Biogeochemical cycle of organic matter in coastal marine environment: the role of viruses in controlling bacterial proliferation
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1. Introduction

Viruses and biogeochemical cycles

Understanding the pathways for the supply and recycling of organic carbon in aquatic systems is crucial for quantifying nutrient and energy flux. Carbon can be considered a general tracer of energy flow through biological systems because all organisms store energy in the form of chemical bonds within carbon-based complexes. Most of the carbon enters the biological pool via photosynthesis (mostly sustained by phytoplankton organisms), whereby it is converted to carbohydrates by plants and algae (Wilhelm & Suttle, 1999). The concept of the marine food web and its link to carbon cycling has changed considerably during the last decades. To the existence of classical food chain model (starting with phytoplankton followed by a series of grazers increasing in size) has been added the microbial food web, recognizing bacterioplankton as its central component. Bacteria take up dissolved organic matter and are consumed by small grazers such as flagellates and ciliates that are in turn consumed by larger grazers, and by that organic matter links to the grazing food chain (fig.1.1). There are numerous sources of dissolved organic carbon (DOC) including sloppy feeding, egestion and excretion by grazers, and leakage from phytoplankton (Fuhrman, 1992).

The theory that viruses infect all components of aquatic food web has been confirmed by many culture and field studies describing specific virus interaction with zooplankton (Sindermann, 1990; Comps et al., 1991), eukaryotic phytoplankton (Pienaar, 1976; Mayer & Taylor, 1979; Cottrell and Suttle, 1991; Van Etten et al., 1991; Milligan & Cosper, 1994; Cottrell & Suttle, 1995; Suttle & Chan, 1995; Jacobson et al., 1996; Nagasaki & Yamaguchi, 1997; Suttle, 1999b), photosynthetic prokaryotes (Safferman & Morris, 1963; Padan & Shilo, 1973; Suttle & Chan, 1994) and bacterioplankton (Moebus & Nattkemper, 1983). These findings resulted in an even more complex concept of aquatic food webs. According to the steady-state energy model proposed by Jumars et al. (1989) and refined by Wilhelm and Suttle (1999) viral lysis contributes for 2-10% loss of photosynthetic carbon fixed by

Fig. 1.1. Marine food webs according to Brathak et al. (1994).
phytoplankton and for 3% to 15% to the loss of carbon derived by bacterial production, accounting that between 6% and 26% of the organic carbon produced by photosynthesis finally ends in the DOC pool due to viral lysis of cells. This flow of energy and matter is also known as viral ‘short-circuit’ or viral ‘shunt’ (Wilhelm & Suttle, 1999), excluding the carbon from transfer to higher trophic levels (fig. 1.2).

Organic carbon in the oceans is generally divided into operational pools of dissolved (DOC) and particulate organic carbon (POC) based on two dimensional separation by 0.2 μm or 0.4 μm pore-size filtration. Although this qualitative separation of different carbon sources is sometimes considered arbitrary, the two pools behave differently. The main biogeochemical function of viruses is thought to act as catalysts for the transformation of particulate (POM) into dissolved (DOM) organic matter as the content of host organisms is released during cell lysis. The lysis of heterotrophic and phototrophic microbes liberates cytoplasmic and structural materials that become immediately available to bacterial assimilation (Bratbak et al., 1990; Proctor & Fuhrman, 1990; Fuhrman, 1992; Middelboe et al., 1992; Gobler et al., 1997; Noble, 1998), even if the formation of more recalcitrant forms of DOC derived by viral lysis have been recently suggested (Noble et al., 2003), being reused several times as they passes through the food web (Cole et al., 1982).

There are generally recognized two modes of control acting upon marine microbial community: ‘top-down’ control is determined by the presence of predators while the ‘bottom-up’ control is given by the availability of nutrients and osmotrophs’ competition

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**Fig. 1.2.** The ‘viral shunt’ in marine food webs. The model has been originally proposed by Jumars et al. (1989) and revised by Wilhelm and Suttle (1999).
for a common limiting substrate. Viral lysis does not only produce a selective ‘top-down’ control, better called ‘side-in’ to distinguish it from predation, but also potentially a ‘bottom-up’ effect by alteration of the substrate spectrum for the osmotrophs (phytoplankton and heterotrophic bacteria).

However, it has yet to be shown, whether viruses have a stabilizing or destabilizing effect on ecosystems or geochemical cycles. Assuming that an increased complexity of a network has stabilizing effects by allowing for alternative routes, viruses should result in the former (Weinbauer, 2004).

On the basis of morphological data and finding that viral infection is driven by host encounter via passive diffusion (Schwalbach et al., 2004), and the greater abundance of bacteria over that of other planktonic hosts, it is generally recognized that bacteriophages make up the majority of viruses within the virioplankton in many aquatic environments (Wilhelm & Suttle, 1999).

Viruses as a cause of microbial mortality

It is important to understand the mechanisms responsible for the mortality of marine bacteria, as different routes of microbial mortality yield different pathways of organic matter flow and oxidation in the ecosystem. Heterotrophic bacteria are not only important in processes but are considered particularly abundant biological entities (Azam et al., 1983), representing a significant proportion of the biomass pool. It has been estimated that they represent up to 70% of the living carbon in the photic zone (Fuhrman et al., 1999), although lower values have also been argued. If photosynthetic prokaryotes are included, then estimates of the contributions of all microbes swell to over 90% of the total biological carbon in the oceans.

Traditionally, grazing by flagellates and ciliates have been considered as the only important factor of bacterial loss (McManus & Fuhrman, 1988; Pace, 1988), acting as ‘top-down’ control upon microbial community; however, attempts to budget bacterial production and removal by protozoan grazers have often led to imbalances, suggestive of unknown mechanisms for the loss of bacteria (Servais et al., 1985; McManus & Fuhrman, 1988; Pace, 1988; Sherr et al., 1989).

Nowadays, also viral lysis has been identified as significant source of marine bacterioplankton mortality (Fuhrman & Noble, 1995; Weinbauer & Peduzzi, 1995). As has been discussed above, whether cells are grazed or lysed implies different biogeochemical consequences with different implications for the flow of energy and matter through the microbial food web (Fuhrman, 1999; Wilhelm & Suttle, 1999). Even if similar in the principle, grazing rather than viral lysis (or vice versa) can imply also different ecological consequences: although grazers have been shown to exhibit a preference for certain prey, this form of selection pressure is minimal compared to that exerted by the highly specific nature of viral infection, therefore named ‘side-in’ control (Hennes et al., 1995; Fuhrman, 1999; van Hannen et al., 1999).
It is generally believed that viruses increase the diversity of host population in different ways, both by preventing permanent dominance of otherwise successful species ("killing the winner" theory) and by increasing the rate of horizontal gene transfer between hosts (Noble et al., 2003; Weinbauer, 2004).

Many studies have examined the degree of mortality caused by grazing or viral lysis (Fuhrman & Noble, 1995; Weinbauer & Peduzzi, 1995; Steward et al., 1996; Weinbauer & Höfle, 1998; Guixa-Boixereu et al., 1999; Tuomi & Kuupo, 1999; Karuza, 2001) indicating that the importance of grazing and viral lysis varies for different environments, host organisms and seasons.

The nutritional or metabolic status of the host is critical for viral infection and proliferation by affecting adsorption, replication, lytic activity and survival of the phage (Farrah, 1987; Moebus, 1987; Williams et al., 1987; Williams, 1994). There is still little known whether or not a phage requires a living or metabolically active cell for adsorption and proliferation. Numerous culture studies have shown that maximum proliferation rates and yield of phages are observed at maximum growth conditions of the host (Lenski, 1988). The finding that host generation times often influence phage latent periods (Proctor et al., 1993; Guixa-Boixereu et al., 1996; Middelboe, 2000) and that a low nutrient availability affects latent periods and burst size (Kokjohn et al., 1991; Proctor et al., 1993; Middelboe, 2000) suggest that phage proliferation strongly depends on host metabolism. It has also been shown that even a long time starvation does not prevent infection and viral production in all host species (Kokjohn et al., 1991; Schrader et al., 1997) and even recently killed cells may still be exploited to produce viral progeny (Anderson, 1948), although in both cases cell lysis is delayed and burst size reduced.

**Life cycles and ecological strategies of bacteriophages**

Viruses show several life cycles: lytic, lysogenic, pseudolysogenic and chronic infections (Ackermann et al., 1987), all of which start with attachment of the virus to a specific site and injection of the viral nucleic acid into the host cell (fig.1.3). In the lytic cycle, the lytic (or virulent) phage redirects the host metabolisms towards the production of new phages (viral progeny) that are released into the environment when the host cell lysis (bursts). The number of virions released per cell into the extracellular environment is called the burst size.

In the lysogenic cycle, the genome of the temperate or lysogenic phage becomes integrated into the host’s genome and typically remains in the host in a dormant stage (prophage) until the lytic cycle is spontaneously activated or until induction by mutagenic agents such as UV-C radiation or mytomicin C occurs (Ackermann & DuBow, 1987; Stopar et al., 2003). This ‘provirus’ is reproduced along with the host DNA for many generations until the survival of the host is threatened, at which time specific biochemical mechanisms can trigger the onset of the lytic cycle. A less well-
defined type of virus-host interaction is termed pseudolysogeny, in which the viral nucleic acid may remain in the host cell for some time before cell lysis (Lenski, 1988) or cell destruction occurs. Also pseudolysogeny may be related to host starvation, in which the virus adopts an inactive state, unable to initiate viral gene expression owing to the low energy state of the host cell. A chronic infection occurs, when a cell is infected and phage progeny is constantly released from the host cell by budding or extrusion of filaments, while host metabolism and reproduction proceed relatively unaltered. Overall, such classifications are probably a simplification of the diversity of phage life cycles (Lenski, 1988).

Several steps during the life cycle of a prokaryotic virus can be distinguished that are common to all viruses: adsorption, separation of nucleic acids from protein coat, expression and replication of the nucleic acids, virion assembly, release and transmission. The latent period is the phase of phage life cycle from adsorption to cell lysis where extracellular or ‘free’ phages cannot be detected.

The variety of viral life cycles is important to provide them a plastic behaviour according to resource availability. Velicer et al. (1999) suggested that viral life strategy concepts might be the result of an evolutionary trade-off between resource abundance and scarcity. Thus, lytic infection can be seen as adaptation to resource abundance, whereas lysogeny, pseudolysogeny or chronic infection might be adaptation to resource scarcity. Pseudolysogeny could be an adaptation 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 & 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 & Colwell, 2000). Lysogeny might prevail...
when the host abundance is below the necessary to sustain lytic infection (Steward & 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. Wilcox and Fuhrman (1994) reported the threshold value of virus-host density (in the present study called VBP from Virus-to-Bacterium Product), i.e. virus and bacteria product of $10^{12} \text{m}^{-2}$ has to be equalled or more to induce lytic proliferation strategy, even if some authors considered lower values as density threshold limit (Wiggins & Alexander, 1985).

Several studies have shown that viral infection increases along with bacterial and system productivity (Steward et al., 1992; Weinbauer et al., 1999). 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. 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 favourable 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.

Although viruses have no metabolism of their own and must rely on a host organism for any energy-requiring process, including reproduction (Fuhrman & Suttle, 1993), a description of prokaryotic viruses as parasites seems to be an oversimplification regarding the sophisticated and diverse interactions with other cellular and acellular organisms. Even if there is no precise and/or stable definition of virus-host interactions, they can be roughly distinguished into several main relationships. Lytic phages infecting prokaryotes are perhaps better described as predators than parasites, since they do not make prokaryotes 'sick' but lyse them, whereas chronic infection is in accordance with the idea of parasitism. Mutualistic or even symbiotic interactions between phages and hosts may exist as well. For example, phage conversion may result in an increased fitness of the host, showed by Edlin et al. (1975; 1977) and Lin et al. (1977) in studies on Escherichia coli. An increased fitness of the host should also result in higher replication and thus survival rates of the phage genome. With the establishing of lysogenic life cycle the incorporation of phage genome into the host one always confers immunity to the lysogenic cell against superinfection with the same or related phage types (Ackermann & DuBow, 1987). Co-infection of cells with two or more phages is not always adverse, but occasionally also beneficial due to the exchange of genetic material, a process that can occasionally contribute to phage diversity and thus enhance the chance to adapt to the environment (DeFilippis & Villarreal, 2000).
**Virus production and decay**

The dependance of virus particles on the hosts' metabolism determines their very short time persistence in the extracellular environment. Viral abundance determining in marine environment provides only a static picture of a dynamic balance between viral decay and viral production. Direct counts (TEM-transmission electronic microscopy and EM-epifluorescence microscopy) measure only net changes in abundance, thus the virus production rates result in certain underestimation due to the simultaneous viral decay (Noble & Fuhrman, 1997). Solar radiation is recognized as the most important single factor causing viral destruction or infection loss (Suttle & Chen, 1992). The UV-B portion of sunlight is probably the most important wavelength range responsible for the loss of viral particles and infectivity (Suttle & Chen, 1992; Noble & Fuhrman, 1997; Wilhelm et al., 1998). However, there is experimental evidence that UV-A and/or visible light can also significantly contribute to the destruction of phage infectivity (Suttle & Chen, 1992; Noble & Fuhrman, 1997; Garza & Suttle, 1998). Resistance mechanisms against DNA photodamage are known. One of the mechanisms to confer resistance is to increase the G+C content of DNA (Kellogg & Paul, 2002) since a protein coat is unlikely to provide protection against damage. Damaged phages can survive as long as they are able to inject their genome into the host, and the DNA damage can be repaired within the host photoenzymatically, by photoreactivation (Dulbecco, 1949; 1950) and by nucleotide excision or dark repair (Karentz et al., 1994; Friedberg et al., 1995). Also temperature affects viral mortality with a negative effect of increasing temperature for viral survival (Garza & Suttle, 1998) whether it is very likely that grazing is not a significant factor for the loss of viruses (Gonzalez et al., 1992; Karuza, unpublished data).

Since the estimate of viral decay can be affected by bias due to repair mechanisms, viral production could more faithfully describe viral dynamics. Therefore, in the present study we focused on the estimate of viral production. Until nowadays many different methods have been proposed but none of these has evolved to a state of a standard method (Noble & Steward, 2001, Weinbauer et al., 2004).

In order to test the reliability of different methodological approaches to determine the intensity of viral production three protocols were simultaneously assayed. These methods do not regard precisely the same measurement but should be considered as integrative approaches that complete each other and where the preference of one of them rather than the others depends on the conditions of the system in a given time.

**Dilution technique for estimating viral production in already infected bacteria**

Virus production was evaluated using a dilution technique described by Wilhelm et al. (2002) that reduces the background of free viruses through the addition of virus-
free seawater, allowing rates of viral production to be readily monitored from small changes in viral concentration. As the viruses produced derive from cells infected prior to the beginning of the experiment, altered contact rates between viruses and bacteria, which are provoked by changes in virus or host abundances, will not affect cell-specific viral production rates. This method provides the minimum estimate of productivity since it is restricted only to incubation periods with increasing trend of viral abundance, unless the viral decay rate is also known.

Serial dilution technique for estimating viral production in manipulated phage-host assemblage

This approach allows estimating net viral production rate over a 24 h incubation period. A manipulated seawater sample with viral and bacterial assemblage was serially diluted to assess the effect of varying encounter rates between viruses and their host.

For the application of this approach two approximations were necessarily considered:
1) we assume that the growth of a single bacterium is not directly affected by the presence or absence of other bacteria. This implication leads that a reduction in the density of host cells in natural seawater will not directly cause a change in the growth rate of remaining cells;
2) assuming that the major part of viruses are lytic, the probability of bacterial mortality is a direct function of the rate of encounter between phages and their host cells. This implies that the number of lysed cells by a given virus is linearly related to host density (Landry & Hassett, 1982; Murray & Jackson, 1992; Murray & Eldridge, 1994) and that the threshold value of virus and bacteria product of $10^{12}$ ml$^{-2}$ has to be equalled or more (Wilcox & Fuhrman, 1994).

It has been assumed that in natural seawater sample without grazers (removed by filtration) bacterial mortality is principally due to viral lysis and, when burst size is known, virus production can be calculated. In our case, burst size was determined using virus release induced by streptomycin. Virions were liberated into extracellular environment by destroying bacterial cell integrity during a short time (1h) antibiotic incubation period in order to minimize viral decay. It has to be considered that the burst size determined by this approach is rough and therefore represents only a minimum estimate (Wommack & Colwell, 2000; Weinbauer et al., 2004) since not all infected cells might have been lysed by streptomycin and since streptomycin might have prematurely stop phage production.

Radiotracer technique for measurement of viral production

The most direct approach but with a major number of steps and certainly the most expensive, regards the detecting of [methyl-3H]thymidine (3H-TdR) within DNA-
containing bacteriophages (the prevalent form of free virus particles in the sea) as described by Steward et al. (1992).

Tracer uptake by the bacteria secondarily labels the viruses, and tracer incorporation into viruses can be calibrated to yield estimates of virus production. This approach, however, involves several technical challenges regarding the choice of tracer, isolation of labelled phage nucleic acid from the much larger pools of labelled contaminants such as intact and lysed bacteria, subcellular organelles, released dissolved nucleic acids as well as the RNA of secondary formation.

The determination of viral production rates from tracer incorporation experiments requires a factor relating label incorporation to viruses produced. The conversion factor is obtained from incubation experiment in which both $^3$H-TdR incorporation and viral abundance increases are followed over time. Since the determination of conversion factors are not trivial to obtain, all studies of viral production by radiolabel have so far relied on the conversion factors of $2 \times 10^{21}$ (Steward et al., 1992) and $6 \times 10^{20}$ viruses per mole $^3$H-TdR (Fuhrman & Noble, 1995; Kepner et al., 1998). Since every study area contains different viral community structure, which could be characterized by different genome length of its groups, conversion factors should be specifically calculated for each study area.

In our study viral counts were determined using epifluorescence microscopy and therefore we are aware that the obtained conversion factors should be considered as an overestimate if compared to the studies performed by authors mentioned above where the viral abundances were observed by TEM. However the TEM approach for counting marine viruses has also some limits, since it seems to underestimate viral abundance caused by technical problems such as uneven staining, washing off of viruses, low detection limit as well as the lack of recognition of non-typical viruses (Weinbauer et al., 2004).

Environmental forcing of food web structure

Coastal ecosystems are characterized by continuous or sporadic nutrient inputs, both inorganic and organic, that favour the occurrence of populations of large cells such as diatoms (Jacquet et al., 2002). These ecosystems display a cascade of trophic relationships and an efficient transfer of matter towards higher trophic levels (Rivkin et al., 1996) In this context, the smaller size class of plankton (picoplankton) is viewed as a background group, although it may dominate these systems under nutrient-depleted conditions (Agawin et al., 2000). Data on the response of the microbial community are still very scarce (Ypma & Throndsen, 1996). Even if often providing precious in situ obtained information, open field studies suffer from inherent problems related to difficulties in interpreting population changes in drifting water masses. Hence, mesocosms, although not perfect representations of natural ecosystems because of their ecological inertia and resilience (Egge & Heimdal, 1994), constitute an interesting
integration to field measurements to study various phenomena occurring in marine environment and investigation of trophic relationships.

The availability of inorganic nutrients to marine organisms is affected by bacterial respiration. Heterotrophic bacteria contain lower carbon to nitrogen (C:N) and carbon to phosphorous (C:P) ratios than phytoplankton (Redfield et al., 1963; Goldman et al., 1987; Whitman et al., 1998). Gobler et al. (1997) suggested that if bacteria obtained all of their nutrients from phytoplankton lysis products, then the bacteria would need to obtain additional nitrogen and phosphorous from other sources to satisfy their requirements for these nutrients. However, bacteria do not convert all of the carbon they assimilate into biomass. A significant amount of this carbon is converted into energy (by respiration) to drive cellular processes. It has been suggested that limitation by elements such as carbon, nitrogen, phosphorous, sulfur and iron can only indirectly influence phage proliferation by controlling the host metabolism (Weinbauer, 2004). Wilson et al. (1996) reported that nitrate and phosphate depletion reduce the burst size and viral titers and, accordingly to the results of Tuomi et al. (1995), they experimentally confirmed that the addition of inorganic nutrients increases phage infection or abundance (Wilson et al., 1996; Williamson et al., 2002). Overall, DOC released by viral lysis of a bacterial host population can be an important substrate source stimulating the growth of non-infected bacterial populations (Middelboe et al., 1996). Hewson et al. (2001) reported the stimulation of viral production by addition of inorganic nutrients also for natural communities. There are two possible explanations for the increased virus relevance in highly trophic systems. First, highly trophic environments allow a higher standing stock of bacteria and consequently higher number of hosts for bacteriophages (Weinbauer et al., 1993; 1995). Second possible explanation is that higher trophic levels can directly stimulate viral development. In fact, Williamson et al. (2002) reported that in 22% of microcosm experiments viruses increased after inorganic phosphorous enrichment.

Moreover, phosphorous limitation has been hypothesized by many authors as one of the causative factors leading to the hyperproduction and massive accumulation of organic matter in the Northern Adriatic Sea during summer (Herndl et al., 1992; Obermster & Herndl, 1995; Degobbis et al., 1999; Myklestad, 1995; Puddu et al., 2000; Pettine et al., 2001). There is evidence that phosphorous limitation affects both primary productivity and bacterial uptake of DOC, the latter due to a low assimilation of dissolved organic substrates when phosphorous is not available for bacterial metabolism (Thingstad & Rassoulzadegan, 1995; 1995; Vaulot et al., 1996; Zohary & Robarts, 1998). Still little is known about the bacterial utilization of extracellular organic matter produced by phytoplankton (Puddu et al., 2003), but there is an evidence that the rates of DOM uptake largely depend on the physiological state of bacteria and the chemical composition of the organic substrate (Moriarty & Bell, 1993). Most DOM in seawater is considered to be resistant to microbial utilization and its labile fraction, usually less than 5% of the total, is confined to the upper levels of the water column.
(Carlson & Ducklow, 1995). Thus, in the present study we followed bacterial uptake of freshly phytoplankton-produced DOM in phosphorous-depleted conditions, bacterial and viral community dynamics and their interactions. Diversion of the energy flux and material from particulate to dissolved fraction also implies a diversion from potentially sinking into non-sinking forms. Bacteria attached to sinking particles are also believed to dissolve the particles through enzymatic activities. Viral lysis of these bacteria thus might 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, nitrogen and phosphorous are transferred from sinking to non-sinking forms.

**Virus distribution in pelagic systems**

Viruses are the most abundant dynamic component among the microorganisms in the surface waters of the world’s oceans (Fuhrman, 1999). Viral abundance, also reported as number of Virus-Like-Particles (VLP; virus that have not been cultured to identify hosts) from all sorts of environments (coastal, offshore, deep sea, and tropical to polar latitudes) have been found to range from $10^7$ particles $l^{-1}$ in oligotrophic systems to over $10^{11}$ particles $l^{-1}$ in eutrophic systems (Wommack & Colwell, 2000). In marine systems they are lower in the deep sea ($10^7$-$10^8$ $l^{-1}$), intermediate in offshore surface water ($10^8$-$10^9$ $l^{-1}$) and higher in coastal environments ($10^9$-$10^{10}$ $l^{-1}$) (Paul, 2000), decreasing along transects from coastal to offshore waters (Boehme et al., 1993; Cochlan et al., 1993; Corinaldesi et al., 2003). The viral number generally varies according to different factors, such as sampling depth, degree of stratification, season and trophic conditions (Fuhrman, 1999).

Viruses represent 5% of the carbon of bacteria, which is 25-fold higher than the carbon pool of protists (Weinbauer & Herndl, 2002). Since only nucleic acid genome encapsulated in a protein coat (capsid) gives virus structure, viruses may also contribute significantly to the biotic nitrogen and phosphorous pool. However, due to their small size, viruses are more important contributors to elemental cycles than to elemental pools.

Virus-to-Bacterium Ratio (VBR) indicates the numerical predominance of viruses over bacteria and has been used to infer the relationship between viruses and bacteria. In marine pelagic waters VBR is typically 5-10 (Wommack & Colwell, 2000).

Early studies have already shown that virus populations are extremely dynamic, and can change quickly over short timescales (Bratbak et al., 1990; Heldal & Bratbak, 1991; Steward et al., 1992; Bratbak et al., 1996). There is increasing evidence that hydrological variations can also play a role in viral abundance and distribution (Weinbauer et al., 1993). As a consequence, information on the relationship between hydrological features and viral distribution is needed for a predictive understanding of viral development in response to environmental changes. The Adriatic Sea with its anti-estuarine circulation, hydrographic mesoscale features with permanent cyclonic
gyres, general west-east trophic gradient (increasingly oligotrophic moving east), seasonal and spatial shifts in pelagic food web structure and postulated phosphorous limitation constitutes a perfect natural laboratory for the study of the interactions between microbial ecology and ocean biogeochemistry.

**Studying viral ecology**

Viruses are a group of biological entities with a genome consisting either of DNA or RNA encapsulated in a protein coat (capsid). In marine systems the majority of phages seem to be of double-stranded DNA nucleic acid.

Recently, a new biological domain beside the "cellular" domains Bacteria, Archaea and Eukarya was proposed, the Akamara (akamara; in greek from "without chamber" or "without void"), i.e., acellular infectious agents possessing nucleic acid genomes (Hurst, 2000). A possible organizational structure of this domain was suggested with two kingdoms, and several phyla and classes. Reconsidering viral taxonomy it has also been considered that there are most likely no hierarchical groups such as families for phages, but due to gene transfer there are reticulate groups of viruses or "modi", which share a particular genetic module or phenotypic character. In this reticulate, multidimensional phylogeny, phages would then be assigned to different but overlapping sets of modi.

The finding that 96% of all isolated phages from prokaryotes have a tail (Ackermann, 1996) suggests that the majority of viruses, according to traditional taxonomy, belong to the monophyletic group of tailed phages (order Caudovirales). The origin of tailed phages occurred before the separation of life into the three domains Bacteria, Archaea and Eukarya, and tailed phages are probably at least 3.5 – 3.7 billion years old (Ackermann, 1999). Species belonging to the order Caudovirales have a double-stranded DNA as genetic material and are divided into three families according to their tail (as well as biochemical and molecular characteristics (Ackermann, 1999; Hendrix et al., 1999): phages with a long flexible tail (Siphoviridae), phages with a contractile tail (Myoviridae) and phages with a very short tail (Podoviridae).

Viral ecology is the study of the interactions of viruses with other organisms and the environment. Typically, two different ways have been used to enumerate viruses: the indirect, 'viable' counts and the direct total counts. 'Viable' counts are obtained as plaque forming units on a lawn of host cells on an agar plate or in liquid medium as most probable number assays. Since 'viable' counts require the isolation of a host in a culture, and only a little portion of host are able to grow in culture, viable counts represent only a small fraction of total counts and thus are indicated only for specific virus-host interaction investigations. Although direct counts of VLP do not indicate if they are infectious particles, they are considered more useful for viral ecology studies (Alonso et al., 2001). Direct total counts can be determined using three different methods, by using transmission electron microscopy (TEM) and uranylacetate positi-
ve staining (fig.1.4), epifluorescence microscopy and flow cytometry. The last two mentioned techniques require staining with fluorochromes such as DAPI, YOPO-1, SYBR Green I or SYBRGold (Weinbauer, 2004). TEM counts of viruses are typically lower than counts determined by epifluorescence microscopy (Hara et al., 1991; Hennes & Suttle, 1995; Noble & Fuhrman, 1998; Chen et al., 2001; Weinbauer & Suttle, 1997), confirmed also in a study conducted in the Gulf of Trieste (Bensi, 2001) whereas flow cytometric counts are at least as high as those determined by epifluorescence microscopy (Marie et al., 1999; Brussard et al., 2000; Chen et al., 2001). Recently there is a tendency of epifluorescence microscopy becoming more frequently used than TEM for estimating total viral abundance (Weinbauer, 2004). SYBR Green I staining technique (Noble & Fuhrman, 1998) became rather successful over years because of its relative simplicity and ability to detect both RNA and double-stranded DNA viruses. However, the prevalent use of epifluorescence microscopy is probably due to the rapidity of the method, by the ability of application during field studies such as onboard a ship and finally lower costs combined with the through-put of a larger data set of samples, which allows the collection of more data including those for increasing statistical accuracy (Proctor, 1997; Fuhrman, 2000). For epifluorescence microscopy and flow cytometry there is a problem that not only virus bind to fluorochromes, but also DNA to colloidal particles. Also, large viruses can be confused with bacteria, but assuming that viruses are approximately 10 times as abundant as bacteria and that about 10% are large viruses, then the potential bias would be only 10% for viral counts while 100% for bacterial counts. Therefore, counting particles overlapping size range should be rather a problem for bacterial estimation than for viruses.

Fig. 1.4. Electron photomicrograph of tailed phage from seawater in the Gulf of Trieste (photo by Paola Ramani).
2. AIM OF THE STUDY

The goal of the present study is an integrated overview of the mechanisms that link microbial populations' dynamics and in particular virus-mediated mortality to trophic interactions, trophic interactions and environmental forcing to food web structure, and food web structure to biogeochemical fluxes.

Nowadays, the knowledge of the role of viruses in aquatic trophodynamics and biogeochemical processes is largely unknown due to the lack of complex studies aimed at verifying how isolated processes observed in laboratory experiments behave in the context of natural ecosystems.

In the framework of the temporal and spatial studies of microbial community dynamics in natural environments, long-term viral ecology studies become particularly important (and potentially informative) in the Northern Adriatic Sea, affected by phenomena regarding alterations of the system functionality such as mucilage formation. Therefore, in field studies of viral dynamics on different spatial and temporal scales, implemented by laboratory experiments, could contribute to our knowledge about environmental alterations.

In order to better understand virus-mediated mortality of bacterioplankton, crucial to define fluxes of energy and matter, it is necessary to develop new methodologies to evaluate the partition among metabolically different populations and to estimate direct impact of viral lysis on their most abundant hosts. Moreover, virus-mediated mortality can be examined also by the estimate of viral production rates. Since it implies the only use of experimental approaches based on mathematical models, which remain however approximate, prove of the reliable results have to be assessed by comparison among different methodological approaches.

Finally, this study is aimed to place viruses into the context of microbial food webs of the Northern Adriatic Sea over different seasonal and trophic conditions (including system alterations), allowing completing our knowledge about control upon bacterioplankton community given by grazers' predation ('top-down'), nutrient availability ('bottom-up') and finally, control determined by virus-mediated mortality ('side-in').
3. MATERIALS AND METHODS

3.1. STUDY AREA

The Adriatic Sea covers about 800 km by latitude and 100-200 km by longitude. On the basis of its hydrological, oceanographic, and bathymetric features, it is usually divided into three distinct sub basins that are characterized by decreasing depth from the southern to the northern one. A deep southern basin (maximum depth greater than 1200 m) is partly separated from the Ionian Sea by an 800-m deep sill. A second sill, of about 130 m depth, separates the southern basin from the central one. This basin, which is characterized by maximum depth of 270 m, is separated from the northern basin by a steep slope, whose bottom gently falls from the 100 m isobath to the 30-40 m mean depth of this shallow area (Fonda Umani et al., 1990). The region adjacent to the Italian coastline forms a shallow strip, with isobaths running parallel to the coast and a topography gently increasing towards the interior of the basin (Krajcar, 2003).

The circulation and the distribution of the water masses are strongly influenced by the morphological features of the Adriatic Sea bottom (Franco et al., 1982b). The sills control the circulation of the dense waters in the deeper parts of the central and the southern basins, while the thermocline in the southern basin is strongly influenced by the exchange with the Ionian Sea, and in the northernmost basin by river inflow, deriving mainly from the western coast.

Water masses closer to the coast show similar characteristics that are related to physical processes of mixing and circulation. These operate on short time- and space scales, particularly evident in the northern basin during certain periods of the year influencing the vertical and cross-frontal exchanges of energy and matter.

3.1.1. NORTHERN ADRIATIC SEA

The oceanography of the northern Adriatic Sea is influenced by the physiography of the basin and by the meteorology of the area (Buljan & Zore-Armanda, 1976; Franco 1973, 1983; Franco et al., 1982). The density structure of the basin varies from complete instability in winter to a highly stratified pattern during the rest of the year. During the winter when the total heat budget is negative (Hendershott & Rizzoli, 1976), the cold waters diluted by the western rivers' inflow remain confined in a coastal belt and are separated from the offshore waters by a frontal system and flow southward. In the offshore area of the basin the waters are highly saline, being advected from the southern basins, actively mixed by wind-driven surface cooling and mechanical stirring.
Under these conditions of vertical instability in the water column, high-density waters are generated and flow towards the central basin especially during strong NE wind jets. This narrow sea surface wind, known as "bora", occurs when cold and dry air masses, spills through gaps in the Dinaric Alps along the Adriatic's eastern shore (Krajcar, 2003). Horizontal variations in these winds drive coastal waters far into the middle basin. The winds also drive intense cooling and overturning, producing a sharp front between dense, vertically homogenous waters (North Adriatic Dense Water) in the north and the lighter (colder, fresher) stratified waters of the Po river plume.

