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ORIGINAL ARTICLE

Proportion of prokaryotes enumerated as viruses by epifluorescence microscopy

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Abstract It is well known that there are prokaryotes small in size (e.g. ultra-microprokaryotes) that pass through a 0.2-μm filter. As bacterial and viral abundances are determined by epifluorescence microscopy and the differentiation between them is based on particle size, some bacteria can be erroneously enumerated as viruses, namely in marine waters where bacteria are small. However, there is no information on the proportion of prokaryotes that could be misidentified as viruses by epifluorescence microscopy. In this work, we assessed, in water samples collected in the estuarine system Ria de Aveiro (Portugal), the proportion of prokaryotes that could be counted as viruses by the current widespread epifluorescence microscopy and, for the first time, by fluorescence in situ hybridization (FISH). The total number of particles was determined on membranes of 0.2 and 0.02 μm after staining with 4′,6-diamidino-2-phenylindole (DAPI), and the number of prokaryotes (Bacteria and Archaea) was determined by FISH for both pore size membranes. The results show that, in the marine zone of the estuarine system, 28 % of particles enumerated as virus-like particles were prokaryotes, but, in the brackish water zone, only 13 % of the particles counted as viruses were actually prokaryotic cells. Epifluorescence microscopy overestimates viral abundance, and also the ratio viruses:prokaryotes, and this error must be taken into consideration because it can vary significantly within a system. In fact, in the marine zone of an estuarine system, the overestimation of viral abundance can be twice as high as in the brackish water zone.

Keywords Epifluorescence microscopy · FISH · Overestimation · Prokaryotes · Viruses

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Introduction

It is currently accepted that bacteria and viruses play important roles in the biogeochemical cycles in the aquatic environment. Bacteria are the most relevant biological component in the turnover (transformation and remineralisation) of organic matter in aquatic systems (Pomeroy 1981). Heterotrophic bacteria often represent 10–30 % of the living carbon biomass (Holligan et al. 1984; Cho and Azam 1990) and may use as much as 40 % of the carbon fixed by the primary producers (Cho and Azam 1990; Ducklow and Carlson 1992). By converting dissolved organic carbon into a particulate form, potentially useable by higher trophic levels, heterotrophic bacteria represent a key link in the cycles of energy and carbon in aquatic systems, as depicted in the concept of the microbial loop proposed by Azam (1983). Studies in several aquatic environments indicate that the microbial loop can process about as much energy as the classical grazing food chain (Riemann and Søndergaard 1986). However, since new players, namely viruses, have been added, the current description of the microbial loop is not a simple task. The importance of viruses in the microbial loop is still uncertain, but it is known that they influence the cycle of organic matter when they infect and destroy bacteria, algae, and cyanobacteria. When cell lysis occurs, particulate organic matter is lost from the detrital food chain but is still available to heterotrophic bacteria.

Viruses are, by far, the most abundant biological entities (around 10^{10}–10^{11} particles L^{-1} of water) in aquatic systems (Suttle 2000, 2007; Wommack and Colwell 2000; Almeida et al. 2001; Fischer and Velimirov 2002; Weinbauer 2006). Most marine viruses are bacteriophages that infect bacteria (Weinbauer 2006), exerting an important regulatory effect on prokaryotic communities (Suttle 2007). Viral lysis in surface waters removes 20–40 % of the standing stocks of prokaryotes each day (Suttle 1994). Consequently, viral lysis plays a significant role on the cycling of nutrients and organic matter.

In addition, viruses may influence the species composition of microbial communities (Wommack and Colwell 2000). They have a restricted range of host cells and, consequently, infection by a particular virus does not act on the total microbial assemblage but rather on specific sub-populations.

