Bottle effects, toxicity and cell viability during incubation experiments to asses community respiration.

Isabel Baños Cerón

Directores académicos: Javier Arístegui Ruíz y María Fernanda Montero del Pino

Instituto de Oceanografía y Cambio Global, Universidad de Las Palmas de Gran Canaria, Campus de Tafira. 35017 Gran Canaria, Spain.

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ABSTRACT

Microbial respiration is a key metabolic process in the ocean carbon cycle. However, so far there is not a "gold standard" method to estimate respiration in the sea. Direct oxygen consumption "*in vitro*" methods rely on long incubations (24h) inside glass bottles, which may enhance changes in the enclosed natural populations. Recently, an enzymatic *in vivo* ETS assay has been proposed as an alternative to estimate "actual" respiration in shorter incubation periods (<6 h). Here we have investigated two main factors that we consider may affect incubations in both the "*in vitro*" oxygen consumption experiments and the "*in vivo*" ETS assays: (i) The effect of enclosing natural population in bottles of different volume (125, 250 and 1000 mL) during 24 h, and (ii) The potential toxic effect of the INT (which is reduced to INT-F during the ETS assay) over natural populations, during the incubation period. We tested the "bottle effect" in the whole planktonic community (C) as well as in the filtered (through 1.2 μm filter) microbial community (F), along three experiments. Our results show variable responses of natural communities during the incubation inside bottles, but without any significant difference between the bottles' volume used. The general pattern of these changes (both in the total and fractionated communities) is a moderate decrease in phytoplankton populations and a sharp increase in bacterial assemblages (favouring the growth of HNA cells), which would presumably affect the final metabolic rates. On the other hand, we tested the toxicity of the INT during a typical *in vivo* ETS assay (6 h incubation, 0.2 mM INT). The results of this single experiment show that, after the INT addition, there is (i) a complete loss of *Prochlorococcus* cells, and a moderate loss of *Synechococcus* and picoeukaryotes, (ii) an increase in HNA bacteria (presumably by the uptake of newly organic substrates from phytoplankton death) but a decrease in LNA

bacteria, and (iii) a decline in bacterial viability, which is produced in the first 0.5 h, superimposed to the growth of HNA bacteria. In summary, our results highlight the importance of monitoring the evolution of microbial communities along incubations (both in the "*in vitro*" oxygen consumption experiments and "*in vivo*" ETS assays) and warn of some important methodological constraints that may drastically influence the actual respiratory rates.

INTRODUCTION

Marine bacteria are considered the greatest contributors to community respiration in the oceans, by uptaking dissolved organic matter and remineralizing into carbon dioxide (Rivkin and Legrende 2001). This role makes them key players in marine carbon fluxes (Gasol et al. 2008), being relevant in the assessment of the global ocean carbon cycle (Azam and Malfatti 2007, Arístegui et al. 2009). Direct measurements of bacterial respiration are however cumbersome and tedious, and require previous separation of bacteria from the rest of the planktonic community (by means of filtration processes), as well as long *in vitro* incubations (generally 24 h or more). Although incubations may last sometimes several days (e.g. del Giorgio and Cole 1998; Sherr et al. 1999), the response of the prokaryotic assemblage incubated inside the bottle has been generally assumed to be representative of the in situ bacterial behaviour (Kirchman et al. 1990). Nevertheless, there is still an unresolved debate on whether metabolic rates obtained by *in vitro* incubations -lasting from hours to days- reproduce realistic *in situ* rates, not only from bacterial assemblages but also from the whole microbial community, due to "bottle effects" (e.g. Serret et al. 2009, Calvo-Díaz et al. 2011). As Robinson and Williams (2005) defined, "bottle effects" include those unavoidable changes that populations enclosed in bottles experience during the incubation process, affecting the abundance (Lee and Fuhrman 1991), bacterivory pressure (Hopkinson et al. 1989, Marrasé et al. 1992, Suzuki 1999), metabolic activities (Sherr et al. 1999), inorganic and organic nutrients uptake (Gasol and Morán 1999), phylogenetic composition (Massana et al. 2001) and community size structure (Ferguson 1984). Due to this, experiments must be handled with extremely care, and microbial abundances and rates monitored through the length of the incubation, to realistically interpret the processes that take

place inside the bottles in order to extrapolate to in situ conditions (Williams et al. 2013 in press). Particularly, in oligotrophic regions where nutrients are scarce and small picoplankton dominate, the presumably non-natural alteration of community structure and metabolism during incubations has received a special attention (e.g. Calvo-Díaz et al. 2011).

Recent studies have brought back into discussion an old scientific dispute that arose in the 80s about quantifying the magnitude of primary production in the oceans based on geochemical versus ecological *in vitro* approaches (Shulenberger and Reid 1981, Platt 1984), to understand its role in the global carbon cycle. This controversy has been translated at present to the study of the metabolic balance (the Production / Respiration ratio) of planktonic communities in open-ocean oligotrophic regions (Ducklow and Doney 2013 in press). Two contrasting views confront the hypothesis of an autotrophic ocean (Production > Respiration)-mostly based on the interpretation of large-scale and long-term biogeochemical estimates (Williams et al. 2013 in press) versus the hypothesis of a heterotrophic ocean (Respiration > Production) -based on short-term oxygen-derived rates obtained from *in vitro* incubations (Duarte et al. 2013 in press).

In the middle of this debate, there still remain large uncertainties on whether "*in vitro*" approaches reproduce reliable estimates of microbial/bacterial respiration due to the extended length (24h or more) needed to obtain significant changes in oxygen consumption. This has fostered the search of alternative non-incubation (or shorter-incubation times) methods to obtain microbial respiration activities in the open ocean. In the 1970s, Packard and co-workers developed an enzymatic *in vitro* assay (ETS*vitro*) to estimate potential respiratory activity (under saturating substrates). Years later, Arístegui and Montero (1995) found a strong global correlation between actual rates of

oxygen consumption and ETS*vitro*, including data from many different surface oceanic regions, spanning from eutrophic to oligotrophic ecosystems; although the correlations were weaker at almost every given particular region. The ETS*vitro* as an estimate of respiratory activity was criticized by Bamstedt (2000), who considered that enzymatic activities should be measured at natural levels of substrates. Recently, Martínez-García et al. (2009) developed an *in vivo* assay of the electron transport system (ETS) method, based on the reduction of the tetrazolium INT salt to INT formazan (INT-F) by natural microbial communities during short incubations (2-6 h), claiming its use to expand the still meagre data base on microbial respiration. They tested the method in a wide range of cultures and surface-water marine environments, finding a strong overall correlation (r^2=0.97) between oxygen consumption (using the Winkler method; R) and *in vivo* INT-F formation rates (ETS), yielding a surprising R/ETS = 12.8. Nevertheless, it must be noticed that most of the R rates used in their study to derive the correlation were several times higher (> 2μM h^{-1}) than published R rates from open-ocean natural communities (e.g. Arístegui and Montero 1995). Moreover, the few R values used in this study from oligotrophic open ocean communities (<2μM h^{-1}) do not seem to correlate the same with the *in vivo* ETS, as observed also in more recent studies from the same authors (Aranguren-Gassis et al. 2011). If the *in vivo* ETS yields reliable and robust estimates of R, the method would definitively allow improving the global data base on R, avoiding "bottle effects" (Robinson and Ramaiah 2011), particularly in the dark ocean, where the Winkler method is close to or under its limit of sensitivity (Arístegui et al. 2005). However, a recent study by Maldonado et al. (2012) questions the validity of the method. These authors show that common cellular substances not involved in the respiratory electron transport system (ETS) may reduce the INT salt to INT-F as well, yielding misleading results that –according to them- would be equivalent to the blank in

an *in vitro* ETS assay (Packard 1971) where NADH, NADPH and succinate are added at saturation levels. Furthermore, the addition of a tetrazolium salt to natural communities may have toxic effects over the cells, reducing their viability and hence decreasing the metabolic rates of the whole community. Indeed, Gasol and Arístegui (2007) observed that the addition of the fluorogenic tetrazolium salt CTC at concentrations close to 5 mM produced a decrease in cell abundance of 22% in coastal picoplankton, after 30 min of incubation. Martínez-García et al. (2009) used in their study concentrations ranging from 0.2 to 1 mM of INT, 5 to 25 lower than those used by Gasol and Arístegui, but at the same time, incubation times 4-12 times longer. Thus, it should be tested whether these lower concentrations of tetrazolium added to cells during longer incubation times have or not a significant toxic effect on microbial populations. If so, independently of the potential bias due to the INT reduction caused by substances not involved in the ETS, the toxicity of the INT would lead to an underestimation of the respiratory activity.