In the early spring the total heat budget becomes positive, and the increase of the incoming thermal flux in the surface layer leads to the generation of thermocline. The vertical stabilization is enhanced by the Po river inflow in the offshore area; the dilution increases the buoyancy of the surface layer that expands to a large part of the northern Adriatic. Successive heating processes, wind mixing and advection from the southern basins in the summer generate a highly stratified water column in which, in general, there are three layers separated by strong density gradients (Fonda Umani et al., 1990). The northern Adriatic is included in the global Adriatic cyclonic circulation only in the cold part of the year. The small cyclonic gyre northern of the imaginary line Po River – Rovinj is present throughout the year and is larger in summer than in the winter (Krajcar, 2003).

3.1.2. GULF OF TRIESTE

The Gulf of Trieste is a large (about 600 Km²) shallow sedimentary embayment in the NE part of Adriatic Sea, thus almost surrounded by land except to the SW, where it is limited by an imaginary line going from cape Tagliamento in Italy to cape Savudrija in Croatia (fig.3.2). The shallowness of the basin is mostly due to riverine inputs. The Isonzo River represents the most important inflow while the inputs from the SE coast (Timavo, Rosandra, Ospo, Risano e Dragonja) are of torrential nature. The intensity of freshwater inputs shows a high interannual variability (Malej et al., 1995), which affects salinity, with the values ranging between 32 and 38 at the surface (Fonda Umani, 1991; Celio et al. 2002). Water temperature follows a regular annual pattern from winter minima, as low as 6 °C in February, to summer maxima > 25 °C (Cardin & Celio, 1997).

The maximum depth is only about 25 m with approximately 10% of area shallower than 10 m (Malej & Malaeiè, 1995). This bathymetric condition distinguishes this basin from the rest of the Adriatic and designs its particular hydrological and oceanographic features. A strong density variability of the water column profile, due mostly to the high temperature reactivity and to riverine inflows, is enhanced by an alternate of cold winds from the ENE (i.e. Bora) described above and mild southern winds (i.e. Scirocco).
3.2. SAMPLING PROGRAM FOR THE MONITORING STUDY

3.2.1. NORTHERN ADRIATIC

Sampling strategy was designed in order to cover an area with the north-south extent between cape Tagliamento (45° 38' N) and a branch of river Po at Goro, (44° 45' N) and within 60 miles from the coastline in the west-east direction. Seawater samples were collected on the seasonal basis during 2004 in May (25th-28th), July (28th-31st) and November (18th-21st) in the framework of Interreg project of Veneto region. Forty-one stations, which were distributed along 6 transects going both perpendicularly and parallel to the western-Adriatic coastline (fig. 3.2). Seawater samples for virio- and bacterioplankton analyses (total bacteria, metabolically active and non viable/dead bacteria) were collected in all stations at surface depth. Temperature, salinity, pH, oxygen saturation and fluorescence data (fluorescence was used as a proxy for phytoplankton chlorophyll concentrations) were obtained using CTD multiparametric probe (Idronaut Ocean Seven 316). Within the transects characterized by major depth and different trophic gradient conditions, also vertical samplings in the water column were performed.

Therefore, during the oceanographic cruises a total of 151 relieves were carried out. The number of transects, stations, vertical profiles and the total number of relieves respectively to each oceanographic cruise are reported in table 3.1.

![Fig. 3.1. Northern Adriatic study area: geographic position and bathymetric features of the basin with distribution of stations along transects are shown.](image-url)
Tab. 3.1. Number of transects, stations, vertical profiles and the number of total reliefs performed for each oceanographic cruise are reported.

3.2.2. GULF OF TRIESTE

Samplings were carried out at a coastal station C1 (C1 45°42'03" N, 13°42'36" E), which has been studied extensively during the last decades (fig.3.2), on a monthly basis from January 2000 to June 2002 and were intensified to a twice a month sampling frequency (with mostly regular 14 days intervals) until October 2005 in the Gulf of Trieste. Samplings were carried out at surface, 5m, 10m and bottom (~16m) depth. Samples were collected using a Carousel water sampler (1016 General Oceanic Inc.) carrying 12 acid-rinsed 5 litres Niskin bottles (fig.3.4), equipped with silicon elastic and red silicon O-rings. Water samples were immediately transferred to a polycarbonate carboy, rinsed with HCl (1 N), and stored in a cooler during transport to the land.
laboratory (fig.3.3), LBM - Laboratorio di Biologia Marina (with the location evidenced in fig. 3.2), which occurred within 1 h.

Fig. 3.3. Laboratory of Marine Biology (Santa Croce, Trieste, Italy).

Hydrological parameters data were obtained using multiparametric probes Idronaut Ocean Seven mod. 316 (from January 2000 to September 2003) and Seabird 19plus (from October 2003 to October 2005).

Fig. 3.4. Marine biology researcher on board of the R/V 'Effevigi' with a Carousel water sampler carrying 12 Niskin bottles (5 litres).
3.3. ANALYTICAL METHODOLOGIES

3.3.1. VIRAL ABUNDANCE

Virus samples were analysed according to slight modification of the Noble and Fuhrman (1998) protocol, which involves the use of SYBR Green I as nucleic acid (both DNA and RNA) fluorescent dye. SYBR Green I has a proprietary formula and its manufacturer (Molecular Probes) does not report its molecular weight. Working solution (200X) of the SYBR Green I (Molecular Probes) was prepared by dilution of the stock solution (10000X) supplied by the manufacturer, in proportion 1:50 with 0.02-μm prefiltered Milli Q. Preparation is done under subdued light and using gloves. The working solution is prepared right before the sample analysis. Optical solution was obtained using 100 μL ascorbic acid solution (w/v 10%) prepared with 0.02-μm prefiltered Milli Q, 4.9 ml PBS and 5 ml of glycerol prefiltered through 0.1-μm pore-size syringe filter (Acrodisc). The solution was 0.2-μm filtered and stored at -20 °C.

Immediately after the deliver in the laboratory sample volume of 10 ml was poured into sterile vial and fixed in 1% final concentration of 0.02-μm syringe (Anotop™, Whatman) prefiltered formalin. Fixed sample was stored at 4 °C in the dark and processed within 1 week. Sample preparation was performed at subdued light. Samples were filtered in triplicate onto 0.02 μm pore-size Al₂O₃ inorganic membrane filters (Anodisc, Whatman) using a vacuum flask and maintaining the filtration pressure < 0.1 atm. Subsamples (varying from 100 to 500 ml according to the virus concentration) were diluted 1:10 in 0.02-μm pore-size prefiltered (virus-free) seawater in order to homogenize virus particles distribution in the glass funnel. The membrane was filtered to dryness and laid on a drop of SYBR Green I (Molecular Probes) reaching a final concentration of 50X of the dye in a sample (Noble and Fuhrman 1998). Glass funnel and filter support were rinsed with 0.02-μm pore-size prefiltered seawater between each subsample filtration. After the staining period of 15 min at dark, the remaining non-incorporate dye was carefully wiped away by touching the backside of the membrane to a Kimwipe towel. The filters were mounted on a glass slide between 2 drops (25 μl each) of antifade solution (50% glycerol, 49% PBS and 1% ascorbic acid). Prepared samples were stored at -20 °C until counting, which occurred within 2 week from the sampling.

Viral enumeration was carried out using an Olympus BX60 epifluorescence microscope equipped with a 100 W high-pressure mercury burner (HPO 100W/2) at 1000X magnification under blue excitation light. Viral particles were distinctly shaped pinpricks that fluoresced bright green and were distinguished from bacterial cells because of their relative size and brightness (fig.3.5). For each filter, 20 to 30 grid (M. Patterson) fields with an area of 9.6 x 10⁻⁴ mm² were selected randomly and a total of about 200 viruses were accounted.
CORRECTION FACTOR FOR VIRAL LOSS IN FORMALDEHYDE FIXED SAMPLES

Since sometimes the collected samples, for instance during oceanographic cruises, cannot be immediately analysed for the determination of virus abundance but preserved in the field and returned to the laboratory, it was necessary to establish the correction factor for sample processing delay for formalin-fixed samples. A 1 week delay (maximum storage period usually requested for our monitoring purpose) was chosen both for oceanographic cruises and single monitoring samplings, in order to obtain comparable data between viral abundances of different sampling programs.

To avoid the underestimation of viral abundance due to viral decay in formaldehyde-fixed samples as reported by Wen et al. (2004), viral abundance data obtained from monitoring studies were recalculated using the in situ determined correction factor. On the contrary, formalin preserved samples for the virus abundance estimate in experimental designs were generally immediately analysed and therefore did not require any correction.

There are several plausible explanations for the decreases in viral abundance in stored fixed samples (Wen et al., 2004). Proteases may break down viral proteins, as has been suggested for bacteria in glutaraldehyde-fixed samples (Gundersen et al., 1996). Alternatively, aldehydes may cross-link the viral proteins and interfere with stain penetration. Finally, viruses may attach to each other, bacterial cells, or other particulates, and this may reduce the number of free viruses. Regardless of the mechanism, the viruses can no be longer be distinguished as individual particles by epifluorescence microscopy or flow cytometry (Marie et al., 1999; Brussard, 2004).

Samples were collected on June 7th 2005 and September 27th 2005 in the C1 station of the Gulf of Trieste at 4 sampling depths, according to the monitoring sampling program described above. Virus abundance was determined both from immediately processed non-fixed samples (real estimation) and from formalin fixed (1% v/v final concentration) and furtherly stored samples (4°C in the dark) that were analysed after 1 wk. The correction factor was determined by the difference in mean virus abundance between non-fixed and 1 wk delayed fixed samples.
3.3.2. TOTAL BACTERIA ABUNDANCE

Total bacterial enumeration was carried out following protocol developed by Porter and Feig (1980). This technique involves staining microbial cells with a fluorescent dye DAPI (4’6-diamidino-2-phenylindole) (Sigma), which stains nucleic acids and therefore relatively specific for biological material.

Samples (10 ml) were preserved with 2% final concentration of formaldehyde at 4 °C and processed within 48 h. After the staining for 15 min with 4’6 diamidino-2-phenylindole (DAPI, Sigma) at 1 mg ml⁻¹ final concentration (Porter & Feig, 1980), subsamples were filtered in triplicate (1-2 ml per subsample) onto 0.2 µm pore-size black-stained polycarbonate filters (Nuclepore, Osmonics). Glass funnels and filter supports were rinsed with Milli-Q between each subsample filtration. Filters were stored at -20 °C until detected, which occurred within 2 weeks from the sampling.

Bacterial enumeration was carried out using an Olympus BX60 epifluorescence microscope equipped with a 100 W high-pressure mercury burner (HPO 100W/2) at 1000X magnification under UV excitation light. Bacterial cells appear as bright blue points and are distinguished from other biological material (viruses and other organisms) greatly on the dimensional basis (fig.3.6). For each filter, 20 to 30 grid (M. Patterson) fields, with an area of 9.6 x 10⁻⁴ mm², were selected randomly and minimum 200 cells were accounted.

3.3.3. ABUNDANCE OF ACTIVE BACTERIA

The abundance of active bacteria was determined according to Choi et al. (1996) with a slight modification of the protocol described by Paoli et al. (2006). The approach for discriminating between active and inactive bacteria is based on the identification of those cells with an active Electron Transport System (ETS) (Rodriguez et al., 1992). Cells able to reduce the membrane penetrable dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) to intracellular red-fluorescing crystals of formazan, are relatively easy to detect and quantify by direct epifluorescence microscopy (del Giorgio et al., 1997).
The term of the cell activity is not so simple to interpret, since there are strong indices that the CTC method detects only bacterial cells with a highly active metabolism (Sherret al., 1999; Ullrich et al., 1999; Karuza et al., 2004). However, for the simplicity of the term we would call the most highly active bacterial population as active bacteria.

CTC (Polysciences) was added to 4 ml of seawater sample to yield a 3 mM CTC final concentration. Samples were incubated in the dark at the in situ temperature for 2 h. Incubation was stopped by adding 5% (final concentration) borate buffered formalin, which was previously prefiltered through 0.2 μm Acrodisc Syringe filters. Aliquots of 1 ml were filtered in quadruplicate onto 0.2 μm black-stained polycarbonate filters (Nuclepore) and mounted onto microscope slides between layers of non-fluorescent immersion oil (Olympus).

Enumeration of active bacteria was carried out using an Olympus BX60 epifluorescence microscope equipped with a 100 W high-pressure mercury burner (HPO 100W/2) at 1000X magnification under a green excitation light filter set. For each filter, 20 to 30 fields with the area given by the visual field (3.8 x 10^-2 mm^2) were selected randomly and a minimum 200 cells were accounted (fig.3.7).

3.3.4. ABUNDANCE OF NON VIABLE BACTERIA

![Fig. 3.7. Active bacterial cells detected by CTC and observed by epifluorescence microscope (Olympus BX60) under green excitation light at X 1000 magnification.](image)

The abundance of non viable bacteria was determined using the Propidium Iodide (PI) nucleic acid staining technique according to the protocol supplied by the manufacturer (Molecular Probes). PI is generally membrane impermeant for viable cells. Thus, it is able to penetrate into cell membranes with compromised functionality and therefore considered non viable or dead. The stain binds by intercalating between nucleic acid bases with little or no sequence preference and with a stoichiometry of one dye per 4-5 base pairs. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20-to 30-fold and the absorption maximum is 535 nm and the emission
maximum is 617 nm. PI also binds to RNA, necessitating treatment with nucleases to distinguish between RNA and DNA staining.

Working solution was prepared diluting 1:3000 stock solution (1.5 mM) with 0.2-μm prefiltered 2X SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0). Preparation is done under subdued light and using gloves. The working solution is prepared right before the sample analysis. The 2X SSC was prepared by dilution of the stable 20X SSC stock solution with 0.2-μm prefiltered Milli Q. RNAse solution (100 μg/ml) was prepared by dissolving 4.5 mg of solid RNAse (Sigma) in 3 ml 2X SSC. If preserved at -20 °C the RNAse working solution is stable for 3-4 days.

Seawater samples were preserved in formaldehyde (2% v/v final concentration) at 4 °C until the analysis. Samples were filtered in triplicate (1.5 ml) onto 0.2 μm pore-size black-stained polycarbonate filters (Nuclepore, Osmonics). Still on the glass filter support and the funnel mounted the sample was equilibrated with the addition of 2 ml 2X SSC and incubated for 5 min. Filtered was dried by filtration, removed to a sterile plastic Petri, and incubated with a drop (100 μl) of RNAse working solution at 37 °C for 20 min. After the RNA enzymatic digestion, filter was repositioned to the glass filter support of the vacuum filtration system and stained with 300 μl PI working solution for 5 min, then filtered to dryness and rinsed twice with 2 ml 2X SSC. Filters were stored at -20 °C until detected.

![Fig. 3.8. Non viable/dead bacterial cells detected by Propidium Iodide and observed by epifluorescence microscope (Olympus BX60) under green excitation light at X 1000 magnification.](image)

The cell enumeration was carried out using an Olympus BX60 epifluorescence microscope equipped with a 100 W high-pressure mercury burner (HPO 100W/2) at 1000X magnification under green excitation light filter set. Non-viable bacterial cells appear similar to red-fluorescent formazan crystals obtained for the identifying of active bacterial cells. For each filter, 20 to 30 fields with the area given by the visual field (3.8 x 10^{-2} mm^2) were selected randomly; a minimum of about 100 cells was accounted (fig.3.8).
3.3.5. ABUNDANCE OF CYANOBACTERIA

The determination of the abundance of cyanobacteria is based on the natural ability of these picophytoplanktonic organisms to emit their proper fluorescence given by the presence of photosynthetic pigments such as chlorophyll a and auxiliary pigments (phycocyanin and phycocerythrin) when illuminated with a suitable light wavelengths.

Samples (50 ml) were fixed with formalin (2% v/v final concentration) (pre-filtered through a 0.2 μm Acrodisc filter) immediately processed. Samples (15 ml) were filtered in triplicate as previously described for heterotrophic bacteria.

Enumeration of cyanobacteria abundance was carried out using an Olympus BX60 epifluorescence microscope equipped with a 100 W high-pressure mercury burner (HPO 100W/2) at 1000X magnification under green excitation light filter set. For each filter, 20 to 30 fields with the area given by the visual field (3.8 x 10² mm²) were randomly selected and a minimum 200 cells were accounted. Cyanobacteria autofluoresced orange and were distinguished from other picophytoplanktonic cells being characteristically circled and bright.

Conversion for the abundance obtained by direct counts into unitary abundance

The organism/particle abundance obtained by direct counting was converted into unitary abundance (present in 1 l sample) following the formula:

\[
\text{cell/particle} \cdot l^{-1} = \frac{N \cdot A}{a \cdot V}
\]

where

- \(N\) = mean value of cell number per visual field;
- \(A\) = filtration area (mm²);
- \(a\) = visual field area (mm²);
- \(V\) = sample filtration volume (l).

Filtration area is equivalent to the internal section of filtration glass funnel, whether the visual field area corresponds to the entire area that can be viewed through the ocular or to the only area designed by the “Patterson” grid.

The abundance obtained for single sample corresponds to the mean value of the abundances of all subsamples.

3.3.6. INORGANIC NUTRIENTS

The analyses of dissolved inorganic nutrients (nitrate, nitrite, orthophosphate and silicate) were performed on seawater samples that were prefiltered through Whatman GF/F filters (0.7 μm pore-size,) following the colorimetric methods of Grasshoff et al., (1983) using a multichannel continious flux analyzer (Braan Luebbe Autoanalyzer III).
3.3.7. DISSOLVED ORGANIC CARBON (DOC)

The Dissolved Organic Carbon (DOC) was measured according to Sugimura and Suzuki (1988) high temperature catalytic oxidation method. Samples (15 ml) were filtered through precombusted (4 h at 480 °C) and acidified (1 N HCl) glass fiber filters (Whatman GF/F) and stored at −20 °C in 20 ml glass vials equipped by teflon-lined screw caps (previously treated with chromic mixture and precombusted for 4 h at 480 °C). Before the analysis, samples were acidified (pH < 2) with 6 N HCl solution and purged for 8 min with high-purity oxygen bubbling (150 ml min⁻¹). The purging stage is required to remove the inorganic and volatile organic carbon present in the sample. DOC concentration was measured using a Shimadzu TOC 5000 Analyzer with a 1.2% Pt on silica as catalyst (Cauwet, 1994) at 680°C. Hundred µl of samples were injected into the instrument port. Carbon concentration was determined by automatic comparison with four-point calibration curves. Standardization was carried out every day using potassium hydrogen phthalate. Each value was determined from a minimum of three injections, with a coefficient variation <2%.

3.3.8. BACTERIAL CARBON PRODUCTION (BCP)

Bacterial Carbon Production was assayed both by the incorporation of [³H]-leucine method (³H-Leu) (Smith & Azam, 1992) and by the [methyl-³H]-thymidine (³H-TdR) incorporation method (Fuhrman & Azam, 1982). The radiolabelled leucine incorporation technique measures the rate of protein synthesis and therefore allows an estimate of biomass increment while the radiolabelled thymidine incorporation allows an estimate of bacterial replication rates.

Triplicate (1.7 ml) samples and one killed control (90 µl 100 % trichloracetic acid - TCA) were amended with 20 nM of ³H-leucine and incubated for 1 h at the in situ temperature in 2 ml Eppendorf microtubes while another set of triplicate (1.7 ml) samples and one killed control (90 µl 100 % TCA) was amended with 20 nM of ³H-TdR and incubated for 1 h at 4 °C. The incubation was stopped with 90 µl 100 % TCA. The samples were centrifugated (Eppendorf 5804R) at 14000 rpm for 10 min at 4 °C. The supernatant was aspirated and the pellet was resuspended in 2 ml of cold (0 °C) 5% TCA. Then the sample was centrifuged again and the pellet was washed with 2 ml of 80% ethanol. The last centrifugation was performed under the same conditions but the pellet was resuspended in 1 ml of scintillation cocktail (Ultima Gold MV, Packard). The incorporated radioactivity was counted using a liquid scintillation counter (Packard Tri-Carb 300) with a 3 min counting time. The incorporation of tritiated labels was measured by the tracer incorporation rate obtained from the Disintegrations Per Minute (DPM):

\[ \text{DPM}_{\text{incorporated}} = \text{DPM}_{\text{sample}} - \text{DPM}_{\text{blank}} \]
The moles of incorporated tracer, per time and volume, was calculated from the formula:

\[
SA = \text{specific activity of the } ^{3}\text{H-TdR or } ^{3}\text{H-Leu solution in nCi pmoles}^{-1};
\]

\[
\text{pmoles incorporated radiotracer } l^{-1} h^{-1} = \frac{DPM_{inc}}{C \cdot SA \cdot t \cdot V}
\]  

(A)

\[C = 2.22 \cdot 10^3, \text{ conversion factor for the transformation of DPM in nCi ;}
\]

\[t = \text{incubation time (h)};
\]

\[V = \text{volume of the incubated sample (l).}
\]

For the transformation of moles $^{3}\text{H-TdR}$ incorporated into bacterial cell number was used the expression (A) following the formula:

\[
text{cell } l^{-1} h^{-1} = (A) \cdot TCF
\]  

(B)

where TCF (Thymidine Conversion Factor) (Riemann et al., 1987) = $1.1 \times 10^{18}$ bacterial cells per 1 mole $^{3}\text{H-TdR}$.

\[
BCP(\mu gC l^{-1} h^{-1}) = (B) \cdot CCF
\]

Finally, Bacterial Carbon Production (BCP) by $^{3}\text{H-TdR}$ was calculated using the expression (B):

\[
BPP(\mu gC l^{-1} h^{-1}) = \text{pmoles } ^{3}\text{H-Leu} \cdot PC_{Leu} \cdot PM_{Leu} \cdot ID \cdot 10^{-6}
\]  

(C)

\[
PC_{Leu} = \frac{100}{\text{% Leu proteic content}} = \frac{100}{7.3} = 13.699
\]

\[PM_{Leu} = 131.2, \text{ Leu molecular weight}
\]

\[ID = 2, \text{ isotopic dilution in the cell (Simon and Azam, 1989).}
\]
\[ BCP(\mu gC l^{-1} h^{-1}) = (C) \cdot R \]  

where CCF (Carbon Conversion Factor) \( = 20 \text{ fgC per bacterial cell.} \)

For the transformation of moles \(^3\text{H-Leu}\) incorporated into Bacterial Protein Production (BBP) was used the following equation:

where

Bacterial Carbon Production (BCP) by \(^3\text{H-Leu}\) was calculated using the expression

where \( R = 0.86 \), conversion factor which represents a C content in the proteins.

3.3.9. CHLOROPHYLL-a

Sample (1 l) were filtered onto glass fibre filters (Whatman GF/F) and stored at \(-20 \, ^\circ\text{C}\). Pigments were extracted overnight in the dark at \(4 \, ^\circ\text{C}\) with 90 % acetone from the homogenized filter and determined fluorimetrically according to Lorenzen and Jaffrey (1980). The Chl \(a\) concentration was measured in triplicate by means of a Perkin Elmer LS 50B spectrofluorometer at 450 nm excitation and 665 nm emission wavelengths.

3.3.10. EXOENZYMATIC ACTIVITIES

Since bacteria can only uptake substrates with molecular weight <600 Da (Weiss et al., 1991), they must use cell-surface or extracellular enzymes to hydrolyse macromolecules such as proteins, polysaccharides, and lipid complexes prior to transport into the cell.

The enzymatic activities were measured from the increase in fluorescence as the non-fluorescent substrates were hydrolysed enzymatically yielding the fluorescent product. The amount of substrate cleaved was equivalent to the amount of highly fluorescent MUF (4-methylumbelliferone) or AMC (7-amino-4-methylcoumarin) anions generated after hydrolysis.

The equation where the fluorogenic substrates \((X)\) was hydrolysed by the exoenzymes \((Y)\) producing the natural bacterial substrate \((Z)\) and the fluorescent compound \((F)\) is represented as following:

\[ X \xrightarrow{Y} Z + F \]

where

<table>
<thead>
<tr>
<th>( X )</th>
<th>( Y )</th>
<th>( Z )</th>
<th>( F )</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUF-(\alpha)-D-(\alpha)-glucoside</td>
<td>(\alpha)-D-glucosidase</td>
<td>glucose</td>
<td>MUF</td>
</tr>
<tr>
<td>MUF-(\beta)-D-(\beta)-glucoside</td>
<td>(\beta)-D-glucosidase</td>
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<td>MUF-(\alpha)-D-galactoside</td>
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<tr>
<td>MUF- phosphate</td>
<td>alkaline phosphatase</td>
<td>phosphate</td>
<td>MUF</td>
</tr>
<tr>
<td>MUF-(K)-olate</td>
<td>leucine aminopeptidase</td>
<td>leucine</td>
<td>MUF</td>
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<tr>
<td>L-Leu-AMC</td>
<td>leucine aminopeptidase</td>
<td>leucine</td>
<td>AMC</td>
</tr>
</tbody>
</table>
The MUF-α-D-glucoside, MUF-β-D-glucoside, MUF-α-D-galactoside stock solutions were made up by dissolving fluorogenic substrates supplied by a manufacturer (Sigma) in ethylene glycol monomethyl ether (Methylcellosolve, Sigma) to a concentration of 10 mM whether the MUF-oleate was brought to a final concentration of 5 mM. The Leu-AMC and the MUF-phosphate substrates were dissolved in Methylcellosolve and Milli Q water yielding final concentrations in the stock solutions, respectively, to 10 mM and 5 mM. The obtained solutions were filtered through 0.2 μm syringe filters (Acrodisc). Standard MUF and AMC solutions were used to establish the calibration curves (500 μM final concentration).

Immediately after the delivery in the laboratory samples were incubated in replicates (2.5 ml) into 100 μM final concentration of MUF-α-D-glucoside, MUF-β-D-glucoside, MUF-α-D-galactoside and leucine-MCA substrates and into 50 μM MUF-phosphate for 1 h in the dark at in situ temperature. Fluorescence in the samples was measured in triplicate immediately after addition of the substrate and after the incubation time. For each sample a blank was assayed in duplicate, as a 0.2 μm filtered seawater sample without substrate added. The spectrofluorimetric measures were obtained at 380/365 nm excitation and 440/455 nm emission for MUF and MCA substrates respectively by using a fluorometer (Shimadzu RF1501). Increase of fluorescence units during the incubation period was converted into activity by preparing a standard curve with the fluorescent product of reaction (F). The standard MUF and AMC titration curves were obtained using the standard MUF and AMC solutions in increasing, but not saturated concentrations.

3.3.11. MARINE BACTERIOPLANKTON COMMUNITY ANALYSIS

DNA extraction

DNA was extracted according to slight modification of Böstrom et al. (2004) protocol. First, in order to lyse bacterial cells, an aliquot (50 ml) of seawater sample was filtered onto 25mm 0.2 μm Supor®, polyethersulfone membrane filters (PALL Corporation) and put in a 2 ml Eppendorf tube containing 525 μl lysis buffer (400 mM NaCl, 750 mM sucrose, 20 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris-HCl, pH 9.0). Sample was stored at -20 °C until processed. Subsequently, 11 μl of lysozyme solution (Sigma Aldrich) prepared with TE buffer (50 mM Tris-HCl and 50 mM EDTA pH 7.5) was added (1 mg ml⁻¹ final concentration) and the sample was incubated for 30 min at 37 °C. To enhance cell lysis, sodium dodecyl sulfate (SDS) (Sigma Aldrich, 1% w/v final concentration) and 3 μl of proteinase K (100 μg ml⁻¹ final concentration) were added. The obtained mixture was incubated at 55 °C for approximately 12 h. At the end of the lysis step, 5 μl of tRNA (Roche Applied Science) and 60 μl of NaAc (3 M, pH 5.2) were added. DNA was precipitated with 1647 μl of ethanol 99% during incubation at -20 °C for 1 h. The pellet was obtained by 14000 rpm centrifugation at 4°C for 20 min and washed with 500 μl of 70% ethanol.
Subsequently, the sample was centrifugated again and the pellet was dried for 1 h, resuspended in 50 μl Milli Q and stored at −20 °C until analysis.

**DNA amplification**

The amplification of a 16 S rRNA bacterial gene was performed using the Polymerase Chain Reaction (PCR). PCR is an *in vitro* technique that allows producing large amounts of essentially identical copies of a specified DNA sequence. PCR exploits certain features of natural DNA replication. The thermostable enzyme Taq DNA-polymerase that was extracted from thermophilic archaeabacterium *Thermus aquaticus* uses single-stranded DNA as a template for the synthesis of a new complementary nucleotide sequence. The Taq DNA-polymerase uses as construction units 4 different deoxyribonucleotides triphosphate (dATP, dTTP, dCTP and dGTP) in equimolar proportion. In this case an universal primer complementary to base positions 517 to 534 (5′-ATTACCGCGGCTGCTGG-3′) and an eubacterial primer complementary to the base positions 341 to 358 with a 40 bp GC clamp (5′-CGCCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCACGGGGGGCTACGGGAGGCAGCAG-3′) (Muyzer et al., 1993). The 40 bp clamp was attached to prevent the complete fusion of amplicons during Denaturing Gradient Gel Electrophoresis (DGGE) (Sheffield et al., 1989; Muyzer et al., 1993). PCR-reaction was performed with Hot Master Taq reagents (Eppendorf). The mixture per sample contained 5 μl of MgCl₂, PCR buffer, 5 μl of each primer at a concentration of 10 μM, 1 μl of dNTP’s (10mM), 0.4 μl of Master Taq and 2 μl of DNA template (final volume 50 μl). Each amplification cycle of rRNA 16S gene fragments generally consists of three steps: strand separation or DNA denaturing step, primer annealing step, and DNA synthesis or polymer extension step. An aliquot (50 μl) of the sample was introduced to a thermocycler (Eppendorf Mastercycler Gradient): first, the double stranded DNA was denaturated at 94 °C for 20 min. Then the primer annealing to the DNA single-stranded template was performed for 10 sec, initially at 65 °C for 10 seconds and decreasing by 0.5 °C for every further cycle yielding finally at 50 °C temperature (Touchdown PCR; Don et al., 1991). Finally the elongation of the DNA sequence occurred at 72 °C for 45 sec. The newly synthesized strands of DNA, starting at each primer, extend beyond the position of the primer on the opposite strand, so that they both contain new primer binding sites for further replication. After 35 cycles the reaction mixture was cooled up to 4 °C. Ideally, the mixture should contain 2³⁵ double-stranded DNA molecules that should be identical to the original sequences between the two primers. To verify that the DNA amplification occurred properly and the absence of eventual contamination, 2 solutions were used as controls in place of template: an aliquot (2 μl) of DNA standard solution as positive control and an aliquot (2 μl) of Milli Q for PCR as negative control. In order to confirm amplification of the desired DNA fragment (~200 bp long), 5 μl of PCR product was analysed by agar gel (1.5%) electrophoresis with the addition of ethidium bromide (final concentration 0.5 μg ml⁻¹),
which has the property to emit fluorescence when intercalated between DNA bases. The fluorescence was observed under UV (312 nm) light produced by an illuminator. A DNA Ladder (Promega) with a known length of 1 kb was used as the reference.

**Denaturing Gradient Gel Electrophoresis (DGGE)**

On the amplified DNA fragments obtained by Polymerase Chain Reaction a molecular biology technique called Denaturing Gradient Gel Electrophoresis (Muyzer et al., 1993) was performed to evaluate modifications of the bacterial community composition. DNA mixture was run across a denaturant polyacrylamide gel (acrylamide-N-N' -methylene bisacrylamide [37:1]). The method is based on the reduction of the DNA fragments mobility in a dense medium when a part of a double strand gets open. The separation of two strands of the double helix is provoked by the increase in the concentration of chemical substances such as formamide and urea. The bands that are positioned on the same distance from the beginning represent the same sequences. On the contrary, different positions of the bands indicate the existence of different bacteria strains. An aliquot (11 µl) of PCR-amplified sample was loaded on a 8% polyacrilamide gel with top to bottom denaturing gradient of 30 to 70% (with 100% denaturant being 40% v/v formamide and 7 M urea). Electrophoresis was conducted at 85 V for 16 h at 60 °C in a hot-bath DGGE unit (CBS Scientific, Del Mar, California) with a running buffer of 0.5X TAE (20 mM Tris-HCl, 10 mM acetate and 0.5 mM Na₂EDTA pH 8.2) After the electrophoretic run, the gel was stained with a SYBR Green I (Molecular Probes) solution (1X) for 30 min in the dark. After the incubation with the dye the bands on the gel containing stained nucleic acids were visualized and documented on a UV illuminator with a charge camera and were taken the pictures.

