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Estimation of bacterioplankton activity in Tasmanian coastal waters and between Tasmania and Antarctica using stains

A. T. Davidson*, P. G. Thomson, K. Westwood, R. van den Enden

Australian Antarctic Division, Department of the Environment and Heritage, and Antarctic Climate and Ecosystems Cooperative Research Centre, Channel Highway, Kingston, Tasmania 7050, Australia

ABSTRACT: Various stains have recently become available that detect bacteria with compromised cell membranes and high esterase or respiratory activity. However, the validity of these stains for determining concentrations of live, dead and active marine bacteria is the subject of debate. We used BacLight™ (Molecular Probes) to stain bacteria with intact membranes green, due to the fluorescence of SYTO®9, while 'leaky' bacteria that were permeable to propidium iodide (PI) stained red. Bacteria with high metabolic activity were stained using 6-carboxy fluorescein diacetate (6CFDA) or 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). Total bacteria concentration was determined using 4′,6 diamidino-2-phenylindole (DAPI) and BacLight™ (SYTO®9 + PI-stained bacteria). Comparison of stains in Tasmanian coastal waters showed that total concentrations of bacteria obtained using DAPI were not significantly different from those using BacLight™ at the concentrations used in our field study. Lower concentrations of BacLight™ reduced the concentration of PI-stained bacteria but not that of those stained by SYTO®9. Concentrations of metabolically active bacteria obtained using CTC and 6CFDA were equivalent to those of viable, culturable bacteria obtained using most probable number. Bacterioplankton activity in the Southern Ocean between Tasmania and East Antarctica during austral winter 1999 and summer 2001 was investigated using these stains. SYTO®9-stained cells commonly comprised <30% of total bacteria, while the remainder stained with PI. CTC- or 6CFDA-stained bacteria comprised around half of the SYTO®9-stained bacterial concentration. Significant positive correlation was observed between concentrations of total bacteria, seawater temperature (mainly due to the correlation between temperature and concentrations of PI-stained bacteria) and *in situ* chlorophyll fluorescence (mainly due to the correlation between *in situ* chlorophyll fluorescence and concentrations of highly active bacteria). Thus, bacterioplankton in the Southern Ocean are not a homogeneous community of active cells. As bacterial metabolism determines the extent to which bacteria are involved in respiration, remineralisation and the microbial loop, our findings are of major significance for understanding pelagic carbon flow and nutrient cycling in the Southern Ocean.

KEY WORDS: Antarctic · Marine · Bacteria · Activity

INTRODUCTION

Bacteria are key components of marine microbial food webs. They play a vital role in nutrient cycling and are the principal route of carbon flow in aquatic ecosystems (Azam 1998, Pomeroy & Weibe 1998). Estimating total bacterial concentration became routine and accurate with the introduction of such nucleic acid stains as Acridine Orange and 4′,6-diamidino-2-phenol indole (DAPI) (e.g. Hobbie et al. 1977, Porter & Feig 1980). However, these stains do not discriminate between active, viable, dormant and dead bacteria. Studies using these stains commonly assumed that natural bacterioplankton assemblages comprised a homogeneous population with uniform physiological and ecological properties (e.g. Smith 1998). It is now appar-

*Email: andrew.davidson@aad.gov.au

ent that such assumptions are invalid (Gasol et al. 1995, del Giorgio et al. 1996, Smith 1998, Choi et al. 1999, B. Sherr et al. 1999, E. Sherr et al. 1999).

The metabolic activity of bacteria largely determines their contribution to respiration, remineralisation and, thus, their role in the microbial loop (del Giorgio et al. 1996, Gasol & del Giorgio 2000). The availability of nutrients for marine bacterioplankton is highly variable in space and time (Azam 1998). As a result, natural bacterial communities exhibit a range of physiological states including dead, live, viable-nonculturable, dormant and active, and can cycle in and out of high metabolic activity (Stevenson 1978, Sieracki et al. 1999, E. Sherr et al. 1999, 2001). Choi et al. (1996) reported that 50 to 93% of marine bacteria have compromised cell membranes and are likely to be dead. However, Schumann et al. (2003) showed that the proportion of the total bacterial population that stained as dead changed due to the molecular size and charge of the stain used to determine membrane permeability. Similarly, the proportion of bacterial communities that are metabolically active varies with the methods used and nutrient availability, but is commonly around 2 to 10% for unpolluted marine waters (Rodriguez et al. 1992, Gasol et al. 1995, Choi et al. 1996, del Giorgio et al. 1996, Ullrich et al. 1996, Yamaguchi & Nasu 1997, Smith 1998, E. Sherr et al. 1999). Recently, Smith & del Giorgio (2003) questioned the validity of restricting the physiological state of bacterioplankton to such categories as active or inactive, instead proposing that they should be viewed as a continuum of physiological states.

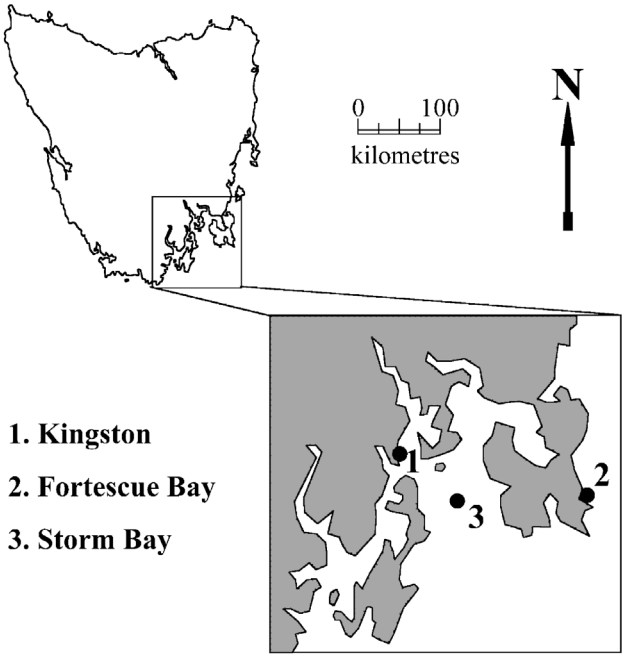

Fig. 1. The 3 sites around southeastern Tasmania, Australia, at which samples were obtained to compare bacterial stains

Metabolically active bacteria are reportedly larger and more heavily grazed by protozoa than dormant cells (e.g. Monger & Landry 1992, Gasol et al. 1995, del Giorgio et al. 1996, Hahn & Höfle 2001). Conversely, bacterial dormancy during nutrient limitation reportedly constitutes a refuge from microheterotrophic grazing and maintains a bacterioplankton community that can capitalise on the chance appearance of nutrients (Jürgens & Güde 1994). This refuge helps bacterial concentrations to remain remarkably constant, despite substantial annual changes in nutrient availability and grazing mortality (Gasol et al. 1995). Thus, determining total bacteria is likely to give a poor approximation of the concentration of bacteria that are contributing to respiration remineralisation and the microbial loop.

