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Frequency, size and distribution of bacteriophages in different marine bacterial morphotypes

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ABSTRACT: The frequencies of cells containing mature phages, the burst sizes, the phage head sizes and the distribution of phages inside cells of different bacterial morphotypes were investigated in the northern Adriatic Sea. Coccoid bacteria more frequently (2.5%) contained mature phages than rod-shaped bacteria (1.2%) and spirillae (1.4%). Including an estimation of non-visible infection we found that up to 27% of rods were infected with viruses, up to 79% of cocci and up to 100% of spirillae. The highest overall infection frequency of the entire bacterial community was 30%. The percentage of rods with mature phages was significantly correlated to increasing rod densities. It is suggested that a threshold density of about 2×10^5 rods ml^{-1} exists that is necessary for infection with phages. No threshold densities could be determined for cocci and spirillae. Burst sizes varied strongly between different host morphotypes. The burst sizes of rods increased significantly with the frequency of rods containing mature phages, probably as a result of superinfection of bacteria with phages. The volume of the host cells seemed to influence the number of phages produced per cell. Most of the phages within rods and all phages within spirillae were smaller than 60 nm, whereas the majority of phages within cocci were larger than 60 nm. Analyses of the distribution of phages inside the cells showed that phages were frequently concentrated in 2 defined areas at the 2 opposite ends of both rods and spirillae. Our results from an *in situ* study suggest that the production of bacteriophages is strongly influenced by the structure of the bacterial community, i.e. by the relative abundances of the various morphotypes.

KEY WORDS: Bacteria · Bacteriophages · Viruses · Infection · Morphotypes · Marine ecosystem

INTRODUCTION

Spencer (1955, 1960) was the first to describe indigenous marine bacteriophages. In subsequent decades phage ecology in aquatic systems dealt mainly with single phage species or with single phage-host systems (e.g. Farrah 1987, Moebus 1987). This approach has provided important information on the distribution of single phage species and on some factors influencing survival and replication of phages. Since 1979, high total numbers of marine viruses have been reported, although the initial method used was not designed for a quantitative collection of viruses (Torrella & Morita 1979). Despite these important findings, bacteriophages were not considered to play a significant role in the dynamics of marine bacterial communities (e.g. Moebus 1987). A major breakthrough in re-evaluating the role of viruses in aquatic systems was presented by Proctor et al. (1988), Sieburth et al. (1988) and Bergh et al. (1989), who found that viral abundances in natural waters are high, usually even exceeding the densities of bacteria. It is supposed that bacteriophages constitute the majority within virus communities in marine systems (Proctor & Fuhrman 1990, Wommack et al. 1992, Cochlan et al. 1993). There is some information accumulating at present on the distribution of viruses in environments of different trophic conditions (Hara et al. 1991, Wommack et al. 1992, Boehme et al. 1993, Cochlan et al. 1993, Paul et al. 1993, Weinbauer et al. 1993). However, in aquatic systems the distribution of lytic versus temperate bacteriophages as well as the role of lysogeny are still poorly understood (e.g. Moebus 1983, Ogunseitan et al. 1990, 1992, Lammers 1992).

Some attempts have been made to quantify the rates and understand the mechanisms of viral decay (Heldal & Bratbak 1991, Suttle & Chen 1992) and also to include viruses into a budget of microbial C-transfer (Bratbak et al. 1992). Infection events have been observed in all numerically important plankton groups, e.g. bacteria and cyanobacteria (Proctor & Fuhrman 1990, 1991, Suttle & Chan 1993) as well as algae (Moestrup & Thomsen 1974, Mayer & Taylor 1979, Waters & Chan 1982, Sieburth et al. 1988, Suttle et al. 1990, Cottrell & Suttle 1991) and heterotrophic nanoflagellates (Nagasaki et al. 1993). The number of phages released during bacterial lysis (burst size) varies strongly; however, most of these data are derived from cultured phage-host systems (see review by Børsheim 1993). Nevertheless, viral infection frequencies and burst sizes are important parameters for estimating virus-mediated mortality of bacteria as well as viral production. There is a considerable lack of *in situ* data relating the viral infection frequencies and burst sizes of bacteria to parameters such as viral and bacterial density. Moreover, almost nothing is known about the potential variations of infection frequency and burst sizes in different bacterial morphotypes (Weinbauer et al. 1993). In the present study we demonstrate that the frequencies of cells containing mature phages, the burst size, the phage head sizes and the distribution patterns of phages inside cells can vary strongly between different bacterial morphotypes.