In the present study we wanted to test two main factors that we considered critical in respiration studies: the effect of enclosing samples inside bottles and the potential toxic effect of the use of formazan salts for the *in vivo* assay of respiratory activity. These represent the main two objectives of this work:

(1) To analyze the changes with time in picoplankton community structure and abundances caused by the enclosure of natural populations in bottles of different volume, in order to elucidate the selective effect of “bottle size” on the growth and mortality of bacteria, cyanobacteria and picoeukaryotes.

(2) To evaluate the potential toxic effects of the tetrazolium salt INT during the length of time of a typical incubation of *in vivo* ETS assay at concentrations reported in the literature.

We have started working on a third objective, but due to timing constraints in the dates scheduled to defend this memory we have been unable to present here the results, which at present are at their initial stage. The objective (which will continue addressing in the near future) is the following:

(3) To look at the variability in the R:ETS*vitro* and R:ETS*vivo* ratios at the start and end of a 24 h incubation, used to estimate oxygen consumption by the Winkler method. Our aim is to see how does the potential growth (or loss) of microbial populations inside the incubation bottles affect the final R/ETS ratios.

MATERIALS & METHODS

Plankton samples for the experiments were collected in the eastern coast of Gran Canaria (27º 51' 26" N, 15º 23'15" W) Spain (Fig.1). These waters are characterized by a picoplankton community presenting populations of heterotrophic bacteria, *Prochlorococcus*, *Synechococcus* and picoeukaryotes, typical of oligotrophic environments (Arístegui and Montero 2005, Baltar et al. 2009). All the samples were collected in 10-liter polycarbonate carboys, at the same time of the day, from 0 to 1m depth. In all the experiments, water was filtered within 1-2h after collection in the laboratory. To separate the bacterial community from the total community (considered it as the water pre-filtered by 100µm mesh), we employed a Polygard-CN cartridge filter (1.2µm final pore size). These filters yield a high performance with a minimum

differential pressure, minimizing cells' breakage. Nevertheless, we realized that many of the picophytoplankton forms also passed through these filters. Before each experiment, all the consumable material and the bottles (glassware and Teflon-coated caps) were cleaned in acid (3% final concentration) to remove any organic matter which could influence the microbial community growth. The cartridge filters were disinfected during 30minutes in water at 80º C and subsequently autoclaved at 126º C during 30 minutes. Only the toxicity assay with INT was performed in a bath water at 18º C. For at least 0.5-1 h, the bottles filled with seawater were temperate in the bath until the desired temperature was reached.

1. Experimental design.

1.1. Volumetric bottle effect.

To assess the volumetric bottle effect we conducted three set of experiments: Expt1(July 2012), Expt2 and Expt3(September 2012). Each of the samples (for both the whole planktonic community and the 1.2μm filtered community) was incubated in three different volumes (125mL, 250mL and 1000mL) at *in situ* temperature (22º C) and in dark conditions. A total of twelve bottles were employed in Expt1 and eighteen in Expt2 and Expt3. Subsamples for Total Organic Carbon (TOC) and picoplankton abundances were obtained at t=0 h (considered as initial) and after 8, 12, 24 hours of incubation, except in Expt1 where the 12 h measurement was not considered. Bacterial viability was also measured but the results at t=24 h are not presented here. The high bacterial abundance at this time caused that more than 1000 events s^{-1} were reached when the sample was counted by cytometry. The dilution required by the sample caused an osmotic shock, and hence an overestimation of the damaged cells.

1.2. Toxicity assay with INT during an "in vivo ETS" assay.

Based on published results of the toxicity of a tetrazolium salt (CTC) on picoplankton communities (Gasol and Arístegui 2007), we designed an experiment to test whether the INT formazan was toxic at the levels used in the *in vivo* ETS assay (0.2mM final concentration), along five different times of incubation (0.5, 1, 2, 4 and 6 h). Control bottles were placed at each time of incubation. Heterotrophic bacteria and picophytoplankton were counted and bacterial viability was determined by using the NADS protocol (Falcioni et al. 2008)(see below). To determine *in vivo* ETS activity, three additional bottles were inoculated with INT (0.2mM of final concentration) and stopped at each time of incubation

2. Heterotrophic bacteria, picophytoplankton and nanophytoplankton abundances and biomass.

To count the abundance of heterotrophic bacteria, samples (800µL) were fixed with 50µl of 20% paraformaldehyde (2% final concentration), kept 30min at 4º C and then preserved at -80º C until being analyzed (<1 month) with a FACSCalibur flow cytometer (Becton Dickinson) equipped with a 488-nm argon laser. Then, 400µL were stained during 15 min at dark with 4µL of 10XDMSO-diluted SYTO 13 (Molecular Probes) at 2.5µM final concentration. Bacteria containing high nucleic acid (HNA) and low nucleic acid (LNA) were detected and separated from photosynthetic prokaryotes (cyanobacteria) by bivariate scatterplots of FL3-H (red fluorescence)-versus-FL1-H (green fluorescence). Picophytoplankton (*Synechococcus, Prochlorococcus* and picoeukaryotes) and nanophytoplankton were identified in samples (1.2 mL) fixed with paraformaldehyde (2% final concentration) and preserved following the same details described above for heterotrophic bacteria samples. Bivariate plots of FL3-H-versus-

FL2-H and FL3-H-versus-SSC-H were used to identify the populations. Nanophytoplankton was also counted by flow cytometry, although abundances were not checked against microscopic counts. As an internal calibration, a 10^6 beads mL^{-1} and 10^5 beads mL^{-1} solutions (1μm size) were added to heterotrophic bacteria and phytoplankton, respectively. Samples were processed using the CellQuest software. Heterotrofic bacteria and picophytoplankton biomass were obtained applying the conversion factors described by Montero (2012) from samples collected at the same location (Table1)

3. Bacterial viability.

Dead (or membrane damaged) and alive (or intact membrane) bacteria were detected by Nucleic Acid Double-Staining protocol (Falcioni et al. 2008). 400 μL of natural samples (no fixation were employed because would alter the membrane state) were simultaneous stained with 4 μL of 10XDMSO-diluted SyberGreen1 (Molecular Probes) and 4 μL of Propidium Iodide (Molecular Probes) at 10μg ml^{-1} final concentration and maintained at dark conditions during 15min. A control sample (killed at 100º C) was stained like the living samples. Intact-membrane cells (alive) and damaged-membrane cells (dead) were identified in scatter plots of FL1-H-versus-FL3-H. The killed sample (control) only exhibited red fluorescence and helped us to draw more exactly the dead bacteria cluster. FL1-H-versus-SSC dot plot was useful in distinguishing photosynthetic prokaryotic, which might appear between the two clusters (alive and dead).

4. Total Organic Carbon.

Water triplicate sample of 10mL were drawn into 13mL polypropylene container tightly closed and stored frozen -20° C. TOC analysis was performed using the high-temperature combustion method on a Shimadzu TOC-5000A. At the beginning of each analysis run, the sample was sparged with CO_2-free air for several minutes to remove the inorganic carbon. The sample was then injected (3 replicates of 100 µl) into a quartz tube with a platinum catalyst and then combusted at 680°C. TOC concentrations were determined from standard curves (30 to 200 µM) of potassium hydrogen phthalate produced every day (Thomas et al. 1995). TOC reference material prepared in the laboratory of Hansell (University of Miami) was analysed every day to check for the accuracy and precision of our instrument. We obtained an average concentration of 44.5 ± 2.7 µM C (n = 31) for the deep ocean reference (Sargasso Sea deep water, 2600 m) and 0.21 ± 0.53 µM C (n = 31) for the blank reference material, whose nominal values were 44.0 ± 2.7 and 0.0 ± 1.5 µM C, respectively.

