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The functional importance of bacteriophages in the microbial loop of an oligomesotrophic lake over a diel cycle

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The abundances of the different compartments of the microbial loop (i.e., viruses, heterotrophic bacteria, heterotrophic nanoflagellates, and pigmented nanoflagellates), total (TPP) and excreted (EPP) primary production, bacterial production (BP), viral lytic activity (LA), and bacterivory by nanoflagellates (FG) were measured on June 15 and 16, 1998, in a moderate-altitude oligomesotrophic lake (Lac Pavin, France), at 5 and 10 m depths. At both depths, losses of the bacterial community by viral lysis ($LA^{5\ m}$ = 1.7 x 10^6 cells.l^{-1}.h^{-1}, $LA^{10\ m}$ = 2.0 x 10^6 cells.l^{-1}.h^{-1}) were, on average, lower than those due to the grazing activity of flagellates ($FG^{5\ m}$ = 10.3 x 10^6 cells.l^{-1}.h^{-1}, $FG^{10\ m}$ = 8.4 x 10^6 cells.l^{-1}.h^{-1}). A carbon budget exercise indicated that, for the sampling period and depths, 17.8 % of C from TPP (= 38.1 % of EPP) was used by bacteria. On the other hand, 52.7 % of BP (= 2.15 % of TPP) was grazed by nanoflagellates, while 11.0 % of BP (= 0.45 % of TPP) was lysed by viruses.

Prise en compte des bactériophages au sein de la boucle microbienne d'un lac oligomésotrophe au cours d'une étude nycthémérale

Mots-clés : lacs, boucle microbienne, virus, nanoflagellés, bactéries, flux de carbone.

L'abondance des communautés de la boucle microbienne (virus, bactéries hétérotrophes, nanoflagellés non pigmentés et nanoflagellés pigmentés), la production primaire phytoplanctonique totale (TPP) et excrétée (EPP), la production bactérienne (BP), ainsi que l'activité lytique virale (LA) et l'activité bactérivore des nanoflagellés hétérotrophes (FG) ont été estimées, les 15 et 16 juin 1998, dans un lac oligomésotrophe de moyenne montagne (Lac Pavin), à 5 et 10 m de profondeur. Au cours de cette étude, quelle que soit la profondeur, les pertes bactériennes moyennes liées à la lyse virale ($LA^{5\ m}$ = 1,7 x 10^6 cellules.l^{-1}.h^{-1}, $LA^{10\ m}$ = 2,0 x 10^6 cellules.l^{-1}.h^{-1}) étaient, en moyenne, inférieures à celles dues au broutage des flagellés phagotrophes ($FG^{5\ m}$ = 10,3 x 10^6 cellules.l^{-1}.h^{-1}, $FG^{10\ m}$ = 8,4 x 10^6 cellules.l^{-1}.h^{-1}). Le bilan estimé des flux de carbone indique qu'en moyenne 17,8 % du carbone issu de la TPP (soit 38,1 % de la EPP) a été utilisé par les bactéries. Par ailleurs, en moyenne 52,7 % de la BP (soit 2,15 % de la TPP) a été consommé par le biais de la prédation des nanoflagellés, alors que 11,0 % de la BP (soit 0,45% de la TPP) a été détruit par la lyse virale.

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1. Introduction

Studies on microbial ecology that have been conducted in aquatic pelagic environments for nearly three decades have shown that the flows of matter and energy are not arranged solely along the linear trophic pathway : large-sized phytoplankton → metazoan zooplankton → fish. These flows also pass through the microbial loop (Pomeroy 1974, Azam et al. 1983) in which the dissolved organic matter (DOM) derived from excretion by phytoplankton or of allochthonous origin is transformed into heterotrophic bacterial biomass. This biomass is consumed by phagotrophic protists, which in turn serve as food for metazoan zooplankton. There is therefore a true microbial trophic network (Amblard et al. 1998) in which heterotrophic bacteria are not only the main agents responsible for recycling matter, but also constitute an essential trophic link that is partly responsible for the transfer of matter and energy to higher trophic levels. Temperature, availability of resources and grazing have long been recognized as the main factors controlling bacterial abundance and production, both in marine and freshwater ecosystems (Fenchel 1982, McManus & Fuhrman 1988, Pace 1988). More recently, the high quantitative abundance and diversity of virus-like particles in fresh waters (Maranger & Bird 1995) and marine waters (Borsheim 1993) has stimulated efforts by planktonologists to understand the role of these bacteriophages in the structure and the functioning of aquatic trophic networks (Fuhrman & Suttle 1993, Murray & Eldridge 1994, Sime-Ngando 1997) and especially in the loss processes that affect pelagic microbial communities. It is therefore necessary to know the relative importance of viral lysis and grazing as mortality factors for the picoplanktonic communities of the «microbial loop».