MATERIAL AND METHODS

Water samples were collected from surface water (~ 0.5 m) at various stations in the northern Adriatic Sea between 1991 and 1993 (Fig. 1). Samples for the analysis of viral and bacterial parameters were fixed immediately after collection with 0.2 µm filtered formalin (for electron microscopy 2 %, for epifluorescence microscopy 4 % final concentration) and stored at 4 °C in the dark until analysis. Virus particle abundance was determined using the ultracentrifugation methodology for harvesting viruses directly onto Formvar-coated, 400-mesh electron microscope grids as described by Mathews & Buthala (1970), Børsheim et al. (1990) and Bratbak et al. (1990) — for details consult Peduzzi & Weinbauer (1993). By using ultracentrifugation methodology, not only viruses but also bacteria are collected quantitatively onto electron microscope grids (Børsheim et al. 1990). Due to the high accelerating voltage of 80 kV used in this study, we were able to identify bacteria which contain mature phages (Weinbauer et al. 1993). In each sample at least 100 cells of each morphotype occurring in the sample were examined for the potential occurrence of mature phages. The number of mature phages occupying the whole cell (burst size), the morphology of the bacteria (rods, cocci or spirillae) as well as the distribution pattern of the phages inside the bacteria were recorded for each sample. Infected bacteria as well as phages were sized from electron micrographs (magnification 50 000×) under a dissecting lens (10×). Volumes were calculated assuming that spheres or cylinders with hemispherical ends are reasonable approximations to the real shape. The mean head size diameters of the phages within the cells were determined separately for the different host morphotypes. The total volume of all phages within a cell was calulated by multiplying the mean head volume of the phages by the burst size. Bacterial densities were determined with epifluorescence microscopy using the

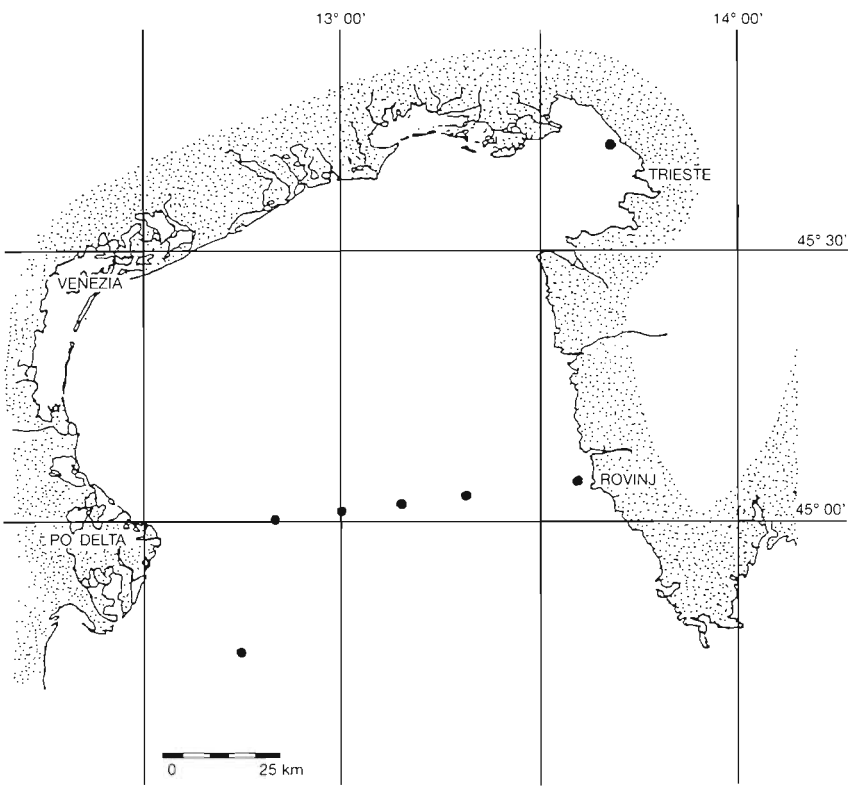

Fig. 1. Location of sampling stations in the northern Adriatic Sea

Table 1 Relative abundance and frequency of bacteria containing mature phages and burst size of various bacterial morphotypes. Values are means calculated from all investigated samples. n: no. of samples (range in parentheses)

Bacterial morphotype	n	% of total	n	Bacteria with mature phages (% of total)	n	Burst size
Rods	53	84.1 (62.5–96.5)	53	1.2 (0–3.8)	43	51 (12–124)
Cocci	53	10.7 (1.1–28.1)	53	2.5 (0–11.1)	36	28 (6–61)
Spirillae	53	5.2 (0–29.0)	53	1.4 (0–14.30)	39	35 (16–58)

Table 2. Distribution pattern of phages inside the different bacterial morphotypes. Values calculated from all investigated bacteria. n: no. of bacterial cells

Bacterial morphotype	n	% of total			
		Whole cell occupied	1 center	2–3 centers	No pattern
Rods	299	44.9	17.5	21.7	15.8
Cocci	238	83.3	8.2	0	8.4
Spirillae	254	59.9	22.0	18.1	0

AODC technique (Hobbie et al. 1977). Ambient temperature and salinity were determined for each water sample. Student's *t*-tests as well as correlation and regression analysis were performed.

