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# High control of bacterial production by viruses in a eutrophic oxbow lake

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ABSTRACT: The aim of the study was to test the hypothesis that the magnitude of viral control on bacterial production in a eutrophic oxbow lake of the River Danube would be higher than all average values reported so far in the literature. This assumption was based on the findings of low grazing of heterotrophic nanoflagellates (HNF) in this system, accounting on average for 5% of the bacterial mortality. Several approaches (viral decay method, estimation of the frequency of infected bacterial cells) to determine viral control of bacterial production were applied on a comparative basis. All system-specific parameters necessary to describe virus-bacteria interactions (burst size, bacterial production, contact rates) were monitored simultaneously. The average viral control of bacterial production determined by the different approaches was similar, ranging from 55.7 to 62.7%, and prevailing over HNF grazing by a factor of more than 11. For individual events, however, we observed large variations between the methods, indicating that the use of one single method is not reliable to decide whether a detected trend is representative of a specific system. We discuss error sources of the applied methods and mathematical models, and accounted for them when calculating the contribution of viruses to bacterial mortality. We demonstrated that viruses could control more than 100% of the bacterial production in the Alte Donau, which implies that occasionally up to 1.6% $h^{-1}$ of the bacterial standing stock was removed from the water column. High bacterial mortality due to viruses indicated that a large amount of dissolved organic carbon might be recycled from bacteria by phage-induced cell lysis. On average 15.2 µg C $l^{-1}$ $d^{-1}$, corresponding to some 46% of the bacterial secondary production (BSP), was released into the water column due to viral lysis of bacterial cells and again became available for microheterotrophic consumption.

KEY WORDS: Virus · Bacteria · Viral decay · Bacterial mortality · Eutrophic oxbow lake

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## INTRODUCTION

Viral infection of bacteria and subsequent lysis of cells accounts for a significant proportion of bacterial mortality in marine and freshwater systems (e.g. Fuhrman 1999, Wilhelm & Suttle 1999, Wommack & Colwell 2000). Viruses were shown to contribute to bacterial mortality at a percentage similar to that of heterotrophic nanoflagellates (HNF) when both sources of bacterial losses were measured simultaneously (Fuhrman & Noble 1995, Giuxa-Boixereu et al. 1996, 1999, Steward et al. 1996, Weinbauer & Höfle 1998a). Viral lysis of bacterial cells results in the release of viruses as well as cell wall and membrane fragments of the cell, other large cellular fragments, and intracellular polymers such as proteins and nucleic acids. Hence, nitrogen- and phosphorus-rich macromolecules such as DNA, RNA and protein might become available for bacterial nutrition (Paul et al. 1987, Rosso & Azam 1987, Karl & Bailiff 1989, Noble & Fuhrman 1999). It has been reported that viral lysis products can strongly stimulate bacterial production and carbon uptake (Fuhrman & Noble 1995, Middleboe et al. 1996). Even unsuccessful infection could be important for carbon and nitrogen cycling because enzymes might destroy the inactivated viruses, and the released products probably are a source of nucleic

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acids and amino acids for bacteria (Proctor & Fuhrman 1990). Hence, most of the carbon release during viral lysis of bacterial cells might be cycled several times in the bacteria-virus-dissolved organic matter (DOM) loop before it gets mineralized, and the transfer of this carbon to higher trophic levels might be reduced (Thingstad et al. 1993).

For the quantification of the viral control of bacterial production, several methods have been used during the last decade. Some of them preclude information on viral production, which can be monitored by viral decay (Heldal & Bratbak 1991), dilution technique (Wilhelm et al. 1998a, Hewson et al. 2001), $^3$H-thymidine incorporation or $^{32}$P incorporation (Steward et al. 1992). Moreover, the frequency of infected bacterial cells (FIC) can be estimated by applying conversion factors presented by Proctor et al. (1993) to the frequency of visibly infected cells (FVIC), which can be determined by transmission electron microscopy (TEM). Assuming that bacteria undergo binary fission (1 daughter cell survives to divide again), the proportion of total bacterial mortality due to viral lysis is equal to the FIC multiplied by a factor of 2. This 'factor-of-2' rule has been used in most studies about viral impact on bacterial production (e.g. Hennes & Simon 1995, Steward et al. 1996, Weinbauer & Höfle 1998a, Guixa-Boixereu et al. 1999, Wilhelm & Smith 2000), but its universal validity for, e.g. limnic systems or marine systems of different trophic status has never been tested. The model of Proctor et al. (1993) fails to take into account the continuous nature of viral infection, growth and mortality of bacterial cells in a population, and the possibility that grazing mortality is an alternative fate for infected bacterial cells. A mathematical model has been developed recently which incorporates grazing of bacterivores in the assessment of the viral impact on bacterial production (Binder 1999).

We monitored the variations in viral and bacterial abundance over a period of 12 mo in a eutrophic oxbow lake of the River Danube and tried to estimate the relative importance of viral lysis as a mechanism of bacterial loss, not expecting that microzooplankton would exhibit the main control of the bacterial production. Hence, the aim of this quantification was to test the hypothesis that the low grazing impact of HNF on bacteria, accounting on average for 5% of the bacterial mortality in our system (Wieltschnig et al. 1999, 2001), resulted in an unproportionally high viral impact on bacterial production. Many studies investigated virus-bacteria interactions, but most of them used only 1 method to determine phage-induced bacterial mortality. As mentioned earlier, the model of Proctor et al. (1993) has been widely used, even though the validity of the conversion factors for different aquatic systems is debatable. In contrast, the decay method has rarely been applied (Heldal & Bratbak 1991, Mathias et al. 1995, Guixa-Boixereu et al. 1996, 1999, Tuomi et al. 1999), and only 2 authors used this method simultaneously with the model of Proctor et al. (1993) (Mathias et al. 1995, Guixa-Boixereu et al. 1996, 1999).

Although our estimations of phage-induced bacterial mortality were based primarily on data from viral decay experiments, mean burst size and bacterial secondary production (BSP), we also calculated the FIC, applying the model of Proctor et al. (1993) and the model of Binder (1999). It was expected that a discussion on the quality of the observed data could be improved by comparing the results of different approaches. Moreover, care was taken to monitor simultaneously the parameters necessary to describe virus-bacteria interactions such as burst size, contact rates (CRs) and BSP (using specific conversion factors calculated for the Alte Donau at different water temperatures).

Besides corroborating the quantification of material flux processes in the microbial and the viral loop, we hope to be able to offer constructive criticism of the value of the above-mentioned models describing the bacterium-virus interrelationships.