5. Measurements of respiration rates.

In vivo electron transport system (ETS) activity

In vivo ETS activity was measured following the method described in Martínez-García et al. (2009). Four 250mL bottles were filled with seawater. One of the bottles was immediately fixed with formaldehyde (4% final concentration) and considered as a killed blank. 0.2mM final concentration of a solution 2-para (iodo-phenyl)-3(nitro-phenyl)-5(phenyl)-tetrazolium chloride (INT) was inoculated to the samples and incubated at 18º C during 0.5, 1, 2, 4 and 6 h (see *Toxicity assay with INT* in *Experimental design*). Samples were fixed in the same way as the killed blank and 15

min later were filtrated through a 25 mm polycarbonate filter (0.2μm final size pore). The filters with INT-Formazan crystals were preserved in cryovials at -20º C until being processed the following days. To extract INT-Formazan from the filters, one millilitre of propanol was added to each cryovial and sonicated at 50º C in an ultrasonic bath (Ultrasonic Cleaner) with a coupled resistance, and subsequently shacked in a vortex during 1-2min. One mililiter of the sample (propanol plus INT-F crystals) was dispensed in 1.5mL vials and centrifugated at 13200 g during 10 min at 18º C. Carefully, the supernatant was separated from the debris and the absorbance at 485nm was then read in a spectrophotometer. The INT- F concentration was calculated by applying a calibration curve obtained previously from seventeen different concentrations, between 0.01μM and 34 μM, of pure INT-F dissolved in propanol.

6. Statistical method.

Statistical analyses were made with SPSS. The Student *t* test for comparisons of means was applied to detect differences for each bottle between the beginning and the end of the incubations in volumetric bottle effect experiments

RESULTS & DISCUSSION

1. Enclosure, filtration and volumetric bottle effects.

Figures 2 to 6 show the evolution of picophytoplankton (*Prochlorococcus, Synechococcus* and picoeukaryotes), nanophytoplankton and heterotrophic bacteria populations enclosed in bottles of different volume (125 mL, 250 mL and l000 mL), during the time evolution (0 to 24 h) of three experiments. We hypothesized that populations changes inside bottles of larger volume would be smaller, and that

increasing the size of the bottles -normally used during respiration experiments (125 mL)- would decrease the potential bias in respiration rates attributed to the confinement of samples in small incubating volumes. The rational is that larger volumes of water would decrease on the one side the death of phytoplankton, and on the other the growth of bacteria attached to the glass walls, due to the reduction of the surface/volume ratio in larger bottles (Zobell 1943, Taylor and Collins 1949, Lee and Fuhrman 1991). However, although we observed sharp changes in the planktonic abundances and biomasses (particularly in bacteria) along the course of the experiments, we did not find any recurrent and significant variability among the different bottle-volumes used. This result might result surprising, but is similar to recent published results by Calvo-Díaz et al. (2011), using the same range of volumes in their experiments with open-ocean communities from the North Atlantic subtropical Gyre. Nevertheless, we cannot disclaim that even larger volumes of water (e.g. 5L or more) would not minimize bottle effects.

In order to see the effect of pre-filtration before running incubation experiments for bacterial respiration, we analysed also the evolution of samples filtered through 1.2 μm, comparing to those samples only pre-filtered through 100 μm (which we considered to represent the "total microbial community"). In theory (as it is considered in most published studies) samples filtered by 1.2 μm would contain only the heterotrophic prokaryotic community (i.e. namely bacteria). However, we realized that, beside bacteria, many picophytoplankton cells, due to their small size, passed through this pore-size filter. Indeed, Montero (2012) reported from an annual monitoring sampling in the same area of study that the mean diameter of the picophytoplankton cells do not exceed 1 μm. This would explain that a significant (but variable) fraction of the

different picophytoplankton groups was always observed in the 1.2 μm filtered sample (see Figs. 2-6). The most striking situation was observed in Expt1, where almost a 100% of the picophytoplankton passed through the 1.2μm cartridge filter (Figs. 2-4). A plausible explanation is that the filtration pressure was higher than desirable during this experiment, damaging the filtrated cells. That could explain the sharp drop in abundances in all the picophytoplankton groups, between the initial conditions and time 8h.

1.1. Time evolution of the phytoplankton groups in the "total microbial community" (C) and the "1.2 μm filtered" (F) samples.

There were differences between groups and experiments, although the general trend was a decrease in the abundances of all groups with time.

Phrochlorococcus decreased only slightly during Expt1, both in C and F (Fig. 2A) excepting the sharp drop from T0 to T8 in the F sample. However, in Expt2 and 3 the decrease ranged from 10-28% to 33-41%, respectively (Figs. 2B-C). After 24 h all the decreases in the *Prochlorococcus* abundances were significant (paired *t*-test $p<0.05$) except in the 1L bottle in Expt2 (paired *t*-test $p=0.71$) (Fig. 2B). It is worth noting the similarity in the declining trends in spite of the large variability in the initial concentrations of cells, with up to 5 times more abundances in Expt1 than Expt3.

Synechococcus showed similar decreasing trends than *Prochlorococcus*, although with a smoother decline (Figs. 3A-C). The sharpest change was found in the F sample of Expt3, between T12 and T24 (Fig. 3C), where cells dropped down from 1500 to less than 300 cells mL^{-1}. Overall, however, the average decrease in the abundance of *Synechococcus* was very similar in the three experiments, around 12-20%. Like with

Prochlorococcus, there were differences in the initial concentrations of *Synechococcus*, with >6000 cells mL^{-1} in Expt2 and <2000 cells mL^{-1} in Expt3 (Figs. 3B-C).

Picoeukaryotes presented an almost stable concentration in the C samples during the first 12h, giving rise to a sharp decline at 24h (Figs 4A-C). This variability was not evident in the F samples (except at T0-8 in Expt1), where cell concentrations remain low but pretty stable.

Nanophytoplankton cells (>2μm) (Figs. 5A-C) did not pass the 1.2 μm filter, but populations in the C sample dropped more sharply than other phytoplankton groups in the first 8 hours of incubation. Although nanophytoplankton presented concentrations (300-500 cells mL^{-1}) much lower than the picoplankton groups, their larger biomasses make them key players in the community metabolism. Thus, the decline with time of their abundances down to half or less the initial concentrations after 24 h would represent a strong handicap when estimating metabolic rates.

There are very few studies looking at the factors that produce the decline in planktonic communities enclosed in incubation bottles. Some of these studies indicate that *Synechococcus* responds with a greater resistance to the manipulation and to a drastic change in the light regime to darkness than *Prochlorococcus* (Binder and Chisholm 1995, Jacquet et al. 1998), while *Prochlorococcus* appeared to be more influenced by a higher grazing pressure (Guillou et al. 2001). Instead, picoeukaryotes and nanophytoplankton, seem to be more sensible to confinement than cyanobacteria due to a higher nutrient request (Raven 1998, Veldhuis et al. 2005).. In any case, it is likely that the exhaustion of inorganic nutrients and the cascading trophic effects (by enhanced grazing and decreasing growth rates) produce changes in all populations of phytoplankton, largely affecting to the most sensible groups (phytoplankton >2 μm).

1.2. Time evolution of the bacterial assemblages in the "total microbial community" (C) and the "1.2 µm filtered" (F) samples.

In contrast to the recurrent decline with time in phytoplankton populations, bacteria show a clear opposite trend with a sharp enhancement, which is more acute after the first 12 h. In all the three experiments, the C samples respond more rapid than the F samples to bacterial increase (Fig. 6). Indeed, after the first 8 h an almost exponential growth of bacterial cells can be observed in the three C experiments, reaching values from 4 to 10 times more after 24 h (Fig. 6). The F samples, however, display a lag-phase (from 8 to 12 h) before growing exponentially to similar or even higher concentrations. This lag-phase is characteristic of new cultures that need some time to adapt before start growing. The impression is that while the bacterial abundances in the C samples seem to tend to stabilize at 24 h, at least in Expts 2 and 3 (Figs. 6B-C), in all three experiments the F samples look like starting the exponential growth phase at 24 h (probably reaching much higher concentrations in the following hours)

By means of flow cytometry, we can identify two broad assemblages of bacteria that Li et al. (1995) described as low-nucleic acid content (LNA) and high-nucleic acid content (HNA). In fact these two categories comprise a complex assemblage of groups: one with less fluorescence and small size (LNA) and the other with higher fluorescence and larger sizes (HNA). LNA uses to be dominated by the small SAR11clade group, whereas the HNA assemblage is more complex, and may contain large cells from the *Rhodobacterales*, *Bacteroidetes* and Alphaproteobacteria group (Baltar et al. 2012, Vila-Costa et al. 2012). Although both assemblages (HNA and LNA) are active, as they may exhibit similar rates of leucine incorporation (Zubkov et al. 2001), HNA are considered the "ready to respond" fraction (Gasol et al. 1999) outcroping LNA bacteria

under favourable conditions (e.g. excess organic matter). This situation is evident along the three experiments and the two treatments (C and F), showing a clear increasing trend in the HNA/LNA ratio with time (Fig. 7). However, the HNA/LNA ratio increases more in the F treatment compared to the C sample (Fig. 7), probably because the availability of organic carbon is even higher. Indeed, the total organic carbon (TOC) data measured at the initial incubation time inside the bottles (Tables 2-4) show that the TOC value in the F sample is consistently higher than in the unfiltered sample (C). It is impossible to ascribe the amount of semi-labile or semi-refractory organic carbon from TOC available to prokaryotes, but the large differences in TOC between F and C samples (at almost all the sampling times) suggest that filtration produces organic debris and dissolved carbon that might be used in part by bacterial cells to grow up. Moreover, the variability in TOC at each sampling period and treatment may result from the balance between the usage of bio-available TOC by prokaryotes and the production of TOC by the death and excretion of microorganisms (Carlson et al. 1998) In any case, to have a more precise estimate of the TOC available to prokaryotes we first should subtract the biomass of picoplankton from TOC at each sampling time, an exercise that we will do in the near future.