RESULTS

Virus abundances from all seasons and stations ranged from 1.2×10^5 to 8.7×10^7 ml^{-1}, thus fluctuating over almost 3 orders of magnitude. Bacterial densities varied over approximately 1 order of magnitude from 1.2×10^5 to 2.4×10^6 ml^{-1}. Rod densities ranged from 1.0×10^5 to 1.9×10^6 ml^{-1}, cocci from 5.1×10^3 to 4.2×10^5 ml^{-1} and spirillae from not detectable to 9.6×10^4 ml^{-1}. Rods were the most abundant bacterial morphotype (>80%), followed by cocci and spirillae (Table 1). The proportion of the different morphotypes within the entire bacterial community did not vary significantly with bacterial density.

Distribution patterns of phages inside infected bacteria

In 53% of the infected bacteria, phages occupied the whole cell, whereas in 18%, phages were concentrated in 2, or rarely even in 3, defined areas of the host. Table 2 shows the distribution patterns of phages inside cells of the different bacterial morphotypes. In all morphotypes the majority of the infected cells were characterized by phages which occupied the whole cell (compare Fig. 2). The percentage of visibly infected bacteria that were filled completely with phages was higher in cocci than in rods and spirillae (Table 2). In approximately 20% of the infected rods and spirillae the phages were concentrated in 2 or rarely even 3 centers, in most cases located at the 2 opposite ends of the cell. Fig. 3 shows several bacterial cells with 2 distinct areas of phage accumulation. This distribution pattern of phages was never observed in coccoid bacteria. In all other infected bacteria the phages were concentrated in 1 defined area or distributed without any recognizable pattern.

Size classes of phages inside infected bacteria

Phages inside cells of all morphotypes were separated into 2 size classes based on the diameter of the heads: 30 to <60 and 60 to <110 nm. Most of the phages within rods and all phages inside spirillae were smaller than 60 nm, whereas the majority of phages inside cocci belonged to the size class 60 to <110 nm (Table 3).

Burst size of phages in infected cells

The mean burst size of the entire bacterial community, corrected for the different relative densities of the morphotypes given in Table 1, was 48 phages released per cell. The burst size of phages was significantly higher in rods than in cocci and in spirillae ($p < 0.05$; Table 1) and showed considerable variability within each morphotype. The total volume of phage material within a cell varied between 0.006 and 0.133 μm^3. The burst size of rods increased significantly with the frequency of rods containing mature phages (Fig. 4). No correlation was found between the burst size of rods and virus or rod abundance. The burst sizes of cocci and spirillae did not vary significantly with any of the above-mentioned parameters. The burst size varied considerably when

Table 3. Size class distribution of phages inside different bacterial morphotypes. Values calculated from all sized bacteria. n: no. of sized bacteria

Bacterial morphotype	n (total)	% of total	
		30 to <60 nm	60 to <110 nm
Rods	31	74.2	25.8
Cocci	29	34.5	65.5
Spirillae	16	100.0	0