3.3.12. DATA ANALYSIS

If not specified otherwise, statistical analyses were conducted using the Analysis Tools package of Microsoft Excel XP. Virus-to-Bacterium Ratio (VBR) was calculated dividing the number of Virus Like Particles (VLP) with the bacterial cell number. VBR is generally used in viral ecology as index of the intensity of virus – bacteria interactions in water environments. Another index not so commonly used in marine ecological studies, which indicates the phage-host density is Virus-to-Bacterium Product (VBP). The VBP index was calculated as the product of VLP and bacterial cell abundances and is usually expressed in order of $10^{12}$ cell/particle density per ml$^{-2}$, the density value that has to be reached to trigger the induction of lytic life strategy within the viral ‘population’.

The number of bacterial species in the Gulf of Trieste was assessed at C1 coastal station before, during and after the mucilage formation occurred in June 2000 in the Gulf of Trieste. As background values, indicating the ‘normality’ of the system, was
used bacterial biodiversity that was monitored at the same station of the Gulf of Trieste with monthly frequency during the year 2003.

For the determination of bacterial biodiversity were used microbial biology techniques for the marine bacterioplankton community analysis.

Distribution patterns of different parameters and vertical profiles of seawater temperature along the transect E02 were obtained by creating contour maps onto given data set using Golden Software Surpher 7.0 Inc. Vertical profiles of biotic parameters at some selected stations were obtained using Golden Software Grapher 3.0 Inc.

Shapiro-Wilk test or quantile-quantile probability plot were used to verify the normal data distribution. Since data within single variables resulted not normally distributed, a non-parametric analysis was used to assess the association among different variables. Spearman rank correlation analysis resulted suitable for our purpose since it formally test the association between two related variables, i.e. variables measured on the same/matched subject, which need not be measured on the same scale. The analysis was conducted using Statistica software (StatSoft 6.0 Inc.). The p-value was computed using the t-approximation and pairwise case elimination of missing data was performed.

Kruskal-Wallis analysis of variance (1 way ANOVA by ranks) was utilized to investigate differences between data distribution (difference between the medians) of the same variable. The p-value was computed using the \( \chi^2 \)-square approximation. The rate of lytic infection was calculated according to Bratbak et al. (1994). The rate of lytic infection onto total bacterial community was obtained using the following equation:

\[
\frac{dP}{dt} = k \cdot V \cdot B
\]  

where

- \( k \) = adsorption constant that has been experimentally determined for T4 phages and corresponds to 0.25 \( \times 10^{-4} \) cm\(^3\) min\(^{-1}\) (Stent, 1963);
- \( V \) = VLP abundance;
- \( B \) = heterotrophic bacteria abundance.

The average rate of lytic infection operated on the single bacterial species was obtained by dividing the expression (A) with the number of bacterial species present in a given moment.

The temporal series of VLP data was assessed using the Fourier transformation in order to verify the existence of periodicity. The Fourier transform, named after Joseph Fourier, is an integral transform that re/expresses a function in terms of sinusoidal basis functions, i.e. as a sum or integral of sinusoidal functions multiplied by some
3.4. EXPERIMENTAL DESIGN

3.4.1. MICROBIAL POPULATIONS’ DYNAMICS IN DIFFERENT AVAILABILITY OF INORGANIC SUBSTRATE

A mesocosm experiment was conducted from 23 July to 1 August 2003 in order to follow viral and bacterial populations’ dynamics as well as to evaluate phage-host interaction in different availability of phosphorus (phosphorus enrichment and phosphorus limitation). The importance of phosphorus in this study area is given by its very often limitation interesting the coastal waters of the entire Adriatic basin.

Mesocosm enclosures consisted in 6 translucent 25 l polycarbonate carboys (Nalgene). On 23 July, they were filled with approximately 20 l of seawater sampled at 10 m of the C1 coastal station of the Gulf of Trieste. Sampled water was previously filtered through a nylon sieve with a 200 μm mesh to eliminate mesozooplankton grazers. The same quantity of inorganic nutrients was added to each mesocosm bottle in order to obtain following nutrient concentrations: nitrates (NaNO₃ final concentration 100.5 μM), silicates (Na₂SiO₃ × 9 H₂O final concentration 100.5 μM), vitamins (vitamin B₁ (Tiamin) × HCl final concentration 0.1 mg l⁻¹; vitamin B₁₂ (Cobalamin) final concentration 0.5 μg l⁻¹; vitamin H (Biotin) final concentration 0.5 μg l⁻¹) and iron (FeCl₃ 9.5 μM). Only to 3 of 6 mesocosm bottles phosphorus addition occurred (NaH₂PO₄ × H₂O final concentration 6.3 μM). Moreover, each mesocosm bottle was enriched with phytoplankton organisms, which were collected at 10 m depth in the station of the sampled seawater provenience (C1) using a plankton mesh (140 μm-pore size). Collected

Fig. 3.9. Marine biology researchers at t₁ incubation time during mesocosm experiment taking seawater samples from incubation carboys.

Materials and methods—experimental design
phytoplankton was resuspended in 1.5 l seawater volume and 200 ml was added to each mesocosm bottle.

Thus, different phosphorus availability conditions were produced in triplicate: phosphorus depleted condition (P-) in 1, 2 and 3 bottles and phosphorus repleted condition (P+) in 4, 5, and 6 bottles.

Free-floating carboys were incubated in the sea, in the front of LBM, for 9 days in situ conditions and sampled initially (t_0), on 25 July (t_1), on 29 July (t_2) and on 1 August (t_3) at the same hour (between 12:00 and 13:00) (fig.3.9). Following parameters were determined from 150 ml of sampled water: primary production, DOC, nutrients, total bacteria abundance, active bacteria abundance, virus abundance, bacterial carbon production (from both ^3H-TdR and Leu-TdR) and exoenzymatic bacterial activity (α-glucosidase, α-glucosidase, β-galactosidase, lipase, alkaline-phosphatase and leucine-aminopeptidase).

3.4.2. MICROBIAL POPULATIONS’ DYNAMICS IN DIFFERENT AVAILABILITY OF ORGANIC SUBSTRATE

3.4.2.1. ORGANIC MATRIX PRODUCED BY PHYTOPLANKTON BLOOM

Seawater contained in incubation carboys produced by mesocosm experiment discussed in Section 3.3.1. was processed further to assess microbial populations’ dynamics with substrate produced by phytoplankton assemblage in different nutrient availability. The seawater was joined together and filtered through 0.2 μm – pore size polycarbonate filter membrane (Nuclepore) in order to eliminate all the organisms present. The sample containing dissolved organic matter was submitted to ultrafiltration procedure using 30 kDa cartridge (Millipore PLTK Prep/Scale™-TFF 6 Ft 2 Unit; Regenerated cellulose). Thus, different samples were produced corresponding to <30 kDa and >30 kDa fractions. All the obtained samples were enriched with marine bacteria, previously collected at C1 station. Collected seawater was transferred into 5 l polycarbonate bottles (Nalgene), previously washed in diluted HCl (10%) and rinsed with Milli-Q water. During the transport, which occurred within 1 h after collection, samples were preserved in 4 °C cooler in the dark. The collected seawater was filtered through 1 μm polytetrafluoroethylene membranes (Millipore) to eliminate major grazers and a sample was incubated in parallel as a control.

Bacteria were concentrated onto 0.1 μm hydrophilic teflon membranes (Millipore) and the retentate was resuspended in the samples.

Bacteria-inoculated samples were incubated at 25 °C in the dark for approximately 2 months. Samplings were performed daily during the first 5 days and afterwards once a week. The following parameters were analysed: DOC, total bacteria abundance, active bacteria abundance and viral abundance.
3.4.2.2. ORGANIC MATTER BY RIVERINE ORIGIN

Finally, a third mesocosm experiment was conducted from 17 June to 1 July 2004 in order to follow dynamics of microbial populations as well as to evaluate phage-host interaction in a presence of organic matter of riverine origin. Since the water masses of the Gulf of Trieste are considerably influenced by the freshwater input, mostly due to the Isonzo River, a possible impact of its organic matter charge has been assayed in this experiment.

Freshwater (10 l) was sampled at the mouth of Isonzo river and mixed with 50 l coastal seawater sampled at C1 station (according to the previously described sampling procedure) into an open-air polycarbonate container up to the salinity that usually characterises surface seawater during intense riverine inflows (30). At the same time, another open-air polycarbonate container was filled with 60 l of seawater sample as a control. Both samples were incubated at 15 °C for 15 days with 14 to 10 light to dark cycles with an illumination of 115 μmol m⁻² s⁻¹. Samples were stirred twice a day with a sterile mixer.

Samplings were performed initially (t₀) and subsequently every three days: at 21 June (t₁), at 24 June (t₂), at 28 June (t₃) and finally at 1 July (t₄). An additional seawater sample was collected in the field the day before (non treated) and the obtained data were used as background values.

Following parameters were determined from 800 ml of the sampled water: total bacteria abundance, active bacteria abundance, virus abundance and bacterial carbon production (estimated by ³H-TdR and ³H-Leu incorporation methods).

3.4.3. ESTIMATE OF VIRAL PRODUCTION

3.4.3.1. PRELIMINARY EXPERIMENTS FOR ESTIMATING VIRUS PRODUCTION USING RADIOTRACER

The method for estimating virus production in seawater (Steward et al., 1992) is similar in principle to the radiotracer method for measuring bacteria production (e.g. Fuhrman & Azam, 1980). Tracer uptake by the host secondarily labels the viruses, and tracer incorporation specifically into the nucleic acid of released viruses can be calibrated to yield estimates of virus production. For the purpose of the study [methyl-³H]thymidine (³H-TdR) was chosen as radiotracer since it is considered to be mainly taken up by bacteria (Fuhrman & Azam, 1982). It can be used to specifically measure the production of phages containing deoxyribonucleic acid (DNA phages).

Solutions were prepared with purified Milli Q water. Phosphate buffer solution (PBS) containing 250 mM NaH₂PO₄·H₂O and 10 mM MgCl₂·H₂O was acidified up to pH 3.5 and filtered through a 0.2 μm pore size membrane filters (Millipore). Saturated 4M (NH₄)SO₄ was prepared by adding 767 g (NH₄)SO₄ to 1 l PBS and stirring overnight at room temperature. Excess (NH₄)SO₄ and particulates were removed by filtration.
through a 0.2 μm pore size membrane filters (HAWP, Millipore). Working (NH₄)₂SO₄ solution was prepared by diluting the obtained solution 1:1 with PBS.

Stock solutions of DNase (Sigma, 1 U μl⁻¹) and RNase (Sigma, 1 U μl⁻¹) were prepared in Milli Q water and stored at −20 °C.

**Experiment 1**

A first experiment was conducted within the phytoplankton bloom artificially induced by nutrient enrichment (see Section 3.4.2.1) carried out on 23 July 2003. Samples were taken in different phases of phytoplankton bloom, initially and after 96 h, both from phosphate-repleted (P+) and depleted (P-) incubation carboys.

For each sample an aliquot (50 ml) was poured into 50 ml sterile translucent polypropylene tube (Nalgene) and incubated in the dark at in situ temperature with 20 nM final concentration of ³H-TdR. Samplings were performed immediately after the addition of the radiotracer (t₀) and after 16 h (t₁₆) and 18 h (t₁₈). The choice of the

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**Fig. 3.10.** Representation of the incubation steps relatively to the radiotracer technique. First, during the bacterial replication radiolabeled nucleotids (³H-TdR) gets integrated into the bacterial genome. Subsequently, bacteriophages attack and penetrate bacterial cell and use the bacterial DNA for the production of their progeny that liberate destroying bacterial cell (lytic life cycle).
duration of the incubation period was based on the Steward et al. (1992) protocol, showing the maximum production rates between 16 h and 18 h incubation time. For each incubation time 2 ml were fixed in formaldehyde (2% v/v final concentration) to estimate viral and bacterial abundances, and 12 ml were filtered through a 0.2 μm pore-size syringe filter (Acrodisc) and split into 5 aliquots (2 ml) and 2 blanks were immediately stopped with formaldehyde (2% v/v final concentration) whether 3 subsamples were processed further. In order to degrade dissolved nucleic acids, samples were incubated for 1 hour at 20 °C with solutions of DNAse (1U μl⁻¹) and RNAse (1U μl⁻¹). The incubation was stopped with formalin (2% v/v final concentration) and the obtained samples were preserved on ice and processed further within 24 hours (fig.3.10).

Virus and protein precipitation was carried out with (NH₄)₂SO₄ with the sample incubation on ice for 20 min in order to concentrate viruses and to remove unincorporated label and any residual, dissolved nucleic acids not degraded by nucleases. Using a centrifuge maintained in a 5 °C refrigerator, the precipitates were pelleted by centrifugation for 20 min at 14000 rpm. The supernatant was then aspirated while the pellet was resuspended in 2 ml of PBS.

The subsamples were vortexed and split into 2 aliquots of 0.9 ml. One set of the aliquots was incubated with TCA (10 % v/v final concentration) at 100 °C in order to hydrolyse both DNA and RNA while the other one was incubated with TCA (10% v/v final concentration) at 0 °C without provoking nucleic acid hydrolysis. After 1 h of incubation samples were cooled on ice for 10 min. Both sets of samples were vortexed to resuspend the precipitates and the samples were filtered onto 0.45 μm pore-size inorganic membrane filters (Millipore). Sample vials, funnel and glass filter holder were washed 3-fold with 1 ml of cold 5% TCA. Filters were put in the scintillation vials, acidified with HCl (1 N) and incubated at 90-100 °C for 1 h. The incorporated radioactivity of cooled samples was measured with a liquid scintillation counter (Packard Tri-Carb 300). The difference in activity between the replicates was taken as the activity in viral DNA.

Viral abundance is plotted versus moles of ³H-TdR incorporated and the conversion factor is determined from the period that corresponded to the maximum slope of the curve.

Some steps reported by the original Steward et al. (1992) protocol were omitted after the personal discussion with the author (Steward, personal communication) such as the addition of micrococcal nuclease (MNase) to the enzymatic cocktail, the treatment with a carrier solution according to Hollibaugh (1988) containing sheared calf thymus DNA, bovine serum albumine, Torula yeast, TdR and NaN₃, was omitted as well.
Virus production (VP) was calculated using the obtained specific conversion factor of viruses' number produced per mole $^3$H-TdR:

$$VP = \frac{TdR_{inc} \cdot CF}{V \cdot t}$$

where

- $CF =$ conversion factor for the number of viruses produced per mole $^3$H-TdR incorporated;
- $t =$ incubation time (h);
- $V =$ volume of the incubated sample (l).

The moles of incorporated radiotracer were calculated using the following equation:

$$TdR_{inc} = \frac{DPM_{inc}}{C \cdot SA}$$

where

- $DPM_{inc} =$ DPM$_{sample} - DPM_{black}$;
- $SA =$ specific activity of the thymidine solution in nCi pmoles$^{-1}$ (reported by the manufacturer);
- $C = 2.22 \cdot 10^3$, conversion factor for the transformation of DPM in nCi.

To derive virus production rates requires a factor relating label incorporated to virus produced. Nevertheless, in the literature actually exist 2 different conversion factors (Fuhrman and Noble, 1995; Kepner et al., 1998) for the estimate of virus production, conversion factors, separately for $P-$ and $P+$ virus 'population' were determined in the field. However, this empirical determination of conversion factors requires a situation where virus production greatly exceeds virus decay so that the net production, as observed by direct counts, will be close to the gross production. To obtain the virus production $CF$, the increase in virus abundance is plotted versus moles of $^3$H-TdR incorporated and the conversion factor is determined from the period that corresponded to the maximum slope of the curve (within $t_1$ and $t_2$ incubation period). Then the conversion factor can be calculated using the following equation:

$$CF = \frac{\text{virus abundance}(t_1) - \text{virus abundance}(t_2)}{TdR_{inc}(t_1) - TdR_{inc}(t_2)}$$
Experiment 2

The second experiment was carried out in order to better define a scale of the incubation period.

Seawater sample was collected at surface on 7 October 2003 at a coastal station C1 in sterile propylene tube. An aliquot (50 ml) was amended with 20 nM final concentration of $^3$H-TdR in the dark and in situ temperature. A time-response curve was defined checking the viral production after 0, 13, 15, 18 and 20 h of incubation. The samples were processed and further analysed as previously reported (Experiment 1).

COMPARISON AMONG DIFFERENT METHODOLOGICAL APPROACHES FOR VIRUS PRODUCTION

3.4.3.2. SERIAL DILUTION TECHNIQUE IN MANIPULATED PHAGE-HOST ASSEMBLAGE

The experiment was set up according to the dilution method proposed by Landry and Hassett (1982) e.g. Landry (1993) for estimating the protistan grazing activity.

This approach allows estimating indirectly net viral production rate over a 24 h incubation period. A manipulated seawater sample with viral and bacterial assemblage was serially diluted to assess the effect of varying encounter rates between viruses and their hosts.

For the application of this approach two approximations were necessarily considered:
1) It was assumed that the growth of a single bacterium is not directly affected by the presence or absence of other bacteria. This implication leads that a reduction in the density of host cells in natural seawater will not directly cause a change in the growth rate of remaining cells;
2) Assuming that the major part of viruses is lytic, the probability of bacterial mortality is a direct function of the rate of encounter between phages and their host cells. This implies that the number of lysed cells by a given virus is linearly related to host density (Murray & Jackson, 1993; Murray & Eldridge, 1994) and that the threshold value of virus and bacteria product of $10^{12}$ ml$^{-2}$ has to be equalled or more (Weinbauer et al., 2002).

It has been assumed that in natural seawater samples where grazers were removed by filtration bacterial mortality is principally due to viral lysis and, when burst size is known, rates of viral production can be calculated.

Equipment used for the experiment was washed in diluted HCl (10%), rinsed with deionized water and sterilized by autoclaving. Only precleaned and rinsed silicone tubes were used. The whole water (assemblage of bacteria and viruses) was obtained by seawater filtration through a nylon sieve with a 20 μm mesh and by a subsequent filtration through 1.0 μm pore-size polycarbonate membrane filter (Durapore, Millipore) in order to eliminate all grazers. Diluting water was obtained by seawater filtration through 0.025 μm pore-size membrane filters (Durapore, Millipore).
Five serial dilutions (20, 40, 60, 80 and 100% whole water) were incubated in triplicate in 17 ml sterile transparent polyethylene vials. To verify the possible presence and growth of viruses in the 0.025 μm filtered water, 3 additional vials were incubated as controls. The incubation was carried out under simulated in situ conditions for 24 h with free-floating vials that were positioned in a container flushed with running seawater.

Special care was taken to control the effect of the manipulations: for each dilution vial, bacterial concentrations were detected in duplicate. The success of the dilution procedure was verified by first order regression analysis of bacterial abundances plotted versus dilution factor.

Bacterial growth and mortality rates were calculated by first order regression analysis of apparent growth against dilution factor (fig.3.11), based on Landry & Hassett (1982) (e.g. Landry (1993)). The apparent host growth is described by

$$ C_t = C_0 e^{(k-g)t} $$

where

- $C_0 =$ number of bacteria at $t_0$;
- $C_t =$ number of bacteria at $t$ incubation time;
- $k$ (intercept on y axis) = growth coefficient;
- $g$ (regression line slope) = bacterial mortality coefficient.

The average bacterial concentration during the course of experiment was calculated as following:

$$ C_e = \frac{C_0 e^{(k-g)t} - C_0}{k - g} $$
whether the bacteria removal rate, assuming that it derived from bacterial lysis, was obtained by equation:

$$LB = C_m \times g.$$  

To determine the burst size (Bratbak et al., 1992) 50 ml whole water sample were incubated with 0.3% (w/v) streptomycin sulfate (Fluka BioChemika) for 1 h in the dark at in situ temperature. Burst size was calculated from the average number of phages released for a single bacterium.

Viral production (VP) was calculated as a product of the burst size and a number of bacterial cells lysed by virus infection (Hennes & Simon, 1995) according to the equation:

$$VP = \frac{LB \cdot B}{t}.$$  

3.4.3.3. DILUTION TECHNIQUE IN ALREADY INFECTED BACTERIA

Viral production was evaluated using a dilution technique described by Wilhelm et al. (2002) that reduces the background of free viruses through the addition of virus-free seawater, allowing rates of viral production to be readily monitored from small changes in viral concentration. As the viruses produced derive from cells infected prior to the beginning of the experiment, altered contact rates between viruses and bacteria, which are provoked by changes in virus or host abundances, will not affect cell-specific viral production rates. The method provides the minimum estimate of productivity since it is restricted only to incubation periods with increasing trend of viral abundance, unless the viral decay rate is also known.

All the equipment in direct contact with the samples was treated with HCl (10%), rinsed with deionized water and sterilized by autoclaving. Only precleaned and rinsed silicone tubes were used. A volume of 50 ml seawater sample was filtered onto 0.2μm pore-size inorganic membrane filter (Millipore) in order to collect bacterial cells whether the viral particles were allowed to pass through. The retentate (bacteria concentrate) was resuspended in 50 ml of virus-free seawater (0.02 μm-filtered) and shaken for 20 min in order to facilitate removal from the filter. The obtained sample was incubated in transparent polycarbonate bottles (Nalgene) in the dark at in situ temperature. Viral and bacterial abundances were determined in triplicate from 5 ml subsamples that were taken at 0, 3, 6, 9, 12 and 15 hours incubation time.

From the initial non-manipulated sample bacterial abundances were also determined in triplicate in order to define the correction factor for the bacterial loss due to the filtration step.

The viral production rates were determined by the first order linear regression analysis of triplicate viral abundances versus incubation time.
3.4.3.4. RADIOTRACER TECHNIQUE

Solutions were prepared following the procedure reported above for the preliminary experiments (see Section 3.3.1.1.) while the analytical procedure underwent slight modification of the protocol previously described (see Section 3.3.1.1. Experiment 1).

An aliquot (100 ml) of the sample was incubated with [methyl-3H]thymidine (20 nM final concentration) in sterile glass bottles at dark and at in situ temperature. Samplings were carried out at the beginning of the experiment and after 12 and 15 hours. For each incubation time 2 ml were fixed in formaldehyde (2% v/v final concentration) to estimate viral and bacterial abundances, and 20 ml were filtered through a 0.2 μm pore-size syringe filter (Acrodisc) and split into 10 aliquots (2 ml): 4 blanks were immediately stopped with formaldehyde (2% v/v final concentration) whether 6 subsamples were processed further. In order to degrade dissolved nucleic acids, samples were incubated for 1 hour at 20 °C with solutions of DNAse (5 U ml⁻¹ final concentration) and RNAse (5 U ml⁻¹ final concentration). The incubation was stopped with formaldehyde (2% v/v final concentration) and the obtained samples were preserved on ice and processed further within 24 hours.

Virus and protein precipitation was carried out with (NH₄)₂SO₄ with the sample incubation on ice for 20 min in order to concentrate viruses and to remove unincorporated label and any residual, dissolved nucleic acids not degraded by nucleases. Using a centrifuge maintained in a 5°C refrigerator, the precipitates were pelleted by centrifugation for 20 min at 14000 rpm. The supernatant was then aspirated while the pellet was resuspended in 2 ml of PBS. The subsamples were vortexed and split into 2 aliquots of 0.9 ml. One set of the aliquots was incubated with TCA (5 % v/v final concentration) at 100°C while the other one was incubated with NaOH (0.5 N final concentration) at 60°C (G. F. Steward, personal communication). After 1 h of incubation samples were cooled on ice for 10 min. Both sets of samples were vortexed to resuspend the precipitates and the samples were filtered onto 0.45 μm pore-size inorganic membrane filters (Millipore). Sample vials, funnel and glass filter holder were washed 3-fold with 1 ml of cold 5% TCA. Filters were put in the scintillation vials, acidified with HCl (1 N) and incubated at 90-100 °C for 1 h. The incorporated activity of cooled samples was measured with a liquid scintillation counter (Packard Tri-Carb 300) and the obtained DPM were converted into virus production (1⁻¹ h⁻¹) according to calculation procedure previously described for the preliminary experiments (see Section 3.3.1.1.). Conversion factor was determined in the field from the period that corresponded to the maximum increase of virus abundance versus moles of incorporated radiotracer.
3.4.4. AN ESTIMATE OF THE IMPACT OF VIRAL INFECTION ON MARINE BACTERIOPLANKTON

To estimate virus impact on the mortality of different bacterial metabolic groups (total, active and non viable bacteria), there were set up 2 experiments following the dilution approach proposed by Landry and Hassett (1982) e.g. Landry (1993) for estimating the protistan grazing activity. A manipulated seawater sample with viral and bacterial assemblage without grazers (removed by filtration) was serially diluted to assess the effect of varying encounter rates between viruses and their host allowing the estimate of bacterial growth and viral induced mortality rates over a 24 h incubation period.

As already reported in Section 3.4.3.2 where the dilution approach has been described, two approximations were necessarily considered:

1) It was assumed that the growth of single bacterium is not directly affected by the presence or absence of other bacteria. This implication leads that a reduction in the density of host cells in natural seawater will not directly cause a change in the growth rate of remaining cells;

2) Assuming that the major part of viruses is lytic, the probability of bacterial mortality is a direct function of the rate of encounter between phages and their host cells.

The first experiment (Experiment 1) was conducted in vernal period (20 January 2004) at the station C1 in the Gulf of Trieste to assess the impact of virus infection on different bacterial groups that were distinguished according to their metabolic (in)activity. Further, at the same station, another experiment (Experiment 2) was performed on 9 August 2005 to assess the impact of virus infection on different bacterial metabolic groups during the summer season. Moreover, in this second experiment, with the refinement of a technique for the identifying of inactive or dead bacterial cells, the impact on virus infection on this bacterial group was also examined.

Equipment used for the experiment was washed in diluted HCl (10%), rinsed with deionized water and sterilized by autoclaving. Only precleaned and rinsed silicone tubes were used. The whole water (assemblage of bacteria and viruses) was obtained by seawater filtration through a nylon sieve with a 20 μm mesh and by a subsequent filtration through 1.0 μm pore-size inorganic membrane filter (Millipore) in order to eliminate all grazers. Diluting water was obtained by seawater filtration through 0.025 im pore-size inorganic membrane filters (Millipore).

Five serial dilutions (20, 40, 60, 80 and 100% whole water) were incubated in triplicate in 17 ml sterile transparent polyethylene vials. To verify the possible presence and growth of viruses in the 0.025 im-filtered water, 3 additional vials were incubated. The incubation was carried out under simulated in situ conditions for 24 h with free-floating vials that were positioned in a container flushed with running seawater. After the incubation time, active, non-viable and total bacterial abundances were determined.

Special care was taken to control the effect of the manipulations: for each dilution vial active, non-viable and total bacterial concentrations were detected in duplicate.
The success of the dilution procedure was verified by first order regression analysis of bacterial abundances (active, non-viable and total) plotted versus dilution factor (see Section 3.4.3.2).

Bacterial growth and mortality rates were calculated by first order regression analysis of apparent growth against dilution factor, based on Landry & Hassett (1982) (e.g. Landry (1993)). The apparent host growth is described by following equation

\[ C_t = C_0 e^{(k-g)t} \]

where

- \( C_0 \) = number of bacteria at \( t_0 \),
- \( C_t \) = number of bacteria at \( t \) incubation time;
- \( k \) (intercept on y axis) = apparent growth coefficient;
- \( g \) (regression line slope) = bacterial mortality coefficient.

Changes in dissolved organic carbon concentration (DOC) as well as the possible impact of viral lysis on the changes in bacterioplankton community were examined from the samples taken prior \( (t_0) \) and after \( (t_4) \) incubation.
4. RESULTS AND DISCUSSION

4.1. DISTRIBUTION OF VIRIOPANKTON IN THE NORTHERN ADRIATIC SEA AND ITS RELATIONSHIP WITH BIOTIC AND ABIOTIC FACTORS

Since the sampling stations covered a Northern Adriatic area with bottom depths increasing notably from the coastal belt towards the central part of the basin, the analysis of spatial distribution of abiotic and biotic parameters as well as the spatial distribution of VBR (Virus-to-Bacterium Ratio) and VBP (Virus-to-Bacterium Product) was limited to surface values to avoid bias due to data averaging and/or integration.

Vertical profile analysis was performed for seawater temperature data collected along the deepest transect E0 to assess the stratification level of water column. Also, distribution of Virus Like Particles (VLP) and the proportion of active bacteria within total bacterial community were analysed along the water column.

During the year 2004 three oceanographic cruises were carried out in different seasons.

4.1.1. May 2004

4.1.1.1. Abiotic factors

The surface temperature reached a mean value of 18.9 °C. The differences among the stations were relatively marked, with the values ranging between 17.6 and 20.2 °C. The minima values characterized the northernmost part of the basin while in the central
part higher values were found (fig.4.1.1). Vertical distribution of the seawater temperature (fig.4.1.2) along the deepest transect of the area (E0) shows the beginning of the stratification process of the water masses with temperature minima (~10 °C) close to the bottom and much higher values, up to 20.2 °C at surface level.

The Po River run-off was high (average May run-off was 2387 m³ s⁻¹, while the long-term average for the same period corresponds to 1910 m³ s⁻¹). The average surface salinity value of the area was 32.6. Beside the evidence of low salinity area (17-23) due to the River Po plume, marked salinity gradient (17-33) was registered in the northern sector (fig.4.1.3). This low salinity belt along the coastline is probably due to the frontal system formed by the fluvial inflows in that part of the basin and counterclockwise circulation of the general Adriatic marine current.

The Po River inflow affected both pH (fig.4.1.4) and oxygen saturation level (fig.4.1.5). For the rest of the study area both parameters resulted quite homogeneous (8.2-8.3 for pH and 100-110 O₂ %).

The fluorescence ranged from 0.2 reaching up to 4.9 arbitrary units, characterizing the entire basin by particularly high values (mean=1.6; median=1.1). A general coastal to offshore decreasing gradient was observed (fig.4.1.6).
4.1.1.2. Biotic factors

The VLP abundances ranged between 3.7 and $3.0 \times 10^9$ l$^{-1}$. Higher values were registered towards the central part of the basin, designing increasing coastal-offshore gradients along C, D and F transects (fig.4.1.7). A slight VLP abundance enrichment was observed offshore Venice lagoon (C4, C1 and C5 stations). Both E transects (E and 2E) were characterized by higher VLP values at nearshore stations, reaching the maximal values of entire area in the southernmost part.

At some selected stations (C10, E06 and 2E02) samplings were performed also along a water column. Values of VLP at the surface differed among the sampled stations whether the abundances became generally lower and more homogeneous with the increase of water depth (fig.4.1.8). Bacterial abundance at the surface ranged from 0.7 to $5.8 \times 10^9$ cells l$^{-1}$. Higher values were registered along the transect D with an
Fig. 4.1.7. Pattern of virophioplankton distribution in the Northern Adriatic Sea at surface level during May cruise.

Fig. 4.1.8. Vertical profiles of VLP distribution at C10, E06 and 2E02 stations.

offshore increasing gradient that affected also C transect (fig.4.1.9). The same kind of gradient was observed from northern coastline southwards (along A and F transects). Vice versa, along the southernmost transect (E), bacteria displayed higher abundance values at the coastal stations.

Active bacteria ranged between 0.2 and 7.3 x 10⁸ cells l⁻¹ showing very pronounced variability over the study area. Higher active bacterial abundances interested the central part of the basin, which slightly decreased towards the western coastline (fig.4.1.10). Other 2 high-density hotspots were registered in the coastal stations of F transect and in 2E03 station. On the contrary, it is interesting to observe 3 very low-density spots, without any logic distribution pattern, which correspond to minimal values of the study area, at C06, F07 and E10 stations.

From the comparison of virio- and bacterioplankton distribution patterns (fig.4.1.7 and fig.4.1.9) it can be observed that the high-density areas for these microbial components generally match while slightly different pattern of active bacteria distribution was observed (fig.4.1.10). From figure 4.1.11, a decrease of the proportion
of active bacterial cells along the water column at offshore stations C10 and E06 can be observed, while the most southern coastal 2E02 station showed an opposite trend with the increase of the water depth.

4.1.2. July 2004

4.1.2.1. Abiotic factors

Seawater temperature in the surface averaged 25.6 °C, ranging between 23.6 and 27.6 °C. The minimal values were observed in the northern half of the basin (with an exception of high temperature values proximal to the C08 station), where the temperature was affected by freshwater discharges derived from rivers and torrents situated
on the northern part of the basin due to snow melting in Julian Alps. Therefore, a general increasing temperature gradient was registered towards the south (fig. 4.1.12).

In July the Po River runoff was low (with an average of 581 m$^3$ s$^{-1}$) and did not at all affect temperature near to the Po River mouth while salinity was slightly influenced by freshwater input with a gradient (17–35) that was limited only to the nearshore area. For the rest of the basin salinity pattern resulted quite homogeneous with an average of 32 (fig. 4.1.14).

