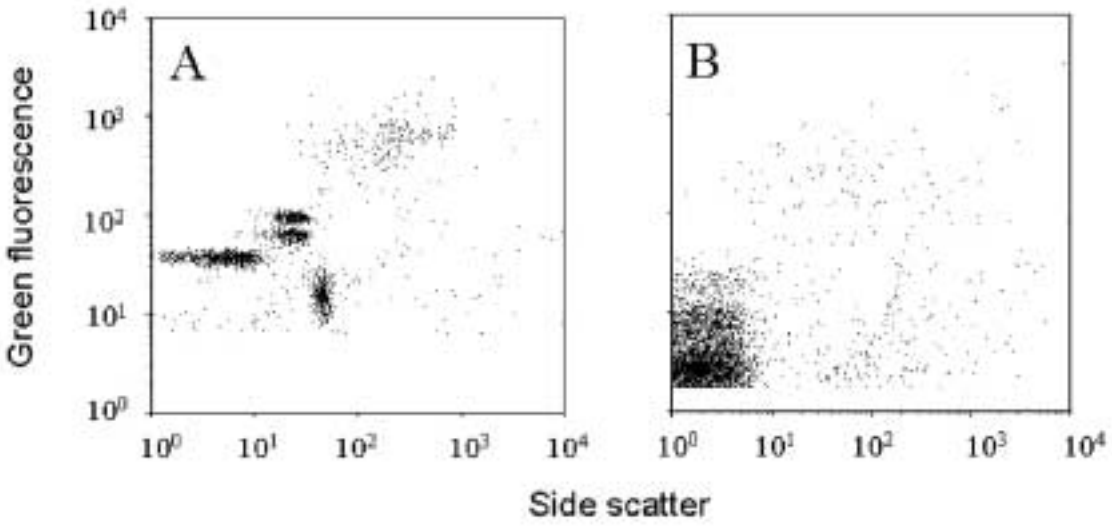

Fig. 1. Cytograms of green fluorescent signal of stained viruses vs side scatter signal. A mixture of 4 different algal viruses **(A)** and **(B)** a natural virus sample from coastal waters was analyzed according to Brussaard *et al.* (2002)

ROLE OF VIRUSES ON MICROBIAL FOOD WEB

With phytoplankton being the base of the marine food web, extensive research has been conducted on the factors affecting succession and biodiversity. Traditionally these studies concentrate on the ability of the species to deal with growth limiting conditions, but lately studies have shown that cell lysis can have a strong impact on phytoplankton population dynamics as well (Brussaard *et al.*, 1995, 1996a; Riegman *et al.*, 2001). Field studies showed, using the dissolved esterase activity assay, that the specific lysis rates of the algal community varied widely in time and space. Cell lysis was found to be a significant, and regularly the major, mortality factor for phytoplankton as compared to other loss factors of phytoplankton studied during these field campaigns. Inclusion of algal cell lysis into mathematical ecosystem models often improves the match between simulation and measured data. Lysis appeared essential for optimal simulation of algal dynamics, species succession and regeneration of inorganic nutrients in ecosystem modelling. Whether phytoplankton sink out, are grazed upon or die due to viral induced cell lysis has major implications for the flow of material and energy cycling in the marine pelagic food webs. The primary result of cell lysis is the release of dissolved organic matter and nutrients into the surrounding water, thereby directly promoting bacterial production (Brussaard *et al.*, 1995, 1996a). The production of DOM is the first step in the microbial food web; DOM is being utilized by heterotrophic bacteria which in turn are controlled by protozoa with the eventual result of linking the microbial food web with the classical food web. Knowledge on processes leading to the production of DOM is crucial for a good understanding of the global carbon cycle, since cell lysis forces the food web towards a more regenerative system (Gobler *et al.*, 1997; Fuhrman, 1999; Wilhelm and Suttle, 1999). Ecosystem models including viral mediated lysis (Fuhrman, 1999; Wilhelm and Suttle, 1999) show that up to 26% of the organic carbon flows through the viral shunt, with bacterial production and respiration increased by 33%.