A critical issue (normally ignored in metabolic studies) is to monitor the abundance of active (vs inactive) cells, which would mostly contribute to the overall microbial metabolism. Some surprising results indicate that although there are changes in bacterial assemblages inside the bottles during long-term incubations (24h or more), constant community metabolic rates can be maintained as a result of shifts in community composition (e.g. Baltar et al. 2012). Whether these shifts reflect variability in the death/growth of certain specific bacterial groups is unclear. Here we have monitored the

viability of bacterial cells along the experiments, looking at their membrane integrity (NADS method), and found consistent responses (Tables 2-4). The initial percentage of live cells (NADS+, with an integral membrane) in Expts 2 and 3 was very similar (91% in the F treatment and 80% in C treatment), keeping also similar trends in both experiments at T12: increasing in C and decreasing in F. Nevertheless, the cell viability in the F treatments showed a more pronounced decrease in Expt3 than in Expt2. This variability might reflect changes also in the shift of bacterial assemblages, but are critical to monitor during metabolic experiments, since probably will give the clue for a possible relationship between active bacteria abundance and metabolic rates.

1.3. Time evolution of the Autotrophic: Heterotrophic biomasses ratio (AB:HB) in the "total microbial community" (C) and the "1.2 µm filtered" (F) samples.

We converted the planktonic cell abundances to biomasses (see methods) in order to estimate the variability in the AB:HB. This ratio provides a balance between the contribution of autotrophic phytoplankton and heterotrophic microbes to the metabolic rates of a given ecosystem (Odum 1971, Arístegui and Montero 2005). Although would be somewhat naïve to equate a biomass ratio to a metabolic proxy (see for instance the discussion above about the active vs inactive bacteria), the AB:HB gives a broad picture of the shift in the metabolic state of the ecosystem along the experiment.

Figure 8 shows a clear decreasing trend of the AB:HB ratio along the experiment, tending to near 0 at T24. At T0 and T8 the ratio is close to 1 (sometimes even higher), suggesting a metabolic balance between autotrophy and heterotrophy. As soon as phytoplankton populations decline and HNA bacteria grow exponentially, the ratio turns out to a strict heterotrophy. Similar results were recently published by Calvo-Diaz et al. (2011), during a set of experiments carried out in the North Atlantic subtropical Gyre. It

remains to know what is the true contribution of the increasing bacteria population (and concomitant decreasing AB:HB ratio) to the bias in actual respiration rates. This topic is part of our future work and needs to be addressed through short-term respiration experiments with natural communities.

Overall, our results evidence different responses of natural communities when they are enclosed and incubated inside bottles of different volume. Nevertheless, the general pattern of these changes (both in the total and fractionated communities), and without any significant difference between volume bottles < 1000 mL, is a moderate decrease in phytoplankton populations and a sharp increase in bacterial assemblages (favouring the growth of HNA cells). It is, however, necessary to monitor the viable bacterial – matching oxygen consumption rates- to see whether or not these cells are responsible of the bulk of respiratory rates.

2. Responses of picophytoplankton and heterotrophic bacteria to INT toxicity during an "*in vivo* ETS" assay.

The response of picoplanktonic (autotrophic and heterotrophic) populations to the addition of INT in concentrations equivalent to those used in "*in vivo* ETS assays" (Martínez-García et al. 2009) varied depending on the groups. The most striking response was that of *Prochlorococcus.* After adding INT to the sample, its populations (with a concentration close to 6 x 10^4 cells mL^{-1}) completely vanished after the first 0.5 h, while in the control sample the populations remained almost stable along the 6 hours of incubation (Fig. 9A). *Synechococcus* abundance experienced an average decrease of 9-12% in the INT sample, with respect to their initial abundance. The decrease was more evident after 1 h of incubation (Fig. 9B). Picoeukaryotes responded similar to *Synechococcus* , with a drop in their abundances of 11-20% along the incubation in the

INT samples (Fig. 9C). It is not clear why *Prochlorococcus* is so sensitive to the toxic effect of INT (compared with *Synechococcus* and picoeukaryotes). Perhaps its perfect adaptation to oligotrophic environments, with a drastic reduction of cell and genome size (Partensky and Garczarek 2010) - also known as "The Black Queen Hypothesis" (Morris et al. 2012)- as well as its dependence on co-occurring microorganisms in cross-protecting from oxidative damage (Morris et al. 2011), makes *Prochlorococcus* a rather susceptible organism under external damage, albeit its perfect adaptation to natural conditions.

Prochlorococcus is the most abundant photosynthetic organism in oligotrophic open-ocean environments (with abundances close to heterotrophic bacteria), and hence must play a key role in the overall microbial metabolism (e.g. respiration). Our results thus alert that the addition of INT for assessing "*in vivo*" ETS activity in natural samples (with the consequent loss of all *Prochlorococcus* cells, and to a lesser extent of *Synechococcus* and picoeukaryotes) might clearly underestimate the actual respiratory activity of the microbial community.

The response of heterotrophic bacteria to the addition of INT was different to phytoplankton, but variable among assemblages (Fig.9 D-F). HNA bacteria responded increasing their abundances in the INT samples (with respect to the control sample), whereas the LNA bacteria slightly decreased in abundance. HNA bacteria increased 23% in abundance in the first 0.5 h after the INT addition, being 11-14% more abundant along the experiment, with respect to the control. The initial increase of HNA could have benefitted of the death (and dissolved organic leaking) of picophytoplankton. LNA bacteria (presumably less opportunistic than the HNA bacteria) didn't seem however to take benefit of the potential increase in organic carbon substrates,

decreasing in their abundances 6-10% after INT addition. Overall, the balance is a slight increase in total bacterial along the 6 h of the incubation experiment (although non significant, except in the first 0.5 h).

Nevertheless, if we look at the viability of bacterial cells after the INT addition (Fig. 10) we observe that just after the first 0.5 h there is a significant 20% decline in the % of viable bacteria cells with respect to the control, which remains constant along the 6 h. Although we haven't had time to search more on the cytometric data, we will be able to discern whether the drop in the viability is more due to HNA or LNA bacteria.

Gasol and Arístegui (2007) found a strong correlation between the increase in the formation of CTC-F (another formazan salt derivative of the reduction of the fluorogenic tetrazolium dye CTC) and the disappearance of HNA bacteria. In their experiments -performed with coastal communities similar to those used in our experiments- they observed that the CTC-F granules formed inside the cells became so large that lasted breaking and killing the cells. In our case, we did not find any correlation between the INT-F formation and the disappearance of bacteria cells (neither HNA or LNA; Fig. 9F), nor with the % of viable cells. Perhaps, the INT-F crystals formed inside the cells were not large enough as to break the cells, but two other reasons may also explain this lack of correlation: (i) The INT concentration was low enough as to affect only to a small proportion of the total cells, and hence the correlation is not evident (which in that case would question the use of 0.2 mM of INT as a saturating level during in vivo ETS assays). (ii) The INT is mostly reduced to INT-F by substrates not involved in the ETS reactions, as Maldonado et al. (2012) argued.

In summary, our results suggest that the addition of INT to the natural samples produce the following effects (mostly in the first 0.5 h): i) A complete loss of *Prochlorococcus*

cells, and a moderate loss of *Synechococcus* and picoeukaryotes, with a leak of dissolved organic substrates that could be used by bacteria. ii) An increase in HNA bacteria (presumably by the uptake of newly organic substrates from phytoplankton death), but a decrease in LNA bacteria. iii) A decline in bacterial viability, which is produced in the first 0.5 h, superimposed to the growth of HNA bacteria.