## MATERIAL AND METHODS

**Study site.** The field work was carried out in the Alte Donau, a eutrophic, stagnant oxbow lake of the River Danube within the city of Vienna, with an area of about 1.6 km$^2$, a volume of $3.7 \times 10^6$ m$^3$ and a depth of 3.5 m.

**Sample collection.** For the seasonal investigation of bacterial and viral abundance, water samples from 4 depths (0.5, 1.5, 2.5 and 3.5 m below the surface) were taken monthly with a Ruttner sampler from January 1996 to January 1997 between 09:00 and 10:00 h. Six subsamples (25 ml) were fixed with electron microscopy grade glutaraldehyde (2% final concentration; Agar Scientific Ltd, Stansted, UK), and were kept at 4°C in the dark until processing. For the decay experiments, triplicate water samples (0.5 l) from 4 depths (0.5, 1.5, 2.5 and 3.5 m below the surface) were pooled in three 2.5 l glass bottles and brought to the laboratory within 1 h. All samples were taken from a pier at the bank of the lake. The bottles were cleaned with acid and rinsed with double-distilled and sample water prior to use.

**Viral decay experiments.** Five separate decay experiments were carried out in triplicate from July 1996 until December 1996 at 5 water temperatures (July, 20°C; August, 23°C; September, 15°C; October, 10°C; December, 2.5°C). The decay, i.e. the decrease in viral

concentration over time, was recorded after inhibiting the production of new viruses by addition of potassium cyanide (KCN) to a final concentration of 2 mM (Heldal & Bratbak 1991). The pH of the KCN stock solution was adjusted to the *in situ* pH. Three experimental containers (2.5 l glass bottles) were subsampled (25 ml) at intervals of 30 min to 9 h for determining the total abundance of free viruses and measuring capsid diameters. Prior to the removal of samples, the bottles were shaken to avoid any counting error caused by the adsorption of viruses to the wall of the bottles. Samples were preserved with glutaraldehyde (2% final concentration). All incubations for decay experiments were done at *in situ* temperature.

**Bacterial production.** BSP was measured at the beginning of each decay experiment through the incorporation of $^{3}$H-thymidine and $^{14}$C-leucine, following the methods of Fuhrman & Azam (1982) for thymidine, and Kirchman et al. (1985) and Simon & Azam (1989) for leucine. Briefly, labeled thymidine (specific activity: 75 to 85 Ci mmol$^{-1}$; NEN Life Science Products, Boston, MA, USA) was added to 4 subsamples (10 ml) and 2 formaldehyde-fixed controls (10 ml) at a final concentration of 30 nM. The incubation at *in situ* temperature was terminated after 30 min by addition of formaldehyde (2% final concentration). The fixed samples were acidified with ice-cold trichloroacetic acid (TCA; 5% final concentration). After 20 min, precipitates were filtered through 0.1 µm pore size cellulose nitrate filters (Sartorius) and treated with 5 ml of phenol-chloroform (50% w/v) solution and 5 ml of ice-cold 80% v/v ethanol (Wicks & Robarts 1987). Scintillation cocktail (4.5 ml; Ultima Gold, Canberra Packard) was added to the dry filters, and after disintegration of the filters, the radioactivity was measured in a liquid scintillation counter (1900 TR, Canberra Packard, Groningen, The Netherlands). Counts were automatically corrected for quenching using a stored standard curve and a machine counting efficiency programme. BSP was calculated using specific conversion factors calculated for the Alte Donau at different water temperatures, ranging from $0.5 \times 10^{6}$ to $7.0 \times 10^{6}$ cells pmol$^{-1}$.

$^{14}$C-leucine (specific activity: 325 mCi mmol$^{-1}$; NEN Life Sciences) was added to replicate subsamples (10 ml) and 1 formaldehyde-fixed control at a final concentration of 40 to 60 nM. The appropriate concentrations were seasonally evaluated by saturation experiments and revealed that saturation occurred at concentrations no higher than 60 nM. Samples were incubated for 30 min at *in situ* temperature. After terminating the incubation by addition of formaldehyde (4% final concentration), proteins were precipitated on ice by adding TCA (5% final concentration) and boiling for 30 min to disintegrate DNA. After filtration through 0.1 µm pore size cellulose nitrate filters (Sartorius) and addition of 5 ml scintillation cocktail (Ultima Gold) the radioactivity was measured in a scintillation counter (1900 TR, Canberra Packard).

**Virus and bacterial counts.** Viruses and bacteria from 12 ml samples were harvested onto carbon stabilized collodium-coated electron microscope grids (400 mesh nickel grids, ATHENE SIRA, 3.05 mm diameter; Smethurst High-Light, Stansted, UK) using a swinging-bucket rotor (Beckman SW 40 Ti) run at 100 000 × *g* for 1 h at 20°C (Børsheim et al. 1990, Bratbak et al. 1990). Grids were stained with 1% uranyl acetate (Agar Scientific) for about 1 min and rinsed 3 times with deionized distilled water. Viruses and bacteria were enumerated on duplicate grids using a Zeiss EM 902 TEM (80 kV). They were counted at 85 000× and 20 000× magnification, respectively. Viruses were identified on the basis of morphology (round or hexagonal capsid structures, tailed and non-tailed), size and staining characteristics. Bacteria were counted only at the beginning of the viral decay experiments. Total counts of 20 to 30 randomly selected fields usually exceeded 250 bacteria and viruses per subsample, respectively. A taper correction factor as described by Suttle (1993) was used to compensate for the non-linear harvesting of particles in the centrifuge tubes. Viral capsid diameters of a minimum of 70 randomly chosen viruses were measured in each triplicate sample, using an Image Analysis System (Nokia Multigraph 449E). They were grouped into the following size classes: <60 nm, 60 to <90 nm, 90 to <150 nm, and ≥150 nm.

**Determination of viral burst size and visibly infected bacteria.** Bacteria were scored as infected when they contained 5 or more intracellular viruses. Viruses inside cells were identified on the basis of structure, size and intensity of staining, and uniformity of structure, size and staining intensity. Burst size was estimated for each triplicate sample by inspecting on average 120 visibly infected cells (VIC) at 30 000× magnification. We distinguished between 2 types of burst size: minimum burst size, estimated as the average number of mature phages in all visibly infected bacteria, and maximum burst size, determined as the average number of viruses in those cells that were completely filled with viruses (Weinbauer & Suttle 1996, Weinbauer & Höfle 1998b). For the estimation of the FVIC, at least 300 bacteria were examined at 20 000× magnification in order to obtain 20 to 60 VIC subsample$^{-1}$. According to the model of Proctor et al. (1993), the FVIC was converted to the FIC using the low (3.70), high (7.14) and average (5.42) conversion factors. A new model was used to calculate the FIC taking into account the effect of grazing mortality on the relationship between FVIC and FIC (Binder 1999). Burst size and FVIC were determined only at the beginning of the decay experiments.