Especially during phytoplankton blooms, when the high algal cell concentration will enhance the virus-host contact rates, viruses can have a significant impact on the development of these blooms (Bratbak *et al.*, 1993; Brussaard *et al.*, 1996b). *Phaeocystis globosa* and *Emiliania huxleyi* (Prymnesiophyceae), for example, can produce very dense blooms in temperate waters, and are considered very important in biogeochemical cycling of organic carbon and nutrients, as well as climate-relevant elements (e.g. by contributing strongly to DMS fluxes). With the high amount of particulate organic matter and nutrients stored in algal blooms, viral lysis of a single dominating phytoplankton population has been shown to significantly affect the transfer and cycling of energy and matter within the pelagic food web (Bratbak *et al.*, 1998; Brussaard *et al.*, 1996b; Gobler *et al.*, 1997; Fuhrman, 1999; Wilhelm and Suttle, 1999). Differences in the magnitude of blooms of HAB-species *Phaeocystis globosa* were indeed reflected in the structuring impact of phytoplankton cell lysis on the microbial pelagic food web. As a result of the remineralized nutrients, virus infection can also be expected to indirectly account for changes in population interactions such as phytoplankton competition and succession.

High total phytoplankton lysis rates (up to 30 % d^{-1}) were, however, not only recorded during the decline of algal blooms (Brussaard *et al.*, 1995, 1996b) but also occur in the open ocean (Agusti *et al.*, 1998). Comparative studies on the ecological role of virus-induced mortality of phytoplankton in ecosystems with contrasting trophic status are of great importance because of the differences in food web structure of eutrophic *versus* oligotrophic ecosystems. With ecosystems along a large trophic gradient being very different in their environmental controlling factors (nutrients, UV), the mechanisms behind phytoplankton cell death are likely to be different too. Generally, in oligotrophic waters the import rate of the controlling nutrient is low and regeneration of the limiting nutrient by members of the microbial food web is found important to sustain high productivity. Picophytoplankton dominate the photoautotrophic community due to their good competitive growth characteristics. As a result of rapid numerical response by heterotrophic nanoflagellates and microzooplankton, phytoplankton biomass is size-selective grazer controlled (Riegman *et al.*, 1993). Although this highly dynamic regenerative system is present in any euphotic zone (Thingstad and Sakshaug, 1990), at increasing import rates of the controlling nutrient (eutrophic waters) microzooplankton set limits to the biomass of the small algae. The primary effect is an increase in the biomass of larger algae escaping the size-selective grazing by

microzooplankton. Control of these larger phytoplankton by mesozooplankton is relatively low due to the relatively long generation times of these grazers. Furthermore, non-edible algal species will be able to increase in concentration, forming temporal algal blooms (e.g. diatoms, *Phaeocystis* spp.). Lysis of an unialgal bloom will affect population interactions (competition, commensalism, antagonism) and may lead to a distortion of the "classical" strucure of the ecosystem. The temperate eutrophic ecosystems seem more likely to be controlled by viruses. Infection of the host organism with lytic viruses leads to cell death while releasing numerous progeny viruses. Infection with lytic viruses is expected to be most prominent in environments with high abundance of the algal host (e.g. algal blooms) because of the increased host-virus contact rates. Phytoplankton at low abundance are not expected to be easily infected, but they may contain temperate viruses. Lysogeny (the viral genome is maintained in the host cells as a prophage without producing progeny viruses) might act as a survival strategy for the virus specific for low abundant algal species. The shift to the lytic mode may occur spontaneously, but at low rate or may be induced by environmental variables such as UV-light, temperature changes, nutrient depletion.