Overall, the effect of adding 0.2mM of INT to a natural sample during an *in vivo* ETS assay would lead to an underestimation of the respiratory activity, although it remains to quantify its magnitude. This will definitively influence also the variability in the R/ETS ratios used to convert ETS activity to actual respiration rates.

CONCLUSIONS AND FUTURE WORK

Our results may be summarized in the following main conclusions:

1. The enclosure of natural populations inside BOD bottles during "oxygen consumption experiments" (along 24 h), produce a moderate (average: 20%) decrease in phytoplankton populations, which is more evident in picoeukaryotes and nanophytoplankton.
2. Heterotrophic bacteria, and particularly the HNA assemblages, increase up to 10 times more, probably favoured by the enhancement in organic substrates available due to phytoplankton mortality.
3. Pre-filtration of natural samples, to achieve bacterial respiration, leads to an enhancement of bacterial growth, which is more evident after a lag phase of about 12 h.

4. The autotrophic/heterotrophic biomass ratio shifts along the experiments, from values >1 to close to 0, due to the moderate decrease in phytoplankton biomass and the sharp increase in bacterial biomass.
5. We did not find any significant variability in the evolution of microbial communities (neither in the whole community nor in the fractionated one) across the different bottles used (with volumes ranging from 125 mL to 1 litre). Nevertheless, we cannot disclaim that larger volumes may reduce "bottle effects".
6. The addition of INT (at 0.2mM final concentration) during an "*in vivo*" ETS assay produced the mortality of all *Prochlorococcus* cells during the first 0.5 h, as well as a moderate loss of *Synechococcus* and picoeukaryotes.
7. Heterotrophic bacteria abundances, however, did not show a clear response to INT additions, although bacterial viability dropped a 20% in the first 0.5 h.

All the above results alert of the importance of monitoring the evolution of microbial populations during incubation experiments to address respiratory rates, and open new research questions that we will address in the near future:

1. Does larger volumes of water minimize the "bottle effects"?.
2. Does pre-filtration increase metabolic rates as seems to do with bacterial abundances in >24 h experiments?.
3. Why *Prochlorococcus* is more sensitive than other picophytoplankton to the toxicity of INT, and how this affect to the final respiratory rates in the "*in vivo*" ETS assays?.
4. Why HNA bacteria increase during the *in vivo* ETS assay?

5. Are the cell membranes of bacteria less permeable than those of phytoplankton cells to INT?.
6. What factors are the main responsible in the variability in the R/ETS*vivo* and R/ETS*vitro* ratios?
7. What are the main constrains to address respiratory rates during incubation experiments?.

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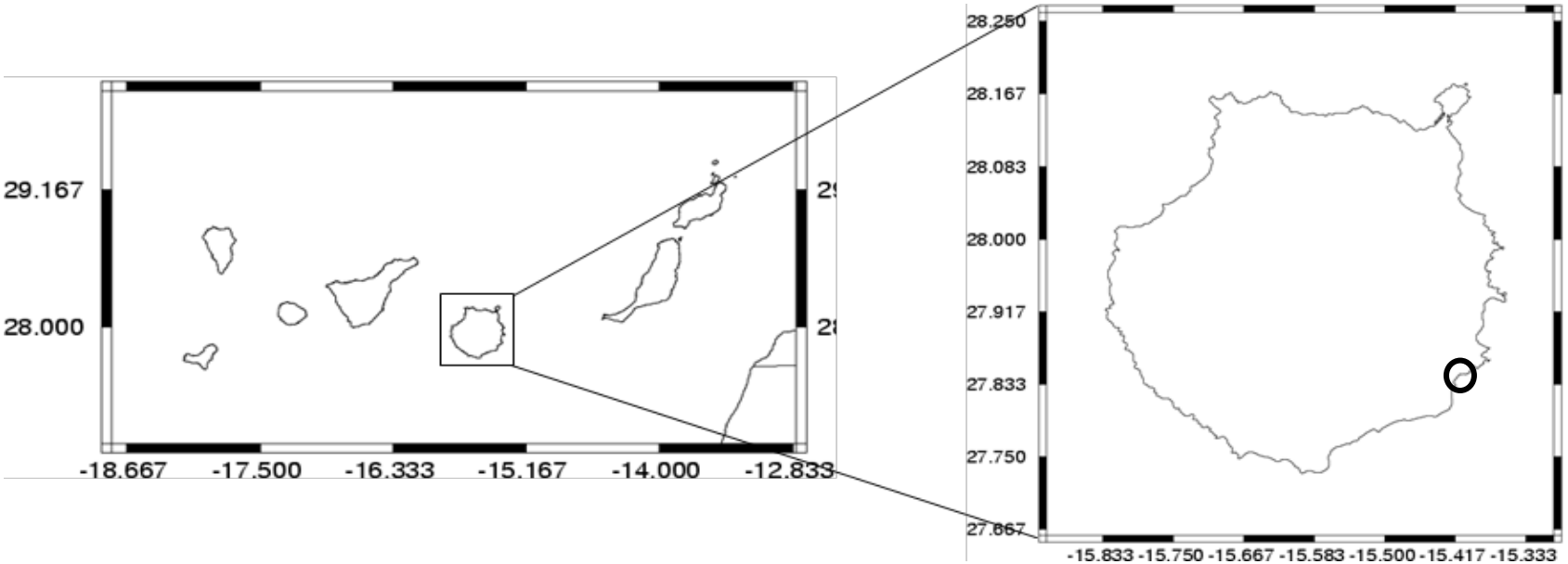

Figure 1. Enlarged map of Gran Canary island showing the study area (circle), east of the island

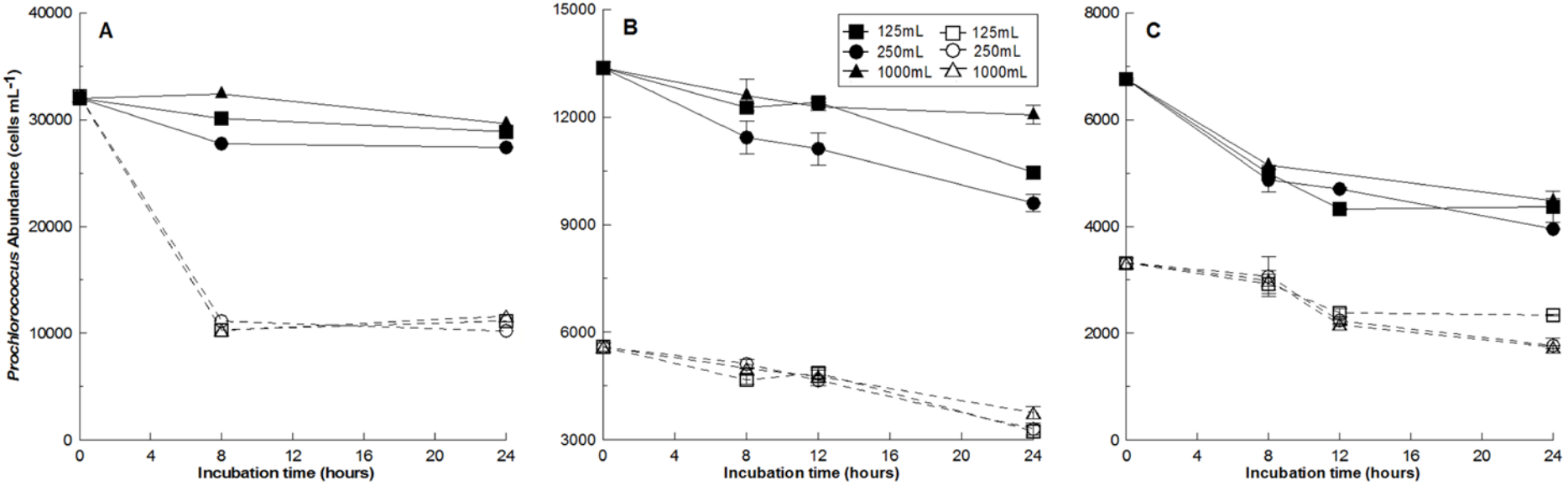

Figure 2. Changes in the abundance of *Prochlorococcus* during volumetric bottle effect experiments in Expt 1 (A), Expt 2 (B) and Expt 3 (C). Continuous line shows the whole planktonic community and dotted line the filtered community. Note the different scales on the *y* axes.