No consensus has been reached on the effectiveness of stains used to discriminate live, dead and active bacteria (Karner & Fuhrman 1997, Yamaguchi & Nasu 1997, Nebe-von-Caron et al. 1998, Ullrich et al. 1999). Here we compared the effectiveness of DAPI and BacLight™ (SYTO®9 + propidium iodide [PI]) staining for determining concentrations of total bacteria. PI is commonly reported to reliably stain cells that are dead and has been widely used to determine concentrations of 'dead' aquatic bacteria (e.g. Choi et al. 1996, Nebe-von-Caron et al. 1998, Williams et al. 1998, Howard-Jones et al. 2001). We compared concentrations of total bacteria that were stained by DAPI and BacLight™. Using BacLight™, we then determined concentrations of total bacteria that were permeable to PI. We also compared concentrations of metabolically active bacteria, obtained using 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 6-carboxy fluorescein diacetate (6CFDA), with the standard microbiological method of most probable number (MPN). We then applied these stains to measure concentrations of total bacteria using DAPI and BacLight™, and concentrations of bacteria stained by SYTO®9, PI, CTC or 6CFDA, in the Southern Ocean between Tasmania and East Antarctica on transects during austral winter 1999 and summer 2001.

MATERIALS AND METHODS

Sampling for stain comparison. Three independent replicate samples of natural marine microbial assemblage were collected into 3 Decon™-washed, Milli Q-rinsed, sterile 1 l Schott™ bottles from surface waters at Kingston Beach, Fortescue Bay and Storm Bay (Fig. 1), southeastern Tasmania, Australia. Samples were

returned to the laboratory at ambient seawater temperature in the dark and, within 4 h of collection, were prepared to determine the concentrations of bacteria stained by SYTO®9, PI, DAPI, CTC and 6CFDA at room temperature (0 to 6°C above ambient). Each replicate sample from each location was mixed and four 10 ml subsamples aseptically transferred to sterile Falcon® tubes and stained (as below). A further eight 100 µl subsamples were obtained to determine the concentration of viable bacteria by MPN (see 'Most probable number' below).

Samples were also obtained (as above) from Kingston Beach to determine the effect of changes in BacLight™ concentration on the concentrations of PI- and SYTO®9-stained bacterioplankton. PI and SYTO®9 were added at a ratio of 7:3 by volume using concentrations of PI from 5 to 200 µg ml^{-1} and of SYTO®9 from 2.9 to 114 µg ml^{-1}. Samples were then incubated for 30 min in the dark at room temperature (–1 to +5°C of ambient). The concentrations of bacteria stained by PI and SYTO®9 were determined by microscopy (see 'Microscopy' below) and plotted against the BacLight™ concentration.

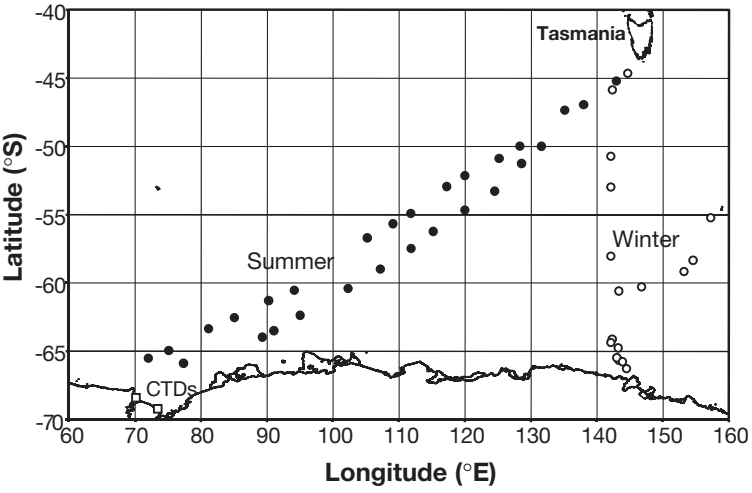

Fig. 2. Sampling sites in the Southern Ocean at which surface samples (○) were obtained during winter (July to September) 1999, and at which surface samples (●) and CTD profiles (□) were obtained during summer (January to March) 2001

Southern Ocean sampling. Measurements of bacterial concentrations and physical parameters were obtained from the RV 'Aurora Australis' in latitudinal transects of the Southern Ocean during winter (July to September) 1999 and summer (January to March) 2001 (Fig. 2). Near-surface bacterial samples were obtained from the laboratory clean seawater supply (intake at 7 m depth). Vertical profiles of bacteria were obtained during summer 2001 by CTD at 2 sites off the Amery Ice Shelf, Prydz Bay, Antarctica (Fig. 2).

At each sample site, replicate samples of known volume (~10 ml) were stained at ±2°C of the ambient seawater temperature and counted by epifluorescent microscopy (see 'Microscopy' below). Temperature, salinity and *in situ* chlorophyll fluorescence were obtained from under-way measurements using a thermosalinograph and a flow-through 10-000R Turner Designs fluorometer which were cleaned and calibrated daily. These measurements were averaged over the 20 min of bacterioplankton sampling.

Staining procedures. To stain bacterioplankton with DAPI, 6 drops (approx. 0.2 ml) of 100 mg l^{-1} DAPI stock solution were added to 10 ml of seawater and incubated for 1 h in the dark at room temperature. Dual staining of bacterioplankton with BacLight™ was achieved by adding 7 µl ml^{-1} of 20 mM PI and 3 µl ml^{-1} of 3.34 mM SYTO®9 and incubating in the dark for 30 min at ±2°C of ambient. Stained samples were then filtered and counted (see 'Microscopy' below).