**CR.** The CR between viruses and bacterial cells was calculated using the formula presented by Murray & Jackson (1992):

$$CR = (Sh \times 2\pi \times d_b \times D_v) \times B_n \times V_n$$

where $Sh$ is the Sherwood number (dimensionless), $d_b$ is the average diameter of bacterial cells ($1 \times 10^{-4}$ cm), $D_v$ is the diffusivity of viruses, and $V_n$ and $B_n$ are the abundance of viruses and bacterial cells, respectively. The Sherwood number depends on the Peclet number ($Pe$), which can be determined using the following equation:

$$Pe = (d_b \times v_b)/D_v$$

where $v_b$ is the swimming velocity of bacteria and $D_v$ is the viral diffusivity, calculated as follows:

$$D_v = (k \times T)/(3\pi \times \mu \times d_v)$$

where k is the Boltzmann constant ($1.38 \times 10^{-23}$ J K$^{-1}$), $T$ is the absolute *in situ* temperature, μ is the viscosity of water, calculated from given values for different water temperatures in Schwörbel (1987), and $d_v$ is the average viral capsid diameter (60 nm in our study). The average swimming velocity of bacteria is dependent on their size, and can be expressed as follows:

$$v_b = (0.15 \times d_b^{0.471})$$

This equation was deduced from swimming speed data of organisms of various sizes from Fig.1 in Murray & Jackson (1992).

The Sherwood number can now be approximated by the following equation:

$$Sh = 0.5 \times [1 + (1 + Pe)^{1/3}]$$

Specific CRs were calculated by correcting the CR for bacterial abundance to estimate the number of contacts per cell and time. Contact success was estimated as the number of cells lysed per time divided by the number of contacts per time × 100 (Weinbauer & Höfle 1998a,b, Wilhelm et al. 1998b).

**Statistical analysis.** All data were log transformed for statistical analysis. Kruskal Wallis 1-way ANOVA was used to test whether BSP and viral decay rates were significantly different between the various sampling events at different water temperatures. A probability of <0.05 was considered significant in all statistical analysis. We used STATISTICA 4.0 software (StatSoft Inc, Tulsa, OK, USA).

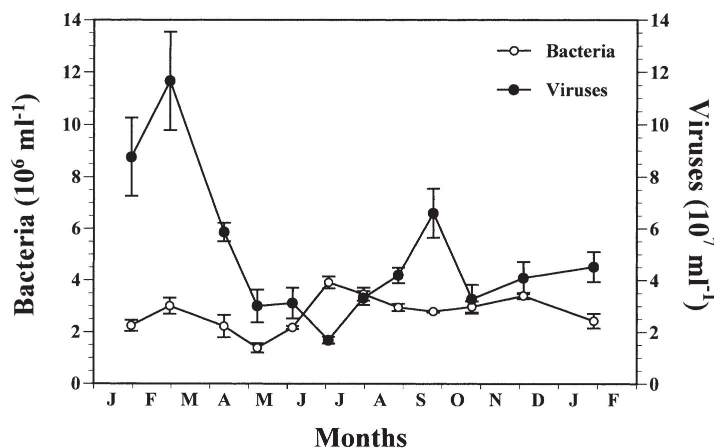


Fig. 1. Seasonal variations in total viral and bacterial counts in the Alte Donau, 1996. Error bars represent standard deviations (SDs) of 6 subsamples

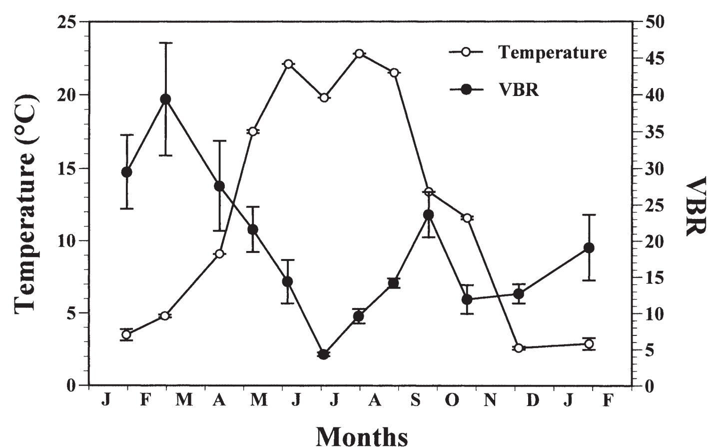


Fig. 2. Seasonal variations in water temperature and virus-to-bacterium ratio (VBR) in the Alte Donau, 1996. Error bars represent SDs of 6 subsamples

## RESULTS

### Viral and bacterial abundance and distribution of virus capsid diameter

Variations in viral numbers exhibited a dynamic pattern of fluctuations during the investigated seasons in 1996. Values ranged from $1.7 \times 10^7$ to $11.7 \times 10^7$ particles ml$^{-1}$, averaging $5.0 \times 10^7$ particles ml$^{-1}$ (Fig. 1). In contrast, bacterial abundance fluctuated less, varying from $1.4 \times 10^6$ to $3.9 \times 10^6$ cells ml$^{-1}$, and averaging $2.7 \times 10^6$ cells ml$^{-1}$. The time course of virus-to-bacterium ratio (VBR) ranged over an order of magnitude from 4 to 39, with an average of 19 (Fig. 2).

Morphologically, both tailed and non-tailed viruses, ranging in capsid diameter from 30 to 180 nm, were observed over the 12 mo sampling period. As a general pattern, viruses <60 nm dominated over all seasons, accounting for 73.7 % of the total (Fig. 3). Viruses with capsid diameters

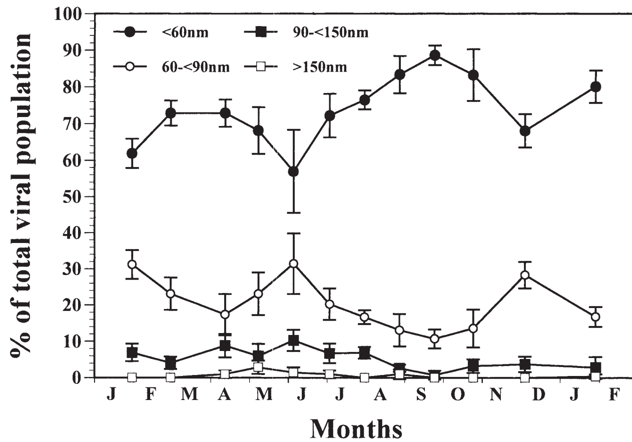


Fig. 3. Seasonal variations in size class distribution of viral capsid diameters in the Alte Donau, 1996. A minimum of 70 capsids was measured per subsample. Error bars represent SDs of 6 subsamples

