Chapter

A FEW TESTS PRIOR TO FLOW CYTOMETRY AND EPIFLUORESCENCE ANALYSES OF FRESHWATER BACTERIO- AND VIRIOPLANKTON COMMUNITIES

Stéphan Jacquet [1,*]***, Ursula Dorigo*** [1] ***And Sébastien Personnic*** [1]
[1]INRA, UMR CARRTEL, Thonon cedex, France

ABSTRACT

Concerns about obtaining accurate determinations of the concentrations of viruses and bacteria in freshwater samples led us to examine a broad battery of counting and storage procedures for use in flow cytometry (FCM) and epifluorescence microscopy (EFM) analyses. Sample preparations were done so as to optimize counts and preservation by using different types and concentrations of aldehyde-based fixatives, stains belonging to the SYBR family, dilution media, temperature and storage conditions. Whenever possible, FCM and EFM counts were compared. Results obtained using FCM supported the addition of fixative for bacteria, preferably glutaraldehyde at a final concentration of 2%, dilution in 0.2-µm or 0.02-µm filtered Tris-EDTA buffer (TE, pH = 7.5), staining with SYBR Green I at a final concentration of 10^{-4} and incubating at ambient temperature for at least 15 minutes. For viruses, there was no need to add fixative, whereas dilution in recently-autoclaved and 0.02-µm filtered TE and incubation with SYBR Gold at a final concentration of 2×10^{-5} at 75°C for 10 minutes is recommended. If possible, FCM samples should be counted on day = 0, although we do show that bacterioplankton samples, at least, may be stored at 4°C and counted at 24 h later but not beyond if samples cannot be frozen in liquid nitrogen. The conditions required for optimum EFM counts of both bacteria and viruses involved were to stain filters with SYBR Gold at a final concentration of 10^{-3}. Slides could be counted for up to 1 month if rapidly frozen and stored at –20°C.

Our results performed on lake samples clearly demonstrate the importance of defining the best conditions in order to get reliable counts of microbial communities such as viruses and bacteria. Each research laboratory should undertake such tests according to the equipment available, and the needs and area of their research.

* E-mail: sjacquet@thonon.inra.fr. Tel: +33.4.50.26.78.12; Fax: +33.4.50.26.07.60.

Keywords: Virus, bacteria, freshwater, enumeration, flow cytometry, epifluorescence microscopy

INTRODUCTION

Bacteria and viruses have been shown to be key components of aquatic microbial communities because of their abundance, ubiquity and impact on nutrient cycling, energy fluxes and microbial food webs (Azam et al. 1983; Fuhrman 1999; Wilhelm and Suttle 1999). Bacterioplankton is mainly responsible for the recycling of nutrients, the decomposition of organic matter and for most of the oxygen uptake in the pelagic zone of freshwater ecosystems (Fisher et al. 2000). Bacterioplankton is also a major food source for small and large protozoa (Berninger 1991; Domazion et al. 2003; Simek et al. 1990). Viruses are important in the control of plankton community composition, diversity and succession, and play a key role in bacterioplankton cell mortality (Sime-Ngando et al. 2003; Weinbauer and Rassoulzadegan 2004; Wommack and Colwell 2000, Jacquet et al. 2010) with impacts that vary according to the ecosystem, time and space. They are responsible for 10-60% of the daily bacterioplankton mortality (Bettarel et al. 2003, 2004; Fischer and Velimirov 2002; Jacquet et al. 2005; Simek et al. 2001), and are the most abundant biological particles in both the marine and freshwater environments, with typically 10^7-10^9 viruses.mL^{-1} (Wommack and Colwell 2000; Jacquet et al. 2010). Bacterioplankton densities typically range from 10^5 to 10^7 cells.mL^{-1} (Berthenuis et al. 2012). These densities can vary considerably both with time and space (Larsen et al. 2004; Ovreas et al. 2003; Schröder et al. 2003) due to the influence of physico-chemical and/or biological parameters.

In order to get a better understanding of the ecology of these microorganisms, and to elucidate their role in aquatic systems, we need high frequency water sampling (in both time and space) and subsequent accurate and rapid determinations of their abundances. In the 1970's bacteria were quantified by transmission electron microscopy (TEM) (Watson et al. 1977) or by epifluorescence microscopy (EFM) on acridine stained samples (Francisco et al. 1973). First estimates of viral numbers were obtained using TEM after ultrafiltration (Paul et al. 1991; Proctor and Fuhrman 1990) or ultracentrifugation (Bergh et al. 1989; Bergström and Jansson 2000; Borsheim et al. 1990, Bratbak and Heldal 1993; Sime-Ngando 1997). However, this technique is not only tedious and very time consuming, but also involves some uncertainties arising from the concentration procedures, still requires expensive equipment and skilled personnel. These features make it unsuitable for routine field analysis.

Since the 1990's, the use of EFM in conjunction with the development of new, highly-fluorescent nucleic acid dyes rapidly supplanted TEM, since it was a quicker and less expensive technology (Hara et al. 1991; Hennes and Suttle 1995; Lisle et al. 2004). Nowadays, aquatic bacteria and viruses may be counted by flow cytometry (FCM) using fluorochromes such as those belonging to the SYBR family (Chen et al. 2001; Marie et al. 1999; Middelboe and Glud 2003; Noble and Fuhrman 1998; Shopov et al. 2000; Wen et al. 2004). FCM can be used to perform very accurate and fast counts (Brussaard 2000; Li and Dickie 2001; Vives-Rego et al. 2000), generally in less than 2 min per sample (Marie et al. 1999). These last two points are important when a large number of samples have to be analyzed and statistically significant data are required. Unfortunately, as counting cannot

always be done on the same day as sampling, reliable fixing and storage procedures may be a critical aspect.

A wide range of procedures, fixatives, dyes and storage has been proposed to date. Historically, considerable efforts have been made to develop procedures that accurately determine and preserve marine bacterioplankton and/or virioplankton in natural samples (Decamp and Rajendran 1998; Lebaron et al. 1998; Trousselier et al. 1995; Turley and Hughes 1992; 1994) or marine viruses in culture (Brussaard 2004; Marie et al. 1999); but only a few studies have been done involving freshwater samples (Lebaron et al. 1998). Some FCM studies have compared various preservatives and storage protocols (Marie et al. 1999; Troussellier et al. 1995; Turley and Hughes 1992), or the use of different nucleic acid stains (Chen et al. 2001; Lebaron et al. 1998; Tomaru and Nagasaki 2007). Different dilution solutions or incubation temperatures have been tested on virus counts by Brussaard (2004). Similar work has also been done for EFM (Ammini et al. 2010; Turley and Hugues 1992; Wen et al. 2004). Some studies have attempted to compare two of the three methods, typically either EFM *vs.* FCM (Gasol et al. 1999; Jochem 2001; Lemarchand et al. 2001) or EFM *vs.* TEM (Bettarel et al. 2000; Hara et al. 1991; Hennes and Suttle 1995; Noble 2001); but very few studies have attempted to compare the effectiveness of all three techniques (TEM, EFM, FCM) for performing direct total counts of bacteria and viruses (Chen et al. 2001; Marie et al. 1999). It would appear that the FCM counts were always correlated to, but slightly higher than those obtained by EFM or by TEM.

In this study, we chose to perform FCM tests with the most popular stains of the SYBR family, and various incubation temperatures, fixatives and dilution solutions and various storage conditions to optimize the counts of viral and non-photoautotrophic (commonly known as heterotrophic) bacterial communities sampled within the three largest natural French lakes (Annecy, Bourget, Geneva). Similar tests were done using EFM, and whenever possible, the two techniques were compared. Our study will highlight that FCM gives better results than EFM (suggesting that the concentrations of the total viruses in aquatic ecosystems are probably higher that many previous VLPs (Virus-like Particles) estimates found in the literature) but also that it may be problematic to believe that only one protocol can exist for all situations.

MATERIALS AND METHODS

Sample Collection

Polypropylene bottles, previously rinsed with water from the collection sites were used to collect water samples, between September 2002 and November 2004 and during summer-fall 2012, from Lakes Annecy, Bourget and Geneva (details in Personnic et al. 2009). Immediately after sampling, water was filtered on board with <2 µm syringe filters to remove all potential grazers (and for keeping only free-living prokaryotes and viruses). Lake water so prepared and kept in cryotubes was then transported in less than 3 hours to the laboratory in refrigerated boxes (kept at 4-6°C to prevent any biological activity), and processed without delay for subsequent experiments. Data from Experiments 1-7 were used to test a wide range of dilution solutions, staining, fixation and storage conditions in FCM (see below).

Experiment 8 was specifically designed to optimize EFM counts of bacteria and viruses (see below). Finally, the data obtained by EFM and FCM were compared. It is noteworthy here that we did not fix samples on board; differently said we did not test transport and storage conditions in affecting the abundances.

All nucleic-acid specific dyes were delivered in dimethyl sulfoxide and stored at -20°C. To avoid contamination, fixatives and dilution solutions, adjusted to a final pH of 7.5 were filtered through 0.2 µm (for bacterial analyses) or 0.02 µm (for viral analyses) using syringe filter (Millipore). Fixation was performed for at least 15 min at ambient temperature (*i.e.* ~20°C), and each sample was vortexed and then diluted 50 fold in the dilution solution. Incubation with the dye occurred at dim light at 20, 45, 65 or 75°C (see later).

Background

We only consider thereafter SYBR dyes that have proved to be very efficient and are the principal ones used nowadays for both freshwater and marine studies. We are aware however that some earlier studies demonstrated that other dyes, for instance Syto, Picogreen or Hoechst, performed very well in other freshwater ecosystems and that new dyes would also deserve to be tested such as the CF™ Dyes (from SIGMA). Originally, SYBR green dyes were preferred in marine studies as they are less sensitive to the salt, but they were also shown to work perfectly in freshwaters. Based on our experience (not shown), SYBR dyes can be used in a variety of freshwater ecosystems (from rivers to mountain lakes). It is possible however that in the case of some high-mountain lakes, none of the *Molecular Probes* green stains may be of any utility when there is glacial silt in the lakes (person. com.). More classical stains such as DAPI should thus be preferred. Also, our study is based on the use of a single laser beam fixed at 488 nm (blue) while less conventional instruments than the flow cytometer used in this study, with lasers at other and/or multiple wavelengths, can offer now the opportunity to use many other dyes (with maybe much higher resolution than the SYBR Green family dyes). Differently said, we are aware that this study is not complete and there no pretention to give the only right solution. Our aim was more to end up with consideration (some possible tests) that should be taken into consideration to perform accurate counts of freshwater bacterio- and virioplankton communities by flow cytometry and epifluorescence microscopy.

It is also noteworthy here that we used the terms heterotrophic bacteria or bacterioplankton while heterotrophic prokaryotes should be preferred to take into account that FCM analyses cannot discriminate between Bacteria and Archea. As authors generally use "bacteria", we also used preferentially this term, even if it is probably not *sensu stricto* true here.

Experiments Conducted

Experiment 1. This experiment was designed principally to choose among a wide panel of dilution solutions (TE, TAE, TBE, lake water, FACSFlow and PBS) for FCM analyses of heterotrophic bacteria. Samples were left untreated or fixed with formaldehyde (FA 1 or 2% final concentration, Sigma), glutaraldehyde (GA 0.25 or 1%, Sigma) or paraformaldehyde

(PF 1 or 2%, freshly prepared home-made solution, (Marie et al. 1999), diluted in each dilution solution and finally stained with either SYBR Green I and II (Molecular Probes) for 15 min at room temperature to be analyzed by FCM.