The vertical profile of seawater temperature along the transect E0 (fig. 4.1.13) evidenced the presence of thermocline that in July settled at 10m-depth. The seawater
temperature above the thermocline was around 25 °C and an increasing gradient reaching the temperature minima of 12 °C resulted near the bottom.

The seawater in the surface layer showed different pH and oxygen saturation patterns: high oxygenated waters, reaching up to 150% of saturation, matched mostly to the Po River freshwater input towards offshore (E transect) and remained quite homogenous, around 100% of saturation, for the rest of the area (fig.4.1.16). On the contrary, higher pH values (8.40–8.58) were limited within offshore area of the transect A while the lowest pH (8.22) values interested the central area of the basin (fig.4.1.15).

Fig. 4.1.15. Seawater pH pattern in the Northern Adriatic Sea at surface level during May cruise.

Fig. 4.1.16. Seawater oxygen saturation (%) pattern in the Northern Adriatic Sea at surface level during July cruise.

The fluorescence values varied from 0.1 to 1.9 arbitrary units. Besides the decreasing gradient towards offshore stations, a fluorescence hotspot was registered around E08 station, which matched exactly with high values of oxygen saturation and the temperature hotspot (fig.4.1.17).
4.1.2.2. Biotic factors

VLP abundances in the surface waters ranged between $2.4 \times 10^9$ and $25.1 \times 10^9$ particles l$^{-1}$ with an increasing surface-bottom gradient that was observed at C10 and E06 stations (fig. 4.1.17). Higher values were limited to the easternmost C station (C15) and to the southern part of the basin with an evident coastal-offshore gradient along D and E transects (fig. 4.1.18).

Total bacterial abundances (fig. 4.1.20), bacterial active fraction (fig. 4.1.21) as well as cyanobacteria distribution (fig. 4.1.22) at surface level showed similar patterns where higher abundances remained limited to the southeastern part of the basin. Thus, along the C, D and E transects a general decreasing coastal-offshore gradient was observed.
Higher cell densities were also registered in the coastal area offshore Venice lagoon. Total bacteria abundance ranged between 0.9 and $6.9 \times 10^9$ cells l$^{-1}$. The variability of active bacterial cells and cyanobacteria resulted much higher with abundances that ranged between 0.1 and $7.2 \times 10^8$ cells l$^{-1}$ but from the median values of their abundance.

Fig. 4.1.20. Pattern of total bacterioplankton abundance distribution in the Northern Adriatic Sea at surface level during summer cruise.

Fig. 4.1.21. Pattern of active bacterioplankton abundance distribution in the Northern Adriatic Sea at surface level during the summer cruise.
Fig. 4.1.22. Pattern of cyanobacteria abundance distribution in the Northern Adriatic Sea at surface level during the summer cruise.

distribution \((0.4 \text{ and } 0.6 \times 10^8 \text{ cells } \text{l}^{-1} \text{ respectively})\) it is evident that the lower abundances were more frequently registered.

4.1.3. November 2004

4.1.3.1. Abiotic factors

The temperature pattern in the surface water layer is shown in figure 4.23. Seawater temperature ranged from 11.3 to 16.6 °C with 14.7 °C and 15.4 °C as mean and median values respectively. Minima values were found in the coastal belt with a temperature increase towards the central part of the basin.

Fig. 4.1.23. Seawater-temperature pattern of the Northern Adriatic Sea at surface level during November cruise.

Fig. 4.1.24. Vertical profile of the seawater temperature along transect EO during November cruise.
The vertical structure of the water column was rather homogeneous in terms of temperature. As can be observed from the vertical profile along transect E0 (fig. 4.1.24), a slight column stratification was registered in the shallower nearshore belt, which completely disappeared in the central, deeper area of the basin.

![Salinity profile](image)

*Fig. 4.1.25. Salinity pattern in the Northern Adriatic Sea at surface level during November cruise.*

The salinity distribution (fig. 4.1.25) displayed rather similar pattern of the seawater temperature, increasing southward from the coast to the central part due to the decreasing influence of riverine outflows. Salinity ranged between 29.7 and 38.4, with the prevalence of high salinity values (mean=36.1; median=37.7).

Finally, the pH (fig. 4.1.26) and oxygen content (fig. 4.1.27) resulted rather homogeneous for the entire study area, displaying a slightly decreasing trend from north to south. Values of pH

![pH profile](image)

*Fig. 4.1.26. Seawater pH pattern in the Northern Adriatic Sea at surface level during November cruise.*
ranged from 8.0 to 8.1 only and the oxygen values remained rather low, not exceeding 93.8% of saturation.

The fluorescence values in November resulted particularly low compared to spring and summer periods of the same year, ranging only from 0.1 to 0.8 arbitrary units. Higher values interested the northern part of the basin, extending towards the central sampling stations of the C and D transects (C12 and D12). Coastal stations of the D transect displayed slightly higher values as well (fig. 4.1.28).

4.1.3.2. Biotic factors

VLP abundances in the surface layer ranged between 3.8 and $19.9 \times 10^9 \text{l}^{-1}$. Higher values were found around Po river mouth (coastal stations of the E transect) and offshore lagoons of Grado and Marano (A3 and A5 stations). With the exception of the hot spot found at C12 station, the central part of the basin (F transect and
central stations of D and E transects) displayed the lowest VLP abundances for the autumn survey (fig. 4.1.29). VLP abundance along the water column showed rather variable vertical profiles. Similar distributions were found at C10, C15 and E06, characterized by lower values in the surface layer, followed by an increase at 5 m depth again decreasing towards 15 m. At the bottom, finally, very variable values were observed. Other investigated stations did not display any common distribution (fig. 4.1.30).

Heterotrophic bacterioplankton displayed quite narrow variability, ranging within 0.9 and $1.8 \times 10^9$ cells $l^{-1}$. Despite mostly homogeneous distribution that characterized the entire study area, maximum bacterial densities were found at C8 and C13 stations.

Fig. 4.1.29. Pattern of virioplankton distribution in the Northern Adriatic Sea at surface level during the autumn cruise.

Fig. 4.1.30. Vertical profiles of VLP distribution at C06, C10, C13, C15, E06, E10 and E12 stations during the autumn cruise.

Fig. 4.1.31. Pattern of total bacterioplankton abundance distribution in the Northern Adriatic Sea at surface level during autumn cruise.
Fig. 4.1.32. Pattern of active bacterioplankton abundance distribution in the Northern Adriatic Sea at surface level during the autumn cruise.

Fig. 4.1.33. Vertical profiles of the proportion of active cells (%) at C06, C10, C13, C15, E06, E10 and E12 stations during the autumn cruise.

The abundance of active bacterial cells varied from 0.1 to $0.6 \times 10^6$ cells l$^{-1}$ and showed completely different distribution pattern with evident coast to offshore decreasing gradient, which resulted particularly marked near the Po river mouth. The external stations of the E transect seem to be affected by another decreasing gradient deriving from the Istrian coast (fig. 4.1.32).

Although any common trend among different vertical profiles relatively to the proportion of the active cells cannot be observed (fig. 4.1.33), the percentage of active cells showed similar vertical distributions to those registered for VLP abundance (fig. 4.1.30), especially regarding C10, C15 and E6 stations.

The spatial distribution pattern of non-viable/dead bacteria displayed wide abundance variability (fig. 4.1.34), with the values ranging between 0.1 and $9.5 \times 10^8$ cells l$^{-1}$. Mean and median values resulted $2.6 \times 10^8$ l$^{-1}$ and $1.7 \times 10^8$ l$^{-1}$ respectively. The proportion of non viable/dead cells within total bacterial population (fig. 4.31) varied between minimum of 0.4% up to 91%. Even if no similarities with bacterial (both total and active) spatial distributions can be observed, quite similar, but opposite pattern characterized VLP abundances, with the areas of lowest non-viable/dead bacteria that corresponded to the highest VLP abundances.

For the 95% of the study area the abundance of cyanobacteria displayed quite homogeneous distribution, without exceeding $4 \times 10^6$ cells l$^{-1}$. The only marked hot spot was registered at E11 station where the cell density reached $34.4 \times 10^6$ cells l$^{-1}$ (fig. 4.1.35).
4.1.4. An overview across different surveys

The virioplankton abundance over three seasonal sampling periods ranged between 0.2 and $3 \times 10^{10}$ L$^{-1}$. The major variability among data distribution was observed in May, while the minor one was registered in November. No particular differences between abundance distributions among spring (mean=$7.5 \times 10^9$ L$^{-1}$; median=$6.3 \times 10^9$ L$^{-1}$), summer (mean=$5.7 \times 10^9$ L$^{-1}$; median=$3.8 \times 10^9$ L$^{-1}$) and autumn (mean=$5.8 \times 10^9$ L$^{-1}$; median=$5.12 \times 10^9$ L$^{-1}$) surveys were observed (fig. 4.1.36).

The active bacteria resulted a particularly variable fraction of the bacterial community with the greatest variability registered over spring and summer surveys (fig.4.1.36c). Particularly high proportion of active cells was observed in May, with 15% as the average proportion of active cells, reaching up to 41% (C12 station) of total bacterial community. Unusually high values for field studies were observed
Results—viral distribution

Fig. 4.1.36. Box plots of (a) VLP, (b) total bacteria and (c) active bacteria–CTC data distributions over May, July and November surveys in the Northern Adriatic Sea.

also along C and E transects (20-35%). In July the active bacterial abundances showed major distribution variability with mean and median values being respectively $0.2 \times 10^9$ cells l$^{-1}$ and $0.4 \times 10^8$ cells l$^{-1}$. In that period the average proportion of active bacteria consistently decreased (6.4%), ranging between 1 and 23% of total bacterial cells. During the November cruise both total bacterial abundance (fig.4.1.36b) and its active fraction varied very little (between 0.8 and $1.9 \times 10^9$ cells l$^{-1}$ and between 0.1 to $1.1 \times 10^8$ cells l$^{-1}$ respectively), with the proportion of the active fraction that remained below 2.4%.

During the spring survey viral abundance was positively correlated with the abundance of total bacteria ($p=0.031; r_s=0.35; n=39$). Lower positive correlation, slightly below the 95% significance limit, was found relatively to active bacteria abundance ($p=0.054; r_s=0.31; n=39$). Viruses were significantly positively correlated with temperature ($p=0.008; r_s=0.42; n=39$), pH ($p=0.0001; r_s=0.58; n=39$) and oxygen saturation level ($p=0.012; r_s=0.40; n=39$) while no significant correlations between viruses, salinity and fluorescence were observed (tab.4.1.1).

<table>
<thead>
<tr>
<th>VIRUSES &amp;</th>
<th>n valids</th>
<th>$r$ (Spearman)</th>
<th>p-level</th>
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<tr>
<td>TOTAL BACTERIA</td>
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</tr>
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<td>ACTIVE BACTERIA</td>
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<td>SALINITY</td>
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<tr>
<td>pH</td>
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<td>FLUORESCENCE</td>
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<td>-0.32</td>
<td>0.0007</td>
</tr>
</tbody>
</table>

Tab. 4.1.1. Correlation analysis between viruses and bacteria and between viruses and main hydrological parameters during May survey. The number of considered data couples (N valids), Spearman correlation coefficient ($r$) and the probability level (p-level) for rejecting $H_0$ hypothesis are shown (*$p<0.05$; **$p<0.01$; ***$p<0.001$).
In the summer survey viral abundances were significantly positively correlated with all bacterial groups examined: total bacteria \((p=0.0001; r_s=0.62; n=33)\), active bacteria \((p=0.0027; r_s=0.51; n=33)\) and cyanobacteria \((p=0.0075; r_s=0.46; n=33)\). Among viruses and hydrological parameters, significant correlation was observed only with temperature \((p=0.0037; r_s=0.49; n=33)\) and pH \((p=0.0027; r_s=0.51; n=33)\), while salinity, oxygen saturation and fluorescence did not show any significant relationship (tab. 4.2).

\[
\begin{array}{ccc}
\text{VIRUSES} & \text{n valids} & r \text{(Spearman)} & p\text{-level} \\
\hline
\text{TOTAL BACTERIA} & 33 & 0.62 & 0.0001 \\
\text{ACTIVE BACTERIA} & 33 & 0.51 & 0.0027 \\
\text{CYANOBACTERIA} & 33 & 0.45 & 0.0075 \\
\text{TEMPERATURE} & 33 & 0.46 & 0.0037 \\
\text{SALINITY} & 33 & -0.25 & 0.1637 \\
\text{OXYGEN SATURATION} & 33 & 0.27 & 0.1960 \\
\text{pH} & 33 & 0.51 & 0.0027 \\
\text{FLUORESCENCE} & 33 & 0.22 & 0.2137 \\
\end{array}
\]

Tab. 4.1.2. Correlation analysis between viruses and bacteria and between viruses and main hydrological parameters during July survey. The number of considered data couples \((N \text{ valids})\), Spearman correlation coefficient \((r)\) and the probability level \((p\text{-level})\) for rejecting \(H_0\) hypothesis are shown (*\(p<0.05\); **\(p<0.01\); ***\(p<0.001\)).

In the autumn survey there were no significant correlations between viral abundances neither with plankton nor with hydrological parameters (tab.4.1.3).

\[
\begin{array}{ccc}
\text{VIRUSES} & \text{n valids} & r \text{(Spearman)} & p\text{-level} \\
\hline
\text{TOTAL BACTERIA} & 30 & 0.30 & 0.1121 \\
\text{ACTIVE BACTERIA} & 30 & 0.03 & 0.7682 \\
\text{CYANOBACTERIA} & 30 & 0.21 & 0.2823 \\
\text{TEMPERATURE} & 30 & -0.15 & 0.4636 \\
\text{SALINITY} & 30 & -0.19 & 0.3117 \\
\text{OXYGEN SATURATION} & 30 & 0.07 & 0.7225 \\
\text{pH} & 30 & 0.07 & 0.7000 \\
\text{FLUORESCENCE} & 30 & -0.04 & 0.9200 \\
\end{array}
\]

Tab. 4.1.3. Correlation analysis between viruses and bacteria and between viruses and main hydrological parameters during November survey. The number of considered data couples \((N \text{ valids})\), Spearman correlation coefficient \((r)\) and the probability level \((p\text{-level})\) for rejecting \(H_0\) hypothesis are shown (*\(p<0.05\); **\(p<0.01\); ***\(p<0.001\)).
Values of virus-to-bacterium ratio (VBR) for all surveys are reported in table 4.1.4. In spring and summer the VBR index fell within almost the same interval, ranging from 1 to 9 in May and from 1 to 10 in July, although if different data distributions were observed. Something higher proportions of virus towards bacterial abundances were found in May (mean=4.5; median=6.2) along the C transect up to offshore C14 station and from the E transect southwards (fig.4.1.37a). In July, within quite homogeneous data distribution (mean=3; median=3), markedly higher VBR values were registered at the most eastern station of the C transect and in the central part of the E transect (fig.4.1.37b). The average and median VBR values observed in November (mean=6; median=5) did not particularly differ from those reported for spring.

Tab. 4.1.4. Virus-to-bacterium ratio (VBR) and virus-host density (VBP) minimum, maximum, mean and median values relatively to May, July and November surveys are reported.

<table>
<thead>
<tr>
<th></th>
<th>VBR</th>
<th>VBP (10^12 ml^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>max</td>
</tr>
<tr>
<td>May</td>
<td>1.3</td>
<td>8.8</td>
</tr>
<tr>
<td>July</td>
<td>0.8</td>
<td>9.6</td>
</tr>
<tr>
<td>Nov</td>
<td>3.3</td>
<td>14.2</td>
</tr>
</tbody>
</table>

Fig. 4.1.37. Pattern of VBR index distribution in the Northern Adriatic Sea at surface level: (a) during the spring cruise; (b) during the summer cruise and (c) during the autumn cruise.
However, in the autumn survey the maximum VBR, reaching up to 14 units was observed in the proximity of the Po river mouth, at the offshore stations of D and E.

Fig. 4.1.38. Pattern of VBP index distribution in the Northern Adriatic Sea at surface level: (a) during the spring cruise; (b) during the summer cruise and (c) during the autumn cruise.
E transect and northern wards close to Grado and Marano lagoons (fig. 4.37c). Mean values of Virus to Bacterium Product (VBP) were 28, 29 and $10 \times 10^{12}$ ml$^{-2}$ respectively in spring, summer and autumn cruises. During spring and summer the ranges (3-175$\times 10^{12}$ ml$^{-2}$ and 2-170$\times 10^{12}$ ml$^{-2}$ respectively), means (28$\times 10^{12}$ ml$^{-2}$ and 29$\times 10^{12}$ ml$^{-2}$ respectively) and medians (11$\times 10^{12}$ ml$^{-2}$ and 10$\times 10^{12}$ ml$^{-2}$) of VBP values resulted quite similar (tab. 4.1.4) while their spatial distributions slightly differed. In May, the highest VBP values corresponded to the coastal area of the southernmost transect (2E02) going towards the offshore D stations (fig. 4.38a). In July, in these D13, D14 and D15 stations the VBP index markedly decreased (from ~100 to ~30) and the only evident gradient remained related to the Po river outflow displaying decreasing gradient along the E transect (fig. 4.1.38b). During the November survey VBP values notably decreased over the entire study area below 27.8$\times 10^{12}$ ml$^{-2}$, showing something higher values near the Po river mouth, in the central part of the basin (C transect) and at the coastal stations of the A transect (fig. 4.1.38c).

Discussion

Virionplankton resulted the most abundant microbial component in the surface waters of the Northern Adriatic Sea with abundances that fall within the range of values published for coastal environments worldwide (Bergh et al., 1989; Proctor & Fuhrman, 1991; Fuhrman & Suttle, 1993) and those reported for the Adriatic Sea (Weinbauer & Peduzzi, 1993; 1995, Corinaldesi et al., 2003; Stopar et al., 2003).

The Northern Adriatic basin displayed clear seasonal scenarios relatively to the hydrological features, thus representing a model for investigating the effects of changing trophic conditions on viral distribution and on microbial loop functioning. The Po River outflow determined evident pattern of oxygen saturation level (decreasing), pH (decreasing) and salinity pattern (increasing) in all seasons, while the seawater surface temperature was affected by riverine discharge only in the autumn survey (verified by the correlation analysis among parameters). In the spring blooming season the temperature began to rise (20 °C) and the high rivers’ inflow, mainly determined by the Po River, induced marked coast-offshore gradient with the stratification of the water column observed over the entire basin. All microbial components resulted particularly abundant, displaying the highest values among all surveys. Both viral and bacterial abundances increases resulted correlated with the increase in water temperature, which was also correlated with the increase in fluorescence. This increase in the trophic state of the basin resulted particularly evident for the entire coastal belt and induced the increment of active bacterial cells (even if not markedly evident from the spatial distribution patterns but confirmed by correlation analysis) up to 41% of the total bacterial population, thus largely exceeding the proportion of active bacteria ever observed in the Gulf of Trieste (Karuza et al., 2004; Paoli et al., 2006). Del Giorgio and Scaraborough (1995) registered proportions >30%
only in highly productive estuarine waters while usually they do not exceed 10% of the total community (Gasol et al., 1995; del Giorgio et al., 1996; Jugnia et al., 2000). Moreover, the active bacterial population in May displayed particularly wide spatial variability, confirming the ability of rapid metabolic shifts as a response to favourable environmental variations, as already reported by Paoli et al. (2006). From the lack of significant correlation between viral abundances and total bacteria, and considering the positive correlation both between viruses and active bacteria (even if on the significance limit, p>94.5%) and fluorescence becomes evident that high levels of active bacteria determine intense viral proliferation sustained by lytic life cycle. In fact, the occurrence of this kind of proliferation strategy (able to rapidly increase viral abundances being characterized by short latent period and high burst size) is certainly present in the highly trophic area, which follows the gradient of Po river plume. This consideration is confirmed by the presence of particularly high virus-bacteria densities (VBP index) observed at nearshore stations of the southernmost transect (up to 1.7×10^{13} ml^{-2}). Since there is evidence that bacteriophages trigger lytic life cycle over approximately 10^{12} cell / particle density (from 1×10^{18} to 9×10^{18}) in a ml of sample (Wilcox & Fuhrman 1994; Weinbauer 2004), viral and bacteria density product results of particular importance for the virus-bacteria encounter rates (Murray & Jackson, 1982; Wiggins & Alexander 1985; Murray & Eldridge, 1994; Maranger & Bird, 1995), determining in our case the environmental factors favouring lytic life strategy. Considering that low values of the VBR should indicate low infectious rates, low numbers of viral particles from each bacterial host (burst size), or much less reliably high decay rate of viruses in the ecosystem (Alonso et al., 2001), high VBR should indicate high infection rates and high burst size. In our case the VBP index resulted more informative than commonly utilized VBR index, which in our case did not evidence any particular pattern and did not reveal high productive areas, being included within values commonly observed for the Adriatic Sea in the absence of mucilaginous formation (Bensi et al. 2003; Corinaldesi et al., 2003; Bongiorni et al., 2005; Karuza & Del Negro, 2005). However, it should be considered that low VBR values that were observed in high productive area of Po plume could be due to the high presence of total bacteria rather than low virus abundance (contrary to the usual observations) suggesting that almost a part of bacterial community is of allochtonous origin. Our consideration that the enrichment in viral abundances from outside sources seems less plausible matches to those reported by other authors, which consider that the major portion of viruses in marine surface waters appears to come from within the system (Wilhelm & Suttle, 1991). Finally, due to lower virus-host density, viruses do not successfully encounter their specific hosts with the consequence of lower infection frequency.

In July further increase of seawater temperature was observed (up to 28 °C in the surface), with the thermocline that settled between 10 and 15 m depth. The abundances of all microbial components examined (viruses, total bacteria, active bacteria and
cyanobacteria) resulted positively correlated with each other and there was an evident match among their spatial distribution patterns, but the dependence on the common factor was excluded: while both heterotrophic and phototrophic bacterioplankton resulted inversely but significantly correlated with salinity, thus suggesting the direct influence of the riverine inflows, viruses resulted the only one component positively correlated with seawater temperature. The exclusive relationship between viral abundance and seawater temperature could suggest more intensive viral proliferation determined by high water temperature, but virus inactivation and lower infection frequencies at lower temperatures might be also a plausible explanation. Even if in the summer microbial abundances slightly decreased relatively to the spring survey, displaying lower variability (particularly evident for active bacteria fraction), the presence of high trophic gradient confined within well-defined area can be observed. Microbial proliferation and activity, with still high proportion of active cells (up to 23%) was certainly stimulated by the nutrient induction since salinity resulted inversely correlated with fluorescence, pH and oxygen saturation that affected only the southernmost part of the basin. Even if VBR index did not involved precious information, in this season VBP index described much faithfully viral interaction with their potential hosts, suggesting the viral lysis occurrence along the southernmost transect. Both active and total bacteria could have represented their most probable host. Based only on the positive correlation between virus and cyanobacteria abundances, the copious presence of cyanophages cannot be excluded.

In November seawater temperature along the coastline strongly decreased to the minimum value of 16 °C and perfectly matched salinity distribution pattern. In fact, temperature resulted positively correlated with oxygen saturation level and pH, and inversely correlated with salinity suggesting the presence of a large amount of freshwater in the coastal belt. The presence of freshwater determined slight water column stratification in the nearshore area, which completely lack for the rest of the basin. Microbial community characteristics during the autumn survey strongly differed from those observed during spring and summer. Virioplankton displayed the lowest differences being characterized by quite similar variability with those observed for the previous surveys. Abundances of both phototrophic and heterotrophic bacteria, including the active bacterial fraction, resulted particularly low with rather homogeneous distribution in the surface layer over the entire basin. Also the proportion of active cells resulted particularly low, without exceeding 4% of the total community with the range of percentage that falls within that commonly observed in the Gulf of Trieste (Karuza et al., 2004; Paoli et al., 2006). Despite the presence of clear hydrological patterns, no relationship with microbial components was observed. Low fluorescence values found in the autumn period were not high enough to induce the microbial proliferation and activity, thus resulting in rather low abundances. There were no significant relationships among viral abundances, total bacteria, active bacteria and cyanobacteria. Although no similarities of virus spatial distribution with bacterial
(both total and active) distribution can be observed, quite similar, but opposite pattern characterized viroplankton distribution, with the areas of lowest non-viable/dead bacteria that corresponded to the highest viral abundances. Unfortunately, no statistically significant confirm was obtained by the correlation analysis, even if the relationship between viruses and non viable bacterial cells could be argued, being p<92% and thus not so far from our confidence level of p<95%. However, it is interesting to observe the presence of highly significant inverse correlation between non-viable/dead cells and total bacterial community (p=0.0006; \( r_s = -0.54 \); n=36), suggesting that the bacterial community includes a variety of metabolic states where the total bacteria are probably only dormant or starving but not non viable or dead.

Conversely to the conditions previously observed for other two surveys, VBR and VBP spatial patterns quite faithfully matched. Virus-bacteria density remained rather low for the entire basin, exceeding the threshold limit only in limited areas, where because of higher proportion of viruses towards bacteria, VBR and VBP increases are observed in parallel. With the exception of these well-defined zones the lysogenic life cycle would be the most plausible viral infection strategy, especially if low bacterial abundances and activity levels are considered. The viral nucleic acid incorporated into bacterial genome could be quiescent together with the bacterial cell and ready to induce rapid lytic proliferation with more favourable environmental conditions. Our considerations match with the results of studies conducted by Stopar et al. (2003) that sustain the high proportion of lysogenic-inducible viruses in the Gulf of Trieste.
4.2. TEMPORAL VARIATION OF VIRIOPHANKTON IN THE GULF OF TRIESTE AND FACTORS INFLUENCING ITS DISTRIBUTION

Vertical profile of hydrological features was followed during the study period. As can be observed from figures 1-6, salinity and temperature displayed similar distribution patterns over years. The main factor influencing salinity variations in the Gulf of Trieste, especially at surface, is the freshwater input due to Isonzo river outflow, which also affected water temperature. The beginning of the stratification of water masses was prevalently registered in March (sometimes delayed to April) and persisted until September with the maximum surface-bottom gradient (7.8 °C) observed in June. Indeed, two salinity minima were generally observed, characterized by following mean values: 32.6 in March-April and 33.0 in October-November periods. Initially, diluted water remained limited only to the surface layer and then progressively sunk to the subsurface level. The salinity of subsurface layer and those proximal to the bottom in some periods of the year were also influenced by advections of highly saline waters from southern basins.

Differences in temperature and salinity patterns among years were mostly due to temporal shifting of season arrivals rather than copious differences of annual values distribution. However, particular thermic conditions regarded the spring-summer period of year 2000 (fig.4.2.1a), when already in February thermocline formation was observed and finally in June markedly enhanced surface-bottom thermal difference up to 13 °C. Another anomaly was recorded during the winter 2001 when the mean temperature during January and February months exceeded by 2 °C mean value usually observed for the same period (fig.4.2.2a). Median seawater temperature value of 14.1 °C observed in 2003 (fig.4.2.4a) and 2004 (fig.4.2.5a) with unusual differing from their respective mean values of 15.2 °C and 14.9 °C, outlined the occurrence of particularly cold periods that were confirmed by low temperature minima (6.5 °C and 6.6 °C). In 2004 the
thermocline formation resulted markedly postponed, with the complete column homogeneity that persisted until June. Similar hydrological condition was registered also over year 2005, characterized by particularly short summer season, thus affecting early mixing of the water layers (fig. 4.2.6a).

Fig. 4.2.2. Temperature (a) and salinity (b) distribution patterns along the water column over the year 2001.

Fig. 4.2.3. Temperature (a) and salinity (b) distribution patterns along the water column over the year 2002.

Fig. 4.2.4. Temperature (a) and salinity (b) distribution patterns along the water column over the year 2003.
Fig. 4.2.5. Temperature (a) and salinity (b) distribution patterns along the water column over the year 2004.

Fig. 4.2.6. Temperature (a) and salinity (b) distribution patterns along the water column from January to October 2005.

The annual distribution of seawater temperature displayed minima values (6.9 °C) in February whether the highest seawater temperatures (26 °C) were observed in the surface during August. Since the major hydrologic variability interested the surface layer of the water column, temporal distributions of temperature and salinity data were additionally considered. However, despite major temperature variability that

Fig. 4.2.7. Temporal distribution of seawater temperature in the surface over 5-years study period. Different years are separated by vertical lines.
affected seawater temperature at the surface, quite regular annual distribution can was observed (fig.4.2.7). Even if periodical salinity oscillations, attributable to the seasonal freshwater input (mostly riverine during spring and rainwater during autumn) can be noted, anomalously low salinity values (from 31 to 33) were registered in December 2000, June 2002 and May 2003 (fig.4.2.8).

Temporal distribution of VLP abundances was followed from January 2000 to September 2005. The sampling strategy over years has been changed and therefore the number of collected samples varied from n=44 and n=48 for the first two years respectively, and was subsequently increased to n=92 and n=88 respectively for years 2003 and 2004. Also during the year 2005 samplings were performed with intensified frequency but due to the interruption occurred in September, the last year data distribution could interfere in the comparison to the other years’ distributions and thus should be considered under reserve.

The distribution of VLP over these 6 years is reported in figure 4.2.9. Virus densities during the entire study period varied from 1.1 to 354.9×10^8 particles l^{-1} with mean and median values of 69.6 and 50.9×10^8 l^{-1} respectively. Only in the year 2000 the abundances were significantly (3 - fold) greater (mean=123.5×10^8 l^{-1}; median=120.5×10^8 l^{-1}) displaying a wide temporal variability (ranging from 14.1×10^8 l^{-1} to 341.0×10^8 l^{-1}) if compared with the abundances reported for the following study period when the mean values over years did not significantly differ (mean_{2001}=69.8×10^8 l^{-1}; mean_{2002}=47.7×10^8 l^{-1}; mean_{2003}=62.9×10^8 l^{-1}; mean_{2004}=70.2×10^8 l^{-1}; mean_{2005}=43.4×10^8 l^{-1}) and with a quite homogeneous data distribution within years (range_{2001}=6.2-341.0×10^8 l^{-1} and median_{2001}=50.2×10^8 l^{-1}; range_{2002}=1.1-175.8×10^8 l^{-1} and median_{2002}=39.4; range_{2003}=2.8-354.9×10^8 l^{-1} and median_{2003}=51.7×10^8 l^{-1}; range_{2004}=2.9-231.8×10^8 l^{-1} and median_{2004}=53.3×10^8 l^{-1}; range_{2005}=7.3-146.8 and median_{2005}=32.5×10^8 l^{-1}). From January 2001 on, the minimum value of 1.1×10^8 particles l^{-1} was registered in the surface during month of December 2002 and the
Fig. 4.2.9. Box plot of viral abundance distribution from January 2000 to September 2005 in a coastal station of the Gulf of Trieste. Single sampling depths data are considered. Crosses represent outlier values.

maximum value was found at 5 m depth in September 2003, the period that corresponded to the late summer, when in a box-plot representation a high number of outliers can be observed.

Temporal distribution of VLP abundances calculated as integrated values of single determinations from 0.5 m, 5 m, 10 m and 16 m samples is reported in fig.10. As it can be observed, within certain periods wide temporal variations up to two orders of magnitude were registered, also between two following samplings. Generally, higher abundances were determined in summer while less number of viral particles characterized the vernal periods. Based on the virus abundance seasonal trend, an

Fig. 4.2.10. Temporal distribution of VLP abundances as integrated values in the water column from January 2000 to September 2005.
anomalously high VLP number reaching $2.0 \times 10^{10}$ particles $l^{-1}$ was encountered in early spring period of the year 2000.

In figure 4.2.11 (a, b, c, d) temporal distribution of VLP abundances ($\pm$ st. dev.) at single sampling depths (0.5 m, 5 m, 10 m e 16 m) is reported. Rather similar abundance oscillations (fig.4.2.11) without particular differences in particle distribution (fig.4.2.12) along the water column were observed.

![Fig. 4.2.11. Temporal variations of VLP abundances for single sampling depths: (a) 0.5 m; (b) 5 m; (c) 10 m e (d) 16 m.](image-url)
In order to verify whether the distribution of VLP abundances depends upon seasonal variations of the main physical parameters that characterize the marine environment, the linear correlation analysis was performed between VLP abundance and temperature and salinity data. Since no significant variation of VLP abundance with water depth was observed, correlation analysis was applied to the entire data set. The normality of data distribution for every single parameter was tested through the quantile-quantile plot analysis (not shown). Since not normally distributed probability plot was produced, the non-parametric Spearman rank correlation analysis was applied. Viral abundance resulted significantly and positively correlated with seawater temperature ($p=0.0002$, $r_s=0.22$, $n=285$) while no significant correlation was found between viral abundance and salinity.