Differences between ecosystems can include variation in nutrient loadings, and it is, therefore, of interest to study the effect of nutrient depletion and different N:P ratios on viral lysis of phytoplankton. Nutrient depletion was sometimes found to affect host-virus interactions (Bratbak *et al.*, 1993; Wilson *et al.*, 1996). Our indoor mesocosm study on *P. globosa* (flagellated single cells and colonial cells) under different N:P ratios did not show obvious differences in their overall population dynamics. This implies a minor role of the macro nutrients (N and P) on the growth dynamics and loss rates of *P. globosa*. However, it was for the first time that viruses were found that infected this HAB-species. Blooms developed in one week, with enhanced rates of total phytoplankton cell lysis during the decline of the *P. globosa* blooms. Viral induced mortality of *P. globosa* caused the decline of the bloom. Viruses seemed an important controlling agent, having strong implications for our understanding of the impact of viruses on the temporal dynamics of *P. globosa*. Microbial dynamics and diversity were strongly influenced by viral induced lysis of phytoplankton, as was found for other mesocosm studies in Norwegian fjords (Castberg *et al.*, 2001).

ROLE OF VIRUSES FOR PHYTOPLANKTON HOST DIVERSITY

Over the last decade, about a dozen model systems of virus and photosynthetic host were isolated and brought into culture. Studies of these systems showed that viral-induced lysis of the entire algal host population usually occurs within one day, thus indicating that viruses can have a major impact on phytoplankton population dynamics (Suttle and Chan, 1993; Bratbak *et al.*, 1998; Brussaard *et al.*, 2001; Sandaa *et al.*, 2001).

In the field, phytoplankton communities differ in species composition through space and time. Especially in temperate coastal waters, a strong succession of phytoplankton species can be observed on a annual base. Similarly, spatial and temporal variations in the composition of the virioplankton community occur as well (Suttle and Chan, 1993; Cottrell and Suttle, 1995; Wommack *et al.*, 1999a; Castberg *et al.*, 2001; Larsen *et al.*, 2001; Short and Suttle, 2002). The use of molecular techniques such as DGGE and PFGE showed the appearance of different virus species after an almost-complete lysis of the blooming photosynthetic host population (Wommack *et al.*, 1999; Steward *et al.*, 2000; Castberg *et al.*, 2001; Larsen *et al.*, 2001; Short and Suttle, 2002). Since viruses have a restricted host range, infection by a particular phytoplankton virus does not act on the total phytoplankton community but will be limited to specific algal species or even different strains. Coexistence of competing phytoplankton species is therefore ensured by the presence of viruses that "kill the winner" (Thingstad, 2000b). Besides this direct role of viruses on interspecies phytoplankton competition, virally regenerated nutrients might stimulate the growth of other algal species (indirect impact; Gobler *et al.*, 1997). The number of studies on this topic is, however, still very limited.

Furthermore, there is increasing awareness that a specific geographical population of one phytoplankton species is not morphologically and genetically homogeneous, but can be rather diverse (Barker *et al.*, 1995; Rynearson and Armbrust, 2000). Thus, besides species-specific control of phytoplankton diversity by viruses, one can also expect viruses to affect phytoplankton

diversity and competition on an intraspecies level. Studies on phytoplankton viruses have revealed strain-specific viral infection in the laboratory and in natural environments (Sandaa *et al.*, 2001; Suttle and Chan, 1993; Cottrell and Suttle, 1995; Sahlsten, 1998; Tarutani *et al.*, 2000). An essential advantage of high diversity in immunity to virus infection and/or in host specificity would be that the potentially regenerated nutrients are not necessarily utilized by other competing phytoplankton species, but are taken up by individuals of the same species. Assuming comparable competitive capacity between the different algal strains, the dominant phytoplankton species will only be reduced in the number of cells of one particular strain and not necessarily in its overall abundance. This suggests the existence of complex and constantly varying host – virus relationships.

In addition to highly diverse individual cross reactions between an algal strain and a virus clone, the challenging issue of the impact of virus infection on competition between non-resistant and resistant phytoplankton strains should be addressed. Very recently, a few studies, including our own, noted recovery of phytoplankton populations upon viral-induced lysis (Thyrhaug *et al.*, 2002). The mechanisms for coexistence of phytoplankton host and virus require further investigation since they are still unknown. It can be hypothesized that resistant host cells may have a reduced competitive fitness as compared to sensitive viruses (Middelboe *et al.*, 2001).