Staining of metabolically active bacteria by CTC was achieved by dissolving 4.7 mg of CTC into the 10 ml subsample and incubating in the dark for 4 h at ±2°C of ambient. Alternatively, subsamples were stained by adding 1 µl ml^{-1} of 1% (w/v) 6CFDA in acetone and incubating as above for 20 min.

Microscopy. Stained samples were filtered to dryness onto a 0.22 µm black polycarbonate Nucleopore™ filter over a 0.8 µm backing filter (Duropore, Millipore) and mounted in p-phenylenediamine antifade (Noble & Fuhrman 1998). Counts of stained bacteria were obtained from a randomly chosen microscope field at 1000× magnification using a Zeiss Axioscop equipped for epifluorescence. On average, ≥20 fields were counted for each sample at each site. However, fewer fields were occasionally counted where significant fading of fluorescence was encountered (minimum 10 fields). UV excitation (filter set 487902 with 365 nm exciter filter, 395 nm chromatic beam splitter and 420 nm barrier filter) was used for DAPI-stained bacteria. Blue light excitation (filter set 487909 with 450 to 490 nm exciter filter, 510 nm chromatic beam splitter and 520 nm barrier filter) was used for SYTO®9, PI, CTC and 6CFDA.

Most probable number. The MPN technique was used as an independent estimate of viable, culturable bacteria in the Tasmanian samples. Various culture media were tested for their capacity to support growth of natural marine microbial communities including half-strength seawater medium, seawater yeast extract medium, ZoBell's 2216 medium (Franzmann et al. 1987) and seawater nutrient medium (Bowman & Nichols 2002). Half-strength seawater medium gave

the most reproducible growth and greatest concentration of culturable bacteria using the MPN technique compared to the other media (Table 1) and was therefore used throughout this study. Using half-strength seawater medium, we then determined the concentration of viable, culturable bacteria by axenically pipetting 900 µl of sterile medium into sterile Falcon® Multiwell™ plates. Eight replicate inocula of 100 µl from each replicate sample from each site were aseptically transferred into wells containing 900 µl of sterile medium, mixed and transferred into the following 8 wells. This serial dilution was repeated, giving 8 replicate well columns with dilutions from 10^{-1} to 10^{-11}. The plates were incubated at room temperature (~20°C) and the positive/negative pattern of culture growth to extinction was recorded after a 2 wk period. The presence of bacterial growth at dilutions between 10^{-3} and 10^{-8} was then used to calculate the concentrations of viable, culturable bacteria using MPN Calculator™ Build 20 (http://members.ync.net/mcuriale/mpn/ VB6 version) and the results were cross-checked using a MPN BASIC computer programme (Koch 1994).

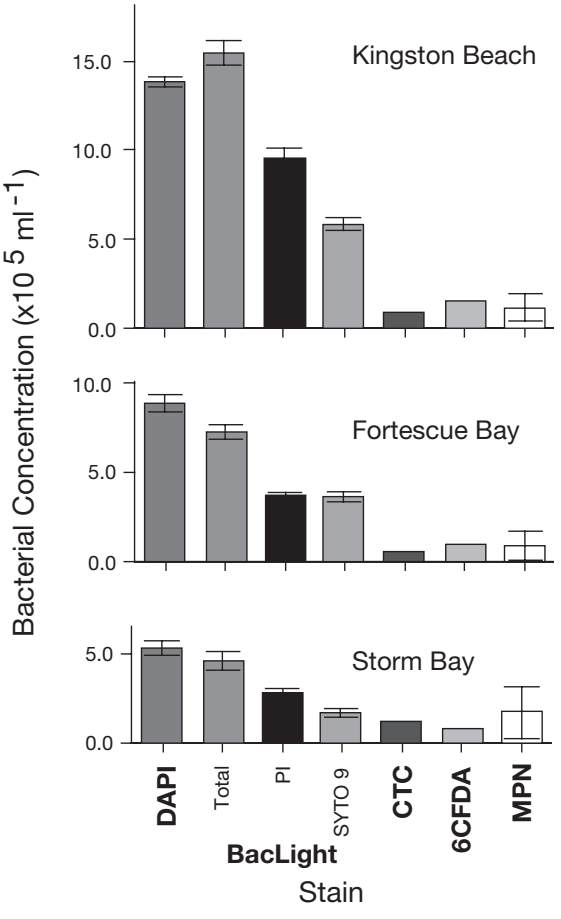

Fig. 3. Concentrations of bacteria from southeastern Tasmania stained using DAPI, SYTO®9 + PI (total), PI (dead), SYTO®9 (live), CTC and 6CFDA (active bacteria), and concentrations of viable, culturable bacteria obtained using MPN. Error bars represent ±1 SE

Table 1. Most probable number (MPN) of viable, culturable bacteria over dilutions from 10^{-3} to 10^{-8} in the media half-strength seawater (½ SW), ZoBell's 2216 (ZoBell's) and seawater nutrients (SW nutrients). No growth was observed at these dilutions using seawater yeast extract medium. UCL and LCL represent 95% upper and lower confidence limits, respectively, using MPN Calculator

Dilution	½ SW	ZoBell's	SW nutrients
MPN ml^{-1}	2.3×10^5	1.3×10^5	1.8×10^4
95% UCL ml^{-1}	5.2×10^5	2.9×10^5	4.0×10^4
95% LCL ml^{-1}	1.0×10^5	0.5×10^5	0.8×10^4

Statistical analyses. ***Stain comparison:*** Concentrations of bacteria stained by PI and SYTO®9 were plotted against the stain concentration. The least squares regression was plotted and the statistical significance of the regression determined from the correlation coefficient (r^2).

Differences in the concentrations of total bacteria using DAPI and SYTO®9 + PI (BacLight™), and active/viable bacteria using CTC, 6CFDA and MPN, were statistically compared by 2-way multivariate analysis of variance (MANOVA) within and between sample locations using Statistica®.

Southern Ocean samples: Concentrations of bacteria obtained using BacLight™ that were stained by SYTO®9 or PI were summed and the total concentration was compared by paired *t*-test with the concentrations of bacteria obtained using DAPI. Linear regressions were also performed to examine correlations between environmental variables and the concentration of bacteria obtained using each of the above stains.

RESULTS

Comparison of stains

Total bacteria. Pooled data for sites in Tasmanian waters showed that total bacterial concentrations obtained using BacLight™ (SYTO®9 + PI) were not significantly different from those obtained using DAPI ($p_F = 0.36$) (Fig. 3). However, significant differences were observed between the concentrations of total bacterial obtained using DAPI and BacLight™ at Kingston Beach and Fortescue Bay ($p_F \leq 0.00014$) (Fig. 3).