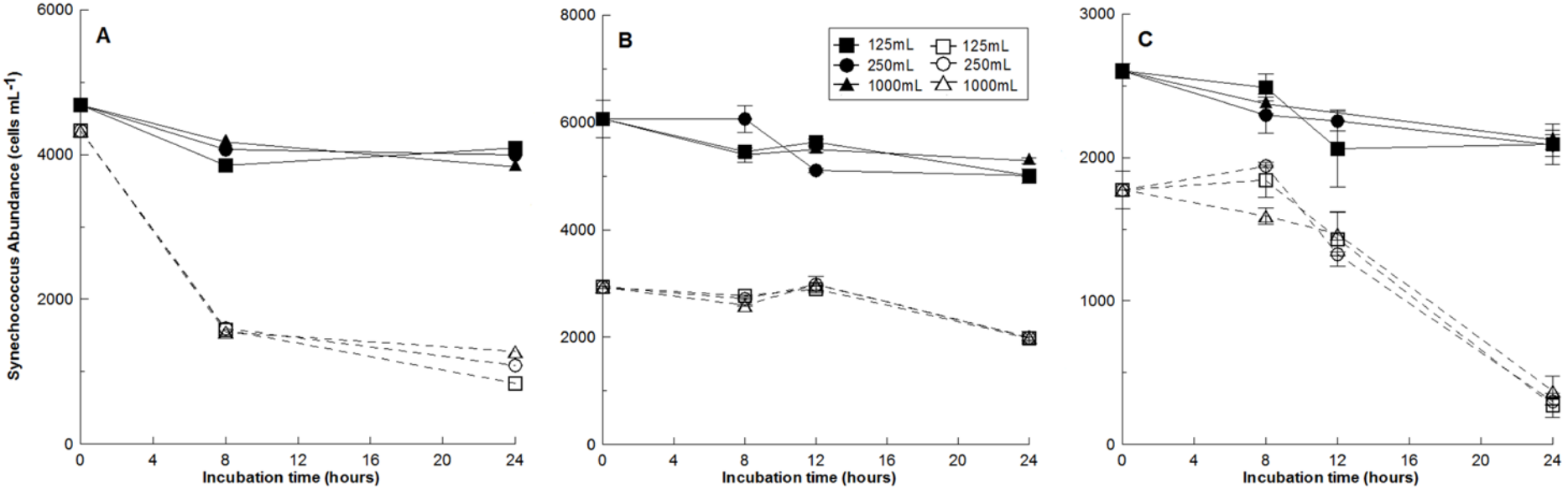

Figure 3. Changes in the abundance of *Synechococcus* during volumetric bottle effect experiments in Expt 1 (A), Expt 2 (B) and Expt 3 (C). Continuous line shows the whole planktonic community and dotted line the filtered bacterial community. Note the different scales on the *y* axes.

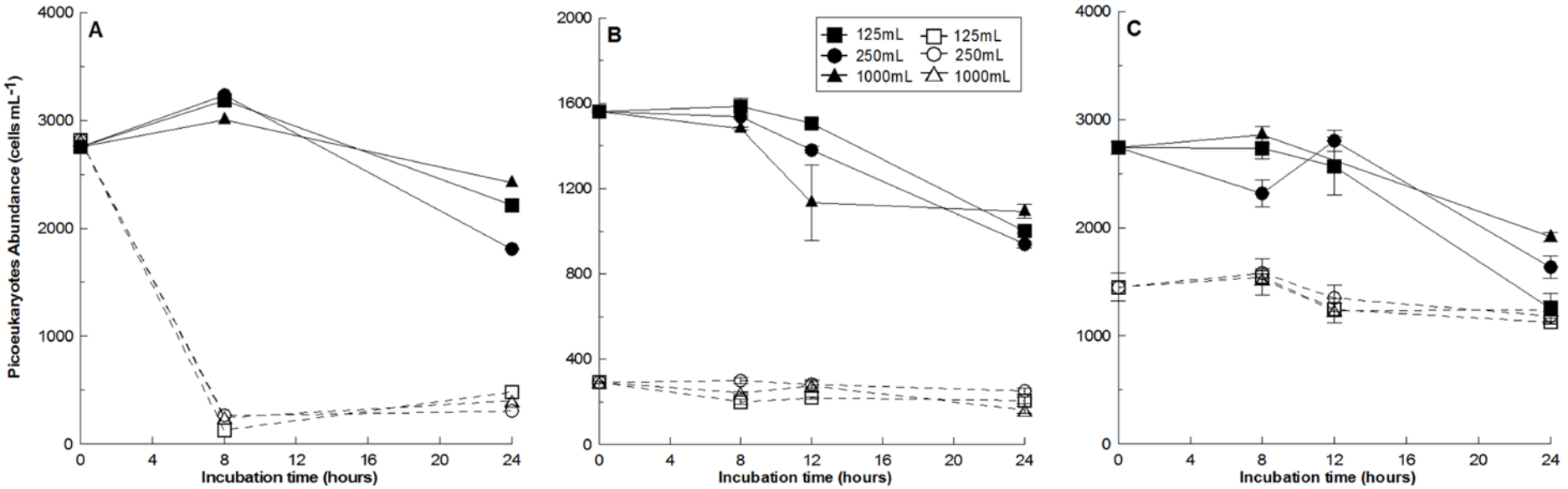

Figure 4. Changes in the abundance of picoeukaryotes during volumetric bottle effect experiments in Expt 1 (A), Expt 2 (B) and Expt 3 (C). Continuous line shows the whole planktonic community and dotted line the filtered bacterial community. Note the different scales on the *y* axes.

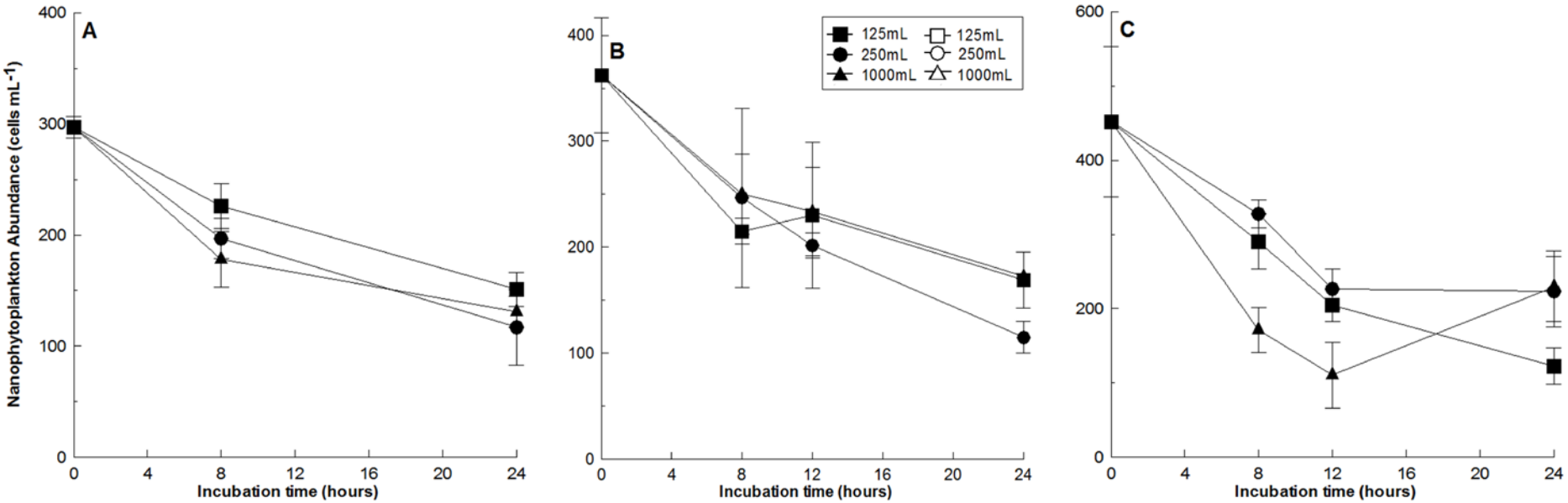

Figure 5. Changes in the abundance of nanoplankton during volumetric bottle effect experiments in Expt 1 (A), Expt 2 (B) and Expt 3 (C). Continuous line shows the whole planktonic community and dotted line the filtered bacterial community. Note the different scales on the *y* axes.