Finally, there are only a few studies that examined the morphologic and genetic diversity of virus strains infecting the same phytoplankton strain (Cottrell and Suttle, 1991; Lu *et al.*, 2001). Diversity in particle size, latent period, genome size, host range, and restriction fragment pattern of the viral DNA was found amongst virus isolates infecting identical host strain, indicating that these viruses are different from eachother and that they are likely competing with eachother to infect the phytoplankton host cells. The factors regulating the competitive fitness of such viruses are largely unknown. Interestingly, despite the differences in basic virological characteristics, a close molecular relationship among the *P. globosa*-infecting viruses has ben recorded.

So far, almost all isolated phytoplankton viruses have a dsDNA genome. Recently, there have been reports by our group and others of ssRNA and dsRNA viruses infecting phytoplankton species (Tai *et al.*, in press; Tomaru, pers. comm.). The dsRNA virus infecting Micromonas pusilla has a relative long latent period (36 h), but was still able to coexist with a dsDNA virus infecting the identical strain of *M. pusilla* with a latent period of only 14 h (15). Nothing is known yet about the interactions between these two different types of viruses. Virus competition is an unexplored field of ecological research, deserving attention since it has the potential to contribute to a comprehensive understanding of phytoplankton successions in marine ecosystems.

CONCLUSIONS

This brief, and incomplete, overview is an attempt to highlight what we presently know and do not know about some important processes in phytoplankton virus ecology. The following issues deserve particular consideration:

- Information on the quantitative significance of the different phytoplankton mortality processes is still lacking. Method that allow the quantification of viral lysis rates specifically phytoplankton under natural conditions need to be developed.
- Insight in the ecological importance of viruses for phytoplankton in ecosystems with different trophic level, as well as the influence of important environmental variables on the interactions between virus and algal host cells.
- Viruses can keep the host at non-blooming concentrations (Larsen *et al.*, 2001), and are often found to be a major cause for phytoplankton decline (Bratbak *et al.*, 1993; Nagasaki *et al.*, 1994; Brussaard *et al.*, 1996). However, blooms do occur and so viruses are unable to prevent blooms at all times. To understand the mechanisms behind this, we need to know what factors are important regulators.
- To allow a proper study of viral diversity under natural conditions, on a spatial and temporal scale, and to obtain insight into the impact of different viruses on phytoplankton dynamics and diversity, virus-specific probes need to be developed.

- Despite the considerable knowledge on phytoplankton dynamics and the increasing number of studies on viral ecology, many aspects of the interactions between virus and phytoplankton host are still poorly understood (only one example is the importance of more than one type of virus in a phytoplankton cell, Brussaard *et al.*, 1996). In awareness of these gaps in knowledge, the ecological role of virus competition and clonal variation on phytoplankton host population dynamics should receive more attention.
- The use of mathematical ecosystem models including a thoroughly tested virus module, and validated against actual data, will add to our understanding of the regulatory role of viruses on phytoplankton dynamics and their impact within the pelagic food web.
- With more virus-phytoplankton host model systems being isolated and brought into culture, we will likely also find “new” virus types. Some of these viruses have been hard to isolate or keep in culture. As was found for the 'non-culturable' bacteria, they might be of significant ecological importance. Comparative studies on the ecological relevance of these viruses is recommended.

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Viral diversity and its implications for infection in the sea

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Host range of individual viruses is complex and driven over evolutionary time by host viral interactions. Despite the complexity, however, there are patterns that can be discerned which allow us to make inferences about the viral infection in natural communities of microbes. For example, some viruses have a broad host range and can infect organisms from different taxa. For example, some cyanomyoviruses (T4-like morphology) can infect phycoerythrin- and phycocyanin-dominant strains of *Synechococcus* (Suttle and Chan, 1993; Waterbury and Valois, 1993) while others infect both *Prochlorococcus* and *Synechococcus* (Matt Sullivan, pers. comm.). Bacteriophages with broad host ranges are typically cyanomyoviruses. Yet, there are many isolates of cyanomyoviruses that have relatively narrow host ranges and there are closely related strains of *Synechococcus* that are not infected by the same viruses. In contrast, cyanopodoviruses (T3-like morphology) typically have a much narrower host range, and the host range of isolates is typically restricted to very closely related hosts. In this case, we can make some loose predictions and testable hypotheses regarding the host range of viral isolates based on their morphotype.