Table 1. Frequency of visibly infected bacterial cells (FVIC) and frequency of infected cells (FIC) calculated after Proctor et al. (1993) using the average factor of 5.42, not considering grazing, and Binder (1999), considering grazing (assumption: specific grazing rate of infected cells equals specific grazing rate of uninfected cells). Values in parentheses are standard errors (SEs) of triplicate samples

| Date (1996) | FVIC (%) | FIC[a] (%) | FIC[b] (%) |
|---|---|---|---|
| July | 7.5 (0.3) | 41 (2) | 41 (1) |
| August | 9 (2) | 48 (10) | 43 (5) |
| September | 3.0 (0.5) | 17 (3) | 19 (3) |
| October | 2.8 (0.1) | 15.2 (0.5) | 18.1 (0.5) |
| December | 3.5 (0.0) | 19.1 (0.1) | 22.2 (0.1) |
| Average | 5 (1) | 28 (6) | 29 (5) |

Following [a]Proctor et al. (1993), and [b]Binder (1999)

in the range from 60 to <90 nm and from 90 to <150 nm accounted for 20.5 and 5.2% of the total numbers, respectively. Viruses with capsids ≥150 nm represented only 0.6% of the total.

### Burst size and FIC

Some 1800 infected bacteria were examined to quantify the number of viruses inside the bacterial cells, and at least 620 (i.e. on average 36%, range 24 to 48% over all sampling events) of all cells were completely filled with viruses. The minimum burst size ranged from 18 to 27, averaging 22 viruses per cell. The maximum burst size was on average twice the minimum burst size and ranged from 34 to 48, with an average of 42 viruses per cell (data not shown).

The FVIC varied between 2.8 and 9%, averaging 5% (Table 1). Using the low (3.70), high (7.14) and average (5.42) conversion factors presented by Proctor et al. (1993) to convert the FVIC to the FIC revealed that on average 28% (using the average factor 5.42) of the bacterial cells were infected. Values ranged from 10% (Factor 3.7) to 63% (Factor 7.14). Additionally, we calculated the FIC taking into account the continuous nature of viral infection, growth and mortality of bacterial cells in a population, and the effect of grazing mortality on the relationship between FVIC and FIC (Binder 1999). We assumed that infected and uninfected bacterial cells are grazed at equal specific rates, and found that on average 29% of the cells were infected, ranging between 18.1 and 43%.

### Viral control of bacterial production

BSP varied significantly between the different sampling events ($p < 0.05$), ranging from 37 to 142 pmol $^3$H-thymidine $l^{-1}$ $h^{-1}$, with an average of 74 pmol $l^{-1}$ $h^{-1}$, and from 98 to 1095 pmol $^{14}$C-leucine $l^{-1}$ $h^{-1}$, averaging 619 pmol $l^{-1}$ $h^{-1}$ (Table 2). To convert the incorporation rates measured via the $^3$H-thymidine method into the number of bacterial cells produced $ml^{-1}$ $h^{-1}$ we used specific conversion factors calculated for the Alte Donau at different water temperatures. BSP ranged between $3.6 \times 10^4$ and $6.2 \times 10^4$ cells $ml^{-1}$ $h^{-1}$, averaging $5.0 \times 10^4$ cells $ml^{-1}$ $h^{-1}$ (Table 3).

The viral decay, used as a measure for viral production, can be described as the negative exponential for all experiments during the first 9 h of the incubation (Fig. 4). The experiments were carried out at water temperatures representative for the seasons. Viral concentrations ranged from $2.3 \times 10^7$ to $5.3 \times 10^7$ particles $ml^{-1}$ at the beginning of the decay experiments and decreased quickly during the first 2 to 3 h. Since

Table 2. Bacterial secondary production (BSP) measured at the beginning of the decay experiments via incorporation of $^3$H-thymidine and $^{14}$C-leucine. n: number of subsamples. Values in parentheses are SEs

| Date (1996) | $^3$H-thymidine incorporation (pmol $l^{-1}$ $h^{-1}$) n | | $^{14}$C-leucine incorporation (pmol $l^{-1}$ $h^{-1}$) n | |
|---|---|---|---|---|
| July | 16 | 142 (10) | 10 | 1095 (58) |
| August | 6 | 61 (10) | 6 | 861 (58) |
| September | 8 | 37 (3) | 6 | 519 (37) |
| October | 8 | 84 (6) | 6 | 521 (42) |
| December | 8 | 44 (14) | 6 | 98 (11) |
| Average | | 74 (17) | | 619 (152) |

Table 3. Viral and bacterial abundance, viral decay rates, percentage of initial viral abundance remaining after 9 h, bacterial secondary production (BSP, measured by the $^{3}$H-thymidine method) and bacterial mortality (calculated by dividing the viral decay rate by the maximum burst size). Values in parentheses are SEs of triplicate samples, with the exception of BSP, where 6 to 16 subsamples (see Table 2) were measured

| Date (1996) | Viral abundance ($10^7$ ml$^{-1}$) | Viral decay rate ($10^6$ ml$^{-1}$ h$^{-1}$) | Viral decay rate (h$^{-1}$) | % of initial viral abundance | Bacterial abundance ($10^6$ ml$^{-1}$) | BSP ($10^4$ ml$^{-1}$ h$^{-1}$) | Bacterial mortality ($10^4$ ml$^{-1}$ h$^{-1}$) |
|---|---|---|---|---|---|---|---|
| July | 2.3 (0.1) | 1.0 (0.1) | 0.055 (0.004) | 61 (2) | 3.9 (0.0) | 5.1 (1.0) | 2.2 (0.2) |
| August | 3.1 (0.1) | 1.3 (0.1) | 0.050 (0.005) | 64 (3) | 3.5 (0.1) | 5.5 (0.3) | 3.1 (0.3) |
| September | 5.3 (0.1) | 1.2 (0.2) | 0.025 (0.003) | 80 (2) | 2.8 (0.0) | 4.8 (0.1) | 2.4 (0.3) |
| October | 3.7 (0.0) | 2.1 (0.1) | 0.077 (0.002) | 50 (1) | 3.0 (0.1) | 6.2 (0.1) | 5.5 (0.1) |
| December | 3.0 (0.1) | 0.5 (0.1) | 0.019 (0.004) | 84 (3) | 3.4 (0.0) | 3.6 (1.1) | 1.5 (0.3) |
| Average | 3.5 (0.4) | 1.2 (0.2) | 0.045 (0.009) | 68 (6) | 3.3 (0.2) | 5.0 (0.4) | 3.0 (0.6) |