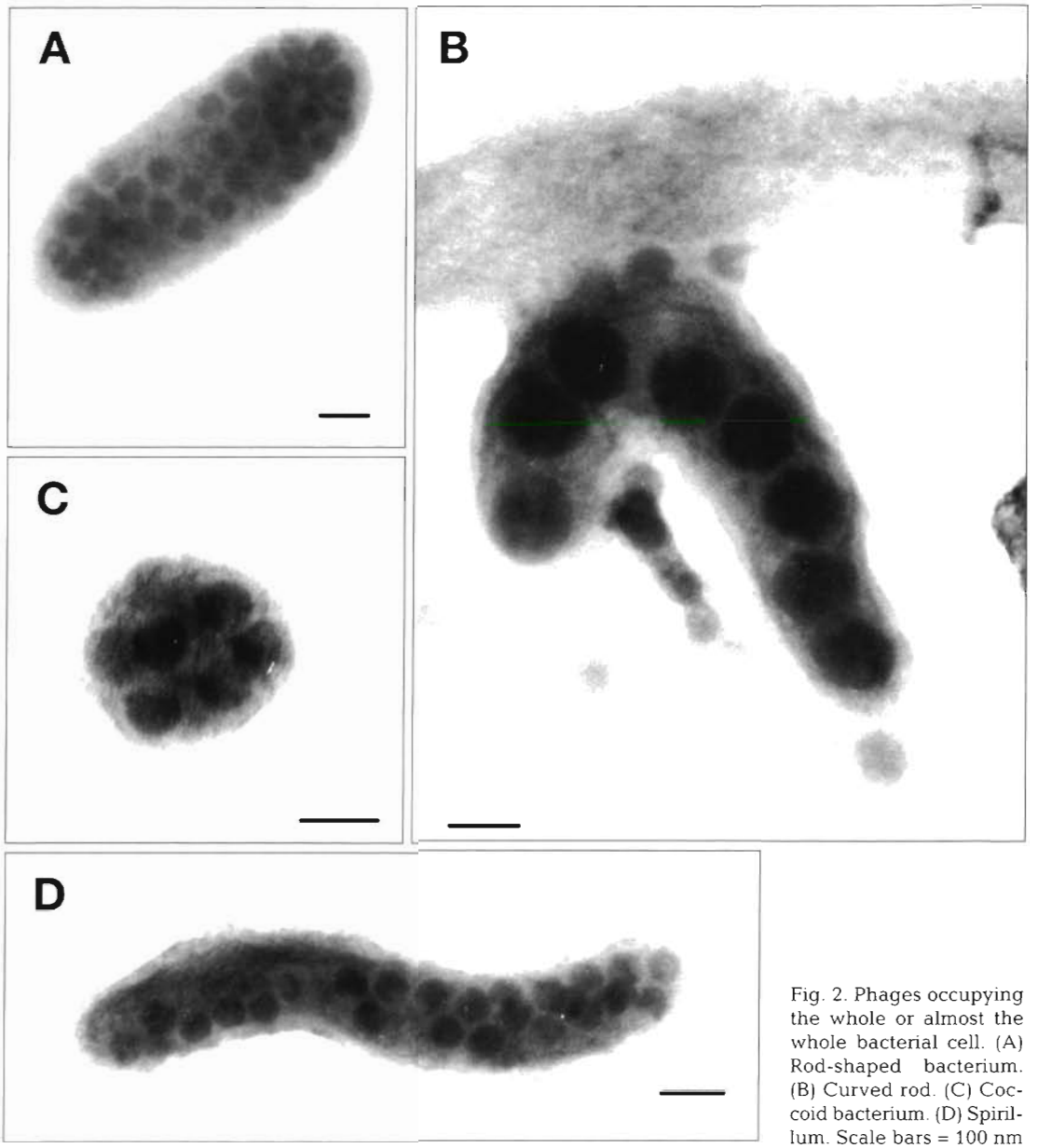

Fig. 2. Phages occupying the whole or almost the whole bacterial cell. (A) Rod-shaped bacterium. (B) Curved rod. (C) Coccoid bacterium. (D) Spirillum. Scale bars = 100 nm

the phages were smaller than 60 nm, whereas in larger phages the burst size was usually lower than 50 (Fig. 5). We found no significant correlation between bacterial cell volume and the burst size. However, when the phages were grouped in 2 size classes based on phage head diameter (30 to <60 and 60 to <110 nm), the burst size in both size classes increased with increasing bacterial volumes (Fig. 6). Fig. 2C shows a coccus with a burst size of less than 10 and a mean phage diameter of 72 nm. The rod-shaped bacterial cell in Fig. 2B is approximately twice as large as the coccoid bacterial cell in Fig. 2C (0.145 vs 0.065 μm^3), but exhibited a low burst size with large phages (mean phage diameter 105 nm). The burst size did not vary significantly with salinity (range 31.0 to 37.9‰) or ambient water temperature (range 7.9 to 28.5 °C) in any of the morphotypes.

Frequencies of bacteria containing mature phages

The mean frequency of cells containing mature phages was significantly higher in cocci than in rods and spirillae ($p < 0.005$; Table 1). The maximum frequency of cells containing mature phages was 3.8% in rods, 11.1% in cocci and 14.0% in spirillae (Table 1). The overall frequency of cells containing mature phages in the entire bacterial community ranged from not detectable to 4.2%. At bacterial densities of less