Experiment 2. The type and the final concentrations of the fixative (GA 1 and 2%, PF 1 and 2%, FA 1 and 2% and PF 1% mixed with GA 0.05%) were tested, as were the storage temperature (4, -20 and -196°C) and duration (t = 0, 1, 8, 30 days) for bacterioplankton and/or virus FCM analyses. From experiment 1, we retained treatments that gave the best statistical results for both cell abundance and FCM signatures (*i.e.* TAE, TE, TBE and lake water among the dilution solutions and SYBR Green I for staining). Briefly, 10 mL subsamples were immediately treated with one concentration of a given fixative. Another subsample was kept without fixative. From each of these subsamples, a set of 1 mL duplicate subsample was kept at 4°C and was then analyzed on days 0, 1, 8 and 30, and 3 sets of 1 mL duplicate subsamples were left for 15 min at 4°C before being stored at –20°C or dove in liquid nitrogen. In this way, each frozen sample was thawed only once just before being analyzed at t = 1, 8 or 30 days. Previous results suggested that repeated thawing clearly has a negative impact on the total counts, and we obviously wanted to avoid this (data not shown). At t0, the samples were diluted as seen above. At t1, 8 and 30 days, refrigerated samples, particularly those stored at –20°C and liquid nitrogen, were warmed to room temperature over 15 min (previous experiments had shown that cold samples had a negative effect on the quality of the FCM signature), vortexed and diluted. The diluted samples were then stained for 15 min at room temperature with the nucleic acid dye SYBR Green I at a final concentration of 10^{-4} for the bacteria and $5x10^{-5}$ for the viruses (Marie et al. 1997).

Experiment 3. The staining characteristics and in particular the staining kinetics of SYBR Green I and SYBR Gold, both at a final concentration of 10^{-4}, were analyzed using bacterioplankton samples which had been fixed with GA 2%. Samples were diluted in TE buffer, and replicate samples were analyzed by FCM at regular intervals after incubation with the dye for 1 min to 1 h.

Experiment 4. This experiment was performed to compare the staining efficiencies of SYBR Green I (final concentration of 10^{-4}) and SYBR Gold (final concentration of 10^{-4}, 5×10^{-5}, 2×10^{-5}) for counting bacteria and viruses by FCM. Briefly, 12 samples which were fixed to a final concentration of 1% GA were diluted in TE or lake water and analyzed by FCM after incubating with the stain for 15 min at 20°C for bacteria, or after incubating at 45°C, 65°C or 75°C for viruses according to Marie et al. (1999) and Brussaard (2004).

Experiment 5. This experiment aimed to test the use of fixatives and dilution solutions on FCM virus counts. Samples were fixed with FA, or GA (1% final concentration) or left untreated and dilution was performed in lake water, in TE and in TBE. All samples were stained with either SYBR Green I or SYBR Gold, at a final concentration of 10^{-4}. Incubation was performed at 75°C (Marie et al. 1999).

Experiment 6. A range of incubation temperatures was also tested. Samples were either left unfixed or fixed with 1% or 2% GA and analyzed at day = 0 or at day = 1 after storage at 4°C or -20°C. For FCM analyses, samples were diluted in TE or in lake water and stained with SYBR Green I (final concentration of 10^{-4}) and subjected to various incubation temperatures 20°C (15 min), 45°C and 75°C, the latter two lasting for 10 min, followed by cooling for 5 min at room temperature.

Experiment 7. In order to test different ways of using TE for FCM virus counts. The conditions tested included the final pH of the TE solution (pH 7 or 8, corresponding to the

natural pH variation observed in our lakes), TE was autoclaved or not, filtered through a 0.02-µm or 0.2-µm filter. The samples were then diluted in the various types of TE buffer and incubated with SYBR Green I or SYBR Gold, both used at a final concentration of 10^{-4}, for 10 min at 75°C, and FCM analyses were done. It is noteworthy here that each test was also performed on a "negative" control (*i.e.* without sample).

Experiment 8. Tests for EFM counts were done by counting both bacterio- and virioplankton in several water samples. Samples were either left untreated or immediately fixed upon arrival in the laboratory for at least 15 min. FA or GA, were added at final concentrations of 1 and 2% or 0.5 and 1%, respectively. 1 mL of the fixed samples was filtered through a 25-mm, 0.02-µm ultra-fine pore size filter (Anodisc, Fisher Scientific), backed by a 25-mm GF/C filter (Whatman), at low vacuum. Each filter was stained either with SYBR Green I (Noble and Fuhrman 1998) or with SYBR Gold (Chen et al. 2001), both at a final concentration of 10^{-3}. The filter was finally mounted on a glass slide and 30 µl of an anti-fading solution was added, that was prepared as recommended by Noble (2001). Briefly, we prepared a solution of 990 µl of a 50% PBS 50% glycerol mixture with 10 µL of 6.6% p-phenylenediamine. Then, a cover slip was placed over the filter. As suggested by Wen et al. (2004), the slides were prepared immediately, and if possible counted on the same day or else stored at – 20°C for no more than a couple of days. In addition to the viral and bacterial counts at t = 0, 10 of the filters (5 filters for bacterial counts, 5 for viral counts), were re-counted after being stored for 16, 29, 53, 68, or 96 days at –20°C in order to assess the importance of counting the slides immediately after they had been prepared. The results have been expressed as a percentage of the counts obtained at t = 0.

Comparison of FCM and EFM

Samples were collected monthly from February to May 2003 from several depths (between 2 and 50 m) in the three lakes. A total of 80 samples were analyzed for bacteria and viruses in order to compare the counts obtained by FCM and EFM. For the FCM analyses, samples were fixed in 0.25% GA, and stained with SYBR Green I at a final concentration of 10^{-4}. Samples for bacterial counts were incubated at room temperature, and samples for viral counts were heated for 10 min to 75°C and then cooled for 5 min prior to analysis. For EFM analyzes, the samples were fixed with 1 % FA and incubated with SYBR Gold at a final concentration of 10^{-3}. The slides were prepared without delay and either counted immediately or stored at -20°C for up to a few days.

Flow Cytometry Analyses

The counts were done using a FACSCalibur (Becton Dickinson) benchtop flow cytometer, equipped with a blue laser beam fixed at 488 nm and with the standard filter setup. The main FCM procedures were the same as those outlined by Marie et al. (1999), and originally devised for marine bacterioplankton and virioplankton. MilliQ water was used as a sheath fluid since no significant differences were recorded with either filtered lake water or the FACSFlow provided by the manufacturer (not shown). Samples were run at medium speed (*i.e.* between 60 and 70 $\mu l.min^{-1}$, the flow rate being checked before each analysis), and

all parameters were acquired in log mode. To avoid coincidence, the number of events was limited to between 100 and 1000 per sec by diluting the sample and/or by raising the threshold of the instrument. Bacterial cell parameters were determined relative to the values found for an internal standard, *i.e*. a solution of 1-µm fluorescent beads (Molecular Probes). Bacteria and viruses were detected from dot plots of right angle light scatter (SSC) versus the green fluorescence of the acid nucleic dye complex (FL1 channel: 530±15 nm) and the red fluorescence of phytoplankton (FL3 channel: >630 nm) versus FL1. Data were collected in listmode files and then analyzed on a separate PC using the custom-designed software CYTOWIN (Vaulot 1989). Abundances are reported as cells.mL^{-1} (heterotrophic bacteria) or particles.mL^{-1} (viruses).

Epifluorescence Counts

Counts were done using a LEICA epifluorescence microscope equipped with a mercury lamp and a blue excitation light (450-490 nm). Around 200 bacterial cells (cyanobacteria were excluded from this counting) were counted in 10 randomly selected fields for each filter, and 400-600 viruses were counted in 20 fields. The viral and bacterial abundances have been reported as particles.mL^{-1} or cells.mL^{-1} respectively, following the procedures outlined by Noble (2001).

Statistics

Bacterial and viral concentrations we obtained following the different treatments were compared and analyzed for significance by using the tests of Mann-Whitney or Kruskal-Wallis with the PAST software package (freely available at http://folk.uio.no/ohammer/past/).

RESULTS

Choice of the Nucleic Acid Stain for FCM Counts

When the dyes were used at a final concentration of 10^{-4}, compared to SYBR Green I stained samples, SYBR Green II yielded 40% less bacterial numbers (Figure 1, experiment 1). This figure also shows that the SYBR Green II counts were correlated to the SYBR Green I counts. The fluorescence of the SYBR Green I stained samples reached maximum and stable bacterial abundances after having been incubated with the dye for 10-12 min, whereas the fluorescence of those stained with SYBR Gold increased less rapidly and was less stable (experiment 3). In the latter experiment the SYBR Green I stained samples showed 16% greater abundances than the SYBR Gold stained ones. From a qualitative point of view, the bacterial signature was easier to interpret when SYBR Green I stain was used (not shown). The 4th experiment, in which we counted bacteria and viruses within various water samples, gave us an indication of the staining efficiency of SYBR Green I compared to that of various concentrations of SYBR Gold.

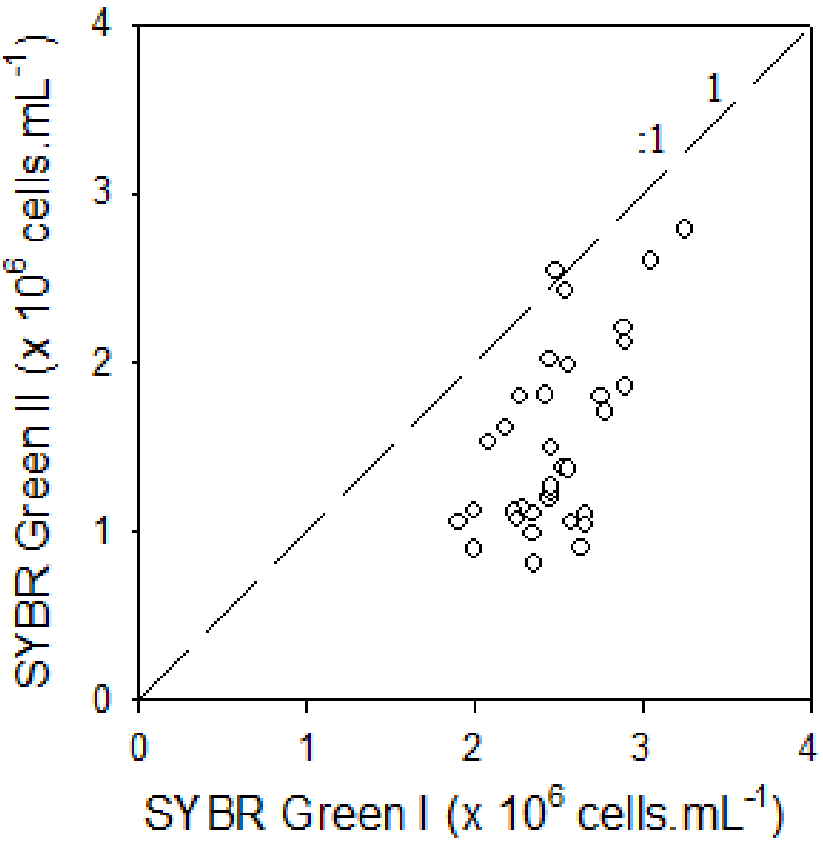

Figure 1. Relationship between SYBR Green I and SYBR Green II, both used at a final concentration of 10^{-4}, for bacterioplankton-stained samples diluted using various dilution solutions (TAE, TE, TBE, lake water, FACSFlow, PBS), fixed with different types and concentrations of fixatives (FA, GA, PF) and analyzed by FCM. $y = 1.11x - 1.23$ ($n = 34$, $r = 0.6$, $p = 0.99$). The dashed line corresponds to the 1:1 relationship. Experiment 1.

Average total bacterial cell count was not significantly different for SYBR Green I used at a final concentration of 10^{-4} and SYBR Gold used at one of the range of concentrations (10^{-4}, 5×10^{-5} or 2×10^{-5}, data not shown).

For virus counts staining with SYBR Green I used at a final concentration of 10^{-4}, rather than with SYBR Gold used at concentrations of 10^{-4}, 5×10^{-5} or 2×10^{-5}, gave virus concentrations that were significantly lower (-28%, Figure 2).

The high standard deviations led us to conclude that the samples were very heterogeneous. In addition, the temperature of incubation had a critical role on viral staining efficiency when SYBR Green I stained (10^{-4}) with mean virus abundances being significantly lower at 45 than at 65 or at 75°C. From a qualitative point of view, the use of SYBR Gold was preferable to SYBR Green I and the lower the concentrations of SYBR Gold, the greater the number of detectable subpopulations within the viral community, whatever the temperatures of incubation (Figure 3).

At 75°C, up to 5 populations could be detected using the lowest concentration of SYBR Gold, versus only 3 populations detected using the highest concentration of SYBR Gold or SYBR Green I.