Table 4. Viral control of bacterial production measured by different approaches: viral decay rate divided by maximum burst size to calculate number of bacterial cells lysed by viruses (expressed as percentage of bacterial secondary production [BSP]); factor-of-2 rule by Proctor et al. (1993), using different factors to convert FVIC to FIC; model of Binder (1999), considering grazing (assumptions: specific grazing rate of infected cells [$g_i$] equals specific grazing rate of uninfected cells [$g_u$]); model of Binder (1999) not considering grazing. All except the first were determined at the beginning of the decay experiments. Values in parentheses are SEs of triplicate samples

| Date (1996) | Viral control of BSP (%) | Viral control of bacterial production[a] (%) | | | Fractional mortality from viral lysis[b] (%) | |
|---|---|---|---|---|---|---|
| | | Factor 3.70 | Factor 5.42 | Factor 7.14 | Considering grazing $g_i = g_u$ | Not considering grazing $g_i = 0$ |
| July | 42 (4) | 56 (3) | 82 (4) | 108 (5) | 100 (7) | 101 (8) |
| August | 57 (5) | 65 (14) | 95 (20) | 126 (27) | 125 (36) | 177 (77) |
| September | 51 (6) | 23 (4) | 33 (6) | 44 (8) | 29 (6) | 29 (6) |
| October | 88 (2) | 20.8 (0.7) | 30 (1) | 40 (1) | 26 (1) | 26 (1) |
| December | 43 (8) | 26.0 (0.1) | 38.1 (0.2) | 50.2 (0.2) | 34.3 (0.2) | 34.1 (0.2) |
| Average | 56 (8) | 38 (8) | 56 (12) | 73 (16) | 63 (18) | 73 (26) |

Following [a]Proctor et al. (1993), and [b]Binder (1999)

Table 5. Viral contact rates and contact success, estimated from a contact model on viruses and bacteria (Murray & Jackson 1992) at the beginning of the decay experiments. Values in parentheses are SEs of triplicate samples

| Date (1996) | Contact rate ($10^7$ ml$^{-1}$ h$^{-1}$) | Specific contact rate (contacts cell$^{-1}$ h$^{-1}$) | Viruses contacting bacteria (% of total h$^{-1}$) | Contact success (% cells lysed contact$^{-1}$) |
|---|---|---|---|---|
| July | 1.90 (0.07) | 4.8 (0.2) | 81 (1) | 0.11 (0.01) |
| August | 2.36 (0.05) | 6.8 (0.3) | 76 (2) | 0.13 (0.01) |
| September | 2.66 (0.03) | 9.6 (0.1) | 50.4 (0.5) | 0.09 (0.01) |
| October | 2.40 (0.02) | 8.1 (0.1) | 64.4 (0.9) | 0.23 (0.01) |
| December | 1.27 (0.03) | 3.8 (0.1) | 43.1 (0.2) | 0.12 (0.02) |
| Average | 2.12 (0.22) | 6.6 (1.0) | 63 (6) | 0.14 (0.02) |

no obvious loss of viruses occurred after 9 h to the end of the experiments, we calculated viral decay rates with data from the initial 9 h period. Decay rates varied significantly ($p < 0.05$) between 0.019 and 0.077 h$^{-1}$ or $0.5 \times 10^6$ and $2.1 \times 10^6$ particles ml$^{-1}$ h$^{-1}$, with an average of 0.045 h$^{-1}$ or $1.2 \times 10^6$ particles ml$^{-1}$ h$^{-1}$, respectively (Table 3). Between 50 and 84 % of the initial population remained after 9 h, averaging 68 %.

To estimate the number of lysing bacteria, we divided viral decay rates by the maximum burst size (Table 3). Between $1.5 \times 10^4$ and $5.5 \times 10^4$ cells ml$^{-1}$ h$^{-1}$ had to be lysed to maintain viral production, with an average of $3.0 \times 10^4$ bacterial cells ml$^{-1}$ h$^{-1}$. Hence, viruses controlled 42 to 88 % of the BSP, averaging 56 %.

Using the range and mean factors proposed by Proctor et al. (1993) to calculate FIC from FVIC, we