Perhaps equally complex is the susceptibility of different host strains to viral infection. If one considers *Synechococcus* strains, some have very little resistance to infection, while others are almost completely resistant to infection (Suttle and Chan, 1993, 1994; Waterbury and Valois, 1993). Similar variations are observed in eukaryotic phytoplankton, as well (Sahlsten, 1998; Nagasaki *et al.*, 1999). An explanation for this variation is that when contact rates are high between host cells and viruses there is strong selection for resistance, resulting in host-cell resistance to co-occurring phage (Waterbury and Valois, 1993; Suttle and Chan, 1994). Conversely, when contact rates are lower the selection pressure is greatly reduced. It has been postulated (Suttle, 2000a) that low contact rates result in cells that have less resistance in oligotrophic oceanic areas (such as the Mediterranean), and high host cell resistance in productive coastal or eutrophic waters. In this instance, inferences can be made on the effects of viral infection on host-cell mortality and hence evolutionary selection pressure based on simple models of transport theory (Murray and Jackson, 1992).

Clearly, as elegantly modeled by Thingstad (2000b) the interactions between viruses and host cells have the potential to strongly influence the diversity of microbial communities. A clear demonstration of this influence can be seen in the results of Tarutani *et al.* (2000), who observed changes in the resistance of host cells to viral infection during the course of a bloom of the toxic phytoplankter, *Heterosigma akashiwo*. These results led them to propose a simple conceptual model of the effect of viral infection on the clonal composition of the host community. Yet, the interactions between viruses and the organisms they infect, and the resulting complex effects on the host range of viruses and the resistance of host populations, make it very difficult if not impossible to generalize the effects of contact rates on patterns of host range and resistance.

Interesting patterns do emerge, however, if one looks at the relationship between virus diversity and host range in more distantly related taxa. For example, if one looks at the phylogenetic relationships (based on the DNA polymerase gene) of the large double-stranded (ds) DNA viruses (Phycodnaviridae) that infect eukaryotic phytoplankton it is apparent that they are monophyletic relative to other viral families (Chen and Suttle, 1996). Moreover, within the Phycodnaviridae other genetically distinct groups (genera) of viruses are clearly resolved and correspond to the host taxon they infect. Hence, although the genetic diversity within an individual viral genus is complex and likely an intractable problem to solve, genetic differences between genera of viruses within a family are clearly resolved and are circumscribed by the host range of the genus (Fig. 1).