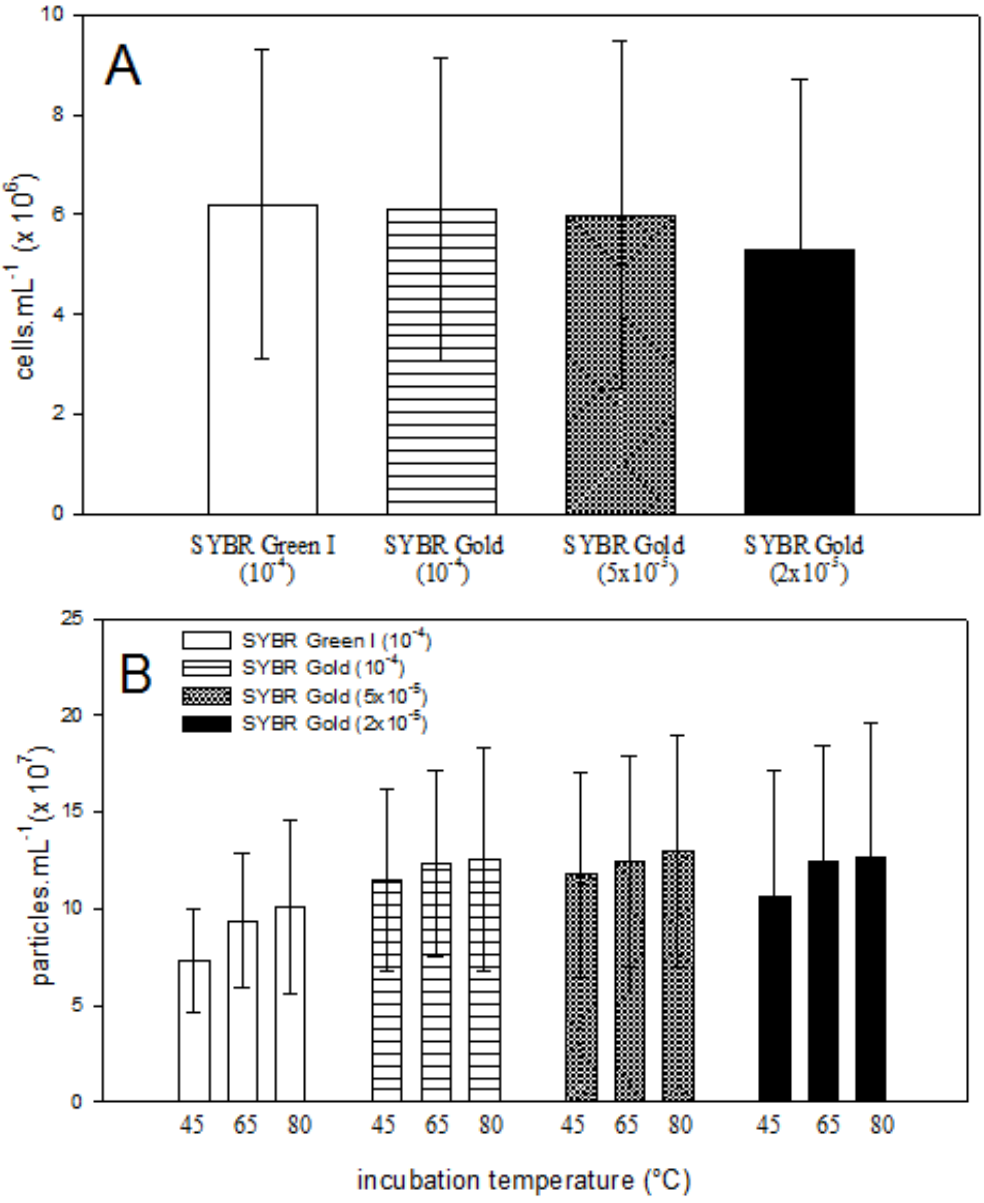

Figure 2. FCM counts of heterotrophic bacteria (A) or viruses (B) stained with SYBR Green I (10^{-4}, white bars) or SYBR Gold at 3 different concentrations (10^{-4}, hatched bars; 5 x 10^{-5}, dotted bars; 2 x 10^{-5}, black bars) for 12 different samples. Viruses were incubated with each dye and concentration tested at 3 different incubation temperatures (45°C, 65°C and 75°C). Error bars are relative to 12 different water samples. Experiment 4.

The results of the 5th experiment, showed again that SYBR Gold and SYBR Green I counts in different water samples, correlate very closely and positively (n = 98, r = 0.42, p = 0.99 for viruses, n = 65, r = 0.94, p = 0.99 for bacteria). In Experiment 4, virus counts were still correlated, but were significantly lower when SYBR Green I was used rather than SYBR Gold (about 20% lower in the case of the 75°C series). Such differences were clearly confirmed here.

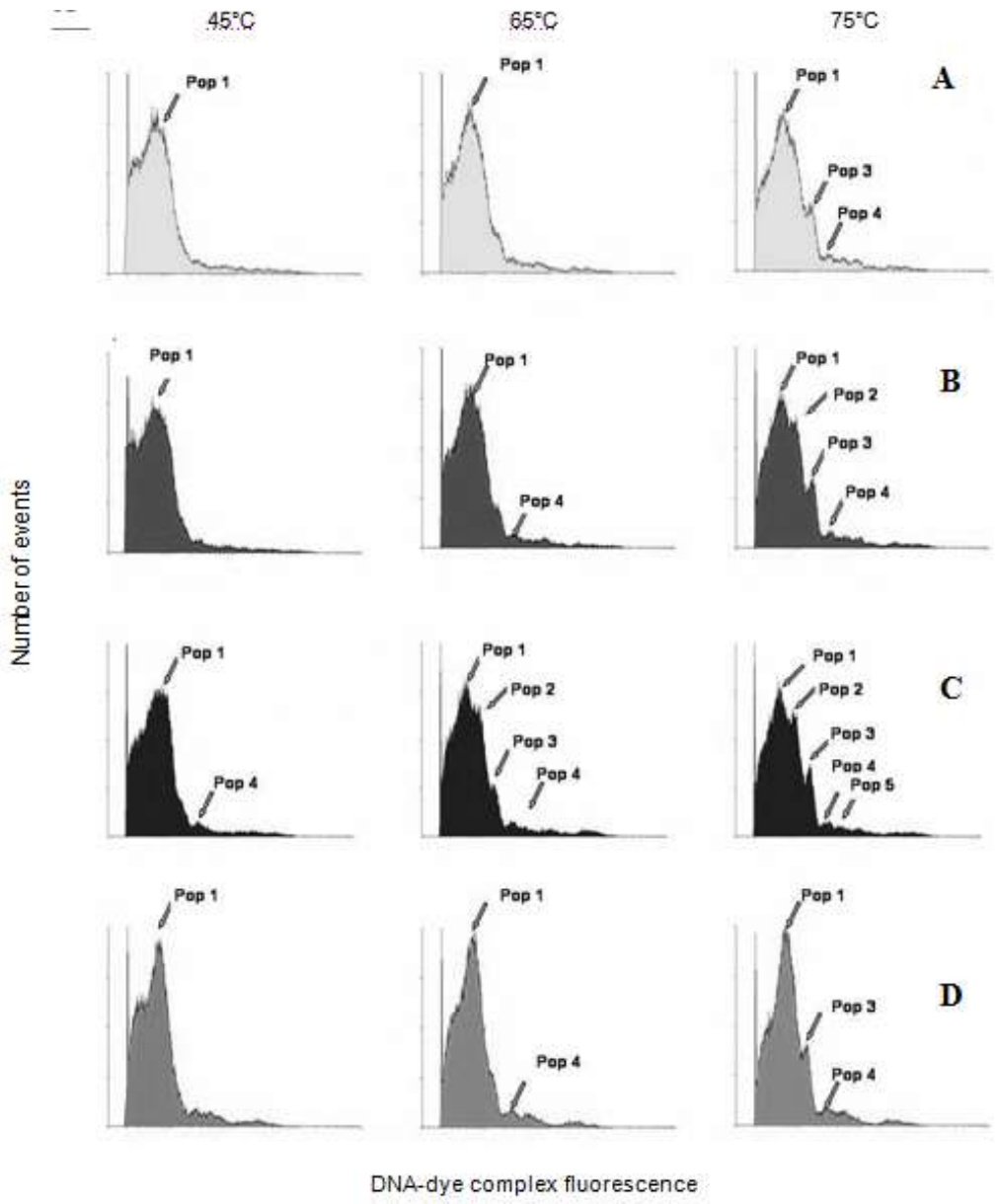

Figure 3. Histograms of virus distributions showing different populations or groups (Pop). Samples were stained with SYBR Gold at a final concentration of 10^{-4} (A), 5 x 10^{-5} (B) or 2 x 10^{-5} (C) or with SYBR Green I at a final concentration of 10^{-4} (D), and incubated at different temperatures (45°C, 65°C or 75°C) for 10 minutes. Experiment 4.

The Choice of the Dilution Solution for FCM Counts

Throughout the first experiment, FACSFlow and PBS used as dilution solution provided the lowest bacterial concentrations, 16% (SYBR Green I) and 52% (SYBR Green II) less than when samples were diluted in TE. From a qualitative point of view, TE, TAE and TBE allowed to distinguish between different populations (Figure 4A).

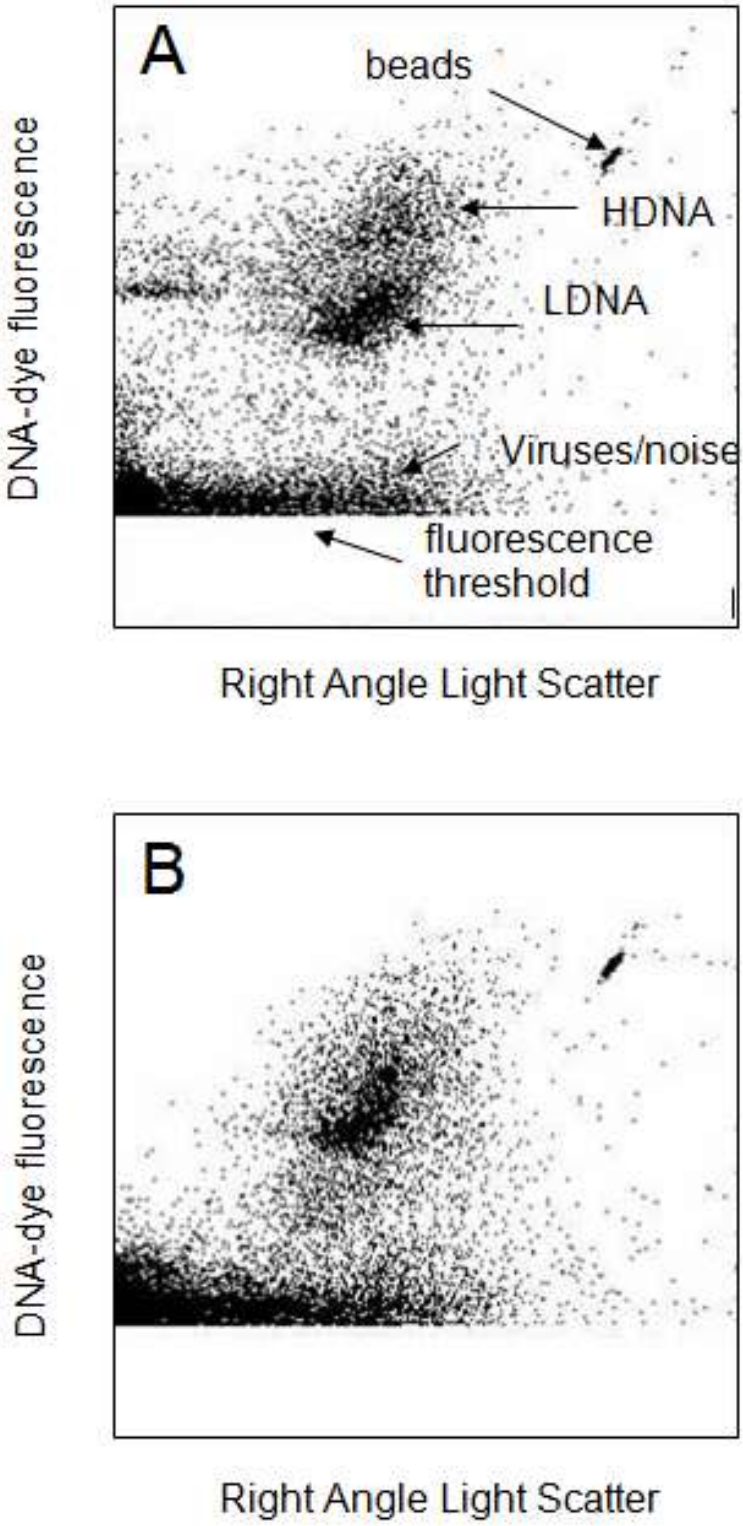

Figure 4. Typical cytograms obtained for bacterioplankton analysis using 0.2 µm filtered TE (A) or lake water (B) to dilute samples.