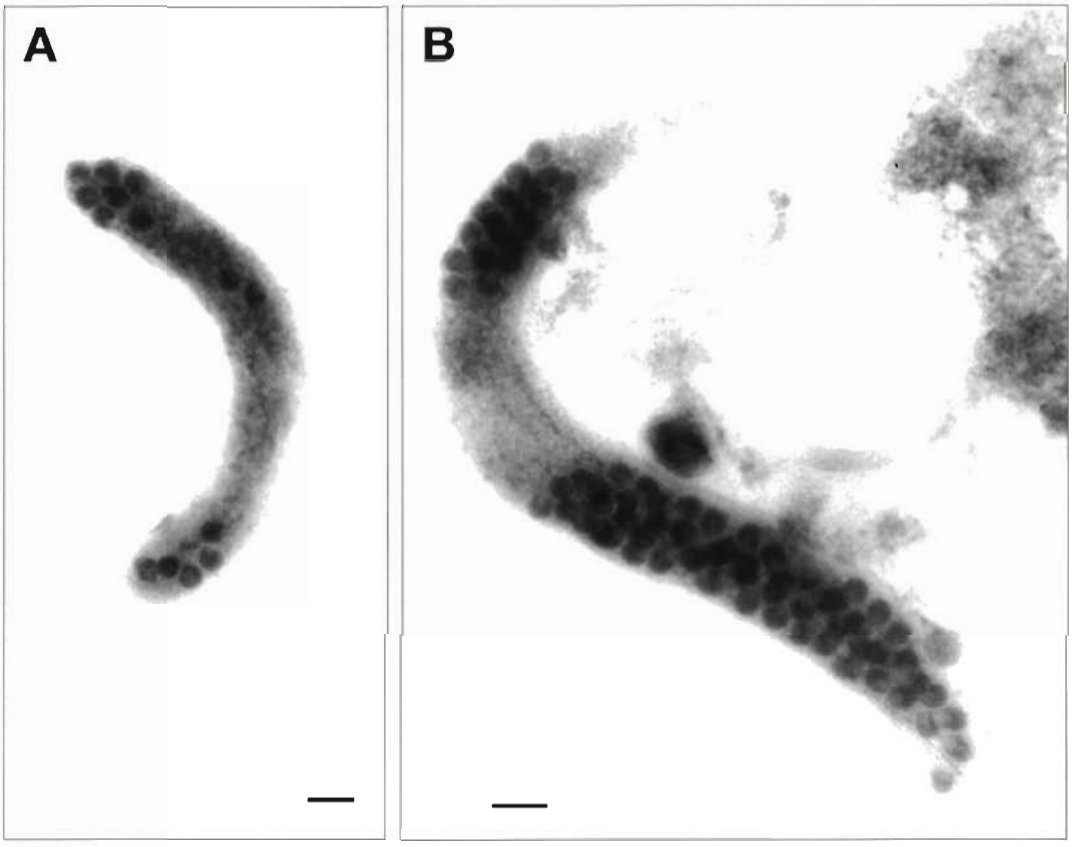

Fig. 3. Phages distributed in 2 defined areas inside rod-shaped bacteria. (A) Curved rod. (B) Spirillum. Scale bar = 100 nm

than 2×10^5 rods ml^{-1} we were unable to observe any rod containing phages even when 800 or more bacteria were inspected. At higher rod densities we found infected cells in almost all samples. Such threshold densities were not detectable for cocci or spirillae. The frequency of rods with mature phages was correlated significantly to increasing rod densities (Fig. 7), but not to increasing viral abundance. The frequency of cocci and spirillae containing mature phages did not vary significantly with the abundances of the respective morphotype or with viral density.

DISCUSSION

The investigated bacterial morphotypes exhibited strong differences regarding the frequency of cells containing mature phages, the burst size (Table 1) and

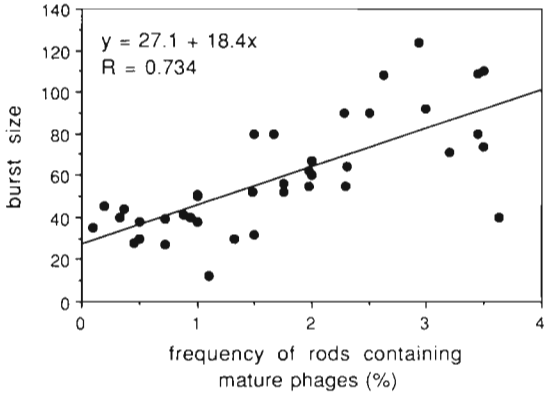

Fig. 4. Correlation of burst sizes with the frequency of rods containing mature phages

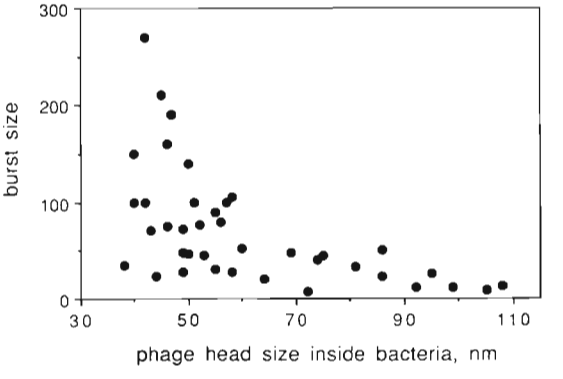

Fig. 5. Dependency of the burst size of phages on phage size

the distribution and size of phages within the cells (Tables 2 & 3). To some extent this might be due to physiological differences associated with the morphotypes. For example, it is known that enhanced growth of cocci due to favourable nutrient supply can result in rod-shaped cells (Amy & Morita 1983, Holmquist & Kjelleberg 1993) and that the impact of phages on bacteria strongly depends on the metabolic activity of the host (e.g. Probst-Ricciuti 1972, Moebus 1987). However, since all marine phages investigated so far are species specific (Børsheim 1993), the different size of phages inside the different morphotypes (Table 3) indicates that rods and cocci were also genetically different in our study.