PI-stained bacteria. Concentrations of PI-stained bacteria were significantly different among sampling

locations ($p_F < 0.01$), accounting for 61.8, 50.5 and 61.6% of the total bacterial concentration at Kingston Beach, Fortescue Bay and Storm Bay, respectively, and their concentrations generally increased with increasing total bacterial concentration (Fig. 3).

PI- and SYTO®9-stained bacteria. Concentrations of PI-stained bacteria increased logarithmically with increasing BacLight™ concentration (Fig. 4). Concentrations of PI-stained cells increased approximately 3-fold between PI concentrations of 5 and 150 µg ml^{-1} and coincident changes in SYTO®9 from 2.9 to 64.3 µg ml^{-1}. However, no significant trend was observed in concentrations of SYTO®9-stained bacteria with increasing BacLight™ concentration (Fig. 4).

CTC and 6CFDA. Bacteria stained by CTC comprised 6.7, 6.8 and 23.1% of the total bacterial concentration at Kingston Beach, Fortescue Bay and Storm Bay, respectively (Fig. 3). CTC stained 11.0 and 11.8% of bacteria (around half the proportion stained by 6CFDA) at Kingston Beach and Fortescue Bay, respectively, but stained 15% of bacteria (more than were stained by 6CFDA) at Storm Bay (Fig. 3). No significant difference was found in the concentrations of bacteria obtained using CTC and 6CFDA at each sampling location ($p_F = 0.14$, 0.27 and 0.31 for Kingston Beach, Fortescue Bay and Storm Bay, respectively) (Fig. 3). Furthermore, concentrations of active bacteria did not differ significantly between sampling locations ($p_F > 0.18$ for CTC and $p_F > 0.10$ for 6CFDA).

Most probable number. We found that there was no significant difference between the concentration of bacteria that were active (see 'CTC and 6CFDA' above) and concentrations of viable, culturable bacteria obtained by MPN ($p_F > 0.316$) (Fig. 3).

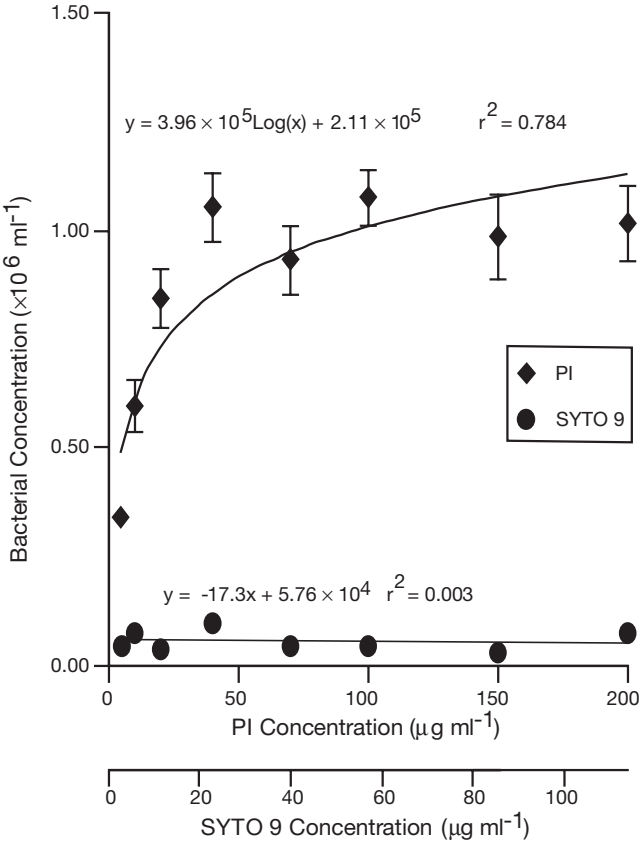

Fig. 4. Concentrations of bacteria stained by PI and with increasing concentrations of BacLight™. Least squares regression formulae for both PI and SYTO® 9 are shown. Error bars represent ±1 SE

Southern Ocean environment

Between Tasmania and the polar frontal zone on the winter voyage, salinity decreased from 34.9 to 33.7 practical salinity units (psu) (Fig. 5A). The salinity then increased south of the polar front, reaching around 34.5 psu in the marginal ice zone (MIZ). Temperature declined from around 11°C near Tasmania to around 6°C in the sub-Antarctic zone (SAZ). South of the polar front, water temperatures declined with increasing latitude from 2°C to a minimum of around −2°C beneath sea ice (Fig. 5B). Unlike the southward transect, temperatures on the northward transect rose to around 5°C at 55° S. Like temperature, *in situ* chlorophyll autofluorescence decreased southward from 24 fluorescent units (flu) at the most northerly station to minimum values around 2 flu beneath the sea ice (Fig. 5C). There was no evidence of enhanced chlorophyll fluorescence at frontal zones or in the MIZ.

Between Tasmania and the polar front on the summer voyage, salinity declined from 34.7 to 33.7 psu (Fig. 5D). Salinity remained relatively constant in the polar open ocean zone (POOZ) south of the polar front but declined in the MIZ, especially on the southward transect between 62 and 65° S (Fig. 5D). Temperature declined southward from around 14°C at 45° S to around 0°C in the MIZ (Fig. 5E). Chlorophyll fluorescence was highly variable with latitude during summer, with high fluorescence around frontal zones and in the MIZ on the northward transect. Otherwise, fluorescence generally decreased with increasing latitude (Fig. 5F).