By comparison, filtered lake water furnished the most compact signatures in combination with unfixed samples (Figure 4B). In addition to the findings of the second experiment, no significant quantitative differences were observed between TE, TAE, TBE or lake water at t0 (data not shown). At t1, t8 and t30, the results were surprisingly different. Bacterial counts were significantly higher when samples that had been stored at 4°C or at –20°C, were diluted in TE or in lake water, than when they were diluted in TAE or TBE. For instance, TBE dilution gave values up to 15% lower than when TE was used. Considering all the viral counts, regardless of whether SYBR Green I or SYBR Gold stain was used, no significant differences were observed after diluting in TE or in lake water. Nevertheless, as for the bacterial counts, TE made it possible to distinguish between various viral subpopulations. Using autoclaved or non-autoclaved TE did not have any influence on the total bacterial counts found (experiment 3, data not shown). This was not the case for viruses (experiment 7). Indeed, when using non-autoclaved TE buffer, some virus populations, which are situated at lowest fluorescence values within the flow cytogram, could overlap with the background noise (corresponding to debris and electronic noise) (Figure 5A, B). Using autoclaved TE buffer circumvented this problem by reducing the noise and by somehow shifting the background noise away from the virus population signatures (Figure 5C). Not to autoclave the TE buffer did significantly influence the total virus counts obtained. The overlapping of the distributions of both signal and cytometric noise fluorescence resulted in possible overestimations of ca. 30% of the viral population 1 (VLP1) and referred to as the bacteriophage community, situated at the lowest fluorescence values within the cytogram (Personnic et al. 2009). When analyzing the controls, we found that the noise within autoclaved controls was reduced up to 7 fold compared to that within the non-autoclaved controls. Total virus counts were not affected by using TE at a pH 7 or 8, nor when filtering the recently made buffer through either 0.2 or 0.02 µm.

Choice of the Fixative for FCM Counts

Experiments 1 and 2 provided useful results concerning the choice of the fixative for bacterial counts (not shown). At t0, the smallest numbers of bacteria were recorded for non-fixed samples, whereas the use of fixatives increased significantly the number of detectable bacteria by an average of 14%. The highest number of bacteria at this time was obtained with a 2% final concentration of GA for all buffers. Up to 34% (on average 21%) more bacteria were detected in GA 2% fixed samples than in fresh ones. In our experiments, GA 1 or 2% gave the highest counts. From a qualitative point of view, fixing sometimes made it possible to distinguish between different bacterial populations, even in filtered lake water. For virus counts, we did not observe any quantitative or qualitative difference weather fixing the samples or leaving the sample unfixed (experiment 5, not shown).

Storage Conditions for FCM Counts

For unfixed samples, a significant increase occurred in bacterial abundance after storage for 8 days at 4°C, with concentrations that could be up to 8 times higher than at t0 (Figure 6A).

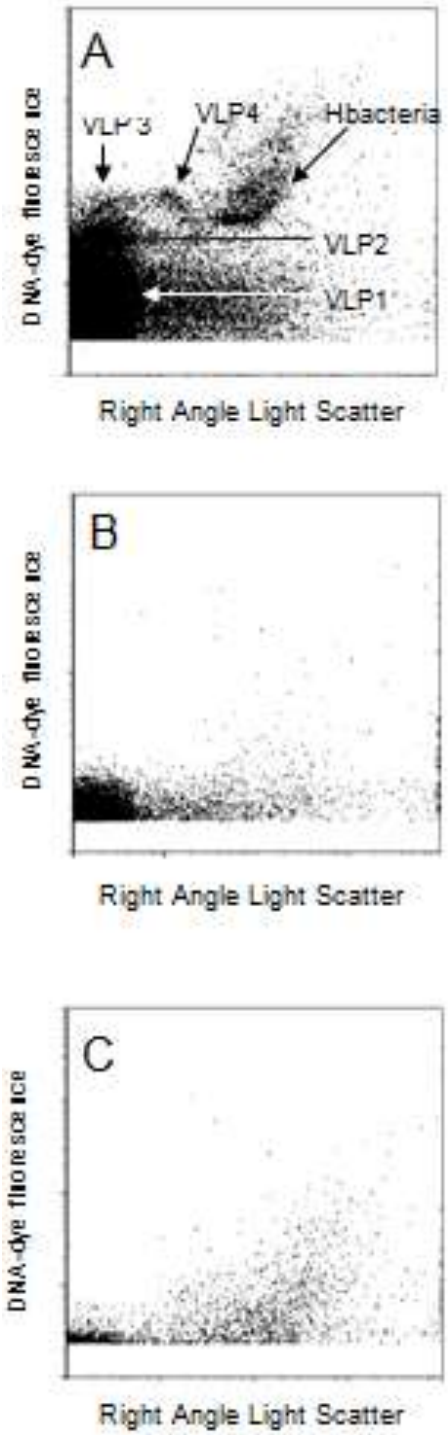

Figure 5. A: Typical FCM cytogram, representing both the heterotrophic bacterial (Hbacteria) community and different viral populations (VLP) stained with SYBR Gold (10^{-4}). B: Control cytogram with no sample and showing the signature of non-autoclaved 0.02 µm filtered TE stained with SYBR Gold (10^{-4}). C: Control cytogram with no sample, showing the signature of autoclaved TE stained with SYBR Gold (10^{-4}). Experiment 7.

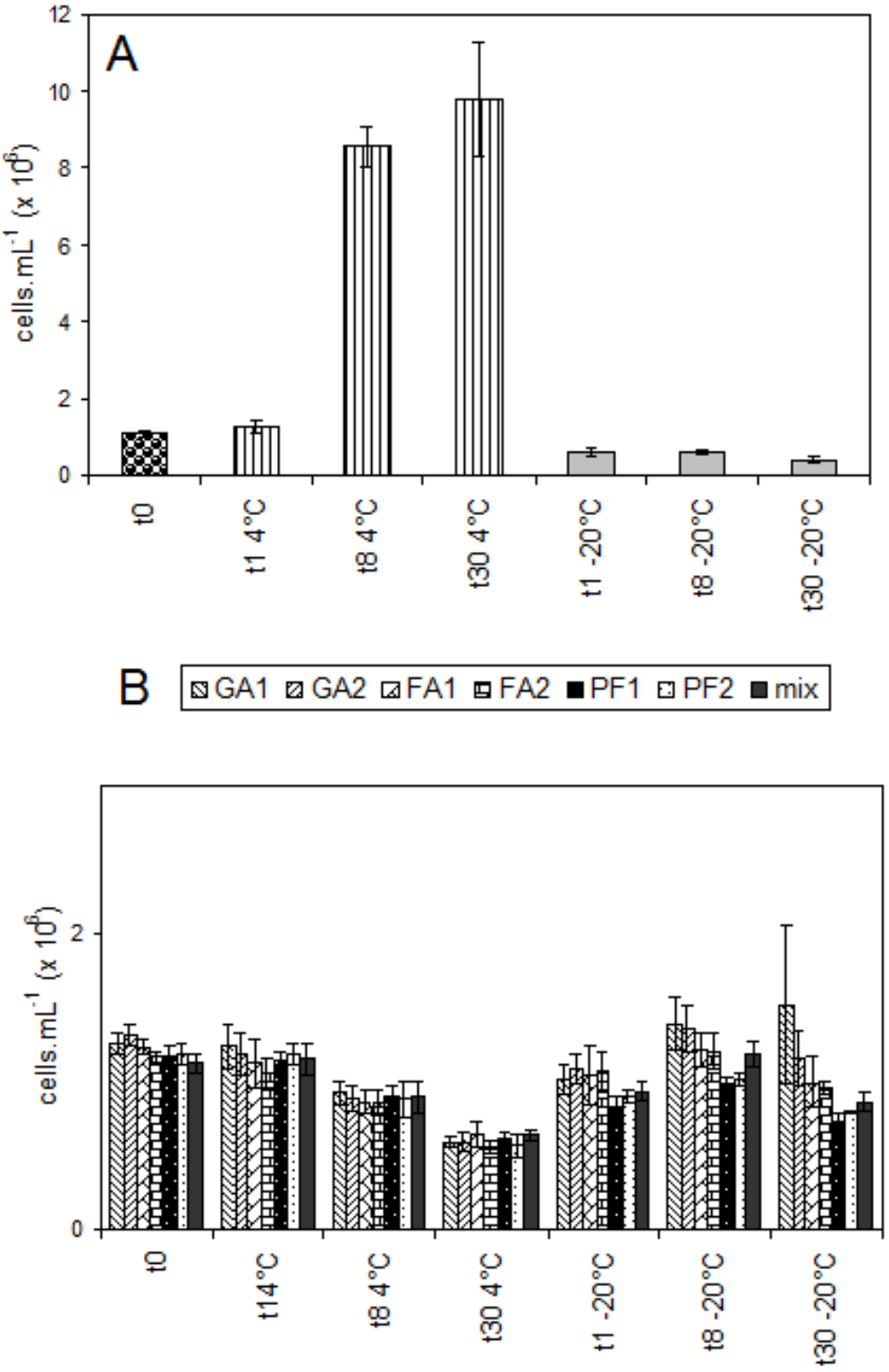

Figure 6. (continued)

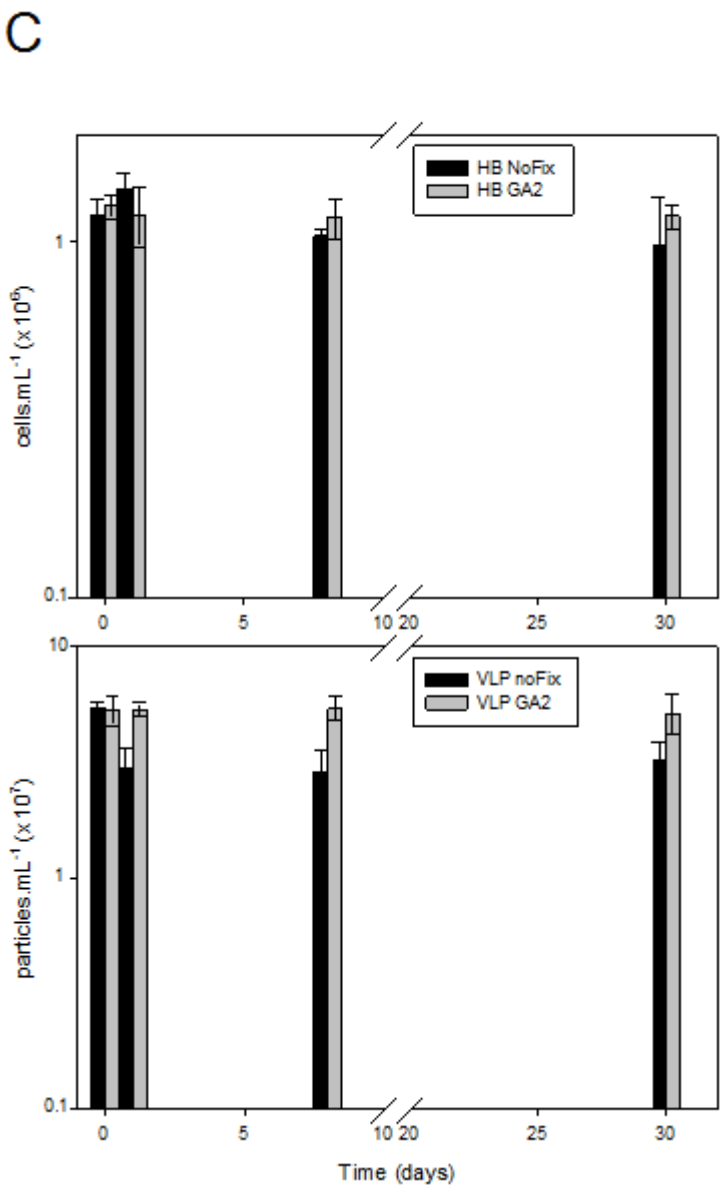

Figure 6. FCM for bacterioplankton and/or viral samples. A: Unfixed samples were analyzed at t = 0 (dotted bar) and at t1, t8 and t30 after being stored at 4°C (hatched bars) or –20°C (gray bars). B: Samples fixed with different fixatives (GA, FA, PF, mix: PF1 and GA 0.05%) and at different concentrations (1 or 2 %) were analyzed at t = 0, and at t1, t8 and at t30 after being stored at 4°C or –20°C. C: Unfixed and fixed samples with 2% glutaraldehye, flash-frozen in liquid nitrogen and analysed after 1, 8 or 30 days. Experiment 2.