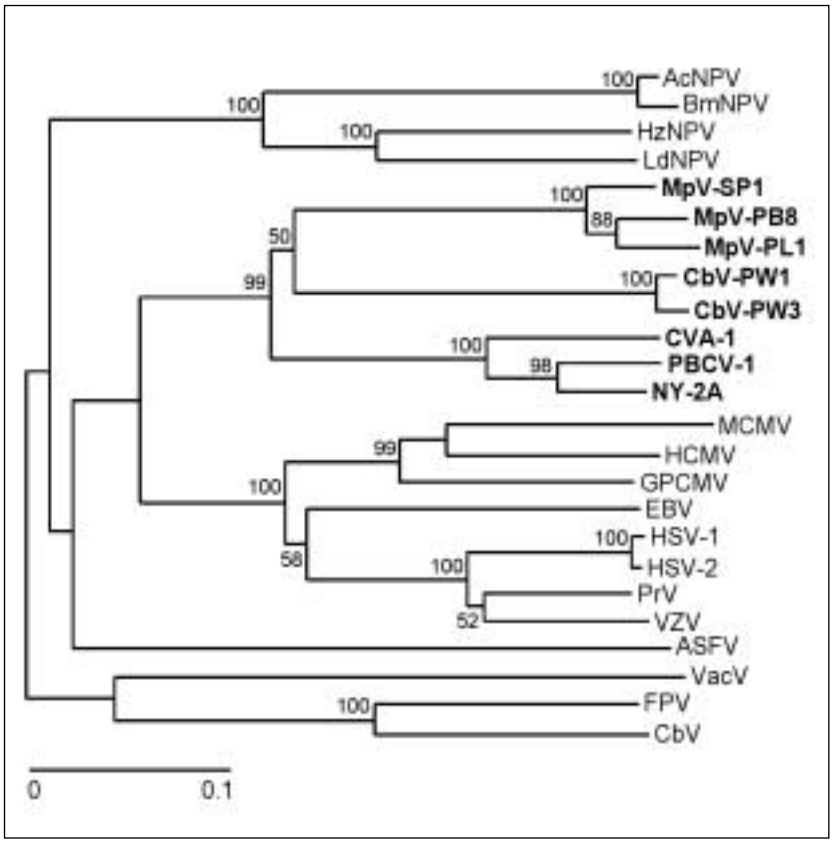

Fig. 1. Phylogenetic tree of 100 bootstrap analyses of DNA polymerase fragments. The neighbor-joining tree was constructed by using 217 of 335 amino acid sites. Virus clones infecting microalgae are indicated by boldface type. The numbers at the nodes indicate bootstrap values, and branches with values less than 75 have been collapsed. The scale bar represents 0.1 fixed mutations per amino acid position. Abbreviations are as follows: AcNPV = Autographa californica nuclear polyhedrosis virus; BmNPV = Bombyx mori nuclear polyhedrosis virus; HzNPV = Helicoverpa zea nuclear polyhedrosis virus; LdNPV = Lymantria dispar nuclear polyhedrosis virus; MpV = Micromonas pusilla virus; CbV = Chrysochormulina brevefilum virus; CVA-1, PBCV-1, and NY-2A = viruses infecting Clorella -like algae; MCMV = Murine cytomegalovirus; HCMV = Human cytomegalovirus; GPCMV = Guinea pig cytomegalovirus; EBV = Epstein-Barr virus; HSV-1 = Herpes simplex virus type 1; HSV-2 = Herpes simplex virus type 2; PrV = Pseudorabies virus; VZV = Varicella-Zoster virus; ASFV = African swine fever virus; VacV = Vaccinia virus; FPV = Fowlpox virus; CbV = Choristoneura biennis poxvirus (From Short and Suttle, 1999).

The importance of viral genera tracking host species arises when we use molecular approaches to examine uncultured viruses from natural waters. When these sequences are examined we see that there are numerous examples of environmental sequences that do not fit within clades that are defined by cultured representatives (Fig. 2). As well, some of the undefined clades contain representative sequences from both the Southern Ocean and north-eastern Pacific coastal waters. These results provide strong evidence of related groups of uncultured viruses that are pathogens of widespread algal hosts.