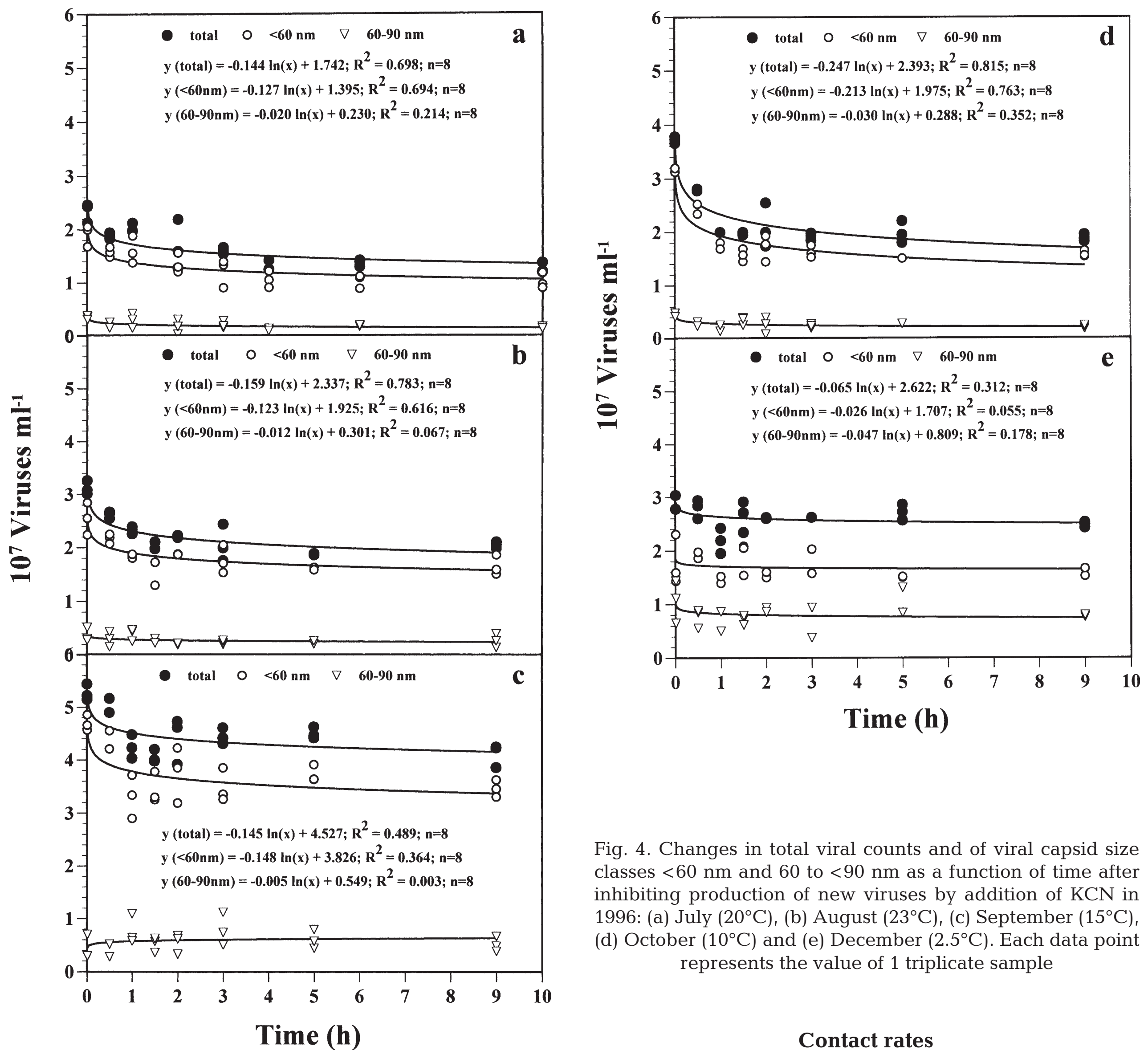


Fig. 4. Changes in total viral counts and of viral capsid size classes <60 nm and 60 to <90 nm as a function of time after inhibiting production of new viruses by addition of KCN in 1996: (a) July (20°C), (b) August (23°C), (c) September (15°C), (d) October (10°C) and (e) December (2.5°C). Each data point represents the value of 1 triplicate sample

found that viruses were responsible for 20.8 to 126% (FIC × 2) of the bacterial mortality, with an average of 56% (Table 4). Considering grazing and assuming that infected bacterial cells were grazed at the same rate as uninfected cells (Binder 1999), the proportion of total bacterial mortality that is due to virus-induced lysis (referred to by Binder as the fractional mortality from viral lysis [FMVL]) ranged from 26 to 125%, averaging 63%. If the grazing mortality was not taken into account, the mortality of bacteria due to viruses was slightly higher, with values varying from 26 to 177% and averaging 73%. Proctor et al. (1993) and Binder (1999) both assumed that the latent period is approximately equal to the generation time of bacterial cells.

### Contact rates

Theoretical CRs, which are necessary to quantify the rate of successful infection, were calculated according to the model of Murray & Jackson (1992). The CR varied from $1.27 \times 10^7$ to $2.66 \times 10^7$ contacts $ml^{-1}$ $h^{-1}$ between bacterial cells and viruses, with an average of $2.12 \times 10^7$ contacts $ml^{-1}$ $h^{-1}$ (Table 5). Between 43.1 and 81% $h^{-1}$ of the total viral community encountered bacterial cells, averaging 63% $h^{-1}$. Comparing the CR with the bacterial mortality necessary to explain the viral production rate indicated that only between 0.09 and 0.23% of all contacts really led to infection of bacterial cells with subsequent development of viruses and final lysis of the bacteria, with an average of 0.14%. The specific CR, i.e. the number of viruses encountering a single bacterial cell per time, ranged between 3.8 and 9.6 $h^{-1}$ and was on average 6.6 $h^{-1}$.

## DISCUSSION

### Viral control of bacterial production

It has recently been shown that HNF grazing accounts on average for only 5% (range 0.3 to 20%) of the bacterial mortality in the Alte Donau (Wieltschnig et al. 1999). We suggested that viruses might play a key role in controlling BSP in this ecosystem, even though a previous study carried out in a mesotrophic oxbow lake of the same backwater system revealed a mean control of the bacterial production due to viral lysis of some 20% (Mathias et al. 1995). Wieltschnig et al. (1999) measured HNF grazing from April 1995 to June 1996, and therefore simultaneously with our experiments over a period of 6 mo. In order to get representative and reliable estimations to assess the viral control of bacterial production we used different approaches. Also, parameters necessary to describe virus-bacteria interactions such as burst size, BSP and CRs were monitored simultaneously (see above).

Using the viral decay data and the maximal burst size, we calculated that viruses lysed between 42 and 88% of BSP in the Alte Donau, averaging 56%. The mean viral control of bacterial production calculated after the model of Proctor et al. (1993) at 56% was identical to the average percentage of BSP being lysed by viruses calculated using the decay method. However, due to the use of the low (3.7) and high (7.14) conversion factor we obtained a broad range from 20.8 to 126%. Since the model of Proctor et al. (1993) fails to take into account the continuous nature of growth and mortality of bacterial cells in a population, we also used the recently presented model of Binder (1999) to calculate the viral impact on bacterial production. This approach revealed a higher bacterial mortality than the results of the Proctor model, at 26 to 177%, averaging 73%. Yet both calculations were made on the assumption that infected bacterial cells were not grazed by bacterivores, which might be unrealistic for natural aquatic environments. Grazing mortality is an alternative fate for infected cells, thus decreasing the FMVL for a given value of FIC and increasing the error of the factor-of-2 rule, relative to the no-grazing case (Binder 1999). Assuming that infected bacterial cells were grazed at the same rate as uninfected cells, between 26 and 125% of the BSP were virus lysed. These values are close to the range calculated after the model of Proctor et al. (1993), but the mean of the calculation after Binder at 63% is slightly higher than the average of the Proctor model (56%). Although the average viral controls determined by the different approaches were similar, the results of the decay method differed distinctly from those of the Proctor and Binder method for single sampling events (Table 4). Even though we cannot address the causes for these discrepancies within samples and between methods, we assume that the latent periods may not always be equal to the generation times of the bacterial host community, thus probably falsifying the calculations of FIC.