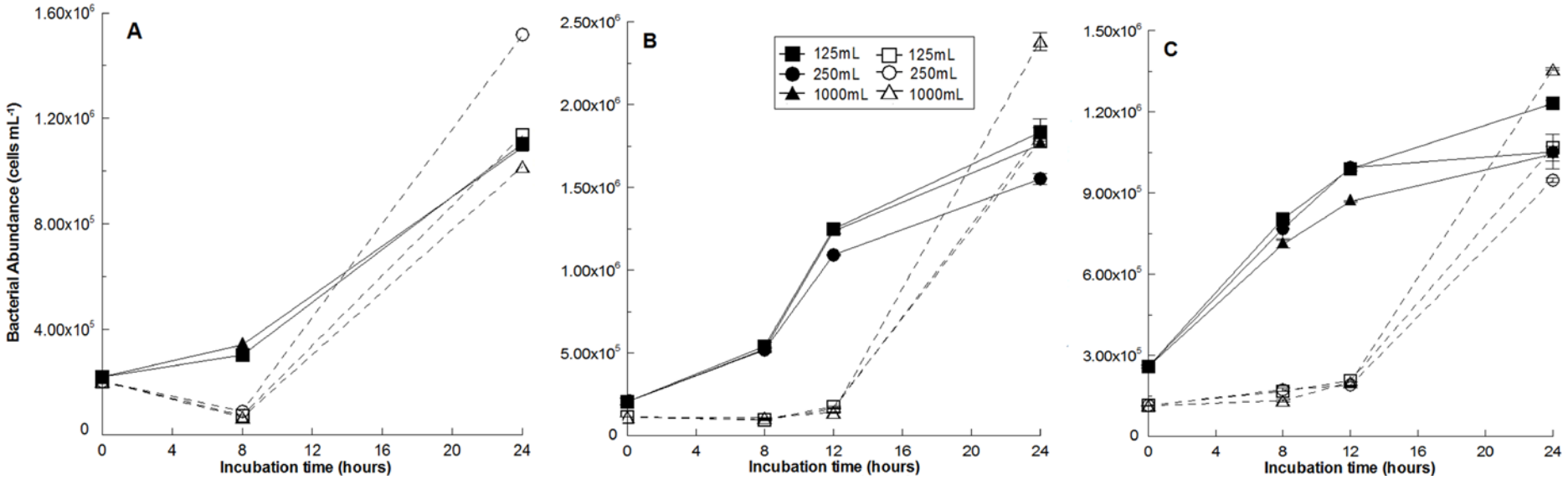

Figure 6. Changes in the abundance of bacteria during volumetric bottle effect experiments in Expt 1 (A), Expt 2 (B) and Expt 3 (C). Continuous line shows the whole planktonic community and dotted line the filtered bacterial community. Note the different scales on the *y* axes.

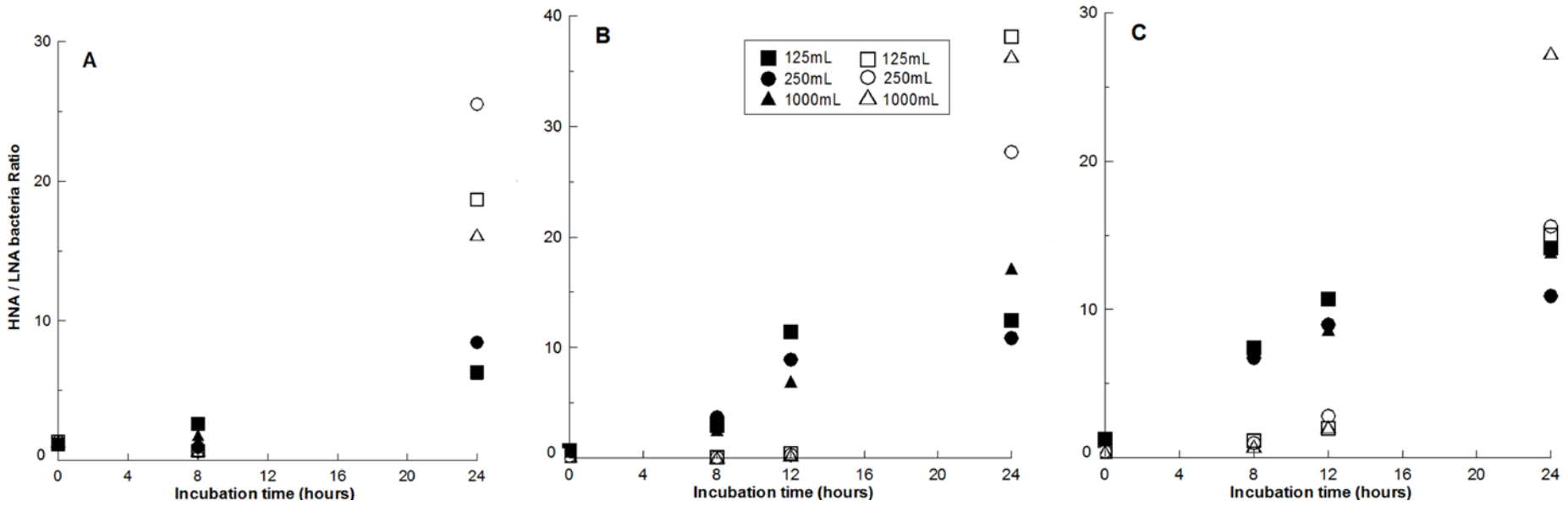

Figure 7.Temporal evolution of the High DNA and Low DNA bacteria ratio (HNA:LNA ratio) during volumetric bottle effect in Expt 1 (A), Expt 2 (B) and Expt 3 (C). Solid symbols refer to the whole planktonic community and empty symbols to the filtered bacterial community. Note the different scales on the *y* axes.

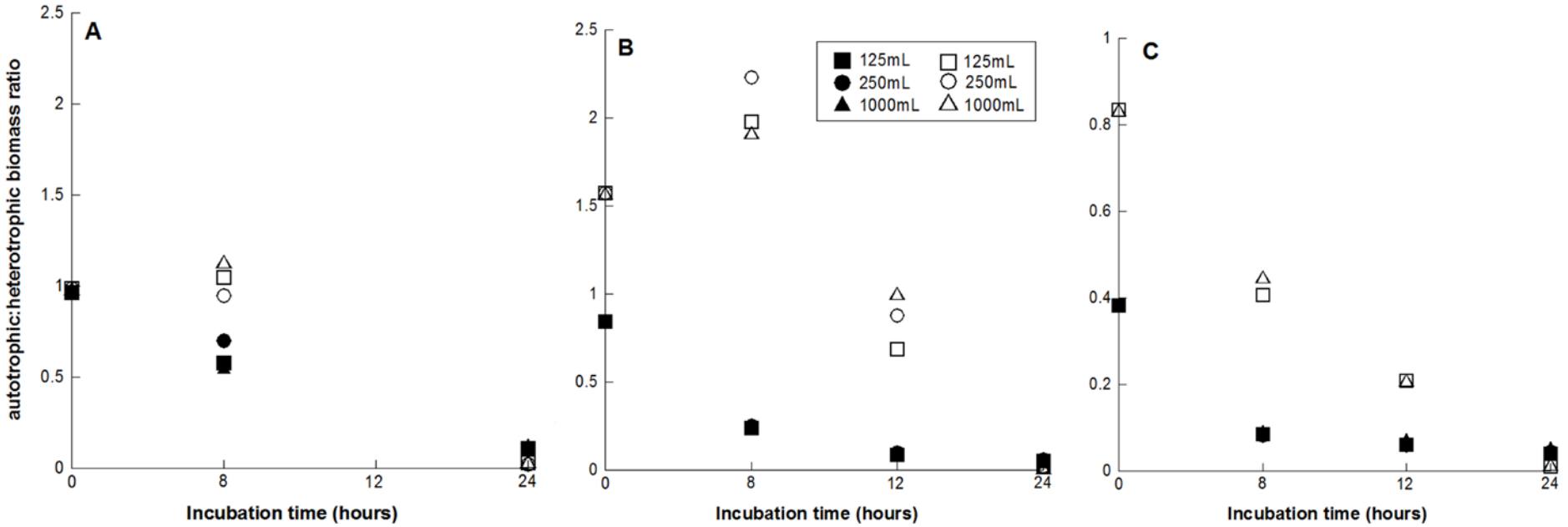

Figure 8.Temporal evolution of the autotrophic: heterotrophic biomass ratio during volumetric bottle effect in Exp1 (A), Expt 2 (B) and Expt 3(C). Solid symbols refer to the whole planktonic community and empty symbols to the filtered bacterial community. Note the different scales on the *y* axes.