The use of reliable methods for determination of bacterial and viral abundance is an essential criterion for establishing the roles of bacteria and viruses in biogeochemical cycles and food chains. In addition, it is also important for understanding the dynamics of bacterial and viral populations in natural systems. The available methods for determining the bacterial abundance in aquatic environments are transmission electron microscope (TEM), epifluorescence microscopy and flow cytometry. The most widely used method is epifluorescence microscopy. By this method, bacteria are concentrated on 0.2-μm membranes, stained with fluorochromes and counted under an epifluorescence microscope (Almeida and Alcantara 1992; Buesing 2005). It is crucial to use fluorochromes specific to nucleic acids, in order to facilitate the differentiation between bacteria and other particles. This is particularly important when the samples are rich in organic matter. Acridine Orange (AO) and 4′,6-diamidino-2-phenylindole (DAPI), which preferentially stain dsDNA, have traditionally been used for estimating the number of bacteria.

Epifluorescence microscopy is also the most popular method for estimating the total viral abundance in aquatic systems. Compared with TEM, this method is faster and more affordable, allowing the processing of a large number of samples and, consequently, to obtain more statistically accurate data. Epifluorescence microscopy is about seven times more accurate than TEM for counting viruses (Danovaro and Corinaldesi 2003). DAPI, YOPRO-1, SYBRGreen and SYBRGold are the most used stains for counting viruses (Weinbauer 2006). Contray to the other fluorochromes, DAPI preferentially stains dsDNA viruses. Although ssDNA, dsRNA and ssRNA viruses are found in aquatic systems and, consequently, are not totally covered by the DAPI staining, in the marine environment dsDNA viruses are the most abundant, representing 96 % of the total number (Ackermann 2007). Although several recently published studies propose new strategies to determine the number of viruses in environmental samples, they are based on the use of specific primers for pathogenic enteric viruses and cannot be applied to determine the total number of viruses in environmental matrices.

As bacterial and viral abundances are determined by epifluorescence microscopy and the differentiation between bacteria and viruses is based on particle size, some viruses can be enumerated as bacteria and some bacteria can also be erroneously considered as viruses, namely in marine waters where bacteria are small. Consequently, the true numbers of bacteria and viruses could be overlooked.

Fluorescence in situ hybridization (FISH) is a cultivation-independent method for in situ analysis that allows the identifying and quantifying of prokaryotes in mixed assemblages (Amann et al. 1990a, b, 1996; Pernthaler et al. 2001). FISH has been frequently used to analyze prokaryotic community structure and to follow the spatial and temporal dynamics of individual microbial populations in their habitat (Amann et al. 1990a, b, 1996; Pernthaler et al. 2001). Most FISH applications target ribosomal RNA (rRNA) with oligonucleotide probes labelled with a fluorescent dye. As each cell contains a high number of ribosomes, a natural signal amplification system is provided. rRNA molecules are suitable for the identification of Bacteria and Archaea (Amann and Fuchs 2008) and therefore this technique can be used to quantify the prokaryotes that are counted as viruses when viral abundances are determined by epifluorescence microscopy. A typical FISH protocol includes four steps: fixation and permeabilisation of the sample; hybridisation; washing of unbound probe; and detection of labelled cells by microscopy or flow cytometry (Amann et al. 2001).

The objective of this work was to assess the proportion of prokaryotes counted as viruses by the current widespread method epifluorescence microscopy used to enumerate viruses.

Materials and methods

Study sites and water sampling

Water samples were collected at Ria de Aveiro which is a coastal lagoon stretching for 50 km along the coast of Portugal. The Ria has a complex topography, with several channels spreading from the mouth towards the different streams, forming a complex and dynamic estuarine system (Lopes et al. 2010).

Two sampling sites were considered in the present study: the station I2 (40°39′29″N, 08°42′12″W), close to the mouth of the estuary, representing the marine zone (maximum depth 30 m, salinity ranging from 10.1 to 36.3 PSU, average 28.5± 8.70 PSU) and the station I6 (40°35′41″N, 08°41′21″W), located in Ílhavo Channel, representing the brackish water zone (maximum depth 2 m, salinity ranging from 0.2 to 36.5 PSU, average 19.9±12.39 PSU) (Fig. 1). All samples were collected at low tide at 0.2 m below the water surface in March 2009, by direct immersion of 0.5-L sterile glass bottles. Water samples were kept cold and in the shade during transport to the laboratory, being processed within 1 h of collection. Three sub-samples were collected for prokaryotes and viruses quantification for each sampling site.