Infection frequencies and bacterial threshold densities

When a cell is infected with a phage, there is a time lag until mature phages are visible and the cell is lysed. Conversion factors for relating the frequency of marine bacteria containing visible phages to the total percentage of bacteria that are phage-infected (infection frequency) are in the range 3.7 to 7.14 (Proctor et al. 1993). Since these conversion factors are derived from investigations using thin sections of cells, they might not be applicable to whole-cell observations as done in our study. However, our own preliminary data, based on the investigation of whole cells, revealed conversion factors between 3.9 and 6.6 (Weinbauer & Peduzzi unpubl. data), well within the range of the published conversion factors. Using the conversion factors given by Proctor et al. (1993) we estimated that from 0 to 30% of the total bacterial community were infected with phages. These estimates are close to infection frequencies of 0 to 31% found in other studies (Heldal & Bratbak 1991, Proctor et al. 1993). The different investigated morphotypes exhibited infection frequencies of up to 27% for rods, 79% for cocci and 100% for spirillae. However, there is the possibility that we lost phage-infected bacteria during the centrifugation process by disruption of cells. Thus the determination of the frequency of bacteria containing mature phages and the estimation of the infection frequency might be conservative.

For some bacteria it is known that their abundance is affected by bacteriophages only when the host density exceeds a threshold density (Wiggins & Alexander 1985). However, in another study no such threshold values were reported (Kokjohn et al. 1991). We did not find any infected cells at rod densities below 2×10^5 ml^{-1}, indicating that a threshold abundance of rods may exist that is necessary for a successful infection. However, since during these low rod densities the viral densities were also the lowest found in our study (less than 5×10^5 ml^{-1}), one can speculate that the threshold densities of rods might be variable depending on the viral abundance. In our study no threshold abundance could be observed for cocci and spirillae, indicating that the production of phages does not depend on the host density in these bacterial morphotypes. One possible explanation for this may be that a high percentage of cells are lysogenic and that virus production does not depend on a recent infection alone. Lysogeny could also be the reason why the frequency of cocci and spirillae with mature phages was similar or even higher than in rods (Table 1), although the host densities were low (together about 15% of the total bacterial community). Another possible explanation for the high frequency of cocci and spirillae with mature phages could be that the species diversity in these morphotypes might be lower than in rods, thus increasing the propagation probability of specific viruses. The finding that the range of phage sizes was lower in spirillae than in rod-shaped bacteria might support the idea of only few phage-host systems (Table 3).

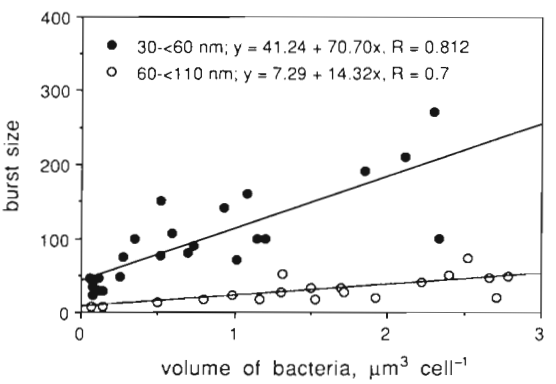

Fig. 6. Correlation of the volume of bacterial cells with the burst size of phages from 2 size classes

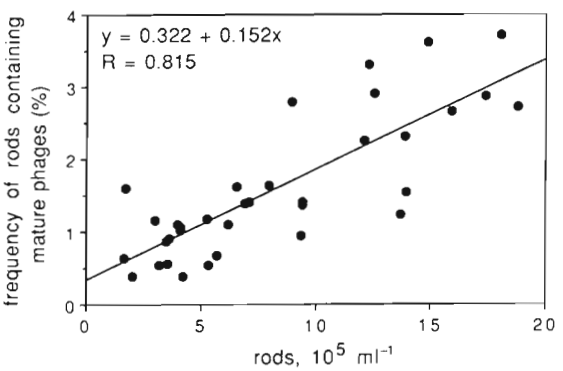

Fig. 7. Correlation of the frequency of rods containing phages with rod density

Distribution patterns of phages inside infected cells

Phages were frequently concentrated in 2 distinct areas within rod-shaped cells and spirillae. These centers of mature phages were generally located at the 2 opposite sides of the cells (Fig. 3) indicating that the development of phages may initiate at the distant parts of a cell and then proceed towards the center until the whole cell is occupied by phages. It might be that the occurrence of these 2 phage clusters is due to beginning cell division, thus originating from the 2 new, recently replicated bacterial genomes. This could happen with lysogenic bacteria (bacteria with viral DNA in their genomes) which have replicated both viral and their own DNA and then entered the lytic cycle producing mature phages at the distant parts of the cell. We never observed 2 phage centers in coccoid bacteria (Table 2). One explanation for this could be that a coccoid cell can turn into a rod shape before it divides again into 2 cocci. Thus some of the rods with 2 defined phage areas could originate from coccoid bacteria that are just dividing. However, it cannot be excluded that the formation of 2 clusters by nontemperate phages is characteristic for some rods and spirillae, but not for cocci.