The values for viral control of BSP in the Alte Donau exceeded those published for most marine and freshwater environments. Only a few studies reported viral control of the bacterial production reaching more than 50% (e.g. Proctor et al. 1993, Fuhrman & Noble 1995, Weinbauer & Peduzzi 1995, Steward et al. 1996, Tuomi et al. 1999, Noble & Fuhrman 2000). The highest mean impact of viruses on bacteria so far reported was 91% for California waters (Noble & Fuhrman 2000) and up to 97% in the eutrophic Lake Plußsee (Weinbauer & Höfle 1998a), thus exceeding the bacterial mortality of the Alte Donau. However, Noble & Fuhrman used a burst size of only 20 to estimate bacterial mortality. These results can therefore be compared only with the viral control in the Alte Donau calculated with the minimum burst size. Under this assumption, the mean for the virus-derived control of BSP accounted on average for 111%. When Noble & Fuhrman used a burst size of 50, viruses lysed only 36% of the bacterial production in California waters. In contrast, the viral control of BSP was 56% in the Alte Donau, when we used the maximum burst size. Similarly, the results of the study of Weinbauer & Höfle (1998a) carried out in Lake Plußsee can not be directly compared to the results of the present investigation because the authors corrected the FVIC for the amount of non-intact bacterial cells in the population. Consequently, the contribution of viruses to the bacterial mortality increased. It has been reported that only 50 to 80% of the bacterial cells in aquatic systems are intact, the rest being damaged or empty (Heissenberger et al. 1996, Weinbauer & Höfle 1998a). Assuming that viruses can be produced only in intact cells and adopting the above frequency of intact cells of 74% (Weinbauer & Höfle 1998a), the percentage of BSP lysed by viruses in the Alte Donau reached up to 129% (average 75%), compared to a maximum of 95% (average 56%) without correction. The maximum viral control of BSP in the Alte Donau was thus about 30% higher than the highest reported control in Lake Plußsee. For this calculation we used the average factor of 5.42 to convert FVIC to the FIC (Proctor et al. 1993). However, data on cell integrity should be viewed with caution, since it is not possible to completely exclude preparation artifacts (Weinbauer & Höfle 1998a).

A comparison between the viral impact on bacteria in the eutrophic Alte Donau (average 56%) and the mesotrophic Kühwörte (average 20%, Mathias et al. 1995) corroborated the assumption that viral control of

bacterial production generally increases with the trophic status of the aquatic system (e.g. Steward et al. 1992, 1996, Weinbauer et al. 1993). Considering the mean bacterial mortality due to viruses and the grazing mortality in the Alte Donau, it can be seen that both parameters determining bacterial mortality could not control BSP. Viruses and HNFs were responsible for only some 61% of the bacterial mortality. Other predators like ciliates and crustacea, autolysis of cells or attachment to sinking particles might be other possible sources of bacterial loss. Yet we cannot rule out the possibility that the methods used to estimate viral control of bacterial production were subject to error.

## Critical view of the methods used to estimate viral control of bacterial production

The assessment of the FVIC obtained by the whole cell method (Weinbauer et al. 1993) presents several problems: Some infected bacterial cells might be lost by disruption during the ultracentrifugation step because of the high centrifugation speed and time. A comparison between the whole cell method and the thin section method carried out by Weinbauer & Höfle (1998a) revealed that on average only 79% of the VIC obtained in thin sections were counted as infected in whole cells (centrifugation at 66 000 × *g* for 20 min). The difference between the results of thin sections and the whole cell method might also result from the different criteria for scoring a cell as being infected: 3 viruses for the thin section method versus 5 viruses for the whole cell method (Weinbauer & Höfle 1998a). Correcting for the findings of Weinbauer & Höfle (1998a), the average FVIC amounted to 6.5% compared to 5% estimated by the whole cell method. Moreover, bacterial cells sometimes appear opaque to the electrons. This may cause some infected cells to be scored as non-infected in whole cells, just because the dark cells mask the viruses (Guixa-Boixereu et al. 1999). Therefore, we suggest that the estimates of VIC determined by the whole cell method are generally underestimates.

Another source of error in the determination of the viral impact on bacteria might be the conversion factors presented by Proctor et al. (1993) to convert the FVIC to the FIC. They were derived from investigations of thin sections of cells and might thus not be applicable to whole cell observations. However, Weinbauer & Peduzzi (1994) revealed conversion factors between 3.9 and 6.6 when investigating whole cells, thus being near to the factors of Proctor et al. (1993) of 3.7 to 7.14. Nevertheless, one should keep in mind that the conversion factors of Proctor et al. (1993), derived from marine vibrio isolates cultured in full strength medium, might not be applicable to natural marine systems or freshwater environments of different trophic status. The mathematical model of Binder (1999) has recently confirmed the uncertainties of the conversion factors presented by Proctor et al. (1993). Even when grazing of flagellates on infected bacteria is considered, the average viral control calculated after Binder was higher than that calculated with the average factor (5.42) of Proctor et al. (1993). All these calculations were based on the assumption that the latent period equals the generation time of bacteria in natural aquatic systems. The validity of this assumption has never been carefully examined, and the results should therefore be considered with caution.

The use of the viral decay method described by Heldal & Bratbak (1991) might also lead to faulty estimates of the viral impact on bacteria for 2 main reasons. (1) It has been suggested that the rate of viral production and decay might follow a diel periodicity (e.g. Heldal & Bratbak 1991, Bratbak et al. 1992, Suttle & Chen 1992, Jiang & Paul 1994, Weinbauer et al. 1995). We can therefore not claim that our viral decay estimates were representative of the daily average because they were all made on water samples taken in the morning. (2) Assuming that bacteria play an active role in viral decay via ectoenzymes and extracellular enzymes, the production of new enzymes is inhibited by cyanide inactivating the bacterial metabolism (Mathias et al. 1995). Hence, not all loss factors occurring *in situ* could be considered in the decay method. Finally, it should be mentioned that bottle incubations might affect viral production as well (Kepner et al. 1998).

Nevertheless, we favor the decay method because the assessment of VIC by TEM provides only a snapshot and fails to take into account the dynamic nature of viral production and bacterial mortality in a microbial system. The decay experiment is a direct access to the dynamic aspect of viral production, while all other methods are extrapolations of assumptions. Since we monitored burst size and BSP simultaneously with the viral decay, we argue that the viral control of BSP measured by decay experiments reflected the virus-bacteria interactions in the Alte Donau more reliably than the data from the models of Proctor et al. (1993) and Binder (1999). Furthermore, BSP was estimated on the same day and $^{3}$H-thymidine conversion factors in the same week as the decay experiment was carried out.