Experimental setup

To determine the number of viruses potentially counted as prokaryotes, membranes of 0.2 μm were used, while 0.02-μm

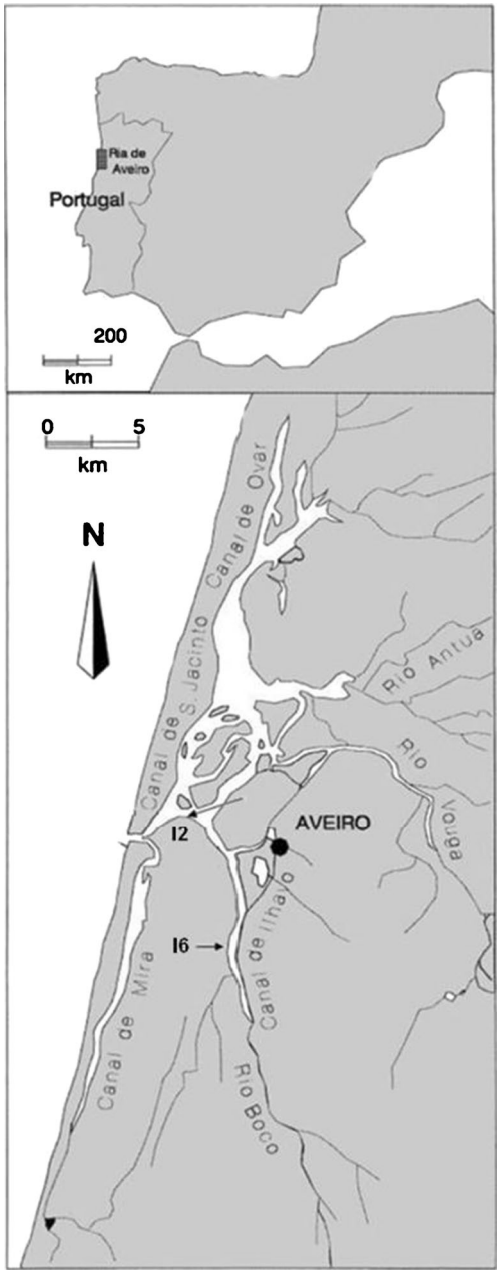

Fig. 1 The estuarine system of Ria de Aveiro. Sampling stations are indicated by *arrows* along Canal de Ilhavo. Station *I2* is in the marine zone and station *I6* in the brackish water zone of the estuarine system

membranes were employed to determine the number of prokaryotes counted as virus-like particles. The total number of particles was determined in both size fractions after staining with 4′,6-diamidino- 2-phenylindole (DAPI), and the number of prokaryotes (Bacteria and Archaea) by FISH, using oligonucleotide probes with Cy3-labelled (Amann et al. 1990a).

Aliquots of 1 mL were filtered through 0.2-μm pore size polycarbonate membrane membranes (Millipore) and the filtrate was further filtered through 0.02-μm pore size membranes (Whatman; Anodisc, 25 mm diameter). Particles retained on 0.2-μm and on 0.02-μm pore size membranes were fixed with 2 % paraformaldehyde for 30 min and rinsed with distilled water and the filters were preserved in the dark until hybridisation.

Domain specific probes were used for Bacteria mix Eub338, Eub338I and Eub 338II, Eub 338III (Amann et al. 1990a) and for Archaea Arc915 (Pernthaler et al. 2001). For each probe, triplicate filter pieces were placed on Parafilm-covered glass slides and overlaid with 30 μL hybridisation solution with 3.0 ng μL^{-1} of probe (final concentration). The hybridisation solution contained 0.9 M NaCl, 20 mM Tris–HCl (pH 7.4), 0.01 % SDS, and the optimum concentration of formamide (35 % for Eubacteria and 20 % for Archaea) (Eilers et al. 2000). Filters were incubated in a hybridisation oven at 46 °C for 90 min. After hybridisation, filters were washed for 15 min at 48 °C in wash solution (20 mM Tris HCl pH 7.4, 5 mM ethylenediamine tetra acetic acid, 0.01 % SDS, and the appropriate concentration of NaCl) (Eilers et al. 2000). Rinsed and dried filter pieces were mounted with 4:1 Citifluor: Vectashield containing 2 μg mL^{-1} 4′, 6-diamidino-2-phenylindole (DAPI). Counting was conducted in a Leica epifluorescence microscope equipped with filter for Cy3 (Cy3–Y3; Leica) and a filter for DAPI (DAPI–A; Leica). For each of the three replicates of each sub-sample, 10 random optical fields were counted. In each optical field, total DAPI-stained particles and labelled cells of Bacteria and of Archaea were counted. The percentages of Bacteria and Archaea were standardised in relation to the total DAPI counts on the 0.2- and 0.02-μm membrane filters. The number of virus-like particles was determined in the 0.02-μm membrane filter by subtracting the number of prokaryotes from the DAPI counts and the results were expressed as DAPI percentage.