There have been very few works that have simultaneously studied these two regulatory factors. Preliminary results, from a study conducted in Californian coastal waters and then in the Eastern Siberian Sea, have suggested that viruses and phagotrophic protists contribute almost equally to bacterial mortality (Fuhrman & Noble 1995, Steward et al. 1996). Weinbauer & Peduzzi (1995) showed, by studying the relations between the abundance of heterotrophic bacteria, viruses and phagotrophic flagellates, that the bacterial mortality caused by viral lysis could be greater than that caused by grazing by protists. More recently, Weinbauer & Höfle (1998) compared these two agents of bacterial mortality in a eutrophic lake. They found that the dominance of one or other of these two factors depended largely on which water layer was studied : the bacterial production tended to be controlled by grazing in the epilimnion, whereas viruses were more important in the metalimnion and hypolimnion. In contrast, Guixa-Boixareu et al. (1996) found no significant effect of viral lysis on bacterial production in coastal Mediterranean waters.

The aim of the present study was to examine, at a time-scale of 24 h, the vertical distribution of the different communities of the microbial loop in a moderate-altitude, oligo-mesotrophic lake, and to determine and compare the impact of lytic activity and of potential grazing activity on bacterial mortality (Bettarel et al., in press). Herein, we estimate, for the first time, a theoretical matter and energy flow budget from the phytoplankton compartment through to phagotrophic flagellates and to bacteriophages, via bacterial production, using 24 h mean values.

2. Material and methods

The study was conducted in Lac Pavin, located in the Massif Central of France at an altitude of 1197 m. It is a dimictic, meromictic oligo-mesotrophic lake. Samples were collected every 2 to 3 h for 24 h, on 15 and 16 June 1998 (except for primary production samples, see later), at depths of 5 and 10 m. From the physico-chemical properties of the lake at the time of sampling, these depths were situated in epilimnion and metalimnion, respectively.

The virus-like particles (VLP), heterotrophic bacteria and nanoflagellates were counted using epifluorescence microscopy, with fluorochromes YOPRO-1 (Bettarel et al. 2000), DAPI (Porter & Feig 1980) and primulin (Caron 1983) respectively. An Olympus BH2 epifluorescence microscope was used. The viruses were examined under blue light, the heterotrophic nanoflagellates (HNF) and the heterotrophic bacteria under UV and the autotrophic and mixotrophic nanoflagellates by autofluorescence of natural pigments. At least 400 bacteria and viruses and 200 flagellates were counted per sample, a number that gives a precision of greater than ± 10 % with a 95 % confidence interval, according to the calculations of Lund et al. (1958). This high precision generally justifies the absence of replicates in most microbial counts conducted in aquatic systems.

Bacteria containing mature viruses were counted after concentrating the samples on grids by ultracentrifugation at 70 000 g for 30 min at 15°C (Sime-Ngando et al. 1996). Each grid was then stained with uranyl acetate for 30 s then examined under a JEOL 1200EX electron microscope at a magnification of 25 000 x, to

count those bacteria that were infected and not infected by viruses. The fraction of mortality from viral lysis (FMVL) was estimated from the frequency of visibly infected cells (FVIC) in the samples analysed, using the model proposed by Binder (1999) : FMVL = (1/g ln 2)*{FVIC/[(1 ñ e) - FVIC]}, where g is the ratio between the latent period and the generation time (g = 1) and e, the fraction of the latent period occurring before the appearance of intracellular viral particles (e = 0.816, see Proctor et al. 1993). At least 600 bacteria were counted per sample for a target number range of infected cells of 20-25 per sample, and the cells considered to be infected contained at least 5 intracellular phages.

The potential grazing activity of nanoflagellates was calculated as the product of bacterial concentration, flagellate concentration and assumed clearances rates of the main HNF. We applied a range of 0.7 to 11.5 nl. flagellate^{-1}h^{-1} (mean = 6.1 nl. flagellate^{-1}h^{-1}) reported from a previous study conducted from June to November 1993 in Lake Pavin (Carrias et al. 1996). Similar approach has been previously used in virioplankton studies (Steward et al. 1996, Weinbauer & Höfle 1998).