In approximately 50% of the visibly infected rods and spirillae and in more than 80% of cocci the entire cell was occupied by mature phages (Table 2). One reason might be that the time span between occurrence of the first mature phages and lysis of cocci is shorter than in the other morphotypes. Since cocci had the lowest burst size of all bacterial morphotypes (Table 1), phage formation could be completed faster than in the other morphotypes, thus resulting in a higher percentage of infected cocci with phages occupying the whole cell. The determination of conversion factors for the estimation of infection frequencies depends on the proportion which the period characterized by visible phages contributes to the entire latent period (see first section of 'Discussion'). If the assumption is true that cocci are characterized by faster phage maturation, the conversion factors should be conservative when applied to coccoid bacteria. This indicates that we need further information on conversion factors for the different morphotypes.

Size distribution of phages inside infected cells

Over the entire bacterial community in this study 71.3% of the phages were smaller than 60 nm. This is well within the range of 81.7% and 54.3% of free viruses smaller than 60 nm found in the years 1991 and 1992 respectively in the northern Adriatic Sea (Weinbauer et al. 1993). A similar distribution of size classes of free viruses is known from other environments (Bratbak et al. 1990, Wommack et al. 1992, Cochlan et al. 1993). The fact that most free viruses as well as phages inside bacteria are smaller than 60 nm may support the speculations of Proctor & Fuhrman (1990), Wommack et al. (1992) and Cochlan et al. (1993) that the majority of marine viruses are bacteriophages. Moreover, since rods are the most abundant bacterial morphotype (Table 1), phage production by rods could be the reason why the size class distribution of free viruses is often skewed towards small viruses. In our study, the majority of the phages inside cocci, a less abundant morphotype, belonged to the size class 60 to <110 nm (Table 3). A head size diameter of approximately 100 nm was also reported for phages of cocci from another environment (Bratbak et al. 1990).

Burst sizes of phages inside infected cells

In the present study the burst size was determined as the number of phages occupying the whole cell. On the one hand, this estimation might be conservative, since phages lying on top of each other are probably counted as 1 phage. On the other hand it could also be an overestimation, if some phages tend to lyse cells before they are completely full. Since almost all bacteria observed in the disruption stage were completely full with phages (unpubl. data), it is unlikely that we overestimated the burst size. However, some uncertainties remain regarding the burst sizes as determined at present.

The burst size varied both between and within the different bacterial morphotypes (Table 1). Temperature and salinity are among the factors that can affect the burst size of marine bacteria (Zachary 1976). However, since in our study salinity and temperature were not correlated significantly with the burst size of the different morphotypes, changes in temperature and salinity were probably unimportant for the variability of the burst size in the investigated environment. Doermann (1948) was the first to describe the phenomenon of 'lysis inhibition' which means that the reinfection (superinfection) of an already infected bacterial cell results in a delay of the lysis and thereby in an increase of the burst size. If a high infection frequency of bacteria indicates a high probability of successful virus attack, then the probability of superinfection should also increase. Therefore superinfection could explain why the burst size increased together with the frequency of rods containing mature phages in our study (Fig. 4). The phages of the most abundant bacterial morphotype, the rods, are probably the only phages that can be produced in quantities large enough to