Statistical analysis

SPSSWIN 15.0 was used for data analysis. The significance of differences in phage and bacteria concentrations between samples was assessed using one-way ANOVA. Only the data with normal distribution (assessed by Kolmogorov–Smirnov test) and with homogeneity of variances (assessed by Levene test) were used. A value of $p < 0.05$ was considered significant. All experiments were conducted in triplicate.

Results

In this study, samples obtained from the I2 and I6 stations, filtered with 0.2-μm pore size membranes, showed $4.6 \pm 0.3 \times 10^9$ and $3.9 \pm 0.2 \times 10^9$ particles (DAPI stain) per litre, respectively (Table 1). In both sampling stations, a clear dominance of Bacteria in comparison to Archaea was observed. The relative abundance of Bacteria (FISH Eub338 probe stain), was 70 % at station I2 and 61 % at station I6, while the relative

Table 1 Total particles, prokaryotes and virus-like particles counts on 0.2- and 0.02-μm pore size membranes by epifluorescence microscopy

		Sampling station			
		I2		I6	
Pore size membranes	Particles type	Number (average ± SD L^{-1})	%	Number (average ± SD L^{-1})	%
0.2 μm	Total (DAPI stain)	$4.6\pm0.3\times10^{9}$	100	$3.9\pm0.2\times10^{9}$	100
	Eubacteria (FISH Eub338 probe stain)	$3.2\pm0.3\times10^{9}$	70	$2.4\pm0.4\times10^{9}$	61
	Archaea (FISH Arc915 probe stain)	$3.0\pm0.1\times10^{8}$	7	$3.9\pm0.04\times10^{8}$	10
	Non-probe labelled (DAPI stain-probes stain)	$1.1\pm0.2\times10^{9}$	23	$1.2\pm0.2\times10^{9}$	29
0.02 μm	Total (DAPI stain)	$1.2\pm0.06\times10^{10}$	100	$1.0\pm0.09\times10^{10}$	100
	Eubacteria (FISH Eub338 probe stain)	$2.6\pm0.6\times10^{9}$	22	$7.5\pm0.2\times10^{9}$	7
	Archaea (FISH Arc915 probe stain)	$7.8\pm0.3\times10^{8}$	6	$6.1\pm0.17\times10^{8}$	6
	Viruses (DAPI stain-probes stain)	$8.5\pm0.6\times10^{9}$	72	$9.0\pm0.8\times10^{9}$	87

Values represent the mean of three sub-samples. For each sub-sample, three replicates were counted

abundance of Archaea (FISH Arc 915 probe stain) was 7 % at I2 and 10 % at station I6 (Table 1). The percentage of non-prokaryote particles tended to be higher at station I6 (average 29 %) than at station I2 (average 23 %) (Table 1) but the difference was not significant (ANOVA, $p>0.05$). When samples were filtered through 0.02-μm pore size membranes, counts of $1.2\pm0.06\times10^{10}$ and $1.0\pm0.09\times10^{10}$ particles stained by DAPI per litre were obtained in samples from stations I2 and I6, respectively (Table 1). The percentage of prokaryotes determined with these membranes was significantly lower than that observed on 0.2-μm pore size membranes. The averaged abundance of Bacteria (FISH Eub338 probe stain) was 22 % at station I2 and 7 % at station I6, while the relative abundance of Archaea (FISH Arc 915 probe stain) was, on average, 6 % at both stations (Table 1). The percentage of particles counted as virus-like particles was higher at station I6 (average 87 %) than at station I2 (average 72 %) (ANOVA, $p<0.05$).