Moreover, the linear correlation analysis was performed on biotic parameters as well in order to test the dependence of virus like particles distribution upon their supposed hosts. After verifying a not normally distribution probability of the given variables (not shown), non-parametric Spearman rank correlation analysis was applied. From the obtained correlation coefficients virus and bacteria abundances did not result significantly correlated while highly significant positive correlations both with cyanobacteria ($p=0.002$, $r_s=0.18$, $n=285$) and heterotrophic nanoplankton abundances were found.

Bacterial relationship with VLP is investigated more in detail observing their simultaneous temporal distributions over years. In figure 4.2.13 VLP and bacterial trends are reported. Although no significant correlation between these two variables was found, for short time sampling periods there was evidence of similar variation trends in particle/cell abundances.
Results—viral distribution

Fig. 4.2.13. Temporal distribution of water column-integrated (a) VLP and (b) bacteria abundances data.

In order to test the periodicity of VLP abundance series, the Fourier analysis was performed. First, a polynomial function that describes VLP temporal trend is obtained onto VLP time series (fig.4.2.14). Then the Fourier transformation produced a decomposition of the function into harmonics of different frequencies (fig.4.2.15), with the major signal corresponding to the period between 10.7 and 12.8 months. This result confirms the existence of the periodicity of VLP abundances with a period that approximates 12 months (i.e. a solar year).

Figure 4.2.16 shows VLP and bacterial distributions over seasons. As there was already mentioned above relatively to the figure 10 and as can be observed from figure 4.2.16a, lower VLP densities were encountered during winter (mean=55.9×10^8 1^-1), the season when, despite quite complete mixing of the water column, higher data dispersion was observed (range=1.9–197.9×10^8 1^-1; median=35.4×10^8 1^-1). Similar data range to the vemal one was registered in autumn with values that ranged between 2.6×10^8 1^-1 and 192.1×10^8 1^-1 but with data distributed towards higher values (mean=68.9×10^8 1^-1; median=55.8×10^8 1^-1). The variability over 2 orders of magnitude
in that period could be perhaps attributable to the stratification of the water layers. On the contrary, lower data variability (which in this case does not match with the column stratification) was observed within spring (range=15.1–146.0×10⁸ l⁻¹) and surprisingly decreased further during summer (12.7–115.7×10⁸ l⁻¹). However, spring and summer periods were characterized by higher VLP abundance values with the averages of 77.6×10⁸ l⁻¹ and 61.4×10⁸ l⁻¹ respectively and the medians of 59.3×10⁸ l⁻¹ and 53.0×10⁸ l⁻¹ respectively.

Regarding the seasonal distribution of bacterial abundances, it displayed much more restricted data variability than VLPs, ranging within one order of magnitude. Observing the figure 16b there can be roughly distinguished two periods: a winter–spring period and a summer – autumn period. During the colder seasons a remarkable decrease of bacterial abundances was observed (mean=7.9±0.4×10⁸ l⁻¹; median=7.7±0.01×10⁸ l⁻¹), with values

Fig. 4.2.15. Harmonics obtained by Fourier transformation onto the polynomial function.

Fig. 4.2.16. Box plot of (a) VLP and (b) bacteria abundance seasonal distribution. Single sampling depths data are considered.
ranging from $2.2\pm0.1 \times 10^8$ to $15.0\pm0.4 \times 10^8$ $1^{-1}$. The evident increase in bacterial abundance was registered generally in the summer-autumn period (mean = $15.2\pm1.1 \times 10^8$ $1^{-1}$; median = $14.8\pm0.4 \times 10^8$ $1^{-1}$) but with different data variability for summer (range = $4.7 - 26.6 \times 10^8$ $1^{-1}$) and autumn (range = $8.9 - 24.1 \times 10^8$ $1^{-1}$, if $32.9 \times 10^8$ $1^{-1}$ outlier omitted) periods were observed.

Although there was not observed any significant evidence of correlation between seawater temperature and VLP abundance, the virus-bacteria interaction seems to be influenced by the seasonality.

Combining VLP and bacterial seasonal distributions’ data, virus-to-bacterium ratio seasonal distribution was obtained. As can be observed from the box-plot reported in figure 4.2.17a, a net difference between winter-spring and summer-autumn periods was registered. In the cold period the average VBR index was about 10, with higher values most frequently occurred in spring (median = 8.4) than during the winter period (median = 5.6). In the winter-spring period results interesting to observe the presence of particularly dislodged outliers, which, since not occasional but related to the subsequent samplings, should not be omitted. These particularly high VBR values up to 64.2 were registered during the year 2000, when the massive mucilage formation was registered at the beginning of June. During the summer-autumn period the average VBR index was halved (mean = $5.0\pm0.3$) and however did not exceed the maximum value of 17.1 that was registered in the autumn. Combining newly VLP and bacterial data to obtain ‘virus-to-bacterium product’, temporal distribution of VBP index was obtained (fig.4.2.18), which can be used as an indicator of the prevalent viral life strategy.

![Box plot of (a) VBR and (b) VBP index seasonal distribution. Single sampling depths data are considered.](image-url)
From the figure 4.2.17b can be evidenced a general increase of virus-bacteria density, which through the increase of their encounter rates increases the probability of successful infection, from 4.2 observed in winter towards 9.9 registered in autumn period when also the major data dispersion was observed.

In figure 4.2.19 VBP and VBR temporal distributions are reported simultaneously in order to verify whether the increase of virus-bacteria density actually matches with the increased number of VLP particles as a consequence of intensive lytic infection. It can be observed a general temporal coupling between VBP and VBR indexes as a sign of a general functionality of the system.

Therefore the VBP threshold value could be used for rather stable host species number. In more variable environments the more accurate estimator can be obtained through the calculation of the rate of lytic infection. Rates of lytic infection onto total bacterial community obtained in the spring and summer months of year 2000, reported in figure 4.2.20, did not particularly differ from the values registered during the year.
Conversely, lytic infection rates on the single bacteria species reported in figure 4.2.21, which can be obtained when the number of bacteria species is known, pointed out remarkable anomaly relatively to the spring-summer period of 2000 reaching infection rates of 40 ml⁻¹ min⁻¹ in May, 81 ml⁻¹ min⁻¹ in June up to 347 ml⁻¹ min⁻¹ in

Fig. 4.2.20. Lytic infection rates onto total bacterial community (ml⁻¹ min⁻¹) relatively to the year 2000 (black histograms) and during the year 2003 (grey histograms).

Fig. 4.2.21. Lytic infection rates for single bacterial species relatively to the year 2000 (black histograms) and during the year 2003 (grey histograms).

Fig. 4.2.22. Number of bacterial species assessed during year 2000 (black histograms) and during the entire year 2003 (grey histograms).
July. These extremely high infection rates per bacterial species outlined the intensity of the infection processes before, during and immediately after the mucilage event, which interested the study area. The number of bacterial species relatively to these distinct periods is reported in figure 4.2.22.

Discussion

Our pluriannual monitoring study confirmed that marine viruses are the most abundant component among microorganisms in the water column of the Gulf of Trieste with abundances that fall within the range of values published for coastal environments worldwide (Bergh et al., 1989; Proctor & Fuhrman, 1991; Fuhrman & Suttle, 1993) and those reported for the Adriatic Sea (Weinbauer & Peduzzi, 1993; 1995, Corinaldesi et al., 2003; Stopar et al., 2003).

Similar to the results obtained by Corinaldesi et al. (2003) relatively to the entire Adriatic basin, the bacterial densities varied within a relatively narrow range (the ratio of the highest value to the lowest value was 34:1), whereas the viral abundance displayed much greater variability (varying up to 322-fold) over the study period. This fact, coupled with the wide range of virus-to-bacterium ratio, confirmed that the virus-host cell system is highly dynamic, and might sign the anomalous functioning of the microbial loop (particularly evidenced during the mucilage period occurred in June 2000).

Conversely to some previous works that report significant decrease of viral abundance with water depth despite nonstratified conditions (Wommack et al., 1992; Cochlan et al., 1993; Guixa-Boixereu et al., 1999) and shallow waters environments (Corinaldesi et al., 2003), investigations carried out in the present study revealed homogeneous viral counts with depth. Also Wommack et al. (1992) found no difference in viral abundance between surface- and bottom-water samples in the mesohaline portion of Chesapeake Bay, also when the water column was stratified. The rather homogeneous virus abundance with water depth in the coastal station of the Gulf of Trieste is almost partially due to particularly shallow waters implied in our study and certainly favoured by the presence of long periods of homogeneous hydrological conditions along the water column. Even if not significant, but higher variability in viral abundance affected the first 10 m of the water column.

Viral distribution resulted independent of salinity gradients, suggesting that virus distribution does not reflect the input of allochtonous freshwater viruses. This result contrasts with the results reported by Weinbauer et al., 1993, who found negative correlation between viral abundance and salinity. A significant positive correlation between viral abundance and seawater temperature suggests an increase of free viral particles and viral production with the trophic state of the system (Bongiorni et al., 2005), generally observed during spring occurrence of phytoplankton blooms stimulated by riverine freshwater inputs and increased irradiance (Bratbak et al., 1990).
According to Weinbauer (2004) phytoplankton blooms are most probably the reason for the high viral abundance (although a significant contribution of algal viruses cannot be excluded). The positive significant correlations between temperature and primary production \((p=0.004, r_s=0.17, n=285)\), chlorophyll-a \((p=9.7 \times 10^{-5}, r_s=-0.23, n=284)\) and DOC \((p=2.8 \times 10^{-19}, r_s=0.50, n=285)\) suggest the general increase in system productivity. Thus, the virioplankton community appears to be favoured by higher energy input and increased biological productivity and likely constitutes an active part of the marine food web (Bratbak et al., 1990; Proctor & Fuhrman, 1990). However, the factor determining increase in viral abundance by prophage induction in lysogenic bacteria relatively to increased irradiation and temperature cannot be excluded (Bratbak et al., 1990; Heldal & Bratbak, 1991).

Even if similar considerations have been already reported (Weinbauer, 2004), the fact that viruses in the present study are not directly correlated with bacteria as their most common host, but with cyanobacteria and heterotrophic nanoplankton could seem surprisingly but might be explained otherwise. The tight statistical relationships between viruses and cyanobacteria, and viruses and nanoplankton are probably due to the strong temperature influence directly on cyanobacterial \((p=4 \times 10^{-4}, r_s=0.67, n=285)\) and nanoplankton development \((p=5 \times 10^{-5}, r_s=0.24, n=284)\). Contrary to several short-term studies that report significant correlation between viral and bacterial abundance (Bird et al., 1993; Cochlan et al., 1993; Weinbauer & Suttle, 1997; Alonso et al., 2001; Stopar et al., 2003), the lack of this kind of correlation relatively to the present study could be almost partially justified by viral and bacterial populations development that do not occur at the same time. Since virus replication tightly depends on the bacterial development, almost slight time delay could be expected for the viral abundance response, as suggested for predator-prey Lotka-Volterra interaction model (Weinbauer, 2004). The oscillations of total bacterial and viral abundance according to predator-prey type of oscillation was already observed by Weinbauer et al. (1995) in the Adriatic Sea during a diurnal study following a drifting buoy. Moreover, the most important reason for the lack of significant correlation between this most numerous virus-host couple in our long-term study is probably due to the variety of interactions (with different virus-bacteria interactions resulting thus in different abundance proportions) that characterizes different study periods.

During massive mucilage formation occurred in June 2000 maximum VBR value of 64.2 was registered in surrounding seawater \((\text{mean}=4.7)\) whereas particularly high average value of 72 was observed inside the aggregates (Del Negro et al., 2005), largely exceeding the VBR range usually registered for the Gulf of Trieste (Stopar et al., 2003) without mucilage formation (Bensi et al., 2003), the Adriatic Sea studies (Fonda Umani et al., 2002, Corinaldesi et al., 2003) and marine pelagic ecosystems elsewhere (Maranger & Bird, 1995; Drake et al., 1998; Wommack & Colwell, 2000; Weinbauer, 2004). Considering that low values of the VBR may indicate low infectious rates, low numbers of viral particles from each bacterial host (burst size), or much
less reliably high decay rate of viruses in the ecosystem (Alonso et al., 2001), high VBR should indicate high infection rates and high burst size. Since there is evidence that bacteriophages trigger lytic rather than lysogenic life cycle over approximately $10^{18}$ cell / particle density (from $1 \times 10^{18}$ to $9 \times 10^{18}$) in a litre of sample (Wilcox & Fuhrman 1994; Weinbauer 2004) average values of their density produce result of particular importance for the virus-bacteria encounter rates (Murray & Jackson, 1982; Wiggins & Alexander 1985; Murray & Eldridge, 1994; Maranger & Bird, 1995), determining thus the prevalent life strategy over different seasons.

In order to verify whether the increase of virus-bacteria density actually matches with increases of VLP particles number as a consequence of intensive lytic infection, VBP and VBR temporal distributions were investigated. As supposed, our results pointed out general coupling between VBP and VBR indexes as a sign of a general functionality of the system. The only exceptions coincide to the early-spring periods of years 2000 and 2005. Despite the evidence of the ‘normality’ of the system it should be considered anyway that the indexes are rather approximate, due to the importance of the number of bacterial species present at a given moment.

Therefore the VBP threshold value would be an excellent index in condition of stable host species richness (i.e. the number of species). In more variable environments the more accurate estimator can be obtained through the calculation of the rate of lytic infection. Rates of lytic infection onto total bacterial community that were registered in the spring and summer months of year 2000, did not particularly differ from the values registered during the year 2003. Conversely, lytic infection rates on the single bacteria species calculated for the same period and known bacterial richness, pointed out remarkable anomaly relatively to the spring-summer period of 2000, when infection rates of 40 ml$^{-1}$ min$^{-1}$ in May, 81 ml$^{-1}$ min$^{-1}$ in June up to 347 ml$^{-1}$ min$^{-1}$ in July were observed. These extremely high infection rates per bacterial species outlined the intensity of the infection processes before, during and immediately after the mucilage event.

Considering viral particles inside mucilage aggregates, their abundances resulted 300-fold higher than in the surrounding water, reaching an average value of $9 \times 10^{11}$ particles l$^{-1}$ (Del Negro et al., 2005). Peduzzi and Weinbauer (1993) previously reported high viral abundances for marine snow. According to Del Negro et al. (2005) viral abundance varied according to aggregate type and size, ranging from 9 orders of magnitude l$^{-1}$ in stringers to 12 orders of magnitude l$^{-1}$ in clouds, affecting therefore also the VBR index that in stringers and cobwebs remained close to the surrounding water while in clouds exceeded 100 arbitrary units, suggesting the extremely high productivity of the mucilage microhabitat (Weinbauer, 2004; Del Negro et al., 2005), confirmed also by TEM inspection of that revealed a significant amount of infected bacteria (Proctor & Fuhrman, 1991). Some experimental studies performed in the Northern Adriatic basin evidenced the increased production up to 3-fold more abundant of algal flocs >1mm when virus concentrates added (Weinbauer & Peduzi, 1993).
4.3. MICROBIAL POPULATIONS' DYNAMICS IN DIFFERENT AVAILABILITY OF INORGANIC SUBSTRATE

Phosphate and nitrate concentrations were followed on alternate days in order to determine nutrient consumption (fig. 4.3.1). In phosphate-depleted enclosures (P-) the phosphate concentration remained stable (setting around 0.2 µM) throughout the course of the experiment. In phosphate-repleted samples (P+), phosphate concentrations markedly decreased over two following samplings from 4.3 µM registered at t₁ to 0.13 µM after 4 days, suggesting the rapid consumption by the planktonic community. Phosphate availability enhanced also the nitrate uptake; nitrate concentrations dropped together with phosphate concentration, from the initial mean value of 93.1 (±35.0) µM below 10 units (7.9±2.0 µM at t₂ and 1.0±0.4 µM at t₃) in the second part of the experiment. Although from figure 4.3.1b could seem that the change in nitrate concentration in phosphate-depleted samples slightly varied over time, the variability among the replicates remains higher than the variation between samples and therefore the nitrate concentration could be considered rather stable with values that approximate 87.0 µM (±16.1).

Completely distinct proportions of inorganic nitrogen to phosphorous characterized two different incubation enclosures: in the phosphate enriched samples (P+) N:P ratio decreased approximately to 40 while in the P(-) samples N exceeded over P for about

![Fig. 4.3.1. Nutrient concentrations over incubation period: a) phosphate (PO₄³⁻) and b) nitrate (NO₃⁻). Mean values (± st.dev.) of non treated (nt), phosphate depleted (P-) and phosphate repleted (P+) triplicate samples are reported for each treatment.](attachment:image.png)

500 fold. Since both treatments showed great variability among the replicates, remaining higher than the variation between samples, no temporal variation of N:P ratio could be observed (fig.4.3.2).

![Graph](image_url)

**Fig. 4.3.2.** Proportion of inorganic nitrogen (approximated to nitrate) to inorganic phosphorous in mesocosm experiment. Mean values (± st.dev.) of non treated (nt), phosphate depleted (P-) and phosphate repleted (P+) triplicate samples are reported for each treatment.

Another chemical analysis, regarding DOC concentration, was followed over time. Up to the second day of incubation (t₁) similar trend of DOC concentrations was found in all enclosures, with or without nutrient addition (fig.4.3.3). If compared to the non treated sample, initial concentration of DOC resulted 50% higher. This increase is probably attributable to the production of dissolved organic matter due to the treatment steps such as filtering and stirring. From the t₂ incubation time on, DOC concentrations clearly differed. Whether DOC concentration in P(-) mesocosm remained rather stable, a consistent DOC enrichment was registered at t₂ in P(+) samples, characterized by 50% higher values than those found in P(-) samples, and finally became 100% higher at the end of the experiment (t₃).

![Graph](image_url)

**Fig. 4.3.3.** DOC concentration over incubation period in non treated (nt), phosphate depleted (P-) and phosphate repleted (P+) samples. Mean values (± st.dev.) of samples incubated in triplicate are reported for each treatment.
Planktonic community was followed over course of the experiment. In P(-) samples, the phytoplankton community was dominated by diatoms and small flagellates, but since the presence of mucus aggregates partially covered phytoplanktonic organisms and therefore could cause a counting bias, their abundances were not determined. The measure of primary production rates did not particularly vary for the entire incubation interval. The maximum rate of 7.0±1.4 μg C l⁻¹ h⁻¹ was observed at t₂ incubation time. In P(+) samples the algal community was sustained mainly by nanoflagellates. The phosphate addition determined an increase in primary production reaching maximum rate at t₂ incubation time equal to 233.2±24.9 μg C l⁻¹ h⁻¹.

Abundance of the entire bacterial community in non treated sample resulted 8.0×10⁸ cells l⁻¹ (nt) and no changes were induced by the initial setting of the experiment (t₀). At t₁ incubation time populations in different phosphate availability began to differ (fig.4.3.4). Total bacteria in P(+) samples increased until t₂ sampling time to a mean value of 2.8×10⁹ cells l⁻¹. Even if bacterial abundance values between triplicates strongly differed (st.dev.=0.4×10⁹ cells l⁻¹), they resulted certainly higher than at the following sampling time (2.1×10⁹ cells l⁻¹). In P(-) incubation bottles bacterial abundances remained rather stable for first 2 days of the experiment, halved subsequently to 6.5×10⁸ cells l⁻¹ at t₂ incubation time and maintained proximal values until the experiment ended (5.7×10⁸ cells l⁻¹).

The abundance of metabolically active bacteria showed similar trend to total bacterial abundance during the entire incubation period (fig.4.3.5). The proliferation in phosphate enriched samples of active bacterial population resulted particularly enhanced. Already after 2 days of incubation (t₁) metabolically active bacteria abundance in P(+) samples increased more than 4 folds, from 4.4×10⁷ cells l⁻¹ recorded at the beginning of the experiment up to 19.7×10⁷ cells l⁻¹. Maximum value of 90.0×10⁷ cells l⁻¹, reaching 42% of total bacteria, was registered at t₂ incubation time (fig. 6). In
Results—substrate availability

P(-) samples active bacteria abundance progressively increased from $3.3 \times 10^7$ cells l$^{-1}$ to $11.8 \times 10^7$ cells l$^{-1}$ at the end of the experiment (fig. 4.3.5) but never exceeding 19% of total bacterial community (fig. 4.3.6).

![Graph showing bacterial abundances over incubation period](image)

Fig. 4.3.5. Abundances of metabolically active bacteria over incubation period in non treated (nt), phosphate depleted (P-) and phosphate repleted (P+) samples. Mean values ($\pm$st.dev.) of samples incubated in triplicate are reported for each treatment.

![Graph showing proportion of active bacteria within total bacterial community](image)

Fig. 4.3.6. Proportion of metabolically active bacteria within total bacterial community over incubation period in non treated (nt), phosphate depleted (P-) and phosphate repleted (P+) samples. Mean values ($\pm$st.dev.) of samples incubated in triplicate are reported for each treatment.

Also virus proliferation resulted strongly affected by phosphorous availability. Viral abundance of approximately $2 \times 10^9$ particles l$^{-1}$ was registered among non treated and treated (P+ and P-) samples. At t$_1$, sampling time the number of VLP within phosphate-enriched samples resulted doubled and further increases on t$_2$ up to the maximum value ($1.0 \pm 0.1 \times 10^{11}$ cells l$^{-1}$) on t$_3$ incubation time were observed (fig. 4.3.7a). Since the difference between viral densities in distinct treatments resulted particularly marked, a two scale graphical representation was additionally proposed (fig. 4.3.7b). As can be observed, in P(-) samples viral abundance over first 2 days strongly decreased to
approximately $5 \times 10^8$ particles $l^{-1}$. Afterwards, similar increasing trend, but characterized by 1–2 lower orders of magnitude to the phosphate-enriched incubation was observed.

![Graph](image)

**Fig. 4.3.** VLP abundances over incubation period in non treated (nt), phosphate depleted (P-) and phosphate repleted (P+) samples: a) the same scale is used for all treatments; b) different scales are used for P(+) and P(-) samples. Mean values (±st.dev.) of samples incubated in triplicate are reported for each treatment.

Although bacterial and viral abundances increased in parallel in both treatments (fig. 4.3.4 and fig. 4.3.7a), the increment in viral abundance in P(+) samples began to exceed the bacterial one at $t_2$ incubation time and finally reached the maximum of 55 at the end of the experiment, as can be observed from VBR trend shown in figure 4.3.8. Virus-to-bacterium ratio in P(-) remained rather constant, displaying low values (1.8–2.8) during the entire experiment duration.

As can be observed from the trend of VBP index shown in figure 4.3.9, the virus–bacteria threshold density (above $10^{12}$ orders of magnitude for 1 ml of sample) was reached only in P(+) enclosures after 6 days of mesocosm incubation, while in the P(-) enclosure early dropped from $2.1 \times 10^{12}$ observed at the very beginning of the experiment ($t_0$) up to $10^{11}$ VBP ml$^{-2}$ levels.

Not only planktonic community dynamics strongly differed between phosphate-enriched and phosphate–limited samples, moreover different nutrient availability...
Results—substrate availability

cauased completely distinct production/degradation processes burned by bacterial population. A confirm that the growth of bacterial population in P(+) samples was mostly directed towards cell replication rather than biomass production is given by BCP (TdR / Leu) ratio that after t, incubation time resulted major than 1 and considerably increased (especially in one of the incubation bottles) over initial ratios that did not display marked differences between distinct nutrient availability (fig. 4.3.10).

![Graph](image)

**Fig. 4.3.8.** Virus-to-Bacterium Ratio (VBR) over incubation period in non treated (nt), phosphate depleted (P-) and phosphate repleted (P+) samples. Mean values of samples incubated in triplicate are reported for each treatment.

![Graph](image)

**Fig. 4.3.9.** Virus-to-Bacterium Product (VBP) over incubation period in non treated (nt), phosphate depleted (P-) and phosphate repleted (P+) samples: a) the same scale is used for all treatments; b) different scales are used for P(+) and P(-) samples. Mean values of samples incubated in triplicate are reported for each treatment.
Fig. 4.3.10. Proportion between bacterial carbon production estimated by radiolabelled nucleotides ($^{3}$H-TdR) and radiolabelled aminoacids ($^{3}$H-Leucine) incorporations in non treated (nt), phosphate depleted (P-) and phosphate repleted (P+) samples. Mean values (±st. dev.) of samples incubated in triplicate are reported for each treatment.

Both replication (fig. 4.3.11a) and biomass growth rates (fig. 4.3.11b) resulted clearly favoured in (P+) incubation condition: the $^{3}$H - TdR incorporation rate reached 20.5±0.5 µgC l$^{-1}$ h$^{-1}$ at $t_2$ sampling time becoming that way by 2 orders of magnitude higher than initially, while the maximum rate of bacterial biomass production at the same time exceeded by 50 fold its initial values, reaching 13.3±1.1 µgC l$^{-1}$ h$^{-1}$. Afterwards, high rates of bacterial carbon production (estimated both by $^{3}H$-TdR and $^{3}H$-Leu methods) lasted until the end of the experiment.

Beside the bacterial production rates, degradation rates sustained by main enzymes were assayed as well. Temporal trends of bacterial exoenzymatic activity rates resulted quite similar among lipidic (fig. 4.3.12a) and all glucidic enzymes ($\alpha$-glucosidase, $\beta$-glucosidase and $\beta$-galactosidase) over the entire duration of the experiment (fig. 4.3.12b-d). No difference in hydrolysis between different treatments was observed until $t_1$ incubation, when the activity rates in phosphate enriched samples sharply increased: 190-fold for lipase (up to 1996 nM h$^{-1}$), 584-fold for $\alpha$-glucosidase (up to 334 nM h$^{-1}$), 852-fold for $\beta$-glucosidase (up to 547 nM h$^{-1}$) and 202-fold for $\beta$-galactosidase (up to 322 nM h$^{-1}$). Afterwards, at the end of the experiment, only the lipidic activity resulted certainly lower while the glucidic hydrolyses were considered unvaried since the variability among replicates was higher than the variation between subsequent samples.

The hydrolysis rates of the same enzymes in P(-) samples also increased within $t_1$ and $t_3$ incubation interval, but with much moderate intensity and showing quite the same order of variation (by 46-fold for lipase, 33-fold for $\alpha$-glucosidase, 26-fold for $\beta$-glucosidase and 37-fold for $\beta$-galactosidase).

The most active enzyme was phosphatase (fig. 4.3.12e), reaching the highest hydrolysis rate, equal to 673 nM h$^{-1}$, in phosphate-limited samples after 6 days of
incubation (t₁), thus suggesting the beginning of nutrient limitation. After further 3 days of incubation the phosphatase activity drop by 1 order of magnitude was observed. During the same incubation interval in P(-) samples the variation in phosphatase activity was also observed decreasing from 410 nM h⁻¹ to 169 nM h⁻¹.

The protease (leucine-aminopeptidase) activity in both treatments increased only during the last days showing particularly high hydrolysis rate of 9567±3498 nM h⁻¹ in P(+) samples towards the end of the experiment while at the same time only a slight increase characterized P(-) environment (fig.4.3.12f).

The particularly high proportion of inorganic nitrogen versus inorganic phosphorous determined in field study in the Gulf of Trieste (C1 station) from January 2000 to September 2005 is reported in figure 4.3.13.
Fig. 4.3.12. Bacterial exoenzymatic activities in phosphate depleted (P-) and phosphate repleted (P+) samples were examined: a) lipase; b) α-glucosidase; c) β-glucosidase; d) β-galactosidase; e) alkaline-phosphatase and f) leucine-aminopeptidase. Mean values of samples incubated in triplicate are reported for each treatment.
Fig. 4.3.13. Time series of dissolved inorganic nitrogen (DIN) to dissolved inorganic phosphorous from January 2000 to September 2005 in the Gulf of Trieste (C1 coastal station). Full line represents the Redfield value for the optimal nutrient proportion specific for phytoplankton community uptake.

Discussion
The results of this mesocosm experiment confirmed that the phosphorous deficiency controls the development of phytoplankton community and primary production. Lower phytoplankton biomass production determines completely different microbial food web functioning if compared to the incubation condition with an external nutrient supply. In nutrient limited enclosures bacterial abundances remained rather stable throughout the entire incubation period without considerable shifts towards active metabolism. Both bacterial production and degradation processes did not display any significant variation throughout the course of the experiment, except bacterial exoenzymatic phosphatase activity, which resulted particularly driven in order to provide inorganic phosphorous. In addition, it has been already documented (Obernoster and Herndl, 1995) that bacteria cannot efficiently utilize extracellular products released from phosphate – limited phytoplankton, since their metabolism is affected by the same phosphorous limitation. Therefore in moderate bacterial abundance condition also the viral population results rather stable and lower viral infection frequencies occur (Steward et al., 1992b; Weinbauer et al., 1993; Steward et al., 1996; Noble and Fuhrman, 2000; Almeida et al., 2001; Guixa-Boixereu, 2001; Middelboe et al., 2002; Weinbauer et al., 2003), unable to sustain lytic proliferation strategy. Wilson et al. (1996) reported the lysogenization of Synechococcus cells triggered by low phosphate availability. The extremely high VBR found in phosphate-enriched samples towards those registered in phosphate-depleted condition, which remained within a range of values usually determined in Adriatic Sea field studies (Bensi et al., 2003; Corinaldesi et al., 2003; Stopar et al., 2003) indicates the presence of particularly abundant viral population in free seawater surrounding and suggests the occurrence of lytic viral proliferation. Moreover, different studies reported (Farrah, 1987; Moebus,
1987; Williams et al., 1987; Williams, 1994) that the nutritional or metabolic status of the host results determining for viral infection and proliferation by affecting its critical vital steps such as adsorption, replication, lytic activity and survival. Lenski (1988) has shown that maximum proliferation rates and yield of phages in culture studies are observed at optimum growth conditions of the host. The strong dependence on host metabolism is probably due to the increased latent periods and to reduced burst size in condition of low nutrient availability (Kokjohn et al., 1991; Proctor et al., 1993; Guixa-Boixereu et al., 1996; Wilson et al., 1996; Middelboe, 2000).

The results obtained from the nutrient enriched enclosures confirmed the potential microbial food web dynamics in high productivity systems: phytoplankton community resulted particularly productive enhancing the development of the bacterial population (especially of its active fraction). Glucidic enzymes of bacterial population operated at high hydrolysis rates in order to recycle extracellular carbohydrate - exudates produced by copious phytoplankton community. Intense bacterial production directed prevalently to replication rather than to biomass production increased the density of bacterial cells on levels that particularly favoured driven lytic virus proliferation, acting thus as positive feedback control on bacterial population. High rates of protease hydrolysis observed towards the end of the experiment suggest the enhanced polipeptidic hydrolysis potential that was probably activated because of the presence of overdeveloped planktonic community that began to starve and degrade. Since the lytic viral proliferation unavoidably destroys bacterial cells, the enrichment in DOC in phosphate-enriched samples is almost partially attributable to enhanced viral proliferation. A part of the DOC increase could be also due to the phytoplankton primary production. However the produced DOC seems to be particularly suitable for the activating and the proliferation of highly active bacteria (up to 42% within total bacterial community) largely exceeding the proportions obtained from field studies (in Adriatic and elsewhere) where active bacterial population remains usually below 10% and never exceeds 20% of total bacteria (Karuza et al., 2004; Paoli et al., 2006). Puddu et al. (2003) observed rapid activation of bacteria in nutrient-balanced mesocosm, even if based on different detection technique. Choi et al. (1999) demonstrated that bacteria are characterized by higher growth rates in enriched environments and become dormant or starving when substrate conditions drop below the optimum amount.

Conversely to high N:P ratios observed throughout the year in the Northern Adriatic Sea relatively to optimal nutrient uptake proportion by phytoplankton community, our study simulated the phosphorous – balanced condition and pointed out that nutrient limitation affects the entire microbial plankton community reducing the eutrophication level through the control of phytoplankton production. On the basis of our results and considerations of other authors (Wilson et al., 1996, 1998) we suppose that the concept of nutrient limitation in this experiment affected also directly bacterial and viral
populations, whether the addition of inorganic nutrients stimulates the virus production (Hewson et al., 2001; Bongiomi et al., 2005). In fact, since bacteria and viruses are structurally characterized by particularly high proportion of nucleic acid versus proteic biomass, they require higher phosphorous supply. Thus, if the optimal proportion for phytoplankton growth is given by Redfield’s ratio, which N:P in 16:1, then the optimal nutrient proportion for microbial components certainly favours major phosphorous availability.
4.4. MICROBIAL POPULATIONS' DYNAMICS IN DIFFERENT AVAILABILITY OF ORGANIC SUBSTRATE

4.4.1. ORGANIC MATRIX PRODUCED BY PHYTOPLANKTON BLOOM

A 45 days lasting experiment was set up in order to follow viral and bacterial populations dynamics in availability of organic matter produced by phosphate - limited P(-) and phosphate - enriched P(+) phytoplankton growth from the experimental design previously described (see Section 3.3.). Moreover, in order to verify the bioavailability of released organic matter by freshly added natural bacterial community (bacterial inoculum), the organic matter was dimensionally (or according to molecular weight) divided into filtered (<0.2 μm) and ultrafiltered (<30 kDa) fractions. In addition, a sample of seawater surrounding (1 μm-pore size prefilted) the bacterium inoculum community was incubated in parallel as a control.