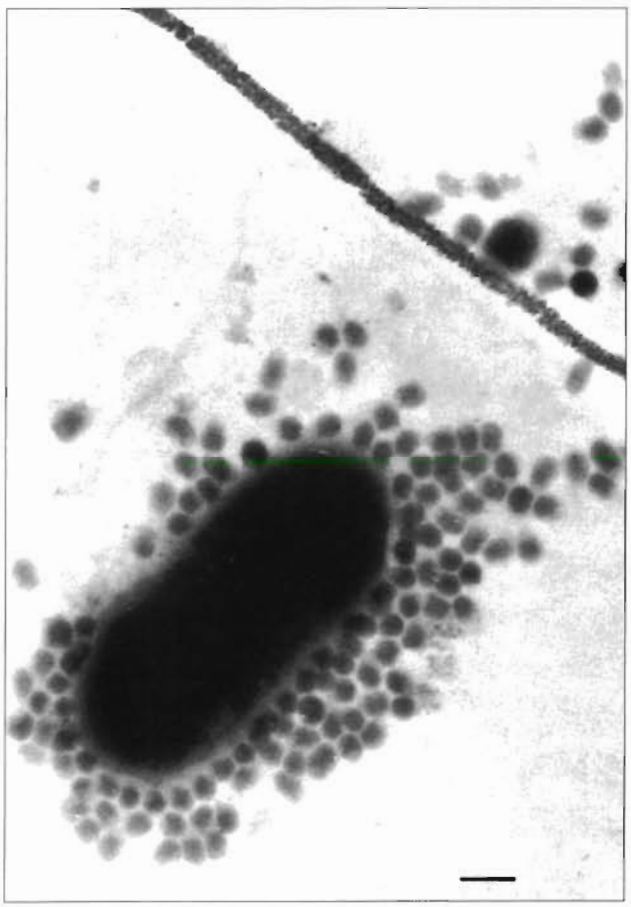

Fig. 8. Rod-shaped bacterial cell surrounded by a cluster of viruses of similar size and shape. Scale bar = 100 nm

cause frequent superinfection events. Since viruses of the same size were frequently observed in clusters or surrounding bacteria (Fig. 8; see also Fig 5B, C in Bratbak et al. 1992), superinfection of bacteria can well be assumed. Other factors such as growth rate (see e.g. Probst-Ricciuti 1972) or bacterial cell volume (Fig. 6) could also have determined the burst sizes of the bacteria. However, we do not know whether the relationship between the frequency of cells containing mature phages and the burst size (Fig. 4) is influenced by bacterial growth rate or bacterial cell volume.

In both of the 2 size classes of phages (30 to < 60 and 60 to < 110 nm) the burst size was correlated significantly with the volume of single infected bacterial cells (Fig. 6), indicating that the size of the infected host might determine the maximum amount of phages produced. This implies that factors determining the size of bacteria, e.g. nutrient supply, might also determine the total amount of phage material set free. Reviewing the literature, Børsheim (1993) calculated a mean burst size of 185 phages in cultured marine bacteria. This burst size is much higher than that determined by electron microscopy in the present study (48 phages per lysed cell) or given in Heldal & Bratbak (1991). Since cultured bacteria are usually larger than indigenous bacterial cells, more phages might be produced in cultured cells (compare Fig. 6) probably leading to higher burst sizes. Small size of bacterial cells in some environments might not only be due to low nutrient supply, but also to size-selective grazing of heterotrophic nanoflagellates on bacteria (Andersson et al. 1986, González et al. 1990). The number of phages produced per cell should then be lower due to the shift towards smaller bacteria. Reduced phage production could result in a lower probability of new infections. Moreover, the ingestion of viruses by heterotrophic nanoflagellates (González & Suttle 1993) is an additional way in which heterotrophic nanoflagellates can influence phage abundance and size distribution.

From our results we conclude that the various bacterial morphotypes may be affected by phages in different ways. We can assume that the impact of phages varies strongly on both a temporal and spatial scale, since the composition of the bacterial community is heterogenous and the relative abundance of the various morphotypes varies even within 1 study site or between different environments (Table 1; Herndl & Peduzzi 1988, Bratbak et al. 1990, Velimirov & Walenta-Simon 1992). This is supported e.g. by the finding that within the total bacterial community, only the numbers of cocci decreased in the slime system of diatoms, which was supposed to be the result of phages specific for these cocci (Bratbak et al. 1990). Based on recent findings using molecular probes it is now suggested that bacterial assemblages are very rich in species, but sometimes only a few of them may form the bulk of the biomass (Lee & Fuhrman 1991, Rehnstam et al. 1993). Since the marine phages investigated so far are species specific (Børsheim 1993), it might well be that temporal changes of dominating bacterial species are caused by the development of specific phages. Our estimation that up to 100% of spirillae can be infected supports the view that specific bacterial blooms can be controlled by viruses. Moreover, it has been suggested that viruses can also terminate monospecific phytoplankton blooms (Suttle et al. 1990, Bratbak et al. 1993). The fact that the frequency of rods containing mature phages increases with the host density (Fig. 7) indicates that the infection and/or phage production is enhanced when the rod density increases. Increasing viral production with bacterial density was also found in another study (Steward et al. 1992). Since the burst size increased together with the increasing frequency of rods containing mature phages (Fig. 4), the release of more phages per cell should result in a higher probability of new infection events, thus accelerating phage production. This might be an important mechanism for phages

to respond quickly to increased host densities, e.g. as in bloom situations. Therefore, further research is strongly urged in order to evaluate the role of virus-mediated mortality for the dynamics of marine bacterioplankton.

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