At t30, bacterial concentrations were up to 10 times higher than at t0. The small increase at t1 compared to t0 was not significant. At –20°C, abundances decreased in a significant way, *i.e.* at t1 (and at t8), and then at t30 these non-fixed, frozen samples showed a decrease in the initial abundance by about 46% and 66%, respectively.

Figure 6B, referring to the fixed samples, clearly shows that at 4°C a gradual and significant decrease in the initial total bacterial counts occurred from t0 (or t1) to t8, and from

t8 to t30, by 23% and 50%, respectively. When stored at -20°C, the concentrations found were significantly lower than those found at t0 for the sets which were thawed both at t1 and at t30 (-20%).

The second frozen set analyzed at t8 did not display any significant change in counts compared to t0 (Figure 6B). When samples were flash frozen (in liquid nitrogen) without fixation, both bacteria and viruses decreased markedly while with fixation (either 1 or 2% glutaraldehyde), no significant differences were recorded for both communities between t0 and t1, t8 or t30 (Figure 6C).

Dye Incubation Temperature for FCM Counts

The results of the incubation temperature experiment for bacterial analyses (experiment 6) have been illustrated in Figure 7. In unfixed samples the number of bacteria detected by FCM decreased significantly at temperatures above 45°C, at 75°C the number of total bacteria being reduced by an average of 22% compared to data obtained at 20°C or at 45°C. For heated and unfixed samples, cell losses were on average greater when samples were diluted in lake water.

Fixing the samples with either GA 1 or 2% yielded significant higher counts than unfixed samples especially at higher temperatures. The efficiency of detecting GA 1 or GA 2% fixed cells was not significantly different if they were heated to 45°C or 75°C, except for the samples diluted in lake water and heated to 75°C, for which we found significant lower concentrations. At temperature exceeding 45°C it appeared that samples which were diluted in TE rather than in lake water were more "protected" from overheating and cell destruction. The results of the incubation temperature experiment for virus samples (experiment 4) have been illustrated in Figure 3. Temperature was proved to be of great importance in the discrimination and the counting of viruses. Two, 3 and 5 viral groups were detected at 45°C, 65°C and 75°C, respectively. At 65 or 75°C, virus counts were significantly higher (+14%) than those at 45°C.

EFM Counts

Each filter was analyzed both for bacteria and viruses. SYBR Gold and SYBR Green I counts ($n = 28$, $r = 0.87$, $p = 0.99$) were positively correlated and showed no significant quantitative differences. The bacterial concentrations found with SYBR Green I and SYBR Gold ranged between $4.28 \times 10^5 - 1.76 \times 10^6$ and $1.84 \times 10^5 - 2.56 \times 10^6$ cells mL^{-1}, respectively.

Viral concentrations displayed a range of $1.09 \times 10^7 - 5.43 \times 10^7$ viruses.mL^{-1} with SYBR Green I, and of $5.01 \times 10^6 - 5.73 \times 10^7$ particles.mL^{-1} with SYBR Gold. At 10^{-3}, SYBR Gold yielded a more stable fluorescence than SYBR Green I. No obvious trend could be discerned related to whether different fixative solutions had been added. As illustrated in Figure 8, the time for which filters can be kept and still yield reliable bacterial and viral counts seems to be limited to 1 month. After 16 days, the estimates were similar to those at time zero (immediately after slide preparation).

After one month storage, there was an important decrease in abundance, estimated to be of 5% to 98% for viruses and 3% to 73% for bacteria. The decrease in viruses occurred faster than that in bacteria.

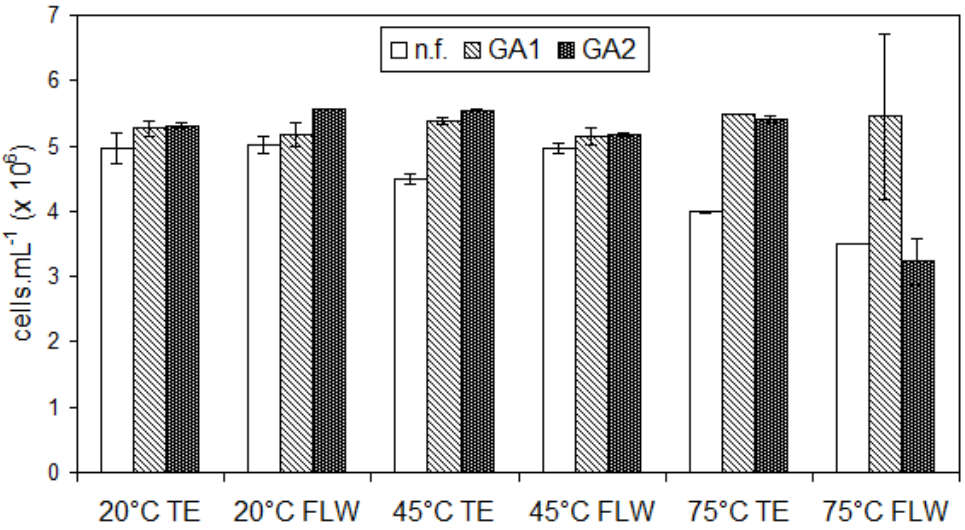

Figure 7. FCM bacterial counts at t = 0. Very similar results were obtained at t=1 (not shown here). Error bars represent standard deviations of duplicate counts. The samples were fixed in GA 1 or 2% or not fixed (n.f.), were diluted in 0.02 µm filtered TE or lake water (FLW) and incubated at temperatures of 20°C, 45°C or 75°C. Experiment 6.

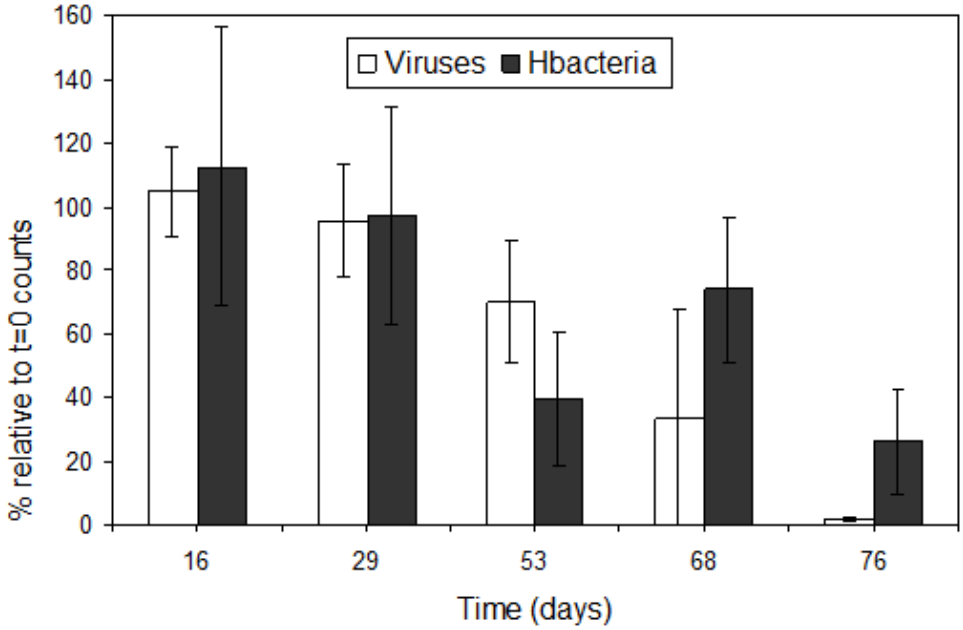

Figure 8. Percentage of bacterial and viral abundances determined by EFM after keeping the sample at –20°C for 16, 29, 53, 68 and 96 days, compared to the values obtained at t = 0. Error bars represent standard deviations of different samples (n = 5). Experiment 8.

Comparison between FCM and EFM Counts

The FCM counts were closely correlated to the EFM counts for both bacteria and viruses (Figure 9A and B). However, with FCM, bacterial estimates were 54% higher and viruses estimates were 32% higher than with EFM. Bacterial counts obtained using EFM ranged from 4.54 x 10^5 to 2.88 x 10^6 (mean 1.19 x 10^6 cells.mL^{-1}), and from 9.48 x 10^5 to 9.36 x 10^6 (mean 2.53 x 10^6 cells.mL^{-1}), using FCM. Viral abundances ranged from 5.54x10^6 to 5.71x10^7 (mean 3.36 x 10^7 particles.mL^{-1}) by EFM, and from 2.7 x 10^7 to 1.32 x 10^8 (mean 4.96 x 10^7 particles.mL^{-1}) by FCM.

DISCUSSION

FCM Analyses

Our results indicate that bacterial and viral counts are quantitatively and/or qualitatively affected by the type and the final concentration of the fluorescent nucleic acid dye used, the incubation temperature and time, whether fixatives and dilution solutions are used and by the storage condition.

Total bacteria counts were highest with SYBR Green I (10^{-4} final concentration) and lowest (40% less) with SYBR Green II (same concentration). These results are not very surprising, as SYBR Green II, unlike the other two dyes tested, preferentially stains single-stranded DNA or RNA, rather than double-stranded DNA, which is the main form present in bacterial cells (indications by the manufacturer). As shown by Lebaron and co-authors (1998) and by our results, bacterial counts obtained by staining the samples with SYBR Green I were closely correlated to those obtained after staining with SYBR Green II. When the staining efficiency of SYBR Green I (10^{-4}) was compared to that of SYBR Gold (10^{-4}, 5 x 10^{-5} or 2 x 10^{-5}) on different natural samples (experiment 4), the mean bacterial abundances found did not differ significantly. On the contrary, when SYBR Gold and SYBR Green I (10^{-4}, in both cases) were tested on replicate water sample (experiment 3), SYBR Green I gave on average 16% higher abundances than SYBR Gold.

These two apparently contradictorily findings indicated that different results may be obtained for different water samples. In addition to the quantitative advantage of using SYBR Green I for bacterial counts, this stain also provides bacterial signatures, which were easier to interpret, especially in organic material rich water samples (data not shown). The kinetics experiment showed that SYBR Green I reached maximum bacterial abundances after only a few minutes, and 15 min was a good compromise before FCM analysis. With regard to the dilution solutions, we strongly advise against using FACSFlow or PBS. They both yielded significantly lower bacterial counts than TE, TAE, TBE or filtered lake water (-16%). Interestingly, at t = 0, no quantitative difference was found between the last 4 dilution solutions mentioned above. At t1, t8 and t30, TE and filtered lake water provided 15% higher abundances than TBE or TAE. From a qualitative point of view, TE, TAE and TBE allowed us to distinguish some bacterial subpopulations, typically two groups that had clearly differing DNA-dye fluorescence. These two groups had already been reported by Gasol et al. (1999) and by Li and Dickie (2001).