In general, the major variations of all parameters assayed were displayed during the first week of incubation and then, for the rest of the experiment remained rather stable.

The DOC concentration in P(+) incubation enclosures, varying from 1.3 to 2.8 mg l⁻¹, remained markedly higher during the entire incubation period, exceeding over the entire experiment by approximately 2-fold the non enriched samples, which remained constantly below 1.2 mg l⁻¹ (fig. 4.4.14). In particular, within the phosphate-enriched mesocosms, sample with higher molecular weight substrate initially displayed particularly high DOC concentrations up to 2.8 mg l⁻¹ registered at t₁ incubation time and afterwards dropped to the values proximal (even if constantly slightly higher) of the low molecular weight DOC.

![DOC concentrations are followed over incubation period in distinct dimensionally-fractioned organic matter availability and in a control. Mean values (±st. dev.) of triplicate determinations are reported for each treatment.](image-url)
Results – substrate availability

With the exception of filtered P(+) sample, where the abundance of total bacteria already after 1 day of the beginning of the experiment (t_1) showed an increasing trend which reached its maximum of $2.6 \times 10^9$ cells l^{-1} after further 3 days (t_4), bacterial abundances in other samples resulted rather similar remaining always at least 1 order of magnitude lower (fig. 4.4.15).

![Graph](image)

**Fig. 4.4.15.** Total bacterial abundances are followed over incubation period in distinct dimensionally-fractioned organic matter availability and in a control. Mean values (± st. dev.) of triplicate determinations are reported for each treatment.

As can be observed in figure 4.4.16, exactly between the second (t_2) and fourth (t_4) day of incubation, bacterial population was largely dominated by its active fraction, which sharply increased and finally culminated at t_4 incubation time when the entire bacterial population displayed highly active metabolism.

![Graph](image)

**Fig. 4.4.16.** Proportion of metabolically active bacteria within total bacterial community is followed over incubation period in distinct dimensionally-fractioned organic matter availability and in a control. Mean values of triplicate determinations are reported for each treatment.
However, the higher levels of DOC concentrations determined enhanced development of metabolically active bacterial population in P(+) - derived samples (fig. ), but with higher shifts towards active metabolism in samples containing DOC substrate of lower molecular weight.

![Graph](attachment://image.png)

**Fig. 4.4.17.** Abundances of metabolically active bacteria are followed over incubation period in distinct dimensionally-fractioned organic matter availability and in a control. Mean values (±st. dev.) of triplicate determinations are reported for each treatment.

Also the trend of viral abundances displayed markedly higher values relatively to the P(+) - derived substrate (fig.4.4.18). Until the first 24 hours of incubation there were no significant differences in viral abundances between samples characterized by distinct organic matter molecular weight. In both samples densities of $10^{11}$ particles $l^{-1}$ were reached at $t_i$ incubation time.

![Graph](attachment://image.png)

**Fig. 4.4.18.** VLP abundances are followed over incubation period in distinct dimensionally-fractioned organic matter availability and a control. Mean values (±st. dev.) of triplicate determinations are reported for each treatment.
From then on, their abundances strongly differed: in samples with ultrafiltered DOC substrate viruses dropped and remained rather stable (varying within this 45-days interval between $1.3 \times 10^{10}$ and $4.5 \times 10^{10}$ particles l$^{-1}$) until the end of the experiment, when completely recovered to the initial densities. In sample with major molecular weight of organic matter high viral abundances (with values above $10^{11}$ particles l$^{-1}$) persisted for further 22 days and sharply decreased (to $1.2 \times 10^{10}$ particles l$^{-1}$) at $t_6$ incubation time. An increasing trend was newly observed towards the $t_8$ sampling time. Finally, at the end of the experiment, viral abundances decreased up to $6.7 \times 10^{10}$ particles l$^{-1}$.

![Graph 1](image1.png)

**Fig. 4.4.19.** Virus-to-bacterium ratio (VBR) over incubation period in distinct dimensionally-fractioned organic matter availability and a control. Mean values of triplicate determinations are reported for each treatment.

![Graph 2](image2.png)

**Fig. 4.4.20.** Virus-to-bacterium product (VBP) over incubation period in distinct dimensionally-fractioned organic matter availability and a control. Mean values of triplicate determinations are reported for each treatment.
Independently of the substrate’s molecular weight, VBR values in P(+) derived samples displayed considerably higher values (mean=63, median=62) up to the maximal 172 than in non enriched samples (mean=10, median=6) over the entire incubation period (fig. 4.4.19). As can be observed from figure 4.4.20, the phosphate enrichment affected also the virus–host density, which was examined by VBP index. The highest VBP trend interested P(+) derived samples characterized by high molecular weight DOC with values that during the entire experiment persisted above $10^{14}$ ml$^{-2}$, with the exception of $t_6$ sampling time when VBP dropped to $2.0 \times 10^{13}$ ml$^{-2}$. VBP trend calculated for ultrafiltered P(+) substrate showed much greater variability ranging between high initial values proximal to $10^{14}$ ml$^{-2}$ and minor densities (of $10^{12}$) that persisted until the final recover observed at the end of the experiment ($1.5 \times 10^{14}$ ml$^{-2}$).

**Discussion**

For all mesocosms in general, the major variations of all parameters assayed were observed during the first week of incubation and then, for the rest of the experiment remained rather stable. Actually, the DOC concentration produced by P(+) incubations remained markedly higher during the entire incubation period, exceeding by approximately 2-fold the non enriched samples. Higher molecular weight sample showed slightly higher DOC concentration values, probably because of the partial DOC retention of the other phosphate-enriched sample during the ultrafiltration step.

Relatively to the higher molecular weight substrate in phosphate enriched sample, it is interesting to outline that the increases of viral abundances observed at $t_1$, $t_6$, and $t_7$ sampling times followed the peaks of abundances in total bacteria. In fact, as can be observed from figures 6 and 7, the trends of VBR and VBP indexes indicated particularly high exceed of viral population over bacterial one with high viral densities certainly derived by lytic viral proliferation. VBP index in ultrafiltered DOC sample displayed something lower values for the entire incubation period but however above $10^{13}$ particles l$^{-1}$, indicating the enhanced lytic proliferation. It is interesting to observe that the VBP matched rather faithfully those observed both for viral and bacterial abundances. From the comparison of viral and bacterial abundances with their abundances product (VBP), the decline of virus abundances at $t$, incubation time was certainly due to the rapid decrease of total bacterial population. Thus, a consequent decrease in virus - host density (observed from VBP index) considerably influenced viral life strategy with minor frequency of lytic proliferations. Therefore, this experiment is the prove that in some cases the encounter rate between viruses and their hosts could be determining for free-virus abundances rather than the metabolic state of host cell. In fact, the active bacterial population just in the same period was increasing its abundances, displaying completely opposite trend to the total bacterial population.

Our results pointed out that in the same condition of nutrient availability the major intensity of degradation processes (Larato, 2005) occurred in incubation with high molecular weight organic matter. On the contrary, the lower weight fraction seemed
able to induce the shift in bacterial physiology state towards active metabolism sustaining enhanced proliferation of the active population. The intensive replication was confirmed also by high bacterial secondary production, which was prevalently directed towards abundance increment (Larato, 2005).

4.4.2. ORGANIC MATTER BY RIVERINE ORIGIN

The mesocosm experiment was carried out in order to verify the impact of freshwater organic matter of fluvial origin onto microbial food web functioning. In particular viral control over bacterial population was followed in freshwater enriched mesocosm and in a control one (natural seawater sample from C1 coastal station) over 2 weeks incubation period. In order to characterize the natural microbial community present in C1 station prior to the beginning of the experiment (t₀), a seawater sample was collected in the field the day before (non treated) and the obtained data were used as background values.

The abundance of total bacteria in non-treated sample with a value of $1.3\pm0.3 \times 10^9$ cells l⁻¹ resulted within range of bacterial density usually registered for the summer season. As can be observed from figure 4.4.21, the initial experimental set up did not affect bacterial abundances, which thereafter remained stable for 4 days (until $t_1$ incubation time). After 7-days ($t_2$) first differences, between mixed sample and a control were registered: in a freshwater enriched treatment bacteria slightly decreased to $8.8\pm0.5 \times 10^8$ cells l⁻¹ ($t_2$) and did not vary until the end of the experiment. In a control sample the abundances remained rather stable over the entire course of the experiment. According to the ANOVA assessment no significant difference between mixed and control bacterial distributions was observed ($F=2.0$, $n=10$).

Fig. 4.4.21. Abundances of total bacteria are followed at the beginning ($t_0$) and throughout the experiment ($t_1, t_2, t_3$ and $t_4$) in the freshwater enriched sample (mixed) and in a control (C1 station). Non-treated sample (nt) is reported as background. Mean values ($\pm$ st. dev.) of triplicate determinations are reported for each treatment.
Mixing with the freshwater sample induced significant changes in active bacterial abundance (F=6.6, p<0.05 according to ANOVA). As can be observed from fig. 4.4.22, treatments at the beginning of the experiment immediately determined an increase of active bacterial cells, from 9.9±0.9 × 10^7 cells l^-1 to 6.9±0.7 × 10^8 cells l^-1 both in the mixed and in the control mesocosms. The density of the active bacteria subsequently remained unvaried until the end of the experiment, when the abundance in the control mesocosm sharply increased up to 5.8±0.3 × 10^8 cells l^-1.

Fig. 4.4.22. Abundances of metabolically active bacteria are followed at the beginning (t₀) and throughout the experiment (t₁, t₂, t₃ and t₄) in the freshwater enriched sample (mixed) and in a control (C1 station). Non-treated sample (nt) is reported as background. Mean values (±st. dev.) of triplicate determinations are reported for each treatment.

Fig. 4.4.23. Proportion of metabolically active bacteria within total bacterial community is followed at the beginning (t₀) and throughout the experiment (t₁, t₂, t₃ and t₄) in the freshwater enriched sample (mixed) and in a control (C1 station). Non-treated sample (nt) is reported as background. Mean values of triplicate determinations are reported for each treatment.
From the comparison of temporal distribution of active bacteria reported in figure 4.4.22 and the trend of the proportion of active bacterial cells within total bacterial population reported in figure 4.4.23, similar temporal distributions can be observed. With the exception of the initial proportion of 17% of active cells within total bacterial population, the percentage of active bacteria in the mixed sample remained rather stable setting around 7%, and matched with the proportion found in the field (nt) prior of the beginning of the experiment and remaining within the values normally observed for the study area (Karuza, 2004; Paoli et al., 2006). If compared to the control sample, the proportion resulted rather similar for the entire incubation period. Only at the end of the experiment marked difference was observed, when the proportion of active cells in the control reached 29% towards 5% in the mixed mesocosm.

Despite lower sampling frequency for the determining of viral abundances, no marked oscillations can be observed during the course of the entire incubation period (fig.4.4.24) and no significant differences (F=2.4, n=7) between the experimental sample and a control estimates were found. Only initially (t₀) a slight increase of viral abundance (5.9±0.07 \times 10^9 \text{ particles l}^{-1}) relatively to the non-treated sample (4.6±0.3 \times 10^9 \text{ particles l}^{-1}) was observed. After 4 days (t₁) the viral abundance resulted around 2.9±0.1 \times 10^9 \text{ particles l}^{-1} and at the end of the experiment both samples recovered to 5.2±0.4 \times 10^9 \text{ particles l}^{-1}.

Also if not statistically significant (according to ANOVA), slightly higher bacterial carbon production rates in mixed rather than in control mesocosm over the entire incubation period can be observed. The production rates initially resulted rather stable
Fig. 4.4.25. Bacterial carbon production assayed by (a) $^3$H-TdR and (b) $^3$H-Leu incorporation is followed at the beginning ($t_1$) and throughout the experiment ($t_2$, $t_3$, and $t_4$) in the freshwater enriched sample (mixed) and in a control (C1 station). Non-treated sample (nt) is reported as background. Mean values ($\pm$ st. dev.) of triplicate determinations are reported for each treatment.

and then slightly increased to the maxima values of $1.0\pm0.1$ igC l$^{-1}$ h$^{-1}$ for the $^3$H-TdR incorporation (fig.4.4.25a) registered at $t_2$ incubation time and $0.9\pm0.04$ igC l$^{-1}$ h$^{-1}$ for $^3$H-Leu incorporation (fig.4.4.25b) at $t_3$ incubation time.

The BCP ratio between $^3$H-TdR and $^3$H-Leu incorporations reported in figure 4.4.26, being lower than 1 over the entire incubation, indicates higher intensity of bacterial biomass production rather than replication processes. However, according to ANOVA no significant differences ($F=1.31$, $n=10$) between mixed and control samples were observed.
Results—substrate availability

Discussion

Our results outlined that neither the enrichment with the fluvial organic matter nor the salinity reduction substantially affected microbial community and the microbial loop functioning. Only the active fraction of bacterial community slightly differed from the control, especially relatively to the final incubation period when the active population in the control recovered exceeding the initial values. This increase of the active fraction towards the end of the experiment was probably driven by the recycled organic matter, even if the estimate of bioavailable DOC (B-DOC) did not evidenced significant increase in refractory concentration within mixed mesocosm incubation (p.c. De Vittor). Bacterial carbon production resulted mostly directed to biomass production rather than replication processes, with peak of $^{3}$H-TdR incorporation rate that matched to the maximum value of the proportion of active cells observed after 1 wk of incubation time. Despite the lack of direct estimates of virus infection rates, the persistence of relatively high viral abundances over the entire incubation period suggest the presence of viral lysis onto bacterial community. Although the virus–bacteria density observed by VBP index resulted above the threshold value of $10^{12}$ ml$^{-2}$ during the experimental lasting, the lytic processes certainly occurred (otherwise could not sustain high abundance levels) but could resulted reduced, due to the slightly different host community structure as reported by Larato (2005) relatively to the same experiment. The change in community composition surely reduced the encounter rates among specific viruses and their hosts, with a consequence of minor infection rates and therefore determining minor control on bacterial population, which however resulted prevalently 'bottom-up'-controlled. Although the DOC concentrations data for this
experiment are not available, we suppose that the increase of active bacterial abundance that matched with the increase of the proportion of active bacteria within total bacterial population (up to 29%) after 2 week of mesocosm incubation was almost partially supported by DOC originated by bacterial lysis. The recycled DOC probably resulted bioavailable for the uptake by active bacterial population that incremented its abundance towards the end of the experiment, as confirmed also by the increase of bacterial replication rate registered at t₃ sampling time. Despite the evidence that microscopic assay of phytoplankton community revealed the dominance of small diatoms within mixed sample and Gonyaulax fragilis in the control (Larato, 2005), different compositions between mixed mesocosm and the control did not affect the functioning of the microbial loop.
4.5. ESTIMATES OF VIRUS PRODUCTION

4.5.1. PRELIMINARY EXPERIMENTS FOR ESTIMATING VIRUS PRODUCTION

Experiment 1

The experiment was set up to determine optimal incubation periods and to verify the influence of inorganic nutrients availability on incubation intervals for the estimate of virus production.

The result of the label incorporation into virus DNA over the incubation period is reported in figure 4.5.1. As it can be observed, the trends of radiolabel incorporation between phosphate-repleted (P+) and phosphate-depleted (P-) incubation bottles completely differ: whether at $t_{16}$ incubation time the virus production in the condition of phosphate limitation just began (34 DPM ml$^{-1}$), reaching its maximum when experiment ended (190 DPM ml$^{-1}$), on the contrary, in the phosphate enriched sample at $t_{16}$ incubation time the incorporation resulted the highest (412 DPM ml$^{-1}$).

Abundances of VLP were determined in parallel at the same sampling periods used for the measurement of radiolabel incorporation. From the results reported in figure 4.5.2 rather similar slopes between VLP abundances dynamics and radiolabel incorporation trends can be observed. In the phosphate enriched sample VLP over the 16 h incubation interval was doubled, reaching $1.1 \times 10^{11}$ particles l$^{-1}$, the value that surely exceeds usual VLP density registered in the field studies. Subsequently, at 18 h incubation time VLP abundance slightly decreased to $1.0 \times 10^{11}$ particles l$^{-1}$. Something similar to the radiolabel incorporation reported above, the dynamics of VLP abundances in the phosphate-depleted (P-) sample showed slightly

Fig. 4.5.1. Comparison of radiolabel incorporation into virus DNA (DPM ml$^{-1}$) between phosphate-repleted (P+) and phosphate-depleted (P-) incubation bottles. Samples were analysed initially ($t_0$) and at 16 h ($t_{16}$) and 18 h ($t_{18}$) time periods.
Results—viral production

Fig. 4.5.2. Change in VLP abundance during incubation of phosphate-repleted (P+) and phosphate-depleted (P-) seawater samples.

increasing trend registering $5.2 \times 10^9$ particles l$^{-1}$ at $t_{18}$ sampling time. The, just two hours later at $t_{18}$ sampling time, abundances increased for about 20% reaching $1.0 \times 10^{11}$ particles l$^{-1}$.

Conversion factors (CF) for the number of viruses produced per 1 mole of $^3$H-TdR were calculated both for phosphate-repleted (P+) and phosphate-depleted (P-) samples. The CF factors resulted of $10^{23}$ orders of magnitude with very little difference between P+ ($1.0 \times 10^{23}$) and P- ($1.2 \times 10^{23}$) sample incubations.

Using the relative conversion factors, rates of virus production were calculated. Considering different incubation intervals virus production rates varied from 2.5 to $3.0 \times 10^{11}$ l$^{-1}$ h$^{-1}$ viruses produced in the phosphate-repleted sample, while in the phosphate-depleted sample production rates were slower, ranging between 3 and $15 \times 10^{10}$ l$^{-1}$ h$^{-1}$ viruses.

Experiment 2

Since the first experiment for the estimate of virus production in the Gulf of Trieste (Experiment 1) did not give any information relatively to the number of viruses produced up to the maximum slope, which corresponded to the first incubation interval, another experiment performed on in field samples, was set up in order to evaluate virus production within the first 16 h of incubation.

Both radiolabel incorporation into viral DNA (fig.4.5.3) and virus abundances (fig.4.5.4), which were followed up to 20 h of incubation at smaller intervals ($t_0$, $t_{13}$, $t_{15}$, $t_{16}$, $t_{18}$, and $t_{20}$), showed a similar trend during the incubation period. The increase in incorporated label was observed already at the first sampling ($t_{13}$), reaching its maximum of 18.4 DPM ml$^{-1}$ after 15 h of incubation. Then, at $t_{15}$ much lower label incorporation (3.3 DPM ml$^{-1}$) was registered, which lasted till $t_{18}$ incubation time (3.7 DPM ml$^{-1}$). At
the end of incubation (t\textsubscript{20}) the incorporation speed recovered reaching 16.6 DPM ml\textsuperscript{-1}. The changes in VLP abundances showed similar trend to the label incorporation for the entire incubation period varying from 2.6 to 2.8 × 10\textsuperscript{6} ml\textsuperscript{-1} between t\textsubscript{13} and t\textsubscript{15} sampling times, followed by a decrease (1.1 × 10\textsuperscript{6} ml\textsuperscript{-1}) that last up to t\textsubscript{18} (1.3 × 10\textsuperscript{6} ml\textsuperscript{-1}) and recovered to 2.4 × 10\textsuperscript{6} ml\textsuperscript{-1} at t\textsubscript{20} incubation time.

Using the conversion factor of 6.7 × 10\textsuperscript{25} viruses produced mole\textsuperscript{-1} \textsuperscript{3}H-TdR, specifically calculated for this experiment, the virus production rates resulted approximately 1.5–1.6 × 10\textsuperscript{12} viruses l\textsuperscript{-1} h\textsuperscript{-1}.

Conversion factors calculated in these preliminary experiments for the estimate of virus production exceeded by 2–4 orders of magnitude those reported by Steward et al. (1992a, 1992b)

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**Fig. 4.5.3.** Radiolabel incorporation into virus DNA (DPM ml\textsuperscript{-1}) at t\textsubscript{13}, t\textsubscript{15}, t\textsubscript{16}, t\textsubscript{18}, and t\textsubscript{20} incubation times.

**Fig. 4.5.4.** Change in VLP abundance during incubation period at t\textsubscript{13}, t\textsubscript{15}, t\textsubscript{16}, t\textsubscript{18}, and t\textsubscript{20} sampling times.
and therefore also the virus production rates resulted distinctly higher than the results published by other authors (Steward et al., 1992a, 1992b; Steward & Azam, 1996; Weinbauer & Höfe, 1998; Noble & Fuhrman, 2000; Wilhelm et al., 2002). The difference could be due to the epifluorescence microscopy determination that evidences not only bacteriophages but virus-like particles as well, although it is generally recognized that the TEM determination probably represents an underestimate of free-virus number (Weinbauer et al., 2002).

Considering that the virus abundance depends on virus production and virus decay rates (virus abundance = virus production - virus decay), similar trends that were registered between changes in incorporated radiolabel and virus abundances over incubation period pointed out that virus decay did not interfere on virus abundance, i.e. was not present (virus abundance = virus production), or was constant during the entire incubation time (virus abundance = virus production \times k).

4.5.2. COMPARISON AMONG DIFFERENT METHODOLOGICAL APPROACHES FOR VIRUS PRODUCTION

Virus production was assayed on the same seawater sample using 3 different experimental approaches in order to compare the accuracy of the obtained results and to establish the usefulness of a single technique.

4.5.2.1. Serial dilution technique for estimating viral production in manipulated phage-host assemblage

The product of virus and bacteria abundances resulted $8.9 \times 10^{12} \text{ml}^{-2}$. Therefore the threshold value of $10^{12} \text{ml}^{-2}$ particles density, which ensures a successful contact rates for the prevalence of lytic life strategy was achieved.

In figure 4.5.5, bacterial abundances in serial dilution performed at the beginning (t₀) of the experiment are reported. The regression coefficient of $r=0.94$ (n=10, p<0.001) calculated on the bacterial data verified the expected proportion of diluted samples towards a whole (undiluted) bacterial sample.

The result of the impact of viral lysis on bacterial community abundance using the dilution approach is presented in figure 4.5.6. The regression coefficient of $r=0.97$ (n = 14, p<0.001) confirmed that as the proportion of a whole sample increases (undiluted) versus 1.0, the difference between bacterial abundance at t₀ (0 h) and t₂₄ (24 h) decreases, showing a higher impact of viral lysis on bacterial mortality.

Burst size obtained from the antibiotic experiment resulted in an average of 13.7 virions released per bacterial cell. During the 1 h incubation period, viral abundance increased from $1.3 \pm 0.1 \times 10^{10} \text{particles litre}^{-1}$ to $1.7 \pm 0.1 \times 10^{10} \text{particles litre}^{-1}$, while the bacterial abundance decreased from $1.2 \pm 0.07$ to $0.94 \pm 0.08 \times 10^9 \text{cells litre}^{-1}$.

Using the obtained burst size, the net production rate calculated over the 24 h of incubation resulted in $1.5 \times 10^9 \text{viruses litre}^{-1} \text{hour}^{-1}$.
4.5.2.2. Radiotracer technique for measurement of viral production

Viral abundance demonstrated an increasing trend over time from $5.4 \times 10^9$ ($t_0$) through $9.8 \times 10^9$ ($t_{12}$) and finally to $21.5 \times 10^9$ particles ($t_{15}$) while bacteria varied consistently only between $t_0$ and $t_{12}$ incubation points (tab.4.5.1).

Since only periods when both virus abundance and incorporated radiolabel (tab.4.5.2.) are increasing can be used for determining a conversion factor, only the $t_0$ and $t_{12}$ points were considered.

The net increase in viruses determined by EM was divided by the net increase in moles of incorporated TdR to obtain a specific conversion factor of $6.1 \times 10^{24}$ during the first 12 hours of incubation.
The increasing slope of $^3$H-TdR incorporation occurred only in the first 12 hours of the experiment, while the increasing trend of viral abundances was found in the entire incubation interval. The virus production rate obtained within the first 12 h using the specific conversion factor resulted in $5.1 \times 10^{11}$ litre$^{-1}$ hour$^{-1}$ while the production rate calculated for the entire incubation period was approximated to $3.6 \times 10^{11}$ litre$^{-1}$ hour$^{-1}$, including also the t$^{12}$-t$^{15}$ period when decay of viral particles occurred.

A comparison of rates of virus production estimated using the specific (A) EM abundance determination and non specific (B, C) conversion factors obtained by TEM counts published by Steward et al. (1992a, 1992b) are reported in table 4.5.2.

<table>
<thead>
<tr>
<th></th>
<th>virus L$^{-1}$</th>
<th>bacteria L$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>5.44E+09</td>
<td>1.06E+09</td>
</tr>
<tr>
<td>12 h</td>
<td>9.77E+09</td>
<td>6.13E+08</td>
</tr>
<tr>
<td>15 h</td>
<td>2.15E+10</td>
<td>6.34E+08</td>
</tr>
</tbody>
</table>

Tab. 4.5.1. Viral and bacterial abundances over a 15 h incubation period. Abundances at 0, 12 and 15 h of incubation periods are reported.

<table>
<thead>
<tr>
<th></th>
<th>$^3$H-TdR incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPM</td>
</tr>
<tr>
<td>0 h</td>
<td>0</td>
</tr>
<tr>
<td>12 h</td>
<td>40.1</td>
</tr>
<tr>
<td>15 h</td>
<td>28</td>
</tr>
</tbody>
</table>

Tab. 4.5.2. Difference of DPM and moles of $^3$H-TdR incorporated at 0, 12 and 15 h incubation points are reported.

### 4.5.2.3. Dilution technique for estimating viral production in already infected bacteria

Bacterial and viral abundances in the natural assemblage resulted $1.1 \pm 0.1 \times 10^9$ cells l$^{-1}$ and $5.4 \pm 0.3 \times 10^9$ particles l$^{-1}$ respectively. After the filtration onto 0.2 μm and subsequent rinsing of the retentate with virus-free water, bacterial and viral abundances were reduced to 37% and 50% respectively.

In Figure 4.5.7 viral and bacterial abundances are reported over the incubation time. Only intervals with an increasing trend of viral abundance were considered for
calculating the rates of virus production. Three different virus proliferation periods can be observed: the first 3 h of incubation time (t₀-t₃), between t₆ and t₉, and finally the last 3 h of incubation (t₁₂-t₁₅). Bacterial abundance showed the highest value \((4.2±0.3 \times 10^8 \text{ cells l}^{-1})\) at the very beginning \((t₀)\) and reached the maximum value after the first 3 h of incubation. During this period the maximum burst size, which corresponds to 57 virions released per bacterial cell, was observed. Subsequently bacteria and viruses showed a similar trend of their abundance.

It can be observed the balancing of viral production followed by an immediate 3 h lasting decay, which maintains the viral standing stock at the average value of \(8.2±0.07 \times 10^9 \text{ particles l}^{-1}\).

For calculating the rate of viral production the correction factor of 2.7 for bacterial loss due to filtration step was applied.

**Fig. 4.5.7.** Viral and bacterial abundances during 15 h incubation period of an experimental design for estimating viral production in already infected samples. Abundances in natural seawater community are reported at nt (non treated) point both for viruses and for bacteria.

**Fig. 4.5.8.** Virus production rate calculated for three incubation intervals in which virus proliferation occurred \((t₀-t₃, t₆-t₉, \text{ and } t₁₂-t₁₅)\).
An attenuation of production/decay processes was registered as the incubation proceeded (fig. 4.5.7), with a decreasing trend of virus production rate from $2.1 \times 10^9$ viruses litre$^{-1}$ hour$^{-1}$, recorded at $t_3$, to $4.8 \times 10^8$ viruses litre$^{-1}$ hour$^{-1}$, observed at $t_{15}$ incubation point (fig. 4.5.8).

Discussion

Our results pointed out the strengths and the lacks of three experimental designs used for the estimate of virus production specifically measured for our study area. The comparison among these methods was possible only in the environmental condition where the occurrence of the lytic phage proliferation strategy was assumed. From the pluriannual monitoring study of virus abundance dynamics in the Gulf of Trieste (Karuza & Del Negro, 2006) we experienced the reaching of phage-host density threshold value for the period that we selected for the present study. On the contrary, lower phage-host densities, which characterize our study area in vernal periods (see Section 4.2), did not provoke bacterial mortality based on the incidence of lytic infection.

Methodological approach of virus production using the incorporation of radiolabelled nucleotides into viral DNA included the highest number of critical steps. Virus production rates obtained using in situ-determined specific conversion factor of $6.1 \times 10^{24}$ virus mole$^{-1}$ $^3$H-TdR, if compared to the other two methods, produced certainly an overestimation. The overestimation is principally due to the EM determination that evidences not only bacteriophages but virus-like particles as well. Although different authors argued that the TEM determination probably represents an underestimate of free-virus number (Weinbauer et al., 2002), when the non specific TEM-determined conversion factors were applied, the value of $5.0 \times 10^8$ litre$^{-1}$ hour$^{-1}$ obtained from our experiment produced much more reliable production rate, especially if compared to results obtained by the other two experimental designs and to the results published by other authors (Noble & Fuhrman, 1998, 2000; Steward et al., 1992, 1996; Weinbauer & Höfle, 1998; Wilhelm et al., 2002).

The experimental technique for the estimate of viral production from already infected bacterial cells gave a detailed scenario of production/decay processes throughout incubation period. However the decrease of free-virus particles probably concerns both virus decay as well as their disappearance due to virus penetration into the hosts' cells. In order to minimize the effect of the UV radiation as the most important virus decay factor (Fuhrman & Suttle, 1993; Wommack et al. 1996; Noble & Fuhrman, 1997; Weinbauer et al. 2002) the sample incubation was carried in the dark. Only the first and at the same time the most marked production event could be considered as the pure production result since the following two abundance increases include, at least partially, the release of particles induced by new viral infection. It is interesting to observe that the virus production occurred already during the first 3 hours of incubation with the rate of $2.1 \times 10^9$ particles litre$^{-1}$ hour$^{-1}$ and that such intensity of the
process was capable to double the abundance of free virus particles. For a correct comparison to the other methods that we simultaneously assayed, the rate of virus production for the entire incubation period should be considered. The net production rate using this experimental design for 15 hours incubation period was approximated to $4.8 \times 10^8$ litre·hour$^{-1}$ while for a $^{3}$H-TdR-incorporation method the same calculation equalled to $3.5 \times 10^8$ litre·hour$^{-1}$. These similar results seem to be reliable although it is quite probable, as it has been already discussed by other authors, that the $^{3}$H-TdR-incorporation approach underestimates the real viral production. The underestimation is mainly due to the loss of material during such numerous treatments and because the method involves only the production carried by DNA viruses. However the virus production assay of already infected bacteria revealed that the stability of free-virus population in a given moment is only apparent and it is the result of extremely dynamic balancing processes determined by virus production and decay.

The technique of serial dilution in manipulated phage-host assemblage is the single approach that measures viral production in the entire incubation period lasting 24 hours and without considering different incubation intervals. The obtained results confirmed our expectations regarding the impact of high viral abundance on bacterial population mortality in the given period, that were based on pluriannual study of viruses and bacteria seasonal fluctuations (Del Negro et al., 1996; Fonda Umani, 2001; Paoli et al., in press) and the threshold value ($10^{12}$ ml$^{-2}$) of phage-host density product. The bacterial mortality in a phage-host assemblage resulted consistent, with a coefficient of bacterial lysis $g=1.94$, which resulted comparable to the bacterial mortality induced by grazing of heterotrophic nanoflagellates reported by Fonda Umani and Beran (2003) for the August 1999 regarding the same study area. The success of the experiment confirmed that in the summer season viruses were characterized by lytic rather than lysogenic proliferation strategy, conversely to the results that were reported by Stopar et al. (2003) where the authors considered the high proportion of lysogens in the Gulf of Trieste obtained from a mytomycin C-induction experiment on the natural bacterial population. According to Evans et al. (2003), the occurrence of lytic cycle may be determinant for the success of the experiment since the duration of the latent period during lytic viral proliferation tends to shorten and thus should fall within the dilution incubation period (in our case 24 h).