Southern Ocean bacterioplankton

Total bacterioplankton. Concentrations of total bacteria, obtained using DAPI or BacLight™, were substantially lower during winter (Fig. 6A,B) than in summer (Fig. 6C,D), particularly south of the polar front. Concentrations north of the polar front during winter (Fig. 6A) (around 2.3×10^5 ml^{-1}) were commonly greater

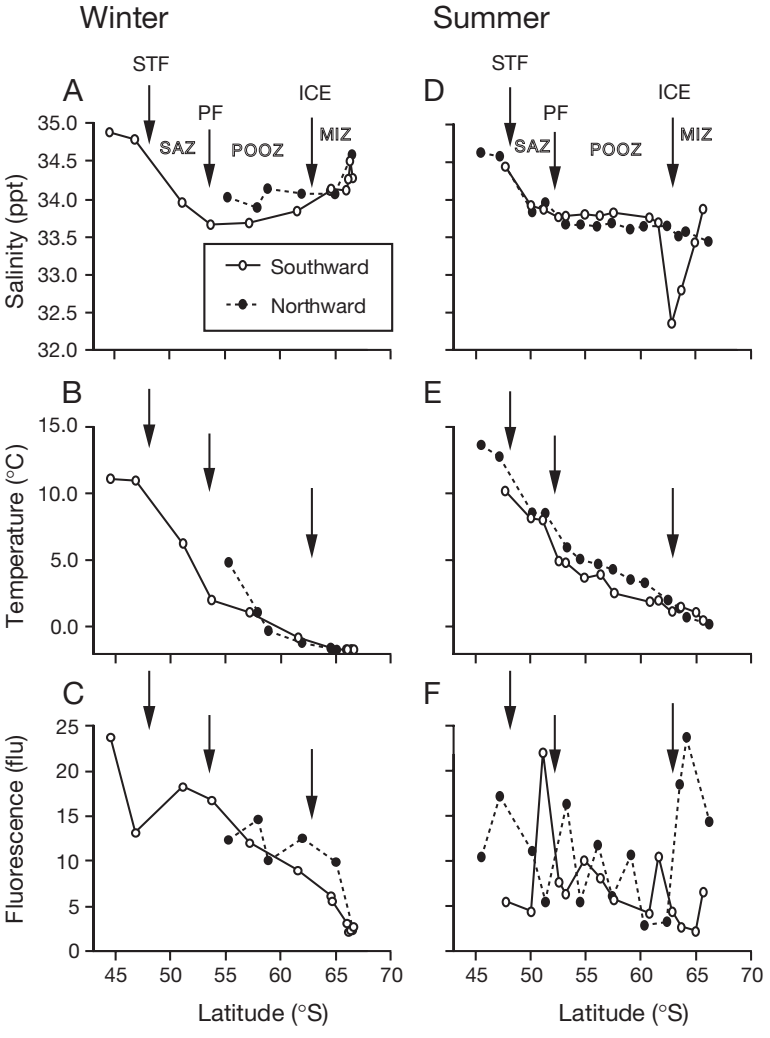

Fig. 5. (A,D) Salinity, (B,E) temperature and (C,F) *in situ* chlorophyll fluorescence (flu: fluorescence units) at sampling stations in northward and southward transects of the Southern Ocean during (A–C) winter 1999 and (D–F) summer 2001. STF: sub-tropical front; PF: polar front; ICE: sea ice; SAZ: sub-Antarctic zone; POOZ: polar open ocean zone; MIZ: marginal ice zone

than those obtained on the southward transect in summer (Fig. 6C), but substantially less than on the northward transect toward the end of summer (up to 8.0×10^5 ml^{-1}) (Fig. 6D). Total bacterial concentrations south of the polar front during winter were low ($<1.2 \times 10^5$ ml^{-1}), especially on the southward transect in July. In summer, concentrations were 2 to 5 times higher, with highest values commonly occurring near the polar front or in the MIZ. The exception was the high concentrations of total bacteria on the southward transect at 60.8° S.

Concentrations of total bacterioplankton were significantly correlated with concentrations of PI-, SYTO®9- and 6CFDA-stained bacteria, explaining 86.6, 28.6 and 45.2% of the variance in total bacterial concentration, respectively (Table 2). Total bacterial concentrations also significantly correlated with temperature and *in situ* chlorophyll fluorescence, explaining 34 and 13.1% of the variance, respectively (Table 2).

SYTO®9- and PI-stained bacterioplankton. Though total bacterial concentrations increased around 2-fold between winter and summer, PI-stained bacteria increased around 3-fold (Fig. 6). The exception was north of the polar front, where concentrations in the southward transect were lower during summer than winter. In contrast, SYTO®9-stained cell concentrations were low in all transects ($<0.5 \times 10^5$ cells ml^{-1}), except on the northward transect during summer, when concentrations were around 1.0×10^5 cells ml^{-1}. Maxima in SYTO®9-stained bacteria occurred at the polar front and in the MIZ (Fig. 6).

Concentrations of PI-stained bacteria were highly variable with location and season and were lower in winter than in summer (Fig. 6). In the southward winter transect (July), concentrations of PI-stained bacteria north of the polar front were $>1.7 \times 10^5$ cells ml^{-1}, with SYTO®9-stained cells commonly comprising only around 20% of the total bacterial concentration (Fig. 6A). South of the polar front, concentrations of PI-stained bacteria in the POOZ decreased to $<0.74 \times 10^5$ cells ml^{-1} and SYTO®9-stained bacteria comprised around half of all bacteria. Concentrations of PI-stained bacteria in the MIZ during winter were around 1.0×10^5 cells ml^{-1}, with SYTO®9-stained bacteria commonly comprising ≤20% of all bacteria (Fig. 6B).

Concentrations of PI-stained bacteria on the summer southward transect were highly variable. High concentrations occurred near Tasmania and at the polar front, and there was also a slight increase in the MIZ (Fig. 6C). However, there was an unusually high value of 4.16×10^5 cells ml^{-1} at the southern limit of the POOZ (60.84° S). Commonly ≤10% of all bacteria on the southward summer transect were stained by SYTO®9 alone. On the northward summer transect, the PI-stained bacterial concentration was highest (7.33×10^5 cells ml^{-1}) at the sub-tropical front, lowest (1.72×10^5 cells ml^{-1}) in the POOZ and increased (4.35×10^5 cells ml^{-1}) in the MIZ (Fig. 6D). SYTO®9-stained cells commonly comprised 20 to 30% of all bacteria.

Pooled data from all transects showed that concentrations of PI-stained bacteria correlated significantly with seawater temperature and *in situ* chlorophyll flu-

orescence (Table 2). Temperature and *in situ* fluorescence explained 34 and 11.2%, respectively, of the variance in the concentration of PI-stained bacteria. The environmental variables we measured did not correlate significantly with concentrations of SYTO®9-stained bacteria (Table 2).