An important parameter in the assessment of the viral impact on bacteria is the viral burst size. It varies widely with regard to location (e.g. Heldal & Bratbak 1991, Weinbauer & Peduzzi 1994, Fuhrman & Noble 1995, Wilhelm & Smith 2000) and depth of the water column (Weinbauer & Höfle 1998a). Therefore, it seems unavoidable to determine the specific burst size for the investigated system in order to

relate estimates of viral production to the level of virus-induced bacterial mortality. We distinguished between 2 types of burst size: minimum and maximum burst size (see above; Weinbauer & Suttle 1996, Weinbauer & Höfle 1998b). Both estimations might be conservative, since viruses lying on top of each other were probably counted as 1 virus. The maximum burst size could be an overestimation of the actual burst size if some viruses induce cell lysis before the bacterial cells are completely filled with new viruses. Nevertheless, Weinbauer et al. (1993) argued that it might be more appropriate to use the maximum burst size for calculations of bacterium-virus interactions because the burst size of the VIC that are not entirely filled with viruses might still increase until the cells are actually lysed. Again, the results should be considered with caution. We therefore also used the minimum burst size to assess the viral control of bacteria. These calculations revealed that between 74 and 178% of the BSP was lysed by viruses, accounting for on average 111%. During 2 sampling events, viruses lysed more than 100% of the BSP, removing 1.6 and 0.5% $h^{-1}$ of the bacterial standing stock, respectively. Hence, we argue that the viral control of BSP in the Alte Donau might be even higher than determined in this study. Most of the bacterial carbon production might be cycled in the bacteria-virus-DOM loop, thereby reducing the availability of bacterial carbon for higher trophic levels to nearly zero.

Overall, the numerous sources of error of the methods and mathematical models to assess virus-induced bacterial mortality and the differences in the results reported in the present investigation clearly show the necessity of using different approaches simultaneously for quantifying viral production. In our opinion, it is the only way to be able to eliminate unrealistic trends or to decide whether detected trends are representative of the specific system.

### Contact rates

The specific CRs presented in this study, ranging from 91 to 230 $cell^{-1}$ $d^{-1}$, were high compared to CRs reported from the Gulf of Mexico, with 0.4 to 10 $cell^{-1}$ $d^{-1}$, and Lake Erie, with 4 to 11 $cell^{-1}$ $d^{-1}$ (Wilhelm et al. 1998b, Wilhelm & Smith 2000). Only CRs of Lake Plußsee were close to those of the Alte Donau, varying from 49 to 180 contacts $cell^{-1}$ $d^{-1}$ at different depths (Weinbauer & Höfle 1998b). A major reason for the high CRs in the Alte Donau is certainly the high viral and bacterial abundance.

Virus-host contact success (the frequency at which a contact resulted in a lytic infection) was low in the Alte Donau, with 0.09 to 0.23% cells lysed per contact, indicating a high host diversity but low frequency of specific host bacteria. Low contact success might also result from low infectivity of free viruses caused by solar radiation (Suttle & Chen 1992, Noble & Fuhrman 1997). Moreover, adsorption to bacterial cells that are lysogenic, inactive or simply not the specific hosts, lowers the percentage of successful infections. Also, grazing of infected bacterial cells by bacterivores and a process called 'lysis from without' might be partly responsible for the low contact success. Lysis from without implies that the cell wall is weakened due to the attachment of numerous viruses, and lysis takes place before the production of new viruses has started (Delbrück 1940, Chiura et al. 2000). Since the frequency of cells lysed per contact was lowest when the number of viruses encountering a single bacterium reached a maximum, we suggest that lysis from without might be a factor deserving further investigation in our system. The highest contact success coincided with the highest value for BSP, demonstrating once more the dependence of viral production on bacterial activity.

### Viral decay and distribution of capsid diameter

Heldal & Bratbak (1991) observed that the majority of the viral community, namely more than 60%, had a relatively short turnover time, whereas a minor portion of 4 to 40% was refractile. In contrast, 50 to 84% of viruses were still detectable 9 h after the start of our experiments. Mathias et al. (1995) reported that 30 to 70% of the initial viral assemblage could be found at the end of the investigation. Besides, viruses sized <60 nm showed the fastest turnover of all size groups obtained in the Kühwörte and in the Alte Donau. The size class distribution of capsid diameters at the end of our experiments suggests that most of the viruses disappearing during the investigation period were indeed bacteriophages (Børsheim et al. 1990, Wommack et al. 1992). However, we are aware of the probability that some of the decaying viruses in the other size classes were algal viruses.

We further demonstrated that the numbers of viruses of different size classes fluctuated with the seasons, with most distinct variations occurring in the size class smaller than 60 nm. Since temporal variations in this size class followed closely the fluctuation patterns in total viral concentrations, we suggested that variations in total viral abundance were mostly due to fluctuations in the abundance of bacteriophages. The proportion of viruses other than bacteriophages among the larger forms remains unknown. Since the viral commu-

nity is a product of the host community, differences in the viral capsid distribution might indicate abundance variations in the dominant active bacterial species or in the bacterial community during different seasons in the Alte Donau.

## Conclusion

Our data reinforce the view that viruses play a major role in the control of the bacterial community in natural aquatic systems. In the Alte Donau, on average 56% of the BSP was lysed by viruses, which prevailed over grazing of HNF by a factor of about 11. If we add up the bacterial mortality due to viral lysis and the mean contribution of HNF (5%), both factors together are on average responsible for only 61% of the total bacterial mortality. We therefore argue that some 40% of the bacterial production may be directly available for micro- and macrozooplankton. However, a critical assumption of the methods used in this study led to the suggestion that the contribution of viruses to the control of BSP might reach more than 100%. Viruses could occasionally remove up to 1.6% $h^{-1}$ of the bacterial standing stock. Hence, viruses seem to be the main factor controlling BSP in the Alte Donau, where HNF were shown to be top-down controlled (Wieltschnig et al. 2001). High bacterial mortality due to viruses indicates that relative to the bacterial carbon production an important amount of organic matter was released into the water column and became available again for microheterotrophs. Using the bacterial biomass in the Alte Donau determined by Dokulil et al. (1997) revealed a bacterial carbon content of 15 to 28 fg $cell^{-1}$ (mean 23 fg $cell^{-1}$) and a release rate of 8 to 44 µg C $l^{-1}$ $d^{-1}$ (mean 18 µg C $l^{-1}$ $d^{-1}$) due to viral lysis. The bacterial biomass was estimated on the same days as the decay experiments were carried out. However, we need to emphasize that these values for the virus-induced recycled bacterial carbon are overestimates. The energetic costs of the infected cells necessary for the replication process of viral DNA and capsid protein synthesis are not accounted for. If we assume a viral carbon content of $10^{-10}$ µg C (González & Suttle 1993), between 1.3 and 5 µg C $l^{-1}$ $d^{-1}$ was needed for the synthesis of viral DNA and capsid proteins (mean 3 µg C $l^{-1}$ $d^{-1}$). The amount of carbon release due to viral lysis of bacterial cells was therefore at most 5 to 39 µg C $l^{-1}$ $d^{-1}$ (mean 15 µg C $l^{-1}$ $d^{-1}$), corresponding to 29 to 79% (mean 46%) of the BSP. Keeping in mind that during the replication process of DNA and capsid protein synthesis a part of the bacterial carbon cell content is lost as $CO_2$, a further reduction of the calculated DOC release into the water column has to be accounted for.

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