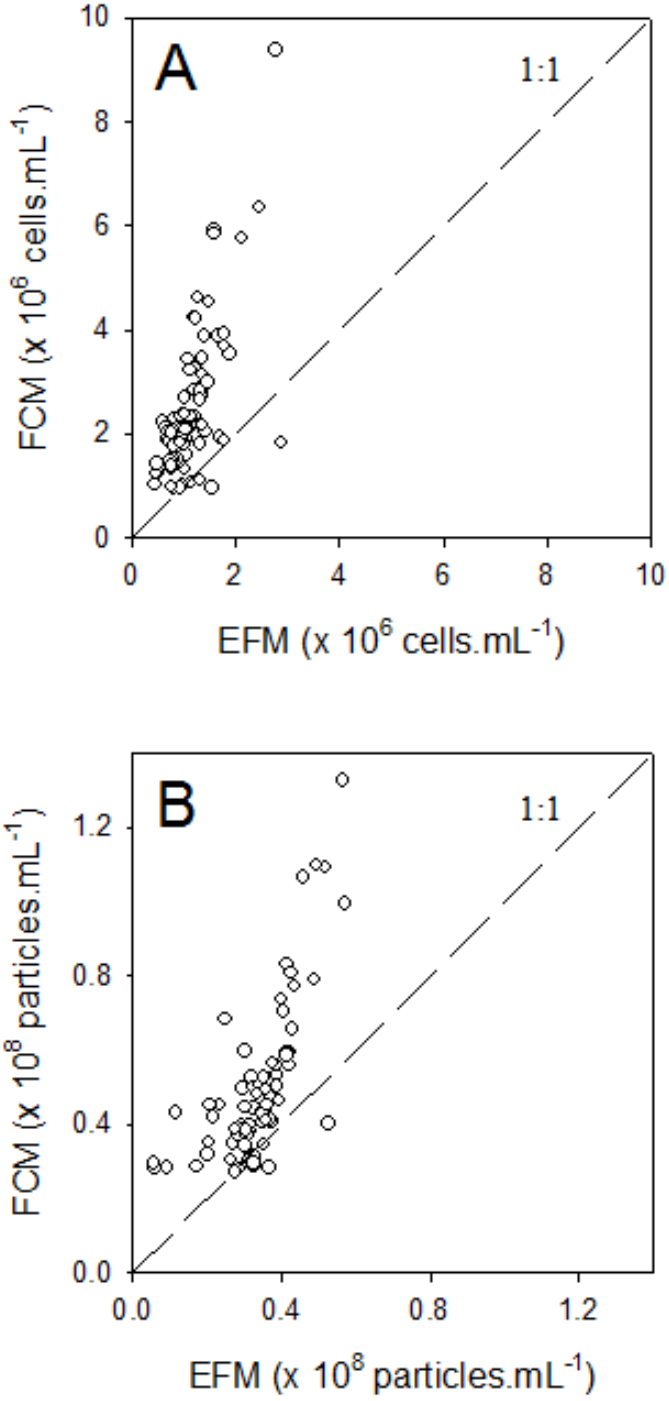

Figure 9. Relationships between bacteria (A) and viral (B) counts assessed by EFM and by FCM. The dashed lines correspond to the 1:1 relationship. A: y = 1.50x – 0.0074 (n = 80, r = 0.7, p = 0.99). B: y = 2.08x + 0.07 (n = 80, r = 0.69, p = 0.99). See Methods for the experimental conditions used.

They named them HDNA (for high DNA containing cells) and LDNA (for low DNA containing cells), or type I and II, respectively. Bacteria belonging to the HDNA or to type-I

group are thought to be metabolically more active than those in the LDNA or type-II group (Gasol et al. 1999; Lebaron et al. 2002) although more recent studies highlighted that such a discrimination was probably not so clear (Bouvier et al. 2007). When samples were diluted in filtered lake water, the signal was generally more compact than when they were diluted in TE, TAE or TBE, likely to be due to the presence of EDTA in the Tris-buffers, which may interact with nucleic acid chains. Sometimes the use of fixatives had a similar effect on the signal, making it possible to distinguish between major subpopulations. One possible explanation for this may be that fixation can sometimes change the refractive index of the cell by affecting the right angle scatter, as well as DNA characteristics and thus fluorescence. At t0, regardless of the fixative used, bacterial abundances were 14% higher for the fixed samples than for fresh samples. This has also been reported by Marie et al. (1999). We may not exclude whether DNA populations observed were artifacts when a given buffer or fixative was used or not.

Generally speaking, fixatives are used to avoid the occurrence of significant changes in the cell counts and characteristics over time. Moreover, fixatives (and also heating treatments), may make the cells more permeable, allowing high-molecular weight molecules (such as the specific nucleic acid stains) to penetrate the cells more quickly and easily (Lebaron et al. 1998, Marie et al. 1999). We tested some members of the aldehyde family (FA, GA, PF), as they are known to penetrate cells rapidly, because of their relative low molecular weights (Hayat 1970; Xenopoulos and Bird 1997). FA is known to crosslink proteins within the cell membrane, and to influence cell morphology (Noble 2001; Vaulot et al. 1989). PF is the polymerized form of FA and unlike FA, PF lacks cross-linking characteristics (Marie et al. 1999). If fixation affects the cell morphology, the forward angle scatter which is related to the size of the cells may also change, thus modifying the signal recorded by FCM (Navaluna et al. 1989). GA is usually used in electron microscopy studies, as the cell shape is little changed even if the stain produces cross links with cell proteins (Vaulot et al. 1989). In our study, GA used at a final concentration of 1 or 2% seemed to be the most appropriate type of fixative. When unfixed or fixed samples were stored at 4°C, abundances found at t = 1 were found to be similar at t = 0, suggesting that analysis could be postponed by one day (see also Jacquet et al. 1998). At 4°C and in unfixed samples, counts dramatically increased between t1 and t8, indicating a rapidly-growing community despite the low temperature. At -20°C, these unfixed samples showed an undoubted decrease in counts, -46% and –66%, after 1 day and 1 month of storage, respectively. One hypothesis is that at very low temperatures and without a gradual temperature decrease, unfixed cells encounter physical problems (*e.g.* intracellular freezing) that result in cell damage. These considerations obviously lead us to discourage the storage of unfixed samples (that was also confirmed when using liquid nitrogen without previous fixation). Then, what occurred when the samples have been fixed? At 4°C, we detected a loss of total abundance at t8 and t30 by 23 and 50% respectively; no loss was detected at t1. When fixed samples were stored at -20°C, we noticed that the concentrations for the sets which have been thawed at t1 and at t30 were significantly lower (by 20%) than the values t0. Generally speaking, a loss in cell numbers may be due to several factors, such as attachment to the wall of the recipient or burst due to virus infection (Turley and Hughes 1992). Cells may encounter uninhibited enzyme activity (Gundersen et al. 1996) causing cell dissolution, or cells may break due to inappropriate physical (temperature) or chemical (fixation) conditions. Gundersen et al. (1996) suggested that major bacterial losses may occur as a result of uninhibited protease activity, even in fixed water

samples. They found bacterial losses of 5% and 50% after 9 and 29 days of storage respectively at -20°C for samples fixed with 2.5% GA. Brussaard (2004) demonstrated that a one month storage at 4°C or -20°C of samples fixed in 0.5% GA led to considerable reductions of viral abundance. Her findings must also be applicable to the storage of bacterioplankton samples. Turley and Hughes (1992) also reported a significant decline in bacterial counts when they analyzed bacterioplankton samples fixed in 1% GA and which had been stored at room temperature – cell numbers were down to 39% of the initial counts prior to storage. Trousselier et al. (1995), comparing the effects of low-temperature storage (5°C or -196°C) on GA, FA, PF bacterioplankton and picophytoplankton cells, found that low but positive storage temperatures resulted in significant and rapid reductions in the total cell count. The study of Wen et al. (2004) has demonstrated the rapid decline in viral numbers over time of viral isolates preserved in aldehyde fixatives (0.5% GA or 2% FA) at 4°C. In their study, viral abundances had decreased by 72% after 16 days. Such results were also confirmed by Ammini et al. (2010) who reported a rapid decline in counts of bacteria and viruses in samples preserved in formaldehyde over a delay of 1 week to 2 months, and they also showed that the decline increased with increase in the final concentration of formaldehyde in the sample. Using liquid nitrogen (blocking all the oxidative reactions responsible for the destruction of organic molecule within cells) and conservation at -80°C provided the best storage conditions on the long-term as already reported elsewhere when samples were fixed (Brussaard 2004). Only a few percentage (<10%) of both cellular and viral loss was indeed recorded between t0 and t30. Our results confirm thus previous studies that reported that preservation of samples at -80°C or in liquid nitrogen (-196°C) is generally much more efficient than preservation at 4°C or -20°C (Brussaard 2004).

In the case of virus counts, the results were somewhat difficult to interpret. When comparing the virus detection efficiency by adding SYBR Green I at a final concentration of 10^{-4} or adding SYBR Gold at a final concentration of 10^{-4}, 5×10^{-5} or 2×10^{-5}, we found that abundances were greatest with SYBR Gold, regardless of the concentration of the stain. The quantitative results were slightly different, if each experiment was considered separately. It appears that at 75°C, SYBR Green I (at a final concentration of 10^{-4}) compared to SYBR Gold (at the three concentrations) incubations, underestimated virus concentrations by 20%, 33% or 35%, respectively. This higher efficiency of SYBR Gold was found at all the incubation temperatures tested (from 45°C to 75°C). Qualitatively, virus populations could be counted more easily when SYBR Gold stained, as the particles yielded higher fluorescence separating them from the low fluorescence background noise of the machine. SYBR Gold also made it possible to distinguish more viral subpopulations if used at low concentrations. The preferential use of low concentrations of SYBR Gold is especially interesting; since SYBR Gold is clearly cheaper than SYBR Green I. Brussaard (2004) tested increasing concentrations of SYBR Gold and SYBR Green I on FCM virus counts, and demonstrated that higher virus counts were obtained by staining the sample with SYBR Green I than with SYBR Gold. She recommended a final concentration of the SYBR Green I stock solution of 5×10^{-5}. However this study was mainly performed using several representatives of different virus families easily distrainable with FCM (and individual populations may react very differently) rather than on natural samples, as in our study. Consistently with her study, we also found that the incubation temperature is very important in order to boost and correctly assess the viral abundance. At low temperatures, there was a significant reduction, and thus a clear underestimation of the total counts.

Taking into account all the dyes and concentrations, we can see that the mean abundances increased significantly by 14% when the temperature was increased from 45°C to 65°C, but there was no significant increase from 65°C to 75°C. It is recognized that heating treatments increase the penetration of the stains by increasing the permeability of the viral capsid and by denaturing the nucleic acids, which may enhance their staining (Marie et al. 1999; Xenopoulos and Bird 1997). Moreover, our study also demonstrated that a higher incubation temperature increased not only the total number of viruses, but also the number of viral subpopulations. It seems that the heat could enhance the "detectability" of different groups of viruses, which might otherwise be invisible because not permeable to the stain. In our study we detected up to 5 different viral populations within the same sample. Analogous to our findings, Chen et al. (2001), revealed the existence of at least four viral subpopulations in a sample from Lake Erie. With regard to the use of dilution solutions, it appeared that no differences were obtained, regardless of whether TE, TBE or filtered lake water was used. Brussard (2004) obtained the highest viral counts when diluting with TE or Tris buffer, and the lowest counts when using distilled water or seawater. We agree that TE is the optimum dilution solution, at least because TE allowed us to differentiate between several subpopulations of viruses. Moreover, regardless of whether the TE buffer had been filtered at 0.02 µm or 0.2 µm, if it was autoclaved, a 30% overestimation of low fluorescence populations, and thus of the total count, could be prevented by removing the background noise in the critical part of the cytogram.

As indicated by Brussaard (2004), over the range tested (between 7 and 8), the pH did not have any influence on the quality of the signature or on the total abundances found. The fixatives tested (GA or FA 1%) did not produce higher final counts of viruses, than unfixed samples. Our findings are consistent with the work done by Wen et al. (2004), which found no significant difference between viral abundance estimates made with fixed (0.5% GA and 2% FA, final concentrations) samples and unfixed samples, provided that the slides were prepared immediately. Brussaard's study (2004) showed, firstly, that there was no significant difference in the FCM signal of fixed and fresh virus samples for fixing lasting up to 1h and, secondly, no conclusive conclusions could be drawn about the use of FA or GA or the best final concentration of the fixative. The two studies indicated above recommended the use of GA at a final concentration of 0.5%, the first one for the reason that occasional reductions in some phytoplankton virus abundances have occurred at higher concentrations. Our study showed that no fixing is necessary if lake water samples are analyzed immediately after their arrival in the laboratory. In addition to this battery of tests, unpublished storage tests performed on some samples stored at 4°C showed that virioplankton abundances had fallen by 40% after being stored for two days. Only fixed and flash-frozen samples can be kept for a long time and delayed analysis.