Bacterial production was determined by measuring the uptake of tritiated thymidine (3.04 TBq.mmol^{-1}) into bacterial DNA (Petit et al. 1999a), after incubating the samples for 45 min. The radioactivity was measured using a Beckman LS 5000 scintillation counter. The quantity of ^{3}H-thymidine incorporated into DNA was converted into bacterial production by using a conversion factor of 2 x 10^{18} cells produced per mole of thymidine incorporated. This value represents a mean value determined from 97 studies conducted in aquatic systems (Bell 1993). To convert the bacterial production in terms of cells into the equivalent amount of carbon produced, we used the equation of Simon & Azam (1989), which includes a value for the mean cell biovolume that we determined using an image analysis system.

Primary production, phytoplanktonic exudation and the subsequent bacterial reassimilation were measured on 15 June 1998. Primary production was measured from ^{14}C uptake according to Steemann-Nielsen (1952). Subsamples (100 ml) from each of the sampling depths were drawn into two transparent and one dark (control) bottles, inoculated each with 0.5 µCi $NaH^{14}CO_3$, and incubated *in situ* for 4 h on either side of solar midday. After incubation, 30 ml of each subsamples were filtered onto 1 µm pore size Nuclepore filter and the radioactivity associated to the photo-assimilated carbon estimated by liquid scintillation counting (counter : Beckman, LS 5000 CE), for the calculation of particulate primary production (PP). The measurements of phytoplanktonic exudation and the subsequent bacterial reassimilation, as well as the calculation of total primary production (TPP), excreted primary production (EPP) and the fraction of EPP that was re-assimilated by heterotrophic bacteria, were done after treatments of the 1 µm filtrates, as described in detail elsewhere (cf. Petit et al. 1999b)

Normal distribution of data was checked by Kolmogorov-Smirnov test. A paired t-test was used to test differences of the abundances of microbial communities between the two sampling depths during the 24 h. Potential relationships between original data sets were tested by Pearson correlation analysis. All statistical analysis were performed using MINITAB 12.

3. Results and discussion

In this study, the viral and bacterial communities were significantly ($P_{viruses}$ = 0.003, $P_{bact.}$ < 0.0001, paired t-test) more abundant in the metalimnion at 10 m (M_v = 3.9 x 10^7 viruses ml^{-1}, M_b = 5.1 x 10^6 bact.ml^{-1}) than in the epilimnion at 5 m (M_v = 3.0 x 10^7 viruses ml^{-1}, Mb = 3.2 x 10^6 bact.ml^{-1}) (Table 1). These observations are in agreement with those reported in various lakes where the density of micro-organisms and viruses is generally highest in the thermocline during the period of thermal stratification (Drake et al. 1998, Weinbauer et al. 1995). But this was not the case for heterotrophic nanoflagellates (HNF) and pigmented nanoflagellates (PNF) which were significantly (P_{HNF} = 0.045, P_{PNF} = 0.049, paired t-test) more abundant in the epilimnion (M_{HNF} = 0.5 x 10^3 cells ml^{-1}, M_{PNF} = 1.3 x 10^3 cells ml^{-1}) than in the metalimnion (M_{HNF} = 0.3 x 10^3 cells ml^{-1}, M_{PNF} = 0.6 x 10^3 cells ml^{-1}) (Table 1).

The calculation conducted using Binder's (1999) model on the data in this study enabled us to estimate that, on average, 6.4 % (range : 3.5 - 10.3 %) of bacterial cells were infected by viruses at depth 5 m and 15.6 % (6.0 - 33.7 %) at depth 10 m (Table 2). These estimates are in agreement with those of Hennes & Simon (1995) who reported values of between 1 and 17 % in the mesotrophic Bodensee, and are lower than those of Weinbauer & Höfle (1998) who estimated that between 7.7 and 28.7 % (epilimnion), and between 19.6 and 46.8 % (metalimnion), of bacteria were infected by viruses in a eutrophic lake. In addition to direct observation of intracellular viruses by TEM, several authors have used other methods to determine *in situ* virus-mediated mortality (radiotracer incorporation,

Table 1. Mean (± SD) abundances of viruses, bacteria, heterotrophic nanoflagellates (HNF) and pigmented nanoflagellates (PNF) in Lake Pavin at 5 and 10 m depths, during a short-term study conducted on June 15-16, 1998 (n = 9).