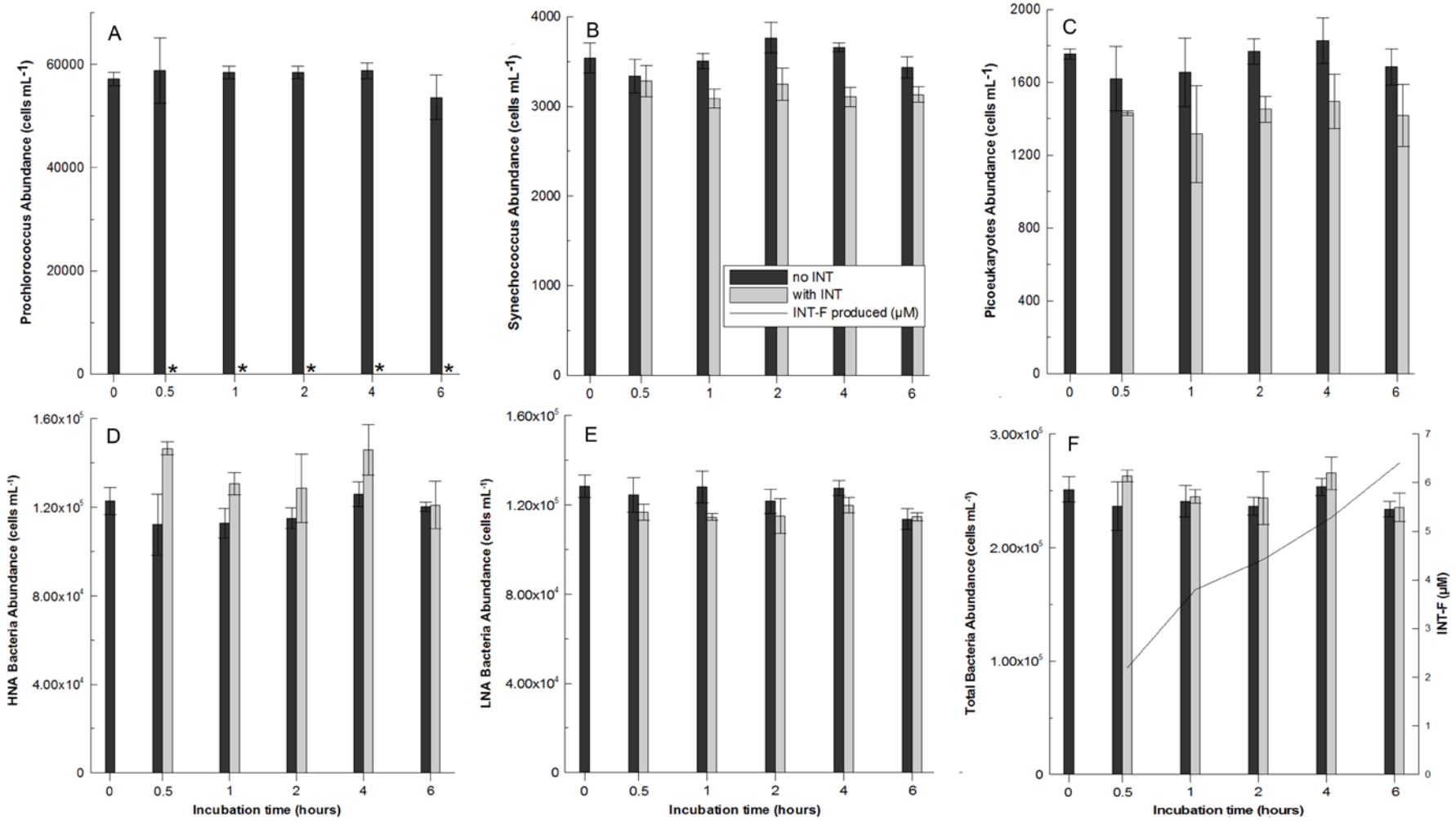

Figure 9. Cellular abundance of picophytoplankton (*Prochlorococcus*, *Synechococcus* and picoeukaryotes) and heterotrophic bacteria (HNA and LNA) during the toxicity assay with INT

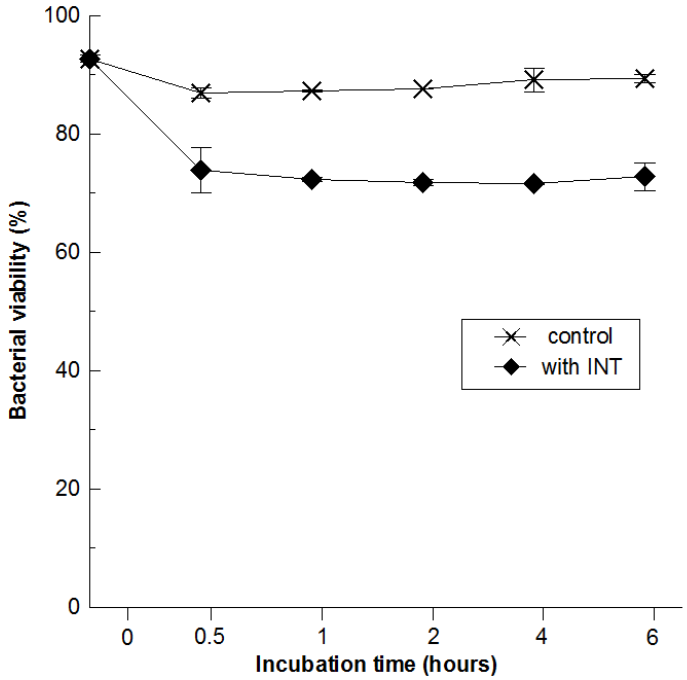

Fig10. Bacterial viability (%) during the toxicity assay with INT

Table 1. Conversion factors used to obtain the picoplankton biomass in our study area, described by Montero (2012)

Cells	Ø (μm)	biovolume (μm^3)	biomass $fgCcel^{-1}$
HNA bacteria	0.46	0.05	10
LNA bacteria	0.63	0.13	26
Prochlorococcus	0.68	0.16	33
Synechococcus	0.97	0.48	96
Picoeukaryotes	0.85	0.32	64

Table 2. Cell viability (NADS) and total organic carbon (TOC) for Expt 1

		WHOLE PLANKTONIC COMMUNITY (C)		1.2µm FILTERED COMMUNITY (F)	
Bottle (mL)	Time (h)	NADS %alive	TOC (µM)	NADS %alive	TOC (µM)
125	0	94.83	87.5	95.78	117.97
	8		70.64		116.38
	24		76.86		82.63
250	0	94.83	87.5	95.78	117.97
	8		81.54		94.65
	24		77.17		111.29
1000	0	94.83	87.5	95.78	117.97
	8		76.71		122.44
	24		72.04		83.39

Table 3. Cell viability (NADS) and total organic carbon (TOC) for Expt 2

		WHOLE PLANKTONIC COMMUNITY (C)		1.2µm FILTERED COMMUNITY (F)	
Bottle (mL)	Time (h)	NADS %alive	TOC (µM)	NADS %alive	TOC (µM)
125	0	80.5	88.16	91.28	110.5
	8	92.41	100.13	86.23	115.64
	12	89.81	92.72	82.85	93.97
	24		65.92		88.29
250	0	80.5	88.16	91.28	110.5
	8	80.72	79.6	89.24	103.57
	12	89.01	67.34	85.61	95.93
	24		73.69		82.09
1000	0	80.5	88.16	91.28	110.5
	8	86.5	83.02	76.8	111.24
	12	85.32	61.33	81.39	86.61
	24		78.75		95.85

Table 4. Cell viability (NADS) and total organic carbon (TOC) for Expt 3

		WHOLE PLANKTONIC COMMUNITY (C)		1.2µm FILTERED COMMUNITY (F)	
Bottle (mL)	Time (h)	NADS %alive	TOC (µM)	NADS %alive	TOC (µM)
125	0	84.89	79.87	91.35	97.45
	8	91.71	81.49	68.71	90.76
	12	94.52	99.51	82.49	107.35
	24		87.92		84.4
250	0	84.89	79.87	91.35	97.45
	8	87.51	79.33	69.01	91.57
	12	86.9	96.66	70.14	84.72
	24		67.53		78.93
1000	0	84.89	79.87	91.35	97.45
	8	81.1	81.04	64.8	108.71
	12	85.94	96.51	71.65	94.52
	24		81.02		94.53