EFM Counts

At a final concentration of 10^{-3} we found that the fluorescence signal of bacteria and viruses were more stable if they had been SYBR Gold-stained rather than SYBR Green I-stained. These results were in agreement with those presented by Noble (2001) and others indicating that 1) the SYBR Green I signal fades within 30 sec, making it necessary to use an anti-fading solution or higher concentrations of SYBR Green I in order to increase stability

(Noble and Fuhrman 1998) and 2) that the fluorescence of SYBR Gold stained viruses is stable for more than 2 min without any anti-fading solution (Chen et al. 2001). Because of the very fast fading of SYBR Green I, Bettarel et al. (2000) recommended that this stain should not be used for viral concentrations higher than 10^8 particles.mL^{-1}. Wen et al. (2004) reported that the suitability of the two stains depended on the sample being analyzed. From a quantitative point of view, we did not observe any significant increase in bacteria or virus counts depending on whether SYBR Gold or SYBR Green I stain had been used. Neither the type nor the concentration of the fixatives tested (GA 0.5 or 1% and FA 1 or 2%) had any influence on EFM estimations.

Our investigation showed that the slides can be stored at -20°C for up to one month and still provide reliable and realistic counts. From 29 days to 76 days of storage, virus counts fell by 5 and 98% respectively, and bacterial counts by 3 and 73% respectively. Noble (2001) limited the storage of frozen slides to 2-3 weeks, and Wen et al. (2004) reported no decline in viral abundance during the 16 days. Furthermore, Turley and Hughes (1992) reported no decrease in bacterial counts of slides counted immediately after preparation of seawater samples and then after being stored, frozen and recounted within 70 days. It is likely that such differences may be related to the chemical characteristics of seawater or lake water, in conjunction with fixation.

Comparison of FCM and EFM Counts

Our data demonstrated that FCM counts were highly correlated to EFM counts, and that FCM counts were generally higher than EFM ones. Typically, FCM counts of bacteria and viruses were 2.13 and 1.47 times higher respectively than the corresponding EFM counts. Another study, conducted by the end of 2004 within our research laboratory confirmed again this trend with factors of 2.42 and 2.07 for bacteria and viruses, respectively (not shown). The findings of other authors agree with our findings even if the multiplication factors they found were different, *e.g.* 1.1 for Chen et al. (2001) and 1.4 for Marie and coworkers (1999), both obtained on virus samples.

What could contribute to such widely different multiplication factors? We can argue that such differences found between the two techniques can be due to the virus types or bacteria strains, the staining characteristics, etc. A possible explanation of the different factors found for viruses and bacteria could be that viruses are significantly smaller than bacteria, and so the signal is likely to fade very rapidly in EFM. The differences observed between the EFM and FCM counts could also be attributable to a lower accurate estimation by EFM, due to the presence of particles in natural samples, to the fact that fewer cells or biological particles were counted by EFM or to the uneven distribution of the biological entities on the filter (Lebaron et al. 1998). As well as being faster and more accurate, FCM makes it possible to distinguish between different populations. The viral and bacterial dynamics were probably more accurately revealed by FCM, which allowed a finer analysis of shifts in abundances due to its higher resolution.

More generally, it is clear that FCM is less operator-dependent and less labor-intensive than EFM, but that EFM can provide additional information, such as information about the presence of different morphotypes, especially in the case of bacteria. Finally, only EFM permits bacterial phylotyping, as discriminated by the FISH technique (Glöckner et al. 1996).

CONCLUSION

The major finding of our study is that no unique and universal method exists to assess reliable counts of natural bacterio- and viroplankton populations and that many factors should be considered. It seems however that the confirmation is made that FCM gives better results than EFM and it suggests typically that the concentrations of the total viruses in aquatic ecosystems is probably higher that many previous VLPs estimated found in the literature.

Concerning bacterial counts with FCM, highest numbers were obtained by fixing the samples with GA at a final concentration of 2%, and by incubating the sample at room temperature for 15 min with SYBR Green I at a final concentration of 10^{-4}. From both the quantitative and qualitative considerations, we suggest diluting the samples in TE (0.2- or 0.02-µm filtered). It is important that analyses be done on the same day than sampling, or no more than one day later after conserving the samples at 4°C.

For viral counts using FCM, we recommend using SYBR Gold at a low concentration (2 x 10^{-5}) in order to obtain high viral counts, and at the same time to be able to access the various subpopulations within the community. For the same reason, the incubation temperature should be 75°C, and the dilution solution recently autoclaved and 0.02 (or 0.2)-µm filtered TE buffer adjusted to a final pH of 7 or 8. Since Marie et al. (1999), FCM analysis has been improved for marine samples by Brussaard et al. (2000), Chen et al. (2001), and Brussaard (2004) proposed the optimized protocol mostly used nowadays for dsDNA viruses on the basis of the analysis of 5 phycoDNAviruses, 1 cyanophage and 6 bacteriophages. In 2007, Tomaru and Nagasaki examined whether this last protocol was also suited for the analysis of small genome viruses with either DNA or RNA genomes using again algal viruses from a marine origin. They could show that the optimum staining protocol may vary significantly among viruses tested. Their article also revealed that for the small viruses (below 40 nm) that harbor either DNA or RNA genomes, FCM counts were similar or lower than values obtained with the Most Probable Number assay, suggesting that only infectious particles could be counted, and more generally speaking that FCM could also sometimes underestimate an important fraction of the smaller viruses in the aquatic plankton. Such a result was also reported recently by Holmfeldt et al. (2011) who revealed that FCM underestimation was clearly obvious for ssDNA. Differently said, the optimum staining protocol may differ among aquatic viruses (i.e. cyanophages, bacteriophages, algal viruses and even in each group, according to the genome type) and all these studies including ours highlight the fact that it may be a non sense to believe that only one protocol exist for all situations. Thus, we have to keep in mind that the protocol we propose here for freshwater viruses is mainly suited for (i) the whole community but that each population/isolate should be tested to find its optimum staining, (ii) dsDNA "large" viruses since it is likely that we miss most of the small containing DNA or RNA particles.

In the case of "our" lake survey, it is important to remind here that we can proceed without fixation that result in considerable eukaryotic cell loss (not shown) and without using liquid nitrogen since we only have a few samples at a time and thus do not need to use extreme temperatures to keep samples. We agree however that both fixation and flash-freezing could be performed as soon as possible after sampling to avoid any evolution of the samples (predation, enzyme activity, and chemical oxidation), particularly if the analyzes cannot be performed the very same day, or last several days or months as usual when

hundreds of samples are collected during a cruise or important field survey. Nowadays, dry containers filled with liquid nitrogen, or even Deware's tanks filled with dry ice (CO_2) are available and affordable. As in most sampling cruises hundreds of samples are brought back to the lab, it is obvious that running all of them the very same day is impossible. The need for long term storage and preservation is thus mandatory and this choice must be chosen.

At last, concerning counts done by means of EFM, we recommend that samples should be processed immediately by filtering 1 mL (without dilution), staining the filters with SYBR Gold at a final concentration of 10^{-3} and storing the slides at a temperature of no more than -20°C for up to one month before counting.

ACKNOWLEDGMENT

Ursula Dorigo and Sebastien Personnic were PhD students funded by the French Research Ministry. Monika Ghosh is acknowledged for revising the English version of the manuscript.

REFERENCES

Ammini, P., Radhakrishnan, S., Sajila, M. P. and Jacob, B. (2007) Study of changes in bacterial and viral abundance in formaldehyde-fixed water samples by epifluorescence microscopy. *Environmental Monitoring and Assessment, 177*, 227-231.

Azam, F., Fenchel, T. Field, J. G., Gray, J. S., Meyer-Reil, L. A. and Thingstad, F. (1983). The ecological role of water-column microbes in the sea. *Marine Ecology Progress Series*, *10*, 257-263.

Bergh, O., Borsheim, K. Y., Bratbak, G. and Heldal, M. (1989). High abundance of viruses found in aquatic environments. *Nature, 340*, 467-468.

Bergström, A. K., and Jansson, M. (2000). Bacterioplankton production in humic lake Ortrasket in relation to input of bacterial cells and input of allochthonous organic carbon. *Microbial Ecology, 39*, 101-115.

Berninger, U.-G. (1991). Protozoan control of bacterial abundances in freshwater. *Limnology and Oceanography, 36*, 139-147.

Bettarel, Y., Sime-Ngando, T., Amblard, C., Carrias, J. F., and Portelli, C. (2003). Virioplankton and microbial communities in aquatic systems: a seasonal study in two lakes of differing trophy. *Freshwater Biology, 48*, 810-822.

Bettarel, Y., Sime-Ngando, T., Amblard, C., and Dolan, J. (2004). Viral activity in two contrasting lake ecosystems. *Applied and Environmental Microbiology, 70*, 2941-2951.

Bettarel, Y., Sime-Ngando, T., Amblard, C., Laveran, H. (2000). A comparison of methods for counting viruses in aquatic systems. *Applied and Environmental Microbiology, 66*, 2283-2289.

Bettarel, Y., Sime-Ngando, T., Carrias, J. F., Sargos, D., Garabétian, F., and Lavandier, P. (2003). Viral lysis, flagellate grazing potential, and bacterial production in Lake Pavin. *Microbial Ecology, 45*, 119-127.

Børsheim, K. Y., Bratbak, G., and Heldal, M. (1990). Enumeration and biomass estimation of planktonic bacteria and viruses by transmission electron microscopy. *Applied and Environmental Microbiology, 56*, 352-356.

Bouvier T., del Giorgio, A.P., and Gasol J.M. (2007). A comparative study of the cytometric characteristics of high and low nucleic-acid bacterioplankton cells from different aquatic ecosystems. *Environmental Microbiology*, 9, 2050-2066.

Bratbak, G., and Heldal, M. (1993). Total count of viruses in aquatic environments, p. 135-138. *In* S. Kemp, Sherr, Cole (ed.), Handbook of methods in aquatic Microbial Ecology. Lewis Publisher, Boca Raton Fla.

Brussard, C. P. D. (2004). Optimization of procedures for counting viruses by flow cytometry. *Applied and Environmental Microbiology, 70*, 1506-1513.

Brussard, C. P. D., Marie, D., Bratbak, G. (2000). Flow cytometry detection of viruses. *Journal of Virological Methods, 85*, 175-182.

Buitenhuis, E.T., Li, W. K. W., Lomas, M. W., Karl, D. M., Landry M., Jacquet, S. (2012). Bacterial biomass distribution in the global ocean. *Earth System Science Data*, 5, 301-315.

Chen, F., Lu, J. R., Binder, B. J., Liu, Y. C., and Hodson, E. R. (2001). Application of digital image analysis and flow cytometry to enumerate marine viruses stained with SYBR Gold. *Applied and Environmental Microbiology, 67*, 539-545.

Decamp, O., and Rajendran, N. (1998). Bacterial loss and degradation of bacterial membrane in preserved seawater samples. *Marine pollution bulletin*, 36, 856-859.

Dingman, C. W., and Peacock, A. C. (1968). Analytical studies on nuclear ribonucleic acid using polyacrylamide gel. *Biochemistry, 7*, 659-668.

Domaizon, I., Viboud, S., and Fontvieille, D. (2003). Taxon-specific and seasonal variations in flagellates grazing on heterotrophic bacteria in the oligotrohic lake Annecy - importance of mixotrophy. *FEMS Microbiology Ecology, 46*, 317-329.

Duhamel, S., and Jacquet, S. (2006). Flow cytometric analysis of bacteria and virus-like particles in lake sediments. *Journal of Microbiological Methods, 64*, 316-322.