Tableau 1. Abondance moyenne (± écart-type) des virus, bactéries, nanoflagellés hétérotrophes (HNF) et nanoflagellés pigmentés (PNF) présents dans le lac Pavin, à 5 et 10 m de profondeur, au cours de la période allant du 15 au 16 juin 1998 (n = 9)

	Viruses (10^7.ml^{-1})	Bacteria (10^6.ml^{-1})	HNF (10^3.ml^{-1})	PNF (10^3.ml^{-1})
5 m	3.0 (0.5)	3.2 (0.6)	0.5 (0.3)	1.3 (0.5)
min-max	2.1 - 3.7	2.5 - 4.0	0.1 - 1.3	0.2 - 2.0
10 m	3.9 (0.6)	5.1 (0.6)	0.3 (0.1)	0.6 (0.3)
min-max	3.2 - 5.0	4.5 - 6.3	0.1 - 0.5	0.3 - 1.0

Table 2. Mean (± SD) values of the fraction of bacterial mortality from viral lysis (FMVL), lytic activity (LA), potential grazing rate by flagellates (FG), bacterial production (BP) and total (TPP) and excreted (EPP) primary production at 5 and 10 m depths in Lake Pavin, during a short-term study conducted on June 15-16, 1998. n = 9 for FMVL, LA and FG, n = 5 for BP. Measurements of primary production were done on June 15 around local noon.

Tableau 2. Valeur moyenne (± écart-type) du pourcentage de bactéries infectées (FMVL), de l'activité lytique des virus (LA), de l'activité bactérivore potentielle des flagellés (FG), de la production bactérienne (BP) et de la production primaire totale (TPP) et excrétée (EPP) dans le lac Pavin, à 5 et 10 m de profondeur, au cours de la période allant du 15 au 16 juin 1998. n = 9 pour FMVL, LA et FG, n = 5 pour BP. Les mesures de la production primaire ont été effectuées le 15 juin autour du midi solaire.

	FMVL (%)	LA (10^6 bact..l^{-1}h^{-1})	FG (10^6 bact..l^{-1}h^{-1})	BP (10^6 bact..l^{-1}h^{-1})	TPP (mg C.m^{-3}h^{-1})	TPP (mg C.m^{-3}h^{-1})
5 m	6.4 (2.5)	1.7 (0.7)	10.3 (6.9)	26.6 (11.1)	5.18	1.60
min-max	3.5 - 10.3	0.9 - 2.7	0.1 - 30.7	20.4 - 46.6		
10 m	15.6 (7.9)	2.0 (1.0)	8.4 (3.2)	12.6 (3.4)	2.74	0.97
min-max	6.0 - 33.7	0.8 - 4.2	0.1 - 12.3	7.5 - 16.6		

viral decay..) but almost all these studies have been conducted in marine systems (Wommack & Colwell 2000).

Using the mean values of bacterial production (BP) obtained at 5 m (M = 26.6 x 10^6 bacteria l^{-1}h^{-1}) and at 10 m (M = 12.6 x 10^6 bacteria l^{-1}h^{-1}) (Table 2), we converted the fraction of mortality from viral lysis (FMVL) into lytic activity (LA) (units : bacteria lysed l^{-1}h^{-1}) by assuming that the duration of the lysis cycle was equivalent to the bacterial generation time (LA = FMVL x BP, Proctor et al. 1993). The mean values of lytic activity obtained at 5 m (1.7 x 10^6 bacteria lysed.l^{-1}h^{-1}) and at 10 m (2.0 x 10^6 bacteria lysed.l^{-1}h^{-1}) were therefore similar (Table 2). These values are much lower than those reported by Mathias et al. (1995) in the River Danube (17.7 to 36.9 x 10^6 bacteria lysed.l^{-1}h^{-1}) and higher than those estimated by Guixa-Boixareu et al. (1996) in the Ebro Delta (0.03 to 0.08 x 10^6 bacteria lysed.l^{-1}h^{-1}).

The ranges of values of the estimated potential bacterial losses caused by flagellates grazing were at 0.1 - 30.7 x 10^6 bact.l^{-1}h^{-1} in the epilimnion, and at 0.1 - 12.3 x 10^6 bact.l^{-1}h^{-1} in the metalimnion (Table 2). The mean potential grazing values lie between the extreme values (0.3 to 19.0 x 10^6 bact.l^{-1}h^{-1}, Table 2) measured previously by Carrias et al. (1996) in Lac Pavin, and also by Bennett et al. (1990) in the eutrophic Lake Oglethorpe (2.1 to 25 x 10^6 bact.l^{-1}h^{-1}).