CTC- and 6CFDA-stained bacteria. In latitudinal transects in summer, concentrations of CTC- and 6CFDA-stained bacteria averaged 11.0 and 13.5% of the total bacterial concentration, respectively. Concentrations of metabolically active bacteria were only determined during summer and only 4 samples were stained using CTC. On the southward summer transect, concentrations were variable and were occasionally higher than those stained by SYTO®9 alone. On the northward transect, concentrations of 6CFDA-stained bacteria, though variable, were always less than the concentrations of SYTO®9-stained cells. CTC- and 6CFDA-stained bacteria comprised >50% of the SYTO®9-stained cell concentration in the MIZ and in waters immediately south of the polar front, but were commonly around 20% of the live cell concentration in the southern POOZ (57.3 to 63.6° S).

Concentrations of 6CFDA-stained bacterioplankton were significantly correlated with *in situ* chlorophyll fluorescence but not seawater temperature (Table 2). The few samples stained by CTC did not significantly correlate with temperature or chlorophyll fluorescence.

Vertical profiles. Vertical profiles of bacterioplankton off the Amery Ice Shelf showed that concentrations of SYTO®9- and PI-stained bacteria varied at depths of <80 m, but that these changes were not consistent with depth (Fig. 7). At the western CTD site (Fig. 2), active bacterial concentrations varied little in the upper water column and concentrations of SYTO®9-, PI- and 6CFDA-stained bacteria at the deepest depth (787 m) were approximately 25% of near-surface maxima. At the eastern CTD site (Fig. 2), 6CFDA-stained bacterial concentrations did not differ significantly from those of SYTO®9-stained bacteria. At the greatest depth sampled (1121 m), SYTO®9-, PI- and 6CFDA-stained bacterial concentrations had declined to 25, 36 and 45%, respectively, of their near-surface concentrations. Subsequent investigations using flow cytometry on a transect from Tasmania to Antarctica along 143° E longitude (data not shown) have shown that there is little change in total, SYTO®9-, PI-, CTC- or 6CFDA-stained bacterial concentrations in the euphotic zone (upper 120 m depth).

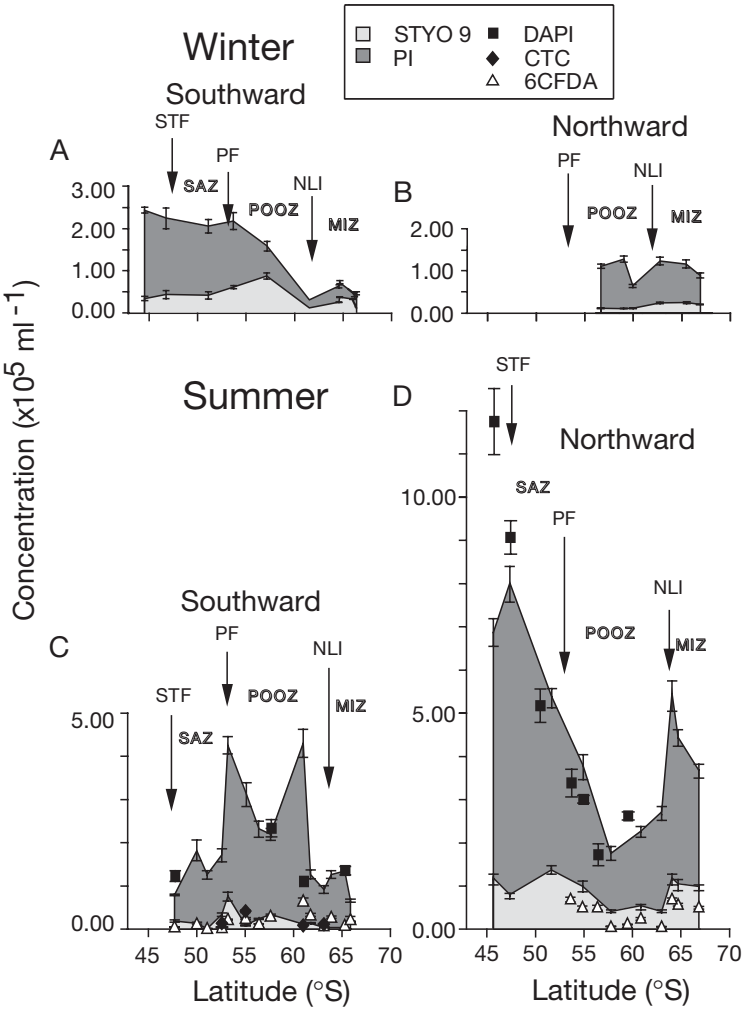

Fig. 6. Changes in the concentrations of bacteria that were stained by SYTO® 9 and PI during (A,B) winter 1999 and (C,D) summer 2001 transects of the Southern Ocean, and (C,D) concentrations of bacteria stained by DAPI, CTC and 6CFDA during summer. STF: sub-tropical front; PF: polar front; NLI: northern limit of ice on the transects. SAZ: sub-Antarctic zone; POOZ: polar open ocean zone; MIZ: marginal ice zone. Error bars represent ±1 SE

DISCUSSION

Each technique for estimating the abundance of viable or active cells in natural bacterial assemblages has advantages and disadvantages. Stains reportedly provide a rapid and ecologically valuable measure of bacterial activity (e.g. del Giorgio et al. 1996, B. Sherr et al. 1999, E. Sherr et al. 1999). However, the efficiency of staining can vary: some stains are toxic or inhibit bacterial metabolism; the concentration of metabolically active bacteria varies with the stain used; and stains reportedly correlate poorly with molecular probes using universal 16S rRNA probes, autoradiography and microbial culturing (Karner & Fuhrman 1997, Yamaguchi & Nasu 1997, Ullrich et al. 1999, Grégori et al, 2001, Howard-Jones et al.

Table 2. Correlation coefficients (r^2), degrees of freedom (df) and probability (p) from regressions of bacterial concentrations stained by DAPI, BacLight™, CTC and 6CFDA against environmental variables. Salinity did not significantly correlate with bacterial concentrations ($p > 0.50$). *Statistically significant probabilities

Regression variables	r^2	df	p
Log Total vs Log Dead	0.866	38	$p \ll 0.001$*
Log Total vs Log Live	0.286	39	$p < 0.001$*
Log Total vs. Log Active	0.452	20	$p < 0.001$*
Fluorescence vs Log Total	0.131	39	$0.01 < p < 0.02$*
Temperature vs Log Total	0.340	39	$p < 0.001$*
Fluorescence vs Log Live	0.025	39	$0.2 < p < 0.5$
Temperature vs Log Live	0.078	39	$0.05 < p < 0.10$
Fluorescence vs Dead	0.112	38	$0.02 < p < 0.05$*
Temperature vs Dead	0.340	38	$p < 0.001$*
Fluorescence vs 6CFDA	0.294	18	$0.01 < p < 0.02$*
Temperature vs 6CFDA	0.019	18	$p > 0.50$
Fluorescence vs CTC	0.118	2	$p > 0.50$
Temperature vs CTC	0.494	2	$0.20 < p < 0.50$

2001, Servais et al. 2001, Smith & del Giorgio 2003). Molecular probes such as universal 16S rRNA probes and autoradiography are more time consuming and technically demanding than staining techniques (e.g. Karner & Fuhrman 1997), thereby limiting their application in extensive field studies. Finally, many fastidious bacteria are 'nonculturable' and estimates of their viability are not possible using microbial culture techniques.