Fischer, U. R., and Velimirov, B. (2002). High control of bacterial production by viruses in a eutrophic oxbow lake. *Aquatic Microbial Ecology, 27*, 1-12.

Fisher, M. M., Klug, J. L., Lauster, G., Newton, M., and Triplett, E. W. (2000). Effects of resources and trophic interactions on freshwater bacterioplankton diversity. *Microbial Ecology, 40*, 125-138.

Francisco, D. E., Mah, R. A., Taylor, D., and Rabin, A. C. (1973). Acridine orange-epifluorescence technique for counting bacteria in natural waters. *Trans American Microscopy Society, 92*, 416-421.

Fuhrman, J. A. (1999). Marine viruses and their biogeochemical and ecological effects. *Nature, 399*, 541-547.

Gasol, J. M., Zweifel, U. L., Peters, F., Fuhrmann, J. A., and Hagström, A. (1999). Significance of size and nucleic acid content heterogeneity as measured by flow cytometry in natural planktonic bacteria. *Applied and Environmental Microbiolology, 65*, 4475-4483.

Glöckner, F. O., Amann, R., Alfreider, A., Pernthaler, J., Psenner, R., Karlheinz, T. and Schleifer, K. H. (1996). An *in situ* hybridization protocol for detection and identification of planktonic bacteria. System. *Applied Microbiology, 19*, 403-406.

Gundersen, K., Bratbak, G., and Heldal, M. (1996). Factors influencing the loss of bacteria in preserved seawater samples. *Marine Ecology Progress Series, 137*, 305-310.

Hall, J. (1991). Long-term preservation of picoplankton for counting by fluorescence microscopy. *British Phycological Journal*, 26, 169-174.

Hara, S., Terauchi, K., and Koike, I. (1991). Abundance of viruses in marine waters: assessment by epifluorescence and transmission electron microscopy. *Applied and Environmental Microbiolology, 57*, 2731-2734.

Hayat, M. A. (1970). Principles and techniques of electron microscopy. Biological Applications. Van Nostrand Reinhold CO., New York, Vol. 1.226.

Holmfeldt, K., Odic, D., Sullivan, M. B., Middelboe, M., Riemann, L. (2011). Cultivated single-stranded DNA phages that infect marine Bacteroidetes prove difficult to detect with DNA-binding stains. *Applied and Environmental Microbiolology, 78*, 892-894.

Hennes, K. P., and Suttle, C. A. (1995). Direct counts of viruses in natural waters and laboratory cultures by epifluorescence microscopy. *Limnology and Oceanography, 40*, 1050-1055.

Jacquet, S., Lennon, J. F., and Vaulot, D. (1998). Application of a compact automatic sea water sampler to high frequency picoplankton studies. *Aquatic Microbial Ecology, 14*, 309-314.

Jacquet, S., Domaizon, I., Personnic, S., Duhamel, S., Pradeep Ram, A.S., Heldal, M., Sime-Ngando, T. (2005). Estimates of protozoan and virus-mediated mortality of bacterioplankton in Lake Bourget (France). *Freshwater Biology*, 50, 627-645.

Jacquet, S., Miki, T., Noble, R., Peduzzi, P., and Wilhelm, S. (2010). Viruses in aquatic ecosystems: important advancements of the last 20 years and prospects for the future in microbial oceanography and limnology. *Advances in Oceanography and Limnology, 1*, 71-101.

Jochem, F. J. (2001). Morphology and DNA content of bacterioplankton in the northern Gulf of Mexico: analysis by epifluorescence microscopy and flow cytometry. *Aquatic Microbial Ecology, 25*, 179-194.

Kiernan, J.A. (2000). Formaldehyde, formalin, paraformaldehyde and glutaraldehyde: What they are and what they do? Microscopy Today, 8-12.

Larsen, A., Fonnes Flaten, G. A., Sandaa, R. A., Castberg, T., Thyrhaug, R., Rune Erga, S., Jacquet, S., and Bratbak, G. (2004). Spring phytoplankton bloom dynamics in Norwegian coatal waters: microbial community succession and diversity. *Limnology and Oceanography, 49*, 180-190.

Lebaron, P., Catala, P., and Parthuisot, N. (1998). Effectiveness of SYTOX green stain for bacterial viability assessment. *Applied and Environmental Microbiolology, 64*, 2697-2700.

Lebaron, P., Parthuisot, N. and Catala, P. (1998). Comparison of blue nucleic acid dyes for flow cytometric enumeration of bacteria in aquatic systems. *Applied and Environmental Microbiolology, 64*, 1725-1730.

Lebaron, P., Servais, P. Baudoux, A. C., Bourrain, M. Courties, C. and Parthuisot, N. (2002). Variations of bacterial-specific activity with cell size and nucleic acid content assessed by flow cytometry. *Aquatic Microbial Ecology, 28*, 131-140.

Lemarchand, K., Parthuisot, N., Catala, P., and Lebaron, P. (2001). Comparative assessment of epifluorescence microscopy, flow cytometry and solid-phase cytometry used in the enumeration of specific bacteria in water. *Aquatic Microbial Ecology, 25*, 301-309.

Li, W. K. W., and Dickie, P. M. (2001). Monitoring phytoplankton, bacterioplankton, and virioplankton in a coastal inlet (Bedford Basin) by flow cytometry. *Cytometry, 44*, 236-246.

Lisle, J. T., Hamilton, W. A., Willse, A. R., and McFeters, G. A. (2004). Comparison of fluorescence microscopy and solid-phase cytometry methods for counting bacteria in water. *Applied and Environmental Microbiolology, 70*, 5343-5348.

Marie, D., Brussard, C. P. D., Thyrhaug, R., Bratbak, G., and Vaulot, D. (1999). Enumeration of marine viruses in culture and natural samples by flow cytometry. *Applied and Environmental Microbiolology, 65*, 45-52.

Marie, D., Partensky, F., Jacquet, S., and Vaulot, D. (1997). Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry using the nucleic acid stain SYBR Green I. *Applied and Environmental Microbiolology, 63*, 186-193.

Marie, D., Partensky, F., Vaulot, D., and Brussaard, C. P. D. (1999). Enumeration of phytoplankton, bacteria and viruses in marine samples, p. 11.11.11-11.11.15., Current Protocols in Cytometry. John Wiley and Sons, Inc, New York.

Middelboe, M., and Glud, R. N. (2003). Distribution of viruses and bacteria in relation to diagenetic activity in an estuarine sediment. *Limnology and Oceanography, 48*, 1447-1456.

Navaluna, N. A., Perry, M. J., Talbot, M. C. (1989). The effects of chemical fixation on some optical properties of phytoplankton. *Journal of Plankton Research, 11*, 15-24.

Noble, R. T. (2001). Enumeration of viruses, p. 43-51. In J. H. Paul (ed.), Marine Microbiology, Methods in Microbiology, vol. 30. Academic Press, NY.

Noble, R. T., and Fuhrman, J. A. (1998). Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria. *Aquatic Microbial Ecology, 14*, 113-118.

Ogden, R., and Deborah, A. (1987). Guide to molecular cloning techniques, p. 75-78. In S. L. a. A. R. K. e. Berger (ed.), Methods in Enzymology. Academic Press Inc., Orlando (Florida).

Øvreås, L., Bourne, D. A., Sandaa, R. A., Casamayor, E. O., Benlloch, S., Goddard,V. J., Smerdon, G., Heldal, M., and Thingstad, T. F. (2003). Response of bacterial and viral communities to nutrient manipulations in seawater mesocosms. *Aquatic Microbial Ecology, 31*, 109-121.

Paul, M. L., Jiang, S. C., and Brose, J. B. (1991). Concentration of viruses and dissolved DNA from aquatic environments by vortex flow filtration. *Applied and Environmental Microbiolology, 57*, 2197-2204.

Personnic, S., Domaizon, I., Dorigo, U. Berdjeb, L., and Jacquet, S. (2009). Seasonal and spatial variability of virio, bacterio- and picophytoplanktonic abundances in three peri-alpine lakes. *Hydrobiologia, 627*, 99-111.

Proctor, L. M., and Fuhrman, J. A. (1990). Viral mortality of marine bacteria and cyanobacteria. *Nature, 343*, 60-62.

Schröder, D. C., Oke, J., Hall, M., Malin, G. and Wilson, W. H. (2003). Virus succession observed during an *Emiliania huxleyi* bloom. *Applied and Environmental Microbiolology, 69*, 2484-2490.

Shopov, A., Williams, S. C., and Verity, P. G. (2000). Improvements in image analysis and fluorescence microscopy to discriminate and enumerate bacteria and viruses in aquatic samples. *Aquatic Microbial Ecology, 22*, 103-110.

Simek, K., Macek, K., and Vyhnalek, V. (1990). Uptake of bacteria-sized fluorescent particles by natural protozoan assemblage in a reservoir. *Archives of Hybrobiologia Beih, 34*, 275-281.

Simek, K., Pernthaler, J., Weinbauer, M. G., Hornak, K., Dolan, J. R., Nedoma, J., Mašín, M., and Amann, R. I. (2001). Changes in bacterial community composition and dynamics and viral mortality rates associated with enhanced flagellated grazing in a mesoeutrophic reservoir. *Applied and Environmental Microbiolology, 67*, 2723-2733.

Sime-Ngando, T. (1997). Importance des virus dans la structure et le fonctionnement des réseaux trophiques microbiens aquatiques. *Annales de Biologie, 36*, 181-210.

Sime-Ngando, T., Bettarel, Y., Chartogne, C., and Sean, K. (2003). The imprint of wild viruses on freshwater microbial ecology. *Recent Research and Development in Microbiology, 7*, 481-497.

Tomaru, Y., and Nagasaki, K. (2007) Flow cytometric detection and enumeration of DNA and RNA viruses infecting marine eukaryotic microalgae. *Journal of Oceanography, 63*, 215-221.

Troussellier, M., Courties, C. and Zettelmaier, S. (1995). Flow cytometric analysis of coastal lagoon bacterioplankton and picophytoplankton: fixation and storage effects. *Estuarine, Coastal and Shelf Science, 40*, 621-633.

Turley, C. M., and Hughes, D. J. (1992). Effects of storage on direct estimates of bacterial numbers of preserved seawater samples. *Deep-Sea Research, 39*, 375-394.

Turley, C.M., and Hughes, D.J. (1994). The effect of storage temperature on the enumeration of epifluorescence-detectable bacterial cells in preserved seawater samples. *Journal of the marine biological association* U.K., 74, 259-262.

Vaulot, D., Courties, C., and Partensky, F. (1989). A simple method to preserve oceanic phytoplankton for flow cytometric analyses. *Cytometry, 10*, 629-635.

Vives-Rego, J., Lebaron, P., and Neve-von Caron, G. (2000). Current and future applications of flow cytometry in aquatic microbiology. *FEMS Microbiology Ecology, 24*, 429-448.

Watson, S. W., Novitsky, T. J., Quinby, H. L., and Valois, F. W. (1977). Determination of bacterial number and biomass in the marine environment. *Applied and Environmental Microbiolology, 33*, 940-946.

Weinbauer, M. G., and Rassoulzadegan, F. (2004). Minireview: Are viruses driving microbial diversification and diversity? *Environmental Microbiology, 6*, 1-11.

Wen, K., Ortmann, A. C., and Suttle, C. A. (2004). Accurate estimation of viral abundance by epifluorescence. *Applied and Environmental Microbiolology, 70*, 3862-3867.

West, C. A., He, C., Su, M., Swanson, S. J., and Mentzer, S. J. (2001). Aldehyde fixation and thiol-reactive fluorescent cytoplasmic probes for tracking cell migration. *Journal of Histochemistry and Cytochemistry, 49*, 511-518.

Wilhelm, S. W., and Suttle, C. A. (1999). Viruses and nutrient cycles in the sea. *BioScience, 49*, 781-787.

Wommack, K. E., and Colwell, R. R. (2000). Virioplankton: viruses in aquatic ecosystems. *Microbiology and Molecular Biology Review, 64*, 69-114.

Xenopoulos, M. A., and Bird, D. F. (1997). Virus à la sauce Yo-Pro: Microwave-enhanced staining for counting viruses by epifluorescence microscopy. *Limnology and Oceanography, 42*, 1648-1650.

L.T.