The values of lytic and grazing activities have enabled us to estimate that, under the conditions of this study, the bacterial mortality caused by viral lysis was generally lower than that caused by grazing by bacterivorous nanoflagellates, namely by 0.1-12.8 (mean = 6.1) in the epilimnion, and by 0.2-16.3 (mean = 5.7) in the metalimnion. Weinbauer & Höfle (1998) showed in a eutrophic lake that bacterial losses caused by flagellate grazing were 3.3 times higher than those caused by viral lysis in the epilimnion, whereas they were

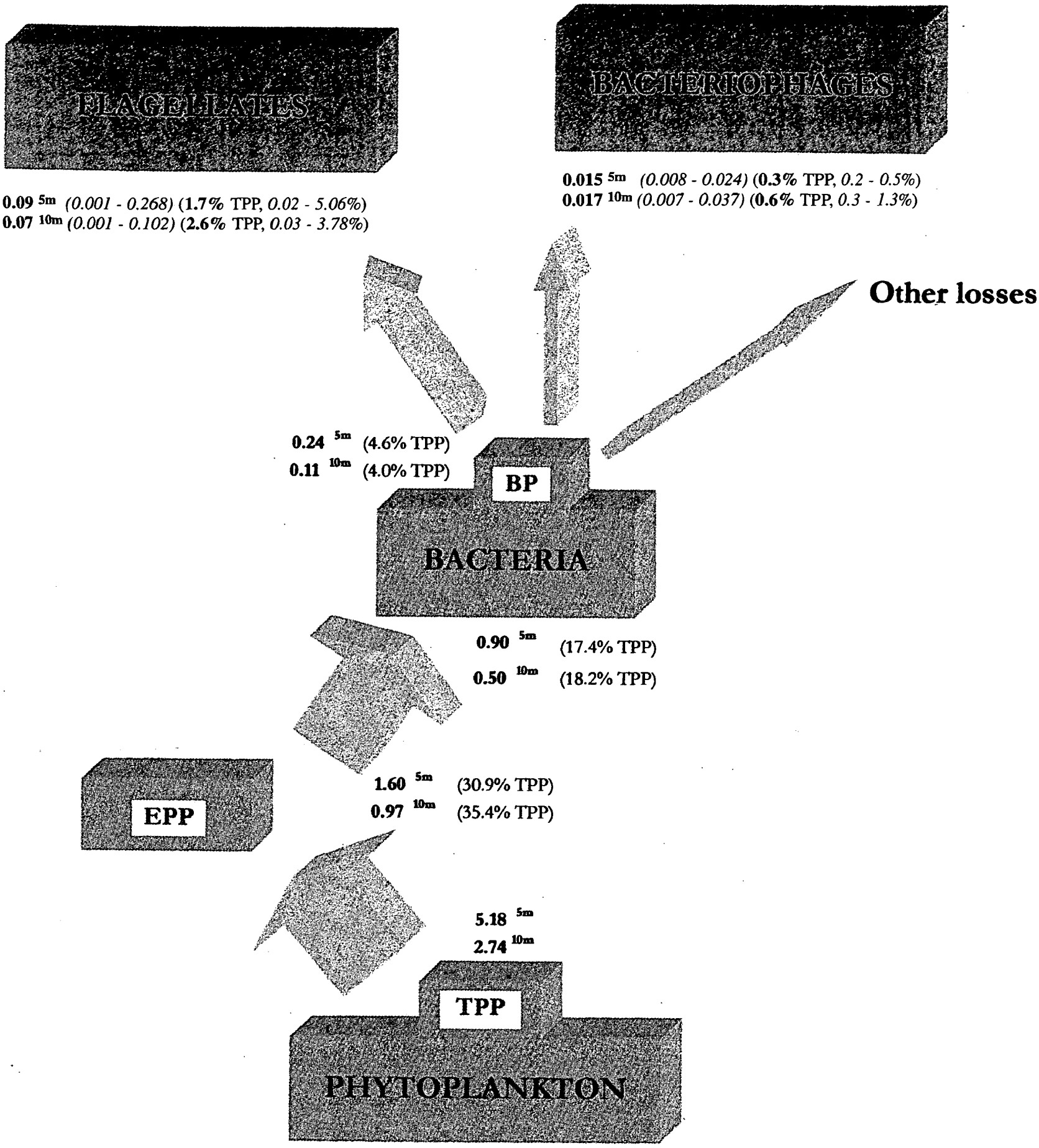

Fig. 1. Theoretical flows of matter and energy within the microbial food web in Lake Pavin, estimated from the results of a short-term study conducted on June 15-16 1998. TPP : total primary production, EPP : excreted primary production, BP : bacterial production. The values in bold are in mg $C.m^{-3}.h^{-1}$.