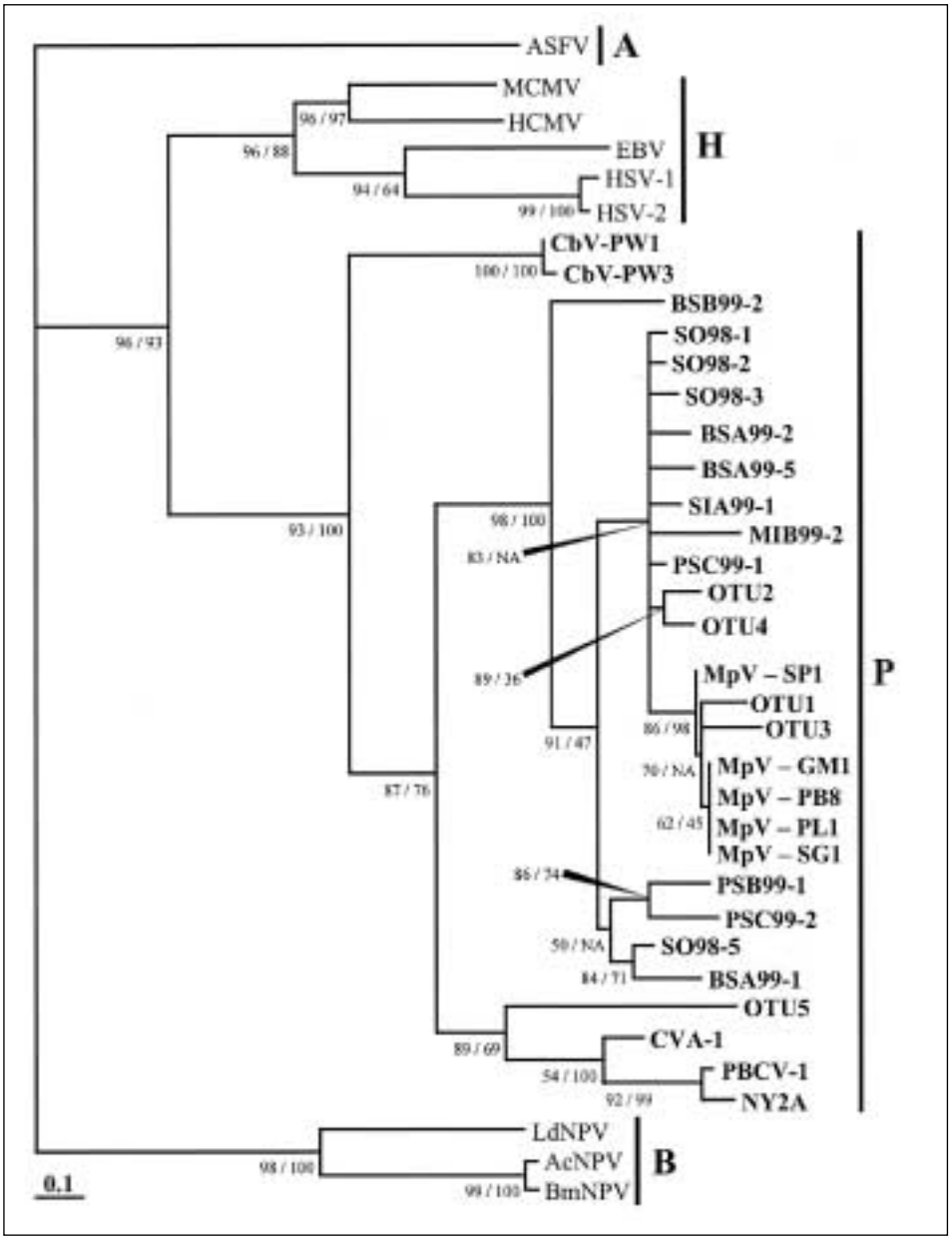

Fig. 2. Maximum-likelihood tree of DNA pol fragments. Quartet puzzling support values for the maximum-likelihood tree and bootstrap values for a corresponding neighbor-joining tree are shown as percentages to the lower left of the appropriate node or as indicated by elongated black arrowheads; NA means the NJ tree topology differs at that node. The vertical black lines and letters to the right of the tree indicate virus families as follows: A, Asfarviridae; B, Baculoviridae; H, Herpesviridae; P, Phycodnaviridae. The Phycodnaviridae are also indicated in boldface lettering. The scale bar represents the number of amino acid substitutions per residue (Short and Suttle 2002).

We have begun to expand this approach to look at other groups of viruses including phage of heterotrophic bacteria and cyanobacteria, as well as RNA viruses of phytoplankton and fish. The results clearly indicate that there are numerous genetically related and widespread groups of viruses in the sea for which there are no cultured representatives. This implies that there are also a corresponding number of taxa of bacteria, cyanobacteria and phytoplankton which are subject to an unknown degree of viral mortality.