The rate of virus production in serial dilution experiment resulted approximately two fold higher than the values obtained by the dilution approaches discussed above. This result could be explained as a consequence of the longer incubation period (24 hours towards previous 15 hours) which certainly obstacles a truthful comparison among the methods, probably enhancing new lytic events. Moreover, at least a part of the particularly driven virus production rate can be attributed to the release of dissolved organic carbon into virus-free (filtered) seawater. In fact, the concentration of DOC in the virus-free seawater resulted 166% higher than a whole undiluted sample collected in this period (data not shown). Therefore in a natural sample minor virus impact on
bacterial mortality has to be expected since in a manipulated sample DOC, which is produced, could stimulate the increase of bacterial abundance both by bacterial cell lysis and by enrichment of a filtration produced virus-free water. The recycled portion of organic carbon potentially becomes available for bacterial consumption and enhances bacterial growth (Fuhrman, 1992; Middelboe et al., 1996; Middelboe, 2000; Karuza, 2001; Middelboe & Lyck, 2002). Another factor that reduces the impact of bacteriophages in a manipulated sample is grazing since a portion of bacterial standing stock in this season is undoubtedly removed by heterotrophic nanoflagellates (Fuhrman & Noble 1995; Fonda Umani & Beran, 2003).

Briefly, the present study pointed out strengths and weakness of a single method providing a range of virus production rates included between $3.50 \times 10^9$ and $1.50 \times 10^9$ viruses litre$^{-1}$ hour$^{-1}$ considering that different techniques tend to over- or underestimate the real process intensity. Moreover, the techniques with different incubation points provided a detailed description of viral production/decay processes. Therefore we conclude that the choice of the technique for the estimate of virus production should be determined principally by the purpose of the study.
4.6. ESTIMATE OF THE IMPACT OF VIRAL INFECTION ON MARINE BACTERIOPLANKTON

In order to simplify the interpretation of the two assays that have been conducted in different seasons, the results were distinguished into winter and summer experiments.

Winter experiment

Bacterial and VLP abundances in the surface seawater resulted 6.1 x 10^8 cells l^{-1} and 7.7 x 10^9 particles l^{-1} respectively, with their density VBP index about 4.6 x 10^{12} ml^{-2}. The proportion of metabolically active bacterial cells resulted 3.1 x 10^7 cells l^{-1} reaching 5.1% of the total bacterial community.

Serial dilutions of total and active bacteria abundances, performed at the beginning (t_0) of the experiment, are reported in figure 4.6.1. The regression coefficients of r=0.86 (n=15, p<0.001)

![Graph](image)

Fig. 4.6.1. Cell abundance of total bacterial cells (a) and their active fraction (b) in a serial dilution of the winter seawater sample at time t_0. Regression line is calculated on triplicate direct count determination and the statistically significant regression line verified a success of dilution procedure. The regression line equation and the R^2 are reported.
for total bacteria and $r=0.73$ (n=12, p<0.01) for their active fraction verified an expected proportion of diluted samples towards a whole (undiluted) bacterial sample.

The results of the impact of viral lysis on total and active bacterial cells are presented in figure 4.6.2. No statistical dependence between the increase of bacterial mortality during 24 h incubation time with the increase of the proportion of a whole sample was found neither for the total bacteria nor for their active fraction.

**Summer experiment**

Bacterial and VLP abundances in the surface seawater resulted $9.9 \times 10^8$ cells l$^{-1}$ and $5.3 \times 10^9$ particles l$^{-1}$ respectively. Virus-bacteria density index (VBP) was about $5.3 \times 10^{12}$ ml$^{-2}$.

In the summer experiment, beside the investigation of the virus impact upon the mortality of total and active bacterial cells, the effect of viruses onto non-viable/dead bacteria and cyanobacteria abundances was also examined. According to the bacterial
metabolic activity, summer bacterial community was divisible into 3% of active bacteria \((2.84 \times 10^7 \text{ cells l}^{-1})\) and 97% of non-viable/dead cells \((9.6 \times 10^8 \text{ cells l}^{-1})\) within \(9.9 \times 10^8 \text{ l}^{-1}\) abundance of total bacterial cells. The abundance of cyanobacteria in the same water sample resulted \(5.3 \times 10^7 \text{ l}^{-1}\).

Cell abundances of total, active, non-viable/dead and cyanobacteria relatively to serial dilutions performed at the beginning \(t_0\) of the experiment, are reported in figure 4.6.3.

The regression coefficients of serial diluted abundances confirmed the expected proportion of diluted samples towards a whole (undiluted) sample relatively to total bacteria \((r=0.65, n=12, p<0.05)\), active bacteria \((r=0.92, n=15, p<0.001)\), non viable-dead bacteria \((r=0.82, n=11, p<0.001)\) and cyanobacteria \((r=0.77, n=25, p<0.001)\).

\[
\begin{align*}
\text{(a)} & \quad y = 4.77x + 5.17 \\
\text{R}^2 & = 0.42 \\
\text{(b)} & \quad y = 2.62x + 0.12 \\
\text{R}^2 & = 0.84 \\
\text{(c)} & \quad y = 100.28x + 0.91 \\
\text{R}^2 & = 0.68 \\
\text{(d)} & \quad y = 5.26x \\
\text{R}^2 & = 0.60
\end{align*}
\]

*Fig. 4.6.3. Cell abundance of total bacteria (a), active bacteria (b), non-viable/dead bacteria (c) and cyanobacteria (d) in a serial dilution of the summer seawater sample at time \(t_0\). Regression line is calculated on triplicate determination for bacteria and on five-fold determination for cyanobacteria. Statistically significant regression line verified a success of dilution procedure for each population. The regression line equation and the \(R^2\) are reported.*
In figure 4.6.4 are reported the results of the impact of viral lysis on total bacterial population (a), active bacteria (b), dead/non viable bacteria (c), and cyanobacteria (d). The obtained correlation coefficients of linear regression analysis indicate significant virus-induced mortality relatively to total \( r=0.98, n=13, p<0.001 \), active \( r=0.96, n=13, p<0.001 \) and non-viable/dead \( r=0.71, n=12, p<0.01 \) bacterial populations. On the contrary, the mortality of cyanobacteria did not increase with higher proportion of whole sample, indicating that viruses did not affect the abundance of cyanobacterial population.

In table 4.6.1 the growth coefficients (k), mortality coefficients (g) and apparent growth coefficients (k - g) of total, active, non-viable/dead bacteria and cyanobacteria populations are reported. The growth of total bacterial community resulted apparently stable, being characterized by apparent growth coefficient proximal to zero (0.03). Actually, different metabolic groups sustained different growth rates and underwent different rates of virus-induced mortality. The entire bacterial population was characterized by quite similar growth and mortality coefficients respectively equal to 4.38 and 4.35. The growth of the active cells resulted particularly intense with growth coefficient of 6.35, while non-viable/dead cells resulted able to grow very slowly (1.79). The mortality rates values between total and active bacterial populations did not significantly differ (4.35 for total bacteria and 4.09 for the active fraction) and resulted rather high, while for the non-viable/dead bacterial population minor virus-induced mortality was found (3.07).

In table 4.6.1 removal (mortality) rates \((L^{-1} d^{-1})\) in terms of cells for total \(4.4 \times 10^9 L^{-1}\), active \(4.4 \times 10^8 L^{-1}\) and non-viable/dead \(1.7 \times 10^9 L^{-1}\) bacterial populations are also reported. If divided by the average bacterial populations density registered during the course of experiment, the obtained values markedly differ according to bacterial metabolic population, reaching 15.5 for the active and only 1.7 for the non-viable/dead bacteria.

<table>
<thead>
<tr>
<th></th>
<th>k</th>
<th>g</th>
<th>removal rate (L^{-1} d^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>total bacteria</td>
<td>4.38</td>
<td>4.35</td>
<td>4.39E+09</td>
</tr>
<tr>
<td>active bacteria</td>
<td>6.35</td>
<td>4.09</td>
<td>4.41E+08</td>
</tr>
<tr>
<td>non viable/dead bacteria</td>
<td>1.79</td>
<td>3.07</td>
<td>1.66E+09</td>
</tr>
</tbody>
</table>

Tab. 4.6.1. Growth (k) and mortality (g) coefficients and removal rates of total, active, non-viable/dead bacteria and cyanobacteria observed during the serial dilution experiment.

In order to assess dissolved organic carbon (DOC) production/consumption processes, DOC concentrations were measured before \(t_0\) and after \(t_24\) the incubation period.
Fig. 4.6.4. Difference of the total bacteria (a), active bacteria (b), non-viable/dead bacteria (c) and cyanobacteria (d) between 0 h and 24 h in a serial dilution incubated samples. Regression coefficient is calculated on triplicate sample determination for each serial dilution. The regression line equation and the $R^2$ are reported.
DOC concentration in whole water sample (1.0) increased from 1.8 to 2.0 mg l\(^{-1}\) over the incubation period. On the contrary, in the most diluted sample, which was obtained by the addition of free-virus seawater in proportion 5:1 towards the whole water sample, the concentration of dissolved organic carbon was reduced over the incubation time, varying from 2.7 to 2.2 mg l\(^{-1}\).

Fig. 4.6.5. Dissolved organic carbon concentrations at the beginning (\(t_1\)) of the experiment and after the incubation time (\(t_2\)) in the most diluted (0.2) and whole water sample (1.0).

Confirm of the successful bacterial DNA amplification by polymerase chain reaction is reported in figure 4.6.6. Since the amount of the amplification product resulted satisfying, the modification in the bacterial community structure was evaluated using denaturing gradient gel electrophoresis. This technique was used to separate bacteria into different bands according to their genomic content. As can be observed from

Fig. 6. Photographic picture of electrophoretic courses conducted on 7 different polymerase chain reaction products. Bands indicated by red arrow correspond to the marine bacterial DNA that was extracted from the sample at \(t_0\) and \(t_2\) incubation time in the summer experiment. The presence of well-defined bands confirms the successful amplification.
figure 4.6.7, no differences between bacterial community structures over 24 h incubation period were recorded. According to the number of bands present in each electrophoretic course, approximately 22 different bacterial species/strains during the summer experiment were accounted.

Virus-host density temporal distribution is reported in figure 4.6.8 in order to examine the most probable phage proliferation strategy that occurred relatively to the examined periods (evidenced by arrows). As can be observed, phage-host density slightly below the density threshold value was registered during the winter experiment ($7.9 \times 10^{12} \text{ ml}^{-2}$), while in the summer experiment quite doubled density was found in the same study area ($14.4 \times 10^{12} \text{ ml}^{-2}$).

**Fig. 7.** Photographic picture of denaturant gradient gel electrophoretic courses of bacterial DNA samples collected at $t_0$ and $t_{24}$ incubation time. $t_0$ and $t_{24}$ incubation time bacterial DNA samples. The presence of well-defined bands confirms the successful bacterial species/strains separation according to their genomic content.

**Fig. 8.** Temporal distribution of phage-host densities (VBP) from January 2000 to September 2005. The arrows indicate period of (below the threshold line) and August 2005 (above the threshold line), evidenced with full-line arrows.
Discussion

In this experiment the virus-induced bacterial mortality resulted particularly enhanced, when examined the effect on total bacterial population, but even on the much less abundant active fraction. Surprisingly, also the non-viable/dead bacterial cells seem to have been affected by the presence of viruses, which slightly reduced they abundance over the incubation period. On the contrary the mortality of cyanobacteria did not increase with higher proportion of the whole sample, indicating that viruses did not affect the abundance of cyanobacterial population.

It is interesting to observe the apparent abundance stability of total bacterial population, for which the apparent growth rate results proximal to zero. Particularly intense growth interested the active cells, which largely exceeded the values of $k$ found among seven different lysis experiments conducted in the same study area on the total community (Karuza, 2001), confirming the presence of enhanced replication processes within the active bacterial population. For the non-viable/dead fraction the absolute absence of growth was initially expected. During the study we realized that, as the active bacterial cells counts constitute only the highly active bacterioplankton (Karuza et al., 2004; Paoli et al., 2006), so the non-viable/dead population surely include almost partially starving or damaged but not completely dead cells which are still able to grow with rather slow rates. Moreover, our experiment pointed out that virus-induced mortality between total and active bacterial populations did not significantly differ and resulted rather high, while for the non-viable/dead bacterial population by approximately 30% minor virus-induced mortality was found.

Mortality rates (l$^{-1}$ d$^{-1}$) on the first sight do not seem particularly informative but when divided by the average bacterial population density registered over the course of experiment, 15-folds higher mortality affected active rather than non-viable/dead bacterial population.

Contrary to my expectations, the incubated sample underwent the consumption of dissolved organic matter rather than enrichment. The particularly high DOC concentration in the diluted sample (0.2) was probably due to the enrichment in DOC that was produced by the filtration step, where the filtration pressure undoubtedly minced a part of particulate organic matter and released it into dissolved fraction.

From the known DOC concentrations of diluted (0.2) sample and undiluted whole water (1.0), the concentration of virus-free filtered seawater (FSW) has been recalculated according to the expression \( \text{DOC (FSW)} = \frac{\text{DOC (0.2 diluted) - 0.2xDOC (undiluted)}}{0.8} \), which derives from the equation that considers the dilution proportions as follows: \( \text{DOC (0.2 diluted)} = 0.2 \times \text{DOC (undiluted)} + 0.8 \times \text{DOC (FSW)} \). Thus, growth process in the present experiment could have been overestimated and therefore should be considered under reserve. In a natural sample minor virus impact on bacterial mortality has to be expected since in a manipulated sample DOC, which is produced,
could stimulate the increase of bacterial abundance both by bacterial cell lysis and by enrichment of virus-free water produced by filtration. The recycled portion of organic carbon probably became available for bacterial consumption and enhanced bacterial growth as already suggested by several authors (Fuhrman, 1992; Middelboe et al., 1996; Karuza, 2001; Middelboe & Lyck, 2002). However the comparison among different metabolically distinguished bacterial populations remains valid since all the samples underwent the same DOC enrichment. Some authors argued that the removal of viruses by 0.02 μm filtration might have changed the bacterial substrate conditions, since the filtration may remove a potential bacterial substrate in the size fraction between 0.02 μm and 0.2 μm (large molecules and colloids). Rather, removal of a potential bacterial substrate would be expected to reduce bacterial growth, unless this size fraction contained some inhibiting factor for bacterial production (Middelboe & Lyck, 2002) such as lysozymes, bacteriocins and other bacteriocidal agents (Moebus, 1972; McCambridge & McMeekin, 1980).

Only for the summer experiment the impact of virus-induced mortality on the bacterioplankton community structure was assayed. No differences between bacterial community structures over 24 h incubation period were recorded. According to the number of bands present in each electrophoretic course, approximately 22 different bacterial species/strains during the summer experiment were accounted. Therefore we deduce that the viral infection pressure occurred in the seawater sample did not modify bacterial community structure. Since viruses have no active form of motility, the encounter rates that are due only to the passive diffusion (Schwalbach et al., 2004), determine the infection probability. Therefore the encounter probability with the entire, metabolically undefined, bacterial population resulted in significant mortality by viral lysis, even if the non-viable fraction, which was encountered with the same rates, often did not result in successful infection. However, our results are in accordance to those reported by several authors that even a long time starvation does not prevent infection and viral production in all host species (Kokjohn et al., 1991; Schrader et al., 1997) and even killed cells may still be exploited to produce viral progeny (Anderson, 1948), although in both cases cell lysis is delayed and burst size reduced. Moreover, we deduce that the estimate of the proportion of non-viable cells results particularly important in order to reduce the uncertainty of the proportion of different metabolic groups, since the detection of active bacteria provides only the estimate of the highly active bacterial cells and still leaves the uncertainty relatively to the metabolic state of other cells within entire bacterial population. Since viruses were able to induce significant mortality of the ‘total’ bacterial population, also the portion of metabolically undefined cells (neither active nor non-viable) is able to sustain lytic viral proliferation. For a general conclusion more experiments should be performed relatively to different seasons in order to investigate virus-bacteria interactions related to distinct environmental and trophic conditions of the area.
On the basis of our experience of viral impact on total bacterial population that was previously assayed in five different periods over year 2000 in the Gulf of Trieste and reconsidering the results of these two experiments reported above, emerged that the serial dilution method produced significant results only for the periods when bacterial and virus abundances were high. Considering the temporal distribution of virus and bacterial abundance dynamics in the Gulf of Trieste, the summer period was purposely chosen for the more detailed virus-bacteria interactions investigation, supposing that the virus-host density threshold value should have been reached. Thus the occurrence of the lytic phage proliferation strategy could have been assumed. On the contrary, lower phage-host densities, characterizing our study area in vernal periods, (during the February 2000 (Karuza, 2001) and also in January 2004) did not provoke bacterial mortality based on the incidence of lytic infection. Moreover, based on the principle of the method, we suppose that the reaching of phage-host density threshold value also in the most diluted sample (1:5) results necessary for the successful experiment performance. According to Evans et al. (2003) the duration of the latent period (i.e. the time between viral infection and host lysis) is likely to be a critical factor in determining the detection of viral mortality by the dilution approach. Therefore the virus-induced mortality should be most easily quantified if the latent period falls within the duration of the dilution incubations. These considerations sustain our concept of lytic life cycle since lytic proliferation tends to shorten the latent period (Weinbauer, 2004).
5. Conclusions

Since viruses act differently according to the substrate availability, host metabolic status and environmental factors, it resulted difficult, to obtain complete frame of viral population’s dynamics and its influence on the energy cycling using single methodological strategy and hence, multi-scalar approach was adopted. In the present study we improved current methods for viral abundance assessment, and developed the existing and new techniques for the estimate of viral production rates and virus-mediated microbial mortality.

This study highlighted the importance of integrative spatial and temporal descriptive-analytical field studies on microbial dynamics. The spatial distribution of different microbial components provided an opportunity to explore the functioning and significance of viruses under contrasting environmental conditions, since the Northern Adriatic Sea displays the most evident trophic gradient in the entire Mediterranean basin. The spatial distribution over seasons outlined different microbial loop functioning and explained the seasonal characteristics from common generic principles.

The results obtained by spatial sampling strategy did not overlap with those obtained by long-term temporal study in a coastal station of the Northern Adriatic, but extended the information: different methodological strategies allowed us to acquire precious findings regarding viral population’s dynamics, since viral interaction with other microbial communities is particularly difficult to define because of the variety of interaction types.

Although viral abundances were constantly monitored for six years in the Gulf of Trieste, the lack of statistical relationship between viruses and bacteria in temporal studies was difficult to explain, even if the prevalence of bacteriophages within total viral community was suspected; now we know that the lack of relationship between their abundances is mostly due to the variety of interaction types, which could not yield to general conclusions. The establishment of lytic rather than lysogenic proliferation strategy is largely driven by the trophic state of the system. Therefore, the evidence of prevalently lytic life strategy determined predator-prey oscillations of viral and bacterial abundances, which did not fluctuate at the same time, contrary to the results obtained from the spatial scenario; viruses resulted mostly represented by bacteriophages, which in spring induce lytic life cycle that persist until high bacterial abundances are maintained. The increase of the bacterial abundance is mostly determined by the trophic state of the system. With lytic induction of phage genome, supported by environmental factors such as substrate availability, host metabolic status or/and irradiance-induction, viruses cause lysis of bacterial cells, reducing the energy and carbon transfer to higher trophic levels and maintaining high concentrations of dissolved organic matter within the euphotic zone. Moreover, high viral and bacterial
abundances require high proportion of phosphorous, which becomes more and more limit and might enhance polysaccharide exudation by microalgae. Bacteria in condition of phosphorous limitation do not grow well but spend their energy mostly to enzymatically hydrolyse organic matter in order to recover organic phosphorous. Our effort to highlight viral role in the functioning of the microbial food web during mucilage formation allowed us to perceive some other peculiarities of viral ecology. Thus, the mucilage formation acted as a natural experiment, which highlighted the usefulness of VBR index, and evidenced the usefulness of VBR and VBP coupling, that was never observed before in other studies. As it usually occurs, the anomalies are more informative than usual oscillations and therefore might evolve to predictive models. Viral and bacterial abundance relationships, assessed by VBR and VBP uncoupling, anomalous relatively to the entire background study period, indicated low bacterial diversity, which was confirmed also by molecular analysis of bacterioplankton community structure. Obviously, these interactions are not completely determinative in predictive models, since also environmental factors play an important role in the mucilage formation.

Our knowledge regarding virus population's dynamics was reinforced by results obtained from experimental approach: limitation by nutrients resulted determinative for controlling of bacterial populations development and therefore strongly influenced viral proliferation. My results also confirmed that, despite copious allochtonous aquatic outflows occurring in certain periods of the year, which cannot be excluded for bacteria, viruses in the Northern Adriatic come from within the system.

Since viral particles are particularly susceptible to decay because of their short-term stability in free-seawater, rates of virus production were examined. Three different methodological approaches were compared: they produced quite similar and therefore reliable results, but allowed us to recognize strengths and limits of the single methods.

Since the methods used for the assessment of viral production indirectly provide the estimate of virus-mediated mortality, assuming the prevalent occurrence of lytic life cycle, there are indices of particularly enhanced bacterial growth and successful bacterial lysis immediately before mucilage formation occurred in June 2000.

My experimental design for the estimate of virus-mediated mortality on metabolically different bacterial populations pointed out that virus infection affects mostly the enhanced growth of non-infected cells, able to proliferate on lysis-derived dissolved organic matter rather than metabolically selective infection and do not directly imply different nutrient cycling.

Therefore, I conclude that this study allowed to understand the overlying mechanisms that control abundance, activity and proliferation strategy of viruses in the Northern Adriatic Sea.
6. REFERENCES


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References


References


References


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7. APPENDIX
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for the moral support; worry, shopping and Basco-tour share;

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thanks to Giunio e Vale for beautiful Sunday on the snow;

the 'Big Blue'

and finally, my dear Massi for all of these and all the rest.

The financial support came from EU and Friuli-Venezia-Giulia Region, within the 'Monitoring project of the Northern Adriatic Sea' (INTERREG II-III).
HIGHLY ACTIVE BACTERIA IN THE SURFACE WATERS OF THE GULF OF TRIESTE (NORTHERN ADRIATIC SEA)

A. Karuza *, P. Del Negro, A. Paoli, S. Comisso, S. Fonda Umani
Laboratory of Marine Biology, Via Auguste Piccard 54, 34010 Trieste, Italy

Abstract
In this study we examined the metabolically active fraction of bacterial community in surface waters of the Gulf of Trieste using the CTC incubation method technique. The results suggested that the CTC+ cells are not responsible for the bulk of bacterial activity. The method seems to be adequate to detect only the cells with very active metabolism but not the cells in a transitory metabolic state. Therefore the CTC reduction method was viewed and interpreted as an efficient method for identifying the most highly active cells in bacterioplankton populations or assemblages.

Keywords: CTC, active bacteria, Gulf of Trieste

In the bulk of bacterial community there are at least three categories of cells that should be of biogeochemical relevance: i) actively growing cells which contribute to production and biomass; ii) living but inactive cells and iii) dead and inactive cells. Although discrimination among these three cellular categories remains unclear (1), some methods have been suggested to determine the fraction of actively growing cells to complex assemblages.

For the purpose of our study we choose the CTC incubation method, a simple and fast technique to determine the number of bacteria that have measurable rates of electron transport system and therefore an active respiration.

Surface (0.5 m) water samples were bimonthly collected in a mesotrophic area of the Gulf of Trieste from June 2002 to April 2003.

Total bacteria abundances were determined using DAPI staining method (2), while metabolically active cells were detected using a CTC incubation technique (3). Bacterial Carbon Production (BCP) was determined by H-3-leucine and H-3-thymidine incorporation (4).

Disolved Organic Carbon (DOC) concentration was assessed by high temperature catalytic oxidation (5). Rates of oxygen utilization were calculated from changes in dissolved oxygen concentration, using the methods of Winder, over a 24 h period in samples incubated in the dark and at in situ temperature. Temperature data were obtained by a Idronaut Ocean Seven (Model 316) multiparametric probe.

Bacterial abundances ranged between 2.51 x 10^8 and 4.78 x 10^9 cells L^-1 whereas the number of active cells (CTC+) fluctuated from 1.72 x 10^7 to 9.92 x 10^7 cells L^-1. The percentage of CTC+ bacteria ranged between 0.03 and 7.41%. The abundance of CTC+ cells was strongly correlated to total bacterial numbers and, better than with total bacteria, it showed strict relationships with temperature and substrate availability, evaluated as DOC concentration. On the contrary, bacterial production, measured as 3H-leucine and H-3-thymidine incorporation, were correlated to total number of bacteria only (Tab 1). Respiration rate within the plankton community resulted strongly correlated to total number of bacteria where the active fraction only partially support the respiration process.

Setting aside methodological differences, our results, like those of many other authors (e.g. 6), show that not all bacteria are metabolically active and that the water temperature appears to have had a profound effect on the pattern of induction of respiration activity. The statistical dependence between CTC+ bacteria and DOC, could have been caused solely by the increase in temperature which is also usually the controlling factor in the phenomena involved in the production of autochthonous organic matter readily assimilable by bacteria.

Although it could seem surprisingly because of the principle of the CTC method based on detecting cell respiratory activity characteristic for growing cells, oxygen consume rate and BCP had better statistical relationships with total bacteria abundances rather than active cells, commonly to the results of Smith (7). This could concern a limit of the method that detects only the cells with very active metabolism but not the cells in a transitory state, between CTC+ and CTC-, that contribute to the total respiration measured and therefore the CTC reduction assay should be viewed and interpreted as identifying the most highly active cells in bacterioplankton populations or assemblages. Indeed, the level of cell activity is what determines the detectability of respiring cells, since even bacteria in a starvation state must sustain certain functions.

Table 1. Parameters of linear regression analysis.

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<tr>
<th>X</th>
<th>Y1</th>
<th>n</th>
<th>P1</th>
<th>Y2</th>
<th>n</th>
<th>P2</th>
<th>Y3</th>
<th>n</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>temperature</td>
<td>CTC+</td>
<td>0.91</td>
<td>&lt;0.001</td>
<td>total bacteria</td>
<td>0.70</td>
<td>&lt;0.001</td>
<td>n=17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3H-Leucine</td>
<td>CTC+</td>
<td>0.70</td>
<td>&lt;0.001</td>
<td>total bacteria</td>
<td>0.90</td>
<td>&lt;0.001</td>
<td>n=16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3H-Label</td>
<td>CTC+</td>
<td>0.24</td>
<td>n.s.</td>
<td>total bacteria</td>
<td>0.77</td>
<td>&lt;0.001</td>
<td>n=17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>respiration</td>
<td>CTC+</td>
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<td>&lt;0.01</td>
<td>total bacteria</td>
<td>0.87</td>
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<td>&lt;0.05</td>
<td>n=18</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

References
A. Bensi, A. Karuza, P. Del Negro, S. Fonda Umani

Laboratorio di Biologia Marina, Via A. Piccard, 54 – 34010 Trieste

VARIAZIONE DELLE ABBONDANZE BATTERICHE E VIRALI IN RELAZIONE ALLA FORMAZIONE DI MUCILLAGINI NEL GOLFO DI TRIESTE (ALTO ADRIATICO)

CHANGES IN VIRUS AND BACTERIA ABUNDANCE IN RELATION TO MUCILAGE FORMATION IN THE GULF OF TRIESTE (NORTHERN ADRIATIC SEA)

Abstract
Viral and bacterial abundances, bacterial carbon production and dissolved organic carbon were analysed from October 1998 to June 2001 in the Gulf of Trieste (coastal monitoring station C1). Three different periods were identified: an extremely high Virus to Bacteria Ratio (VBR) and the significant correlation between viral and bacterial abundances, BCP and DOC during the second period, which ended with the mucilage formation, confirmed the intense processes of bacterial lyses.

Keywords: viruses, bacteria, aggregate, Northern Adriatic

Introduzione

Materiali e Metodi

Risultati
Dall’osservazione dell’andamento temporale delle abbondanze batteriche e virali è stato possibile identificare tre periodi distinti (fig. 1). Nel primo periodo (ottobre 1998-giugno 1999) le abbondanze virali si mantengono su valori minimi (1,9-2,9 \times 10^9 virus L^-1).

Fig. 1. Box plot delle abbondanze batteriche e virali nei 3 periodi considerati.

Box plots of the bacterial and viral abundances in the three considered periods.

Fig. 2. Rapporto tra abbondanze virali e batteriche (VBR).

Virus to Bacteria Ratio (VBR).

L’andamento delle abbondanze batteriche risulta specular a quello dei virus (fig. 1).

Durante il periodo precedente le mucillagini (2° periodo) il rapporto virus:batteri (VBR) risulta estremamente elevato raggiungendo il valore di 70 (fig. 2). Secondo Wommack e Colwell (2000) il VBR risulta normalmente compreso tra 3 e 20. Le abbondanze virali rilevate nei quattro mesi caratterizzati dai più alti VBR (agosto e novembre 1999, febbraio ed aprile 2000) (indicati dalle frecce in figura 2) risultano correlate significativamente (n=16, r=0.77) alle

Tab. 1. Coefficienti di correlazione lineare con i rispettivi livelli di significatività tra abbondanze virali, BCP e DOC.

<table>
<thead>
<tr>
<th>Virus / BCP</th>
<th>Virus / DOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.77</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>0.68</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>0.6</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

abbandonze batteriche (fig. 3) sostenendo la teoria che i virus aumentano in relazione all’aumento di batteri metabolicamente attivi (Fuhrman, 1992). Le abbondanze virali rilevate in questi periodi risultano correlate significativamente anche con DOC e BCP (tab. 1) mentre quando il VBR è basso e paragonabile ai valori riscontrati in letteratura non sono evidenti relazioni significative tra i parametri.

Conclusioni

Nel periodo precedente la comparsa delle mucillagini i virus risultano estremamente infettivi: i valori di VBR sono, infatti, elevatissimi (60-70) e le abbondanze virali sono correlate significativamente alle abbondanze batteriche, alla produzione batterica (BCP) ed al DOC. La lisi delle cellule batteriche indotta dai virus favorisce, infatti, l’incremento di sostanza organica disciolta (DOC) e ne modifica la composizione arricchendola in molecole labili, particolarmente appetibili per il popolamento batterico che risponde aumentando la propria biomassa (BCP). L’utilizzazione della porzione più labile della sostanza organica favorisce l’accumulo di quella refrattaria che, in condizioni meteo-climatiche particolari potrebbe andare incontro a processi di aggregazione.

Questi primi dati sembrano, pertanto, sostenere l’ipotesi che i virus giochino un ruolo importante nei processi di produzione della matrice organica che darà origine alle mucillagini.
A seguito della comparsa dei macroaggregati si osserva, invece, un netto cambiamento nelle condizioni trofiche che porta a modificazioni nella dinamica dei popolamenti batterici alterando anche la distribuzione dei virus.

Rinvenzioni

Gli autori desiderano ringraziare la dott.ssa C. De Vittor per le analisi del DOC ed i colleghi E. Crevatin, C. Larato e P. Rossin per il prezioso aiuto tecnico e scientifico. Il presente studio è stato finanziato dal progetto Interreg II Italia - Slovenia (CEE e Regione FVG).

Bibliografia

Daily variations of highly active bacteria in the Northern Adriatic Sea

A. PAOLI, A. KARUZA, C. DE VITTOR, P. DEL NEGRO* AND S. FONDA UMANI
LAbORATORY OF MARINE BIOLOGY 2,5M, VIA A. PICCARDI, 54, 34016 TRIESTE, ITALY
*CORRESPONDING AUTHOR: pidelargo@atigros.it

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Nowadays, it is recognized that only a fraction of aquatic bacteria are actively growing, but there is little information about the factors constraining their metabolism. Marine bacterioplankton can rapidly modify their metabolic activity level in response to environmental changes. In this study, we focused on the daily changes in abundance and activity of active bacterial fraction over a 20-day period preceded by intense rainfalls which slightly modified water column conditions. Cells capable of reducing the membrane-penetrable dyes 5- (and 2,3-)-diaminofluorescein (DAF) estimated by epifluorescence microscopy, are considered very active (CTC+ bacteria). Total bacterial abundance (TBA) ranged from 0.8 to 2.4 \times 10^{8} cells L^{-1}, whereas CTC+ bacteria were more variable (1.6–9.2 \times 10^{4} cells L^{-1}), accounting for 1.2–4.4% of TBA. Bacterial activity (BA) as the incorporation of [3H]-leucine varied by more than one order of magnitude over the period (25.0–66.2 pmol L^{-1} h^{-1}). BA was strongly related to CTC+ bacteria, suggesting that they were mainly responsible for the bacterial community metabolism. Nevertheless, cell-specific activity, scaled to only CTC+ cells, was very high, suggesting that a fraction of cells not detectably CTC+ may be able to assimilate [3H]-leucine. The correlation between salinity and TBA, CTC+ bacteria and BA supported the hypothesis of the active role of freshwater input in enhancing cell activity. Our results suggest that freshwater input rather than phytoplankton blooms are able to induce shifts in bacterial metabolism over a time scale of days in the area studied.

INTRODUCTION

In natural ecosystems, bacteria play an important role in driving fluxes of material and energy. Bacteria abundance in the ocean is generally much more uniform than in other systems (del Giorgio and Scarahorough, 1995). Conversely, bacterial activity (BA) shows wider variability and is positively related to ecosystem trophic states (Ducklow and Carlson, 1992; del Giorgio and Gasol, 1993). However, there is often only a weak relationship between bacterial standing stocks or activity and environmental trophic status (Sherr et al., 2001).