Fig. 1. Bilan théorique des flux de matière et d'énergie au sein de la boucle microbienne du Lac Pavin, estimé à partir de prélèvements effectués du 15 au 16 juin 1998. TPP = production primaire totale, EPP = production primaire excrétée, BP = production bactérienne. Les valeurs en gras sont exprimées en mg $C.m^{-3}.h^{-1}$.

6 times lower in the metalimnion. In the marine environment, Guixa-Boixareu et al. (1996) also reported that viral lysis was up to 30 times lower than the grazing activity of flagellates. However, two studies conducted in the marine environment have shown that these two factors had a similar impact on bacterial mortality (Fuhrman & Noble 1995, Steward et al. 1996). More recently, Guixa-Boixareu (1999) concluded from a study conducted in a marine mesocosm that the bacterial mortality caused by viruses could be higher than that related to grazing. By adding the bacterial losses caused by viral lysis and by nanoflagellate grazing, the total losses recorded in this study amounted to 4.8-125.7 % (mean = 45.1 %) of bacterial production in the epilimnion, and 10.3-103.5 % (mean = 82.5 %) in the metalimnion. It therefore seems that, in this study, these two bacterial mortality factors accounted for a substantial proportion of bacterial production, that apparently increased with depth. Other grazers such as ciliates, rotifers and cladocerans certainly had also an important impact in controlling bacterial production, especially in the epilimnion.

To produce a matter and energy flow budget in this study, the primary production was measured at 5 and 10 m around solar midday on 15 June. (Table 2). These measurements did not take into account any allochthonous inputs and considered that the system was in a steady state. The proportion of the total primary production (TPP) that was excreted (EPP) was 30.9 % at 5 m and 35.4 % at 10 m (Fig. 1). With values of 0.90 mg C $m^{-3}h^{-1}$ in the epilimnion and 0.50 mg C $m^{-3}h^{-1}$ in the metalimnion, the reassimilation by heterotrophic bacteria therefore accounted on average for 17.8 % of the TPP, i.e. 53.8 % of the total excreted primary production (EPP). This value is close to that (mean = 42 % ± 22) reported previously by Maurin et al. (1997) in Lake Pavin, during an annual study. If we assume that phytoplankton exudates are the main organic nutritional resources for bacteria, the mean bacterial growth yield value (bacterial production/quantity of C reincorporated) was 24.3 % in this study. This value is similar to the estimates of Del Gorgio et al. (1997) who showed that the bacterial growth yield increases with increasing trophic status of the environment, ranging from values of usually less than 10 % in oligotrophic waters to the highest values of about 40 % in the most productive environments. The mean percentage of bacterial production in this study was 4.3 % of the total primary production (TPP), a value lower than that calculated by Cole et al. (1988) for marine and lacustrine ecosystems (mean = 20 %), and that (mean = 16 %) reported by Jugnia et al. (2000) in the oligo-mesotrophic Sep Reservoir. It should however be emphasized that the values obtained from sampling conducted over a single 24 hour cycle are difficult to extrapolate to the overall functioning of Lac Pavin. The quantity of carbon transiting through bacteria as far as bacterivorous flagellates amounted, on average, to 0.09 mg C $m^{-3}h^{-1}$ at 5 m and 0.07 mg C $m^{-3}h^{-1}$ at 10 m, i.e. 1.7 % of the TPP in the epilimnion and 2.6 % in the metalimnion. The proportion of carbon destroyed by the viral lysis varied from 0.3 % of the TPP in the epilimnion to 0.6 % in the metalimnion (Fig. 1).

Since lytic activity leads to an enrichment of the medium in organic matter should it now be envisaged that the involvement of the «viral loop» (bacteria → bacteriophages → DOM → bacteria) has a significant influence on the carbon budget, and more generally on the yield of aquatic microbial trophic networks ? Studies at much greater spatial and temporal scales would be needed to answer this question.

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