Discussion

In order to evaluate the ecological significance and role of viruses in the aquatic environment, a method that is simple, accurate and suitable for routine environmental analysis is required. The high counting efficiency, simplicity of preparation, modest equipment requirements, and the possibility of preparing specimens for long-term storage, make epifluorescence microscopy the preferred method for routine environmental analysis. The results obtained with epifluorescence microscopy indicate that the concentration of viruses in natural waters is higher than previously recognized and imply that the TEM-based method significantly underestimates virus abundance (Hennes and Suttle 1995). However, epifluorescence microscopy is also used to enumerate prokaryotes in the aquatic environment, and sometimes it is difficult to distinguish prokaryotes from viruses. In fact, in the marine environment, where prokaryote cells are small (Almeida et al. 2001, 2002; Baptista et al. 2011), an important fraction may pass through the 0.2-μm pore size membranes used to concentrate them, and are then collected in the 0.02-μm pore size membranes used to concentrate viruses.

The results of this study show that, using epifluorescence microscopy, some prokaryotes are counted as viruses, namely in the marine zone, overestimating viral density and underestimating prokaryotes density. The ratio viruses:prokaryotes, which is determined using the particles counted on the 0.2- and 0.02-μm pore size membranes is, consequently, also affected by a larger error than absolute viral abundance. In the estuarine system Ria de Aveiro, a considerable fraction of particles enumerated as viruses were found to be prokaryotes when the FISH approach was used, being this number significantly higher in the marine zone than in the brackish water zone (28 and 13 %, respectively), where the bacterial size is smaller than that observed in the brackish water zone (Almeida et al. 2001, 2002; Baptista et al. 2011). FISH results using probes for Bacteria and Archaea show that the majority of these fluorescent particles counted as viruses are indeed bacteria.

As the samples are filtered sequentially by 0.2- and 0.02-μm pore size membranes, the prokaryotes that are retained by the 0.02-μm membranes are those that passed the 0.2-μm pores and are not included in the prokaryotes counts, underestimating their number by similar values. However, the non-probe labelled particles stained by the DAPI and enumerated on 0.2-μm pore size membranes can include some viruses, namely the larger ones, which can overestimate prokaryotes counts. Moreover, some of these non-probe labelled particles can also comprise some prokaryotes not detected by the Bacteria and Archaea probes used, as observed in previous studies (Pereira et al. 2011; Oliveira et al. 2012).

As DAPI is a non-selective fluorochrome for nucleic acids, it is able to bind to any free DNA resulted from cell lysis that would pass the 0.2-μm membranes. However, the prokaryotes retained by the 0.02-μm membranes and detected by FISH correspond, in fact, to intact Bacteria and Archaea cells, and eventually to functional cells, and not cell debris or free prokaryotic DNA released by cell lysis since FISH targets 16S rRNA gene sequences. In contrast to DNA, short-lived RNA molecules, such as rRNA 16S, is known to reflect cell activities (Kemp et al. 1993) being used as a marker for bacterial activity. Their content is proportional to cell activity- and they are degraded rapidly in living bacterial cells by enzymes (RNase), which are very stable even in harsh environments (Sheridan et al. 1998).

Since the epifluorescence microscopy would only allow the determination of the fluorescence particles, but not the characterization of their morphologic traits as a virus, it would be essential to check, in a near future, if a DNase treatment would be important to clean up free DNA in the samples, avoiding virus overestimation due to fluorescent particles resulting from free DNA. It would also be important to perform a more controlled experiment, including the addition of a known number of virus particles to the samples to evaluate the method reliability.

Conclusion

In conclusion, it can be said that the determination of the virus-like particles by epifluorescence microscopy can overestimate the density of viruses and, consequently, the ratio viruses:prokaryotes, and the margin of error can vary considerably within a single ecosystem. In estuarine systems, the overestimation of viral abundance in the marine zone can be double that of the brackish water zone.

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Competing interests The authors declare that they have no competing interests.

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