In the ocean, we do not yet fully understand the causes and the extent of bacterioplankton community variability on fine temporal or spatial scales. Samplings for estimating bacterial abundance and activity cover kilometre-scale distances over time scales of weeks to years. Azam (Azam, 1988) suggested that marine bacteria can aggregate rapidly into small-scale patches or ‘hot spots’ in response to favourable microenvironmental conditions. More recently, Sherr et al. (Sherr et al., 2001) and Zubkov et al. (Zubkov et al., 2002) confirmed that natural bacterioplankton assemblages could undergo relatively rapid metabolic ‘shifts-up’ or ‘shifts-down’, depending on local environmental conditions. Thus, while pelagic bacteria are relatively inactive, at least a small percentage of the community can respond to an increase in substrate availability on a time scale of hours by increasing their rates of cell-specific activity and growth (Sherr et al., 1994; del Giorgio and Cole, 2000). In the Gulf of Trieste, Del Negro et al. (Del Negro et al., 1996) and Turk et al. (Turk et al., 2001) have already described bacterial annual dynamics by using a monthly sampling strategy but have yet to report information about bacterial abundance and activity level variability over shorter...
METHOD

Study area

The Gulf of Trieste (Fig. 1) is the northernmost part of the Adriatic Sea. The surface area of the Gulf is about 600 km² with maximum depths of 26 m (Olivotti et al., 1986).

The main freshwater input comes from the Isonzo River. Rivers from the southeastern coast are of a torrential nature. Freshwater inputs show a high interannual variability (Malej et al., 1995), which affects salinity, whose values range from 32 to 38 at the surface (Fonda Umani, 1991; Celio et al., 2002). Water temperatures show a regular annual pattern from winter minima, as low as 6°C in February, to summer maxima >25°C (Cardin and Celio, 1997). A high variability of the water column profile, due to riverine outflows and temperature variations, is enhanced by an alternance of cold winds from the north–northeast (i.e. Bora) and mild winds from the south (i.e. Scirocco).

Sampling

Water samples were collected daily from 12 February 2002 to 11 March 2002 (interrupted from 15 February to 17 February and from 22 February to 24 February because of strong northern winds) at a coastal station (C1 43°42'03" N, 13°42'36" E) in the Gulf of Trieste (Fig. 1). Temperature and salinity data were collected by a multiparametric probe (Idronaut mod. 401). Samples for the chemical and biological analyses were collected with a 10 L Niskin bottle, equipped with silicon elastic and red silicon O-rings, at the surface (−0.5 m) and at the bottom (−16 m). Samples were stored in freezing bags (6 ± 2°C) and processed at the Laboratory of Marine Biology (LBM) within 2 h after collection (Fig. 1).

Chlorophyll a (Chl a)

Samples (1 L) were filtered onto glass fibre filters (Whatman GF/F) and stored at −20°C. Pigments were extracted overnight in the dark at 4°C with 90% acetone from the homogenized filter and determined fluorimetrically according to Lorenzen and Jaffrey (Lorenzen and Jaffrey, 1980). The Chl a concentration was measured in triplicate by means of a Perkin Elmer LS 50B spectrofluorometer at 450 nm excitation and 665 nm emission wavelengths. The replicate samples showed a dispersion <4%.
Dissolved organic carbon (DOC)

Samples 15 mL were filtered through precombusted (4 h at 480°C) and acidified (1 N HCl) glass fibre filters (Whatman GF/F) and stored at −20°C in 20 mL glass vials (previously treated with chronix mixture and precombusted for 4 h at 480°C).

Before the analysis, samples were acidified (pH < 2) with 6N HCl solution and purged for 8 min with high-purity oxygen bubbling (150 mL min⁻¹). DOC concentration was measured using a Shimadzu TOC 5000 Analyzer with a 1.2% vial~ (previous) curves. Sample~ potassium hydrogen phth~alate. Each value was~ stained with l,1(Whannan GF~ Sample~

Total bacterial abundance

Samples (10 mL) were fixed with 2% final concentration of borate-buffered formalin (prefiltered through a 0.2 μm Acrodisc filter) and stained for 15 min with 4',6-diamino-2-phenylindole (DAPI, Sigma) at 1 μg mL⁻¹ final concentration (Porter and Feig, 1980). Subsamples were filtered in triplicate onto 0.2 μm black-stained polycarbonate filters (Nuclepore). Filters were mounted on microscope slides, between layers of nonfluorescent immersion oil (Olympus), and counted within a few hours under a UV filter set, using an Olympus BX 60 F3 epifluorescence microscope at ×1000. A minimum of 300 cells were counted for each filter.

Optimization of CTC protocol

To maximize the identification of CTC+ cells, we carried out some preliminary experiments to assess their percentage as a function of dye concentration and the best incubation time.

As some authors have suggested that high concentrations (≥5 mM) of CTC (Polysciences) are toxic to bacteria (Lovejoy et al., 1996; Ulrich et al., 1996; Karner and Fuhrman, 1997), we defined a dose–response curve using increasing concentrations of CTC. A 64.2 mM CTC stock solution was prepared according to Choi et al. (Choi et al., 1996). Seawater samples (10 mL) were stained with 1, 3, 5, 7 and 9 mM final concentration of CTC. The samples were incubated at the in situ water temperature for 2 h in the dark. On the basis of the results of this experiment, we used a final concentration of 3 mM CTC to carry out the second experiment to test the optimal incubation period.

As it has been observed that incubation time also influences the fraction of CTC+ bacteria (del Giorgio and Scaraborgough, 1995; Choi et al., 1999), we checked their percentage after 1, 2 and 4 h of incubation time.

Metabolic active bacteria

Active bacteria were determined as described by Choi et al. (Choi et al., 1996). CTC was added to 4 mL of seawater samples to yield a 3 mM CTC final concentration. Samples were incubated in the dark at the in situ temperature for 2 h. Incubation was stopped by adding 5% final concentration of borate-buffered formalin (pre-filtered through 0.2 μm Acrodisc filters). Aliquots of 1 mL were filtered in quadruplicate onto 0.2 μm black-stained polycarbonate filters (Nuclepore) and mounted onto microscope slides between layers of nonfluorescent immersion oil (Olympus). The filters were immediately observed by means of epifluorescence microscopy (Olympus BX 60 FS) under a green light excitation filter set at ×1000. A minimum of 300 cells were counted for each filter.

Bacterial activity

BA was assayed by the incorporation of [³H]-leucine by the microcentrifuge method (Smith and Azam, 1992). Triplicate (1.7 mL samples and one killed control [90 μL 100% trichloracetic acid (TCA)] were amended with 20 μM of [³H]-leucine and incubated for 1 h at the in situ temperature in Eppendorf 2 mL vials. The extraction was carried out after the addition of 90 μL of 100% TCA and washing with 5% TCA and 80% ethanol. The incorporated radioactivity was counted using a liquid scintillation counter (Packard Tri-Carb 300). One milliliter of scintillation cocktail (Ultima Gold MV; Packard) and a 3-min counting time were used.

Statistical analysis

DOC, Chl a, TBA, CTC+ bacteria and BA data sets were discussed considering the range and mean values, and the dispersion over the period was estimated by means of the coefficient of variation (CV = standard deviation/mean) × 100). The Shapiro–Wilks test (Shapiro and Wilk, 1965) was used for assessing normality of data sets to choose between parametric and nonparametric statistics. Relationships between all parameters were evaluated by means of the Pearson’s correlation coefficient (r) and the Kendall’s coefficient (τ) of rank correlation. The t critical values, based on a two-tailed test, were derived from those in Table XI of J. V. Bradley,
RESULTS

CTC concentration and incubation time

In the experiment carried out to evaluate the relationship between the abundance of metabolically active bacteria and CTC concentration, CTC+ cells increased from 1 to 3 mM concentration, remained constant from 3 to 7 mM and then sharply declined at 9 mM CTC final concentration (Fig. 2A).

Time-course experiments showed that bacterial cells were already labelled after 1 h of incubation, as reported by other authors (Rodriguez et al., 1992; Schaele et al., 1999; del Giorgio and Scaraborough, 1995), and their abundance increased until the second hour when it reached a plateau (Fig. 2B).

Environmental conditions

The study was carried out at the end of the winter period. The water temperature progressively increased throughout the study period, from 7.1 to 8.6°C at the surface and from 7.1 to 7.8°C at the bottom (Fig. 3A). The period before sampling, characterized by intense rainfall, influenced surface salinity, which varied from 37.1 to 38.0 (Fig. 3B). Salinity did not vary at the bottom, showing a mean value of 38.1 ± 0.0. The freshwater surface content contributed to slightly stratifying the water column, which was then mixed by wind blowing in the periods when sampling was interrupted (from 15 February to 17 February and from 22 February to 24 February).

Chl a concentration (Fig. 4) ranged from 0.4 to 2.5 µg L⁻¹. Initially, Chl a values were two times higher at the bottom of the water column than at the surface. After the first period of wind, Chl a strongly decreased both at the surface and at the bottom; it increased after the second period of wind, reaching the maxima at the end of the study period. A strong increase in Chl a of more than 3-fold occurred from 28 February to 1 March at the surface, while a more than 2-fold increase took 1 day more at the bottom.

DOC ranged between 0.6 and 1.2 mg C L⁻¹, reaching the highest concentrations during the first few days (Fig. 5). A decreasing trend with no significant differences between surface and bottom values was observed. After the first period of wind, DOC reached low values (≤0.8 mg L⁻¹), which rapidly increased up to more than 1 mg L⁻¹ on 21 February. On 28 February, another significant DOC increase, of ~0.4 mg L⁻¹ compared to the day before, was observed at both sampling depths.

Total and active bacteria

TBA ranged from 0.8 to 2.4 × 10⁹ cells L⁻¹ (mean = 1.3 ± 0.5 × 10⁹ cells L⁻¹ at the surface and 1.3 ± 0.4 × 10⁹ cells L⁻¹ at the bottom) as shown in Fig. 6A. During the first sampling period (11–17 February), TBA showed maxima, which were slightly higher at the surface than at the bottom. After 17 February, TBA slightly increased, and after some days, the bottom community reached higher abundances than the surface one. The highest daily variation in TBA was observed at the bottom between the first two days during which values reached a 2-fold decrease, from 2 to 1 × 10⁹ cells L⁻¹.

Surface and bottom CTC+ bacteria abundances clearly differed (Fig. 6B). At the bottom, CTC+ cells averaged 2.2 ± 0.3 × 10⁷ cells L⁻¹, while surface abundances widely varied [3.7 ± 2.1 × 10⁷ cells L⁻¹], showing a general decreasing trend over the period of study. The highest abundances were observed during the first few days. After the first period of wind, there was a relative
increase. The highest CTC+ bacterial daily variations occurred at the surface and reached a more than 4-fold decrease and a more than 2-fold increase between 13 and 14 February and 18 and 19 February, respectively. CTC+ cells were generally two orders of magnitude lower than DAPI-stained bacteria and accounted for 1.2-4.4% of TBA (Fig. 6C).

**Bacterial activity**

BA (Fig. 7) quantified as rates of leucine incorporation is a proxy for bacterial production. BA showed considerable daily variations (the highest was a more than 6-fold decrease) at the surface (mean = 160.8 ± 168.4 pmol L$^{-1}$ h$^{-1}$), which was particularly evident until 21 February and a decreasing trend that led to a value similar to the mean bottom value (84.5 ± 44.6 pmol L$^{-1}$ h$^{-1}$). Rates of bacterial biomass produced varied by more than one order of magnitude, from 25.0 to 662.5 pmol L$^{-1}$ h$^{-1}$. Rates of substrate incorporation were higher at the surface than at the bottom.

**Statistical analysis**

The Shapiro–Wilk ($W$) normality test (Table I) showed different distribution of the two sampling depth's data sets. $W$ is a measure of the straightness of the normal probability plot, and small values indicate departures
from normality. If the $P$-value was smaller than the critical value (0.05), the hypothesis of normality was rejected. At the surface, the hypothesis of normality was rejected for all the parameters except the temperature ($W = 0.96; P = 0.80$). Vice versa, at the bottom, the hypothesis of normality was rejected only for the temperature ($W = 0.82; P = 0.01$) and accepted for all the other parameters. Considering the results of the Shapiro-Wilk test, the relationships between all parameters were estimated differently by using Kendall's coefficient ($\tau$) of rank correlation and Pearson's correlation coefficient ($r$) at the surface and at the bottom, respectively. The correlation analyses showed several significant relationships. At the surface (Table II), BA resulted significantly correlated to all other parameters: negatively to temperature, salinity and Chl a and positively to DOC, TBA and CTC+ bacteria. The highest $r$ values resulted between TBA/CTC+ bacteria ($r = 0.77; P < 0.01$) and BA/CTC+ bacteria ($r = 0.74; P < 0.01$). TBA, CTC+ bacteria and DOC concentration showed similar negative correlations to salinity. DOC concentration also showed a strong negative correlation.
with temperature and a weak positive one with Chl a. At the bottom, the only significant correlation resulted between CTC+ and TBA ($r = 0.70$, $P < 0.01$).

**DISCUSSION**

**Protocol experiments**

The use of CTC as a marker of metabolic activity in individual bacterial cells is subjected to similar limitations described for tetrazolium salts in general, although CTC has a greater sensitivity than the nonfluorescent tetrazolium salts (Burton and Lanza, 1986; Thom et al., 1993; Sherr et al., 1999a). One of the main concerns regards the concentration of CTC. The intracellular reduction of CTC seems to be concentration dependent (Rodriguez et al., 1992), and several authors have shown that the highest percentage of CTC+ cells is obtained with CTC concentrations ranging from 2.5 to 5 mM (Rodriguez et al., 1992; del Giorgio and Scaraborough, 1995; Choi et al., 1999; Sherr et al., 1999a). Below this range, the number of stained cells is usually lower. Above concentrations of 5 mM, the number of stained cells tends to decrease, presumably due to the toxicity of CTC or of its formazan products (Choi et al., 1999). Another concern is the incubation time. Incubation times used by different authors vary greatly. del Giorgio and Scaraborough (del Giorgio and Scaraborough, 1993) used 8-h incubations for lake samples, whereas del Giorgio et al. (del Giorgio et al., 1996) found that longer incubations were needed to attain a maximum number of CTC+ cells in oligotrophic Mediterranean samples. Other authors used shorter incubation times (Rodriguez et al., 1992; Schaule et al., 1993; Choi et al., 1996; Lovejoy et al., 1996).

In the present study, we examined the effect of CTC concentration on the proportion of CTC+ cells. Maxima corresponded to 3–7 mM. Toxicity was observed by a decreased percentage of CTC+ cells at 9 mM. We used a lower concentration than 5 mM according to Karner and Fuhrman (Karner and Fuhrman, 1997) because differences in active cells between 3 and 7 mM were not statistically significant (CV = 2.9%). By adopting a < 5 mM concentration, toxic effects were lowered. We also determined the incubation times from a preliminary time-course experiment. The number of CTC+ cells did not continue to increase indefinitely but stabilized after 2 h, indicating that in every sample tested, there was a finite number of cells able to reduce CTC.

**Field study**

The sampling period was within the vernal blooming season (Fonda Umani and Beran, 2003) when surface temperatures begin to rise and rainfalls usually increase riverine outflow and consequently nutrient availability at the surface, inducing the first phytoplankton response. Unfortunately, because of the high annual variability of the system (Mozetič et al., 1998), the timing of the first bloom can shift from January to April, or as in 2002, not develop at all. Our results suggest that a short-duration, low-intensity bloom was just ending when we began the daily sampling.

The values of biological and biochemical parameters (Table III) analysed in our daily sampling period showed different rates of variability but remained in the annual range of values detected during 2002 (P. Del Negro, Trieste, personal communication) and the years before (Fonda Umani, 2001), deriving from a monitoring project carried out at the same coastal station with a biweekly sampling frequency. Despite the low GV for DOC and
Table II: Relationship between surface parameters as Kendall's coefficient (τ) of rank correlation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Temperature</th>
<th>Salinity</th>
<th>Chl a</th>
<th>DOC</th>
<th>TBA</th>
<th>CTC+</th>
<th>BA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salinity</td>
<td>ns</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chl a</td>
<td>ns</td>
<td>ns</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DOC</td>
<td>-0.59***</td>
<td>-0.47**</td>
<td>0.46**</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBA</td>
<td>-0.39*</td>
<td>-0.59***</td>
<td>ns</td>
<td>ns</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTC+</td>
<td>ns</td>
<td>-0.49***</td>
<td>ns</td>
<td>ns</td>
<td>0.77***</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>BA</td>
<td>-0.57***</td>
<td>-0.49***</td>
<td>-0.50**</td>
<td>0.52***</td>
<td>0.67***</td>
<td>0.74***</td>
<td>1</td>
</tr>
</tbody>
</table>

P < 0.01, **P < 0.05, *P < 0.1, ns = P > 0.10; n = 14.
BA: bacterial activity; Chl a: chlorophyll a concentration; CTC, 5-cyano-2,3-ditolyl tetrazolium chloride; DOC, dissolved organic carbon; TBA, total bacterial abundance.

TBA, results for Chl a concentration, CTC+ bacteria and particularly BA showed considerable variability, with high CV values, even in a short-term (20 days) sampling.

Estimates of TBA (Table III) fall within the range reported by Del Negro et al. (Del Negro et al., 1996) and Turk et al. (Turk et al., 2001) for the Gulf of Trieste. The proportion of CTC+ cells, accounting for 1.2-4.4% of TBA (Fig. 6C), showed values typical of nearly oligotrophic natural seawater environments (<5%) (del Giorgio and Scaraborough, 1995). Smith (Smith, 1998) found extremely higher abundance of CTC+ cells, of approximately one order of magnitude, and percentage of active bacteria, ranging from 3.5 to 47.4%, in a coastal planktonic community. Our results support the recent findings of a general oligotrophy characterizing the pelagic system in the Gulf of Trieste (Fonda Umani et al., 2004) nevertheless the high trophic level of coastal waters of the northwestern Adriatic basin (Degobbis et al., 2000).

The close positive correlations between CTC+ and TBA (τsurf = 0.77, P < 0.01; τbottom = 0.70, P < 0.01) might confirm that CTC+ bacteria, as the highly active fraction of the bacterial community (Karzuza et al., 2004), are those mainly responsible for the TBA variations. No significant differences between surface and bottom TBA distribution were observed, while CTC+ cell abundance and BA (τCTC+/BA = 0.74, P < 0.01) followed a different distribution pattern with a higher variability at the surface than at the bottom (Table III). We suppose that this is due both to different substrate composition and inorganic nutrient availability, as a consequence of the rainfalls occurred in the days preceding the sampling period. Choi et al. (Choi et al., 1999) demonstrated that bacteria are characterized by higher growth rates in enriched environments and become dormant or starving when

Table III: Range, mean and coefficient of variation values of DOC, Chl a, TBA, CTC+, percentage of active bacteria (% CTC+) and BA for the surface, bottom and all data combined during a daily sampling period from 12 February to 3 March 2002

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Range</th>
<th>Mean</th>
<th>CV %</th>
<th>Range</th>
<th>Mean</th>
<th>CV %</th>
<th>Range</th>
<th>Mean</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chl a (μg L⁻¹)</td>
<td>0.4-2.5</td>
<td>1.1</td>
<td>69.4</td>
<td>0.4-2.0</td>
<td>1.1</td>
<td>46.7</td>
<td>0.4-2.5</td>
<td>1.1</td>
<td>58.2</td>
</tr>
<tr>
<td>DOC (mg C L⁻¹)</td>
<td>0.7-1.1</td>
<td>0.9</td>
<td>17.1</td>
<td>0.8-1.2</td>
<td>0.9</td>
<td>19.8</td>
<td>0.6-1.2</td>
<td>0.9</td>
<td>18.2</td>
</tr>
<tr>
<td>TBA (10⁶ cells L⁻¹)</td>
<td>0.8-2.4</td>
<td>1.3</td>
<td>34.7</td>
<td>0.8-2.0</td>
<td>1.3</td>
<td>28.0</td>
<td>0.8-2.4</td>
<td>1.3</td>
<td>31.2</td>
</tr>
<tr>
<td>CTC+ (10⁶ cells L⁻¹)</td>
<td>1.7-8.2</td>
<td>3.7</td>
<td>55.9</td>
<td>1.8-2.6</td>
<td>2.2</td>
<td>15.4</td>
<td>1.6-9.2</td>
<td>5.4</td>
<td>55.4</td>
</tr>
<tr>
<td>CTC+ (%)</td>
<td>1.6-4.4</td>
<td>2.7</td>
<td>32.7</td>
<td>1.2-2.2</td>
<td>1.8</td>
<td>17.7</td>
<td>1.2-4.4</td>
<td>2.2</td>
<td>35.2</td>
</tr>
<tr>
<td>BA (pmol L⁻¹ h⁻¹)</td>
<td>40-602</td>
<td>161</td>
<td>105</td>
<td>25-203</td>
<td>84</td>
<td>53</td>
<td>25-602</td>
<td>123</td>
<td>104</td>
</tr>
</tbody>
</table>

BA: bacterial activity; Chl a: chlorophyll a concentration; CTC, 5-cyano-2,3-ditolyl tetrazolium chloride; DOC, dissolved organic carbon; TBA, total bacterial abundance.
substrate concentrations drop below the optimum amount. Despite the similar surface and bottom DOC concentrations, the DOC fraction available for bacterial uptake may be differed significantly in the two layers. Surface DOC values were inversely related to salinity (r = -0.47, P < 0.05), suggesting a role of freshwater input in increasing DOC concentrations. As reported from Becquevort et al. (Becquevort et al., 2002), almost a part of the land-derived DOC is labile and hence it can be easily utilized from bacteria. Terrestrial input also carried inorganic nutrients, which probably sustained the development of an algal bloom. Phytoplankton exudation usually relates to salinity (Obernoster and Herndl, 1997), which bacteria can uptake directly or after enzymatic hydrolysis. The Chi a concentration detected during the first few days suggests that a short bloom had developed prior to our study (Fig. 4). Chi a concentration resulted higher at the bottom than at the surface. Surface bacteria, even in the presence of a minor phytoplankton biomass, were more active than the bottom ones. This suggests that the labile fraction of land-derived DOC together with the inorganic nutrients carried by freshwater inputs, rather than the small algal bloom, was the main cause of the increase in active bacterial abundance and their activity level.

CTC+ abundances and BA were inversely related to salinity but not to temperature. While other studies have found that the proportion of active bacteria was strictly dependent on temperature (Lovejoy et al., 1996; Kirchman and Rich, 1997; Smith, 1998; Juggins et al., 2000), in our work, carried out in winter season, the effect of temperature was less important, according to Sondergaard and Danielsen (Sondergaard and Danielsen, 2001), and hidden by a much stronger signal related to salinity.

The variability of BA (CV = 103.5%) over the sampling period was not completely explained by the abundance of CTC+ cells alone (CV = 55.4%). Nevertheless, the abundance of CTC+ bacteria varied significantly more than TBA (CV = 31.2%). The large amount of unexplained variability in BA led us to the hypothesis that changes in the rates of BA should be explained by the size and specific activity of CTC+ cells, combined with their abundance, more than by the abundance of CTC+ cells alone (del Giorgio et al., 1997). The strong correlation we found between BA and CTC+ cells (Table II) confirms that the CTC+ cells are the most active cells in the bacterioplankton assemblages even if the number of active cells is underestimated by CTC assay (Sherr et al., 1999a; Ulrich et al., 1999). Other studies have shown a positive relationship of abundance of CTC+ cells with rate of [3H]-leucine incorporation (Lovejoy et al., 1996; Sherr et al., 1999b). The cell-specific activities scaled to only CTC+ cells reached very high values (average value 3875 × 10^{21} mol cells^{-1} h^{-1}) within the order of magnitude showed by Sherr et al. (Sherr et al., 1999b) reporting a value of 2640 × 10^{21} mol cells^{-1} h^{-1} for midshelf ocean system. The cell-specific incorporation rates we found scaling to total bacteria reached 90 × 10^{21} mol cells^{-1} h^{-1}. The values for cell-specific activities must lie between the two estimates since a fraction of bacteria not detectably CTC+ may be able to assimilate substrate (Sherr et al., 1999b).

During the study period, many significant (more than 2-fold) daily variations in biological parameters occurred. In particular, CTC+ bacteria and BA showed respectively a 4-fold and a 5-fold variation between two consecutive samplings. These changes over a time scale of days support the idea that in situ bacterioplankton assemblages can undergo rapid shifts up or shifts down in metabolism, depending on local environmental conditions. It is freshwater input rather than temperature modification, which can be enhanced to disable percent CTC+ bacteria. Those cells were induced to high ETS activity (Choi et al., 1997, 1999) maybe in relation to substrate and nutrient availability on a very short time scale. The supply of bacterial substrate is not only a function of phytoplankton densities (Coveney and Wetzel, 1995) but may be influenced by allochthonous inputs of organic substances used by bacterioplankton, even though their nutritional quality may be low (Tranvik, 1988).

Concluding remarks

In the present study, we have shown that daily fluctuations in environmental parameters affect CTC+ abundances. The CTC method, even with its uncertainties and limitations (Sherr et al., 1999a), provides information that is relevant for evaluating the bacterial community response to freshwater inputs. Bacterial cells responded over a short time scale to environmental modification. According to Murrell (Murrell, 2003), in coastal areas, freshwater DOC together with land-derived inorganic nutrients, rather than autochthonous DOC, controlled the development and activity level of the bacterial community. Grazing by protozoa might also play a role in regulating the abundance and the proportion of active bacteria. There are evidences that highly active cells and dividing bacteria appear to be preferentially consumed (Gonzales et al., 1990; del Giorgio et al., 1998). Further short-term studies in different annual periods are needed to better understand the environmental regulation of bacterial growth and activity level, and consequently the temporary variations in carbon flux pathways.
ACKNOWLEDGEMENTS

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A. KARUZA, P. DEL NEGRO*

Laboratorio di Biologia Marina, Via A. Piccard, 54 – 34010 Trieste
*OGS, Borgo Grotta Gigante, 42/C – 34010 Trieste

VIRIOPLANCTON NEL GOLFO DI TRIESTE

VIRIOPLANKTON IN THE GULF OF TRIESTE

Abstract
Viruses represent the smallest and the most abundant biological entities in marine environments. In the present study we reported the temporal variability of VLP abundances along the water column in a coastal station (C1) of the Gulf of Trieste (Northern Adriatic). The abundance variations ranged by over three orders of magnitude reaching the maximum value of 3.5 × 10^5 mL^-1. Virus-to-Bacterium Ratio (VBR) over this five years monitoring study have also been observed. The highest VBR index resulted in the period preceding mucilage event occurred in June 2000. Although this study concerns only the static picture of virioplankton abundance and its numerical relationship with bacterial one, it represents the first continuous long time data series in the Adriatic Sea and could be used as the basic knowledge of seasonal fluctuations and virus-host densities for planning of further researches.

Key-words: viruses, bacteria, Northern Adriatic Sea, mucilage

Running head: Virioplankton nel Golfo di Trieste

Introduzione
I virus sono le entità biologiche più rappresentate nell’ambiente planctonico (Bergh et al., 1989; Noble e Fuhrman, 2000; Steward et al., 1996; Wommack e Colwell, 2000) con abbondanze solitamente più elevate in superficie e decrescenti con la profondità (Weinbauer, 2004). La loro densità, soggetta a variazioni stagionali di circa 2 ordini di grandezza, risulta significativamente influenzata dalla presenza dei batteri (Wommack e Colwell, 2000), loro ospiti preferenziali. Diversi studi hanno dimostrato, infatti, la prevalenza numerica dei batteriofagi all’interno del virioplancton (Cochlan et al., 1993; Hara et al., 1996; Jiang e Paul, 1994; Weinbauer et al., 1995).

In Adriatico, bacino caratterizzato da una serie storica di dati relativi alla componente planctonica, lo studio dei virus è stato eseguito in modo sporadico e puntiforme (Stopar et al., 2003; Weinbauer et al., 1993), o comunque su scale temporali molto limitate (Bensi et al., 2003; Corinaldesi et al., 2003; Weinbauer e Peduzzi, 1994, 1995).

Il presente studio, seppur non fornendo informazioni sull’infettività, dà una descrizione delle abbondanze virali lungo il profilo verticale di una stazione costiera del Golfo di Trieste per 5 anni consecutivi. Insieme ai dati relativi alle abbondanze batteriche, può costituire la base per gli studi volti all’acquisizione delle conoscenze più approfondite relative alle interazioni tra le componenti del comparto microbico dell’Alto Adriatico, sistema in cui è stata già ipotizzata l’importanza del ruolo che i virus potrebbero svolgere a seconda delle diverse condizioni di trofia (Corinaldesi et al., 2003; Weinbauer et al., 1993).

Materiali e metodi
I campioni d’acqua sono stati raccolti in superficie (~0.5m), a 5m, a 10m ed al fondo in una stazione costiera del Golfo di Trieste (C1, 45°42’03”N, 13°42’,36” E),

Aliquote pari a 10 ml di campione sono state fissate con formaldeide (conc. finale 1%), precedentemente filtrata su filtri di porosità pari a 0.02 μm (Whatman Anotop), e conservate a 4 °C ed al buio per non più di 7 giorni. Al momento della preparazione del campione per l’analisi microscopica del virioplancton, condotta in microscopia ad epifluorescenza (Olympus BX 60, HBO 100 W), aliquote di campione sono state diluite 1:10 in acqua di mare, prefiltrata su membrane di porosità pari a 0.02 μm (Whatman Anotop), e filtrate su membrane in allumina di porosità pari a 0.02 μm (Whatman). Le membrane sono state quindi appoggiate su una goccia di SYBR Green I (Noble e Fuhrman, 1998) (conc. finale 50X) e riposte al buio per 15 min. Terminata la colorazione i filtri sono stati montati su vetrino utilizzando una soluzione di montaggio (50% glicerolo, 49% PBS, 0,5% acido ascorbico). I vetrini sono stati conservati a -20°C fino al momento della lettura avvenuta entro 2 settimane dal campionamento. Per l’analisi della componente batterica è stato seguito il protocollo di Porter e Feig (1980). La stima delle abbondanze virali, espresse come Virus Like Particles (VLP), e batteriche è stata effettuata analizzando 20 campi scelti casualmente ed osservando tre repliche per ogni campione.

Il rapporto tra virus e batteri (Virus to Bacterium Ratio – VBR) è stato calcolato utilizzando i valori di abbondanza integrati lungo la colonna d’acqua.

I dati relativi alle abbondanze virali sono stati corretti per evitare la sottostima dovuta alla conservazione del campione in formaldeide (Wen et al., 2004). E’ stato utilizzato un fattore di conversione pari a 1.34 derivato da una sperimentazione effettuata su campioni dell’alto Adriatico fissati con formalina (conc. finale 1%) ed analizzati dopo 7 giorni dal campionamento.

Risultati

Le abbondanze virali sono risultate comprese tra $1.0 \times 10^5$ e $3.5 \times 10^8$ VLP ml$^{-1}$ mentre il valore medio ed il valore mediano hanno raggiunto $7.5 \times 10^6$ e $5.9 \times 10^6$ VLP ml$^{-1}$ ($n=227$). Il massimo è stato registrato all’inizio di settembre 2003 alla profondità di 5 m mentre il minimo in dicembre 2002 in superficie. La distribuzione lungo la colonna risulta piuttosto omogenea e non sono state evidenziate differenze significative tra le profondità rilevate (CV%=6.4).

Dall’analisi delle distribuzioni annuali, espresse come valore integrato lungo la colonna d’acqua, si evidenzia come il 2000 sia stato caratterizzato da abbondanze relativamente elevate (media = $12.3\pm0.9 \times 10^6$ VLP ml$^{-1}$; mediana = $12.0 \times 10^6$ VLP ml$^{-1}$; $n=11$) mentre durante il 2002 il virioplancton è risultato poco rappresentato (media = $4.7\pm0.5 \times 10^6$ VLP ml$^{-1}$; mediana = $3.4 \times 10^6$ VLP ml$^{-1}$; $n=15$). Nel 2001 (media = $7.3\pm0.7 \times 10^6$ VLP ml$^{-1}$; mediana = $4.8 \times 10^6$ VLP ml$^{-1}$; $n=12$), nel 2003 (media = $6.5\pm0.3 \times 10^6$ VLP ml$^{-1}$; mediana = $4.9 \times 10^6$ VLP ml$^{-1}$; $n=23$) e nel 2004 (media = $7.6\pm0.4 \times 10^6$ VLP ml$^{-1}$; mediana = $6.5 \times 10^6$ VLP ml$^{-1}$; $n=23$) le abbondanze sono risultate tra loro confrontabili (Fig. 1).