We incubated bacteria with the various stains at *in situ* temperatures. Chattopadhyay (2000) suggested that aquatic Antarctic bacterioplankton may adopt a viable but nonculturable state to survive low ambient temperatures. However, using 3 h incubations with the same stain concentrations as our study, Yager et al. (2001) showed that up to 84% bacteria could be stained by CTC during Arctic phytoplankton blooms at –1.5°C. Thus, bacterioplankton can be stained as metabolically active at low temperatures. Our results represent the concentrations of bacteria that were permeable to PI or had sufficient metabolic activity to be stained by CTC or 6CFDA at near-ambient temperatures, rather than those capable of activity at near-optimal temperatures.

Comparison of stains

While samples from around southeastern Tasmania were maintained at ambient seawater temperature in the dark after collection and analysed within ≤4 h of collection, changes in the number and/or physiological state of bacteria may have occurred during that time.

Total bacteria

Like Choi et al. (1996), we found that bacteria stained by DAPI were stained equally well by BacLight™ at the stain concentrations used in this study. Others have reported that BacLight™ only stains around 60% of bacteria (Gasol et al. 1999, Schumann et al. 2003). Product information for BacLight™ indicates that PI can quench the green fluorescence of SYTO®9-stained bacteria and may cause them to fluoresce red (www.probes.com/servlets/product?item=7012). We found that increasing concentrations of BacLight™ lead to an increase in the concentration of PI-stained bacteria but caused no significant trend in the concentration of bacteria that were impermeable to PI. Thus, lower concentrations of BacLight™ than those used here are likely to underestimate the total bacterial concentration.

The significant differences in total bacterial concentrations among sampling locations around southeastern Tasmania were likely due to environmental differences. The differences in total bacterial concentrations at Kingston Beach and Fortescue Bay found between DAPI- and BacLight™-stained samples were likely due to the high variance between independent replicates.

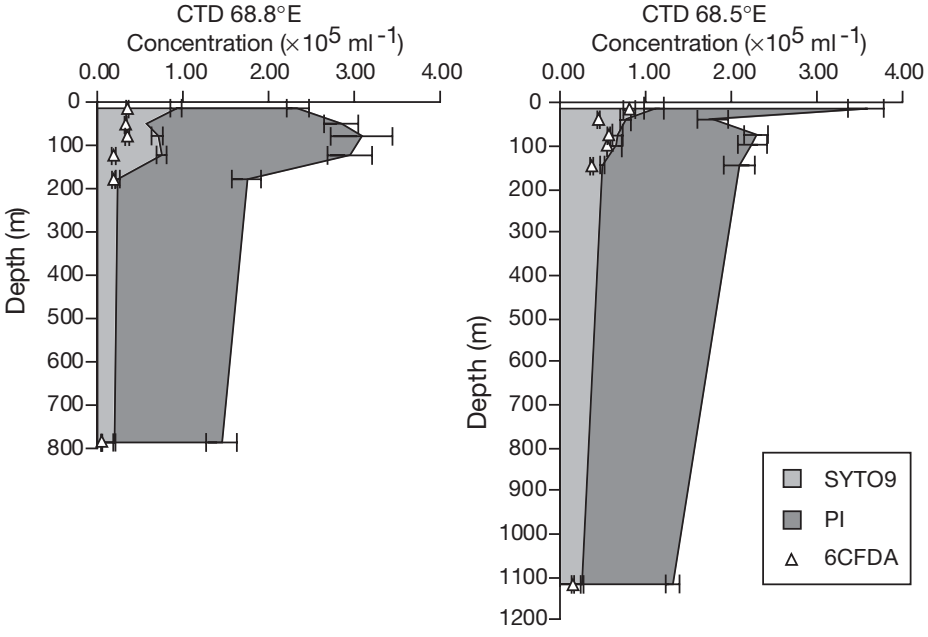

Fig. 7. Vertical profiles of concentrations of bacteria stained with SYTO®9 (live), PI (dead) and 6CFDA (active). Error bars represent ±1 SE

BacLight™-stained bacteria

Though used to determine 'dead' bacteria, PI can permeate the membranes of cells that have recently divided or been exposed to physical, chemical and mechanical changes or starvation (e.g. Choi et al. 1996, Nebe-von-Caron et al. 1998, Williams et al. 1998, Boulos et al. 1999, Howard-Jones et al. 2001). Many studies using PI have been performed on cultured aquatic bacteria (e.g. López-Amorós et al. 1995, Nebe-von-Caron et al. 1998, Williams et al. 1998, Howard-Jones et al. 2001), but only a few have reported using this stain on natural communities (Choi et al. 1996, Boulos et al. 1999, Schumann et al. 2003).

Williams et al. (1998) showed that the proportion of bacteria stained by PI was influenced by temperature, pH, staining time and PI concentration. In the same way as Williams et al. (1998), we stained the samples with PI for 30 min at the ambient pH of seawater (~pH 8). Though Williams et al. (1998) recommended staining at pH 6.5, their concentration at this pH did not differ significantly from that at pH 8. We used concentrations of BacLight™ similar to those of Boulos et al. (1999) and Schumann et al. (2003), thereby staining bacteria at a PI concentration of 93.6 µg ml^{-1} (around 20 times the concentration recommended by Williams et al. [1998] and approximately twice that used by Choi et al. [1996]). We found that lower concentrations caused faint PI fluorescence that faded too quickly to obtain field counts, despite use of the antifade p-phenylenediamine (Noble & Fuhrman 1988). This fading may have been due to our lack of preliminary rinsing with buffer (Choi et al. 1996, Williams et al. 1998). However, we were unable to perform this rinse on the research vessel and Boulos et al. (1999) did not rinse BacLight™-stained samples. Omission of this rinse step also avoided what Porter et al. (1995) described as a potentially damaging and inefficient centrifugation and/or resuspension.

At the high BacLight™ concentrations in our study, PI stained most bacteria in Tasmanian coastal waters. Concentrations of PI-stained bacteria were lower than those reported by Choi et al. (1996) from Oregon coastal waters. However, we found significant correlation between concentrations of PI-stained and total bacterial concentrations. Such results are unlikely if all PI-stained bacteria were dead. Bacterial permeability to PI is reportedly higher than some other stains that detect compromised membranes (Schumann et al. 2003). However, concentrations of bacteria stained by PI were similar to those obtained from a subsequent transect of the Southern Ocean using a different stain that does not permeate intact cell membranes (SYTOX®, Molecular Probes) (data not shown).

Williams et al. (1998) reported that high concentrations of PI caused some viable cells with 'leaky' membranes to be weakly stained. We also observed weak PI-staining of bacteria at low BacLight™ concentrations that may have resulted in quenching of SYTO®9 fluorescence, making them undetectable by microscopy (see 'Total bacteria' above). Boulos et al. (1999) reported that, in the absence of stress, BacLight™ gave SYTO®9-stained cell concentrations that were comparable to those stained by CTC. In addition, studies have shown that a significant proportion of 'dead' bacteria can be reactivated by addition of nutrients (e.g. Stevenson 1978, Luna et al. 2002). At the BacLight™ concentrations we used for our field samples, it is likely that PI stained 'leaky' bacteria, which were neither dead nor highly metabolically active. Our results showed that the concentrations of bacteria that were impermeable to PI were approximately twice that of active bacteria, indicating that BacLight™ detected bacteria at a lower metabolic activity than either CTC or 6CFDA. Thus, the high proportion of PI-stained cells in Tasmanian waters suggests that only a small fraction of bacterioplankton contributed to respiration, remineralisation and the microbial loop.

Active bacteria

CTC is widely and increasingly used to determine concentrations of bacteria with high metabolic activity in natural marine communities (e.g. Rodriguez et al. 1992, Gasol et al. 1995, Smith 1998, Choi et al. 1999, B. Sherr et al. 1999, E. Sherr et al. 1999). Some researchers find that CTC gives a sensitive, robust and ecologically meaningful estimate of bacterial activity (e.g. del Giorgio et al. 1996, B. Sherr et al. 1999). Others report that CTC can inhibit bacterial metabolism and may not permeate some bacterial membranes (Ullrich et al. 1996, 1999, Yamaguchi & Nasu 1997), resulting in a low proportion of active bacteria using CTC (Karner & Fuhrman 1997, Ullrich et al. 1999, Servais et al. 2001). However, B. Sherr et al. (1999) found that CTC is not toxic to bacteria at concentrations of 2 to 5 mM and most strains isolated from seawater are permeable to CTC. The low proportion of active bacteria obtained using CTC in comparison with other techniques (Karner & Fuhrman 1997) was likely due to the high metabolic activity required for bacteria to become stained by CTC (B. Sherr et al. 1999).

As a more sensitive indicator of metabolic activity, Yamaguchi & Nasu (1997) claimed that 6CFDA readily penetrated cell membranes and, in active cells, was hydrolysed by non-specific esterases to form the green-fluorescing 6-carboxy fluorescein. Like Yamaguchi & Nasu (1997), we found that concentrations of

CTC-stained bacteria were commonly less than those of 6CFDA-stained ones, but their concentrations were not significantly different. Thus, our results support the findings of B. Sherr et al. (1999) that 6CFDA principally stains bacteria with high metabolic activity and that high levels of esterase activity do not persist long after electron transport declines.

In Tasmanian coastal waters, concentrations of CTC- and 6CFDA-stained bacteria were not significantly different from viable concentrations obtained using MPN, indicating that CTC and 6CFDA appeared to stain viable, culturable bacteria. MPN is a standard microbiological technique for determining the concentration of viable bacteria in environmental samples (American Public Health Association 1980). However, MPN requires bacteria to be cultured and is therefore reliant on the suitability of the medium or media used in the study to support growth of the diversity of bacterial species existing in natural samples. Fastidious species can be difficult or impossible to grow (Nebe-von-Caron et al. 1998), and the resulting estimates of bacterial viability are characteristically lower than those obtained by other methods (Yamaguchi & Nasu 1997). It remains possible that the growth medium used in this study did not support growth of some bacteria, and the number of bacteria that are able to contribute to the bacterial activity is greater than we observed. However, our finding that concentrations of bacteria obtained using MPN, CTC and 6CFDA were similar indicates that a consistent proportion of the bacterial community were both active and culturable.

Smith & del Giorgio (2003) indicated that the widely held perception that most bacteria in natural aquatic communities are active is not uniformly supported in the literature, even using such sensitive techniques as microautoradiography and fluorescent *in situ* hybridisation. They persuasively argue that the differences in the percentage of active cells observed among methods occur because each method gives the number of active bacteria at different points along the continuum of metabolism from active to inactive. Our results indicate that CTC stains those bacteria with the greatest metabolic activity, followed by 6CFDA, SYTO®9 (PI-impermeable using BacLight™ then PI.

Southern Ocean bacterioplankton

Our results show that the metabolic activity and membrane permeability of natural bacterioplankton communities in the Southern Ocean are heterogeneous. To our knowledge, despite extensive study of bacterioplankton in the Southern Ocean (e.g. Rivkin et al. 1989, Cota et al. 1990, Bjørnsen & Kuparinen 1991, Moriarty et al. 1997, Carlson et al. 1999), there are no other published studies that estimate concentrations of viable or active bacterioplankton in these waters. Rather, estimates of bacterial activity in the Southern Ocean are derived from incorporation rates of radiolabelled amino acids. Rates of production are then normalised to concentrations of bacteria obtained using Acridine Orange or DAPI to determine cell-specific rates of bacterial production (e.g. Rivkin et al. 1989, Billen & Becquevort 1990, Lochte et al. 1997). We show that the inherent assumption that all bacteria contribute equally to this production is invalid.

Total bacteria

The biotic and abiotic environment directly or indirectly affects the abundance of bacterioplankton in the Southern Ocean. Like Sherr et al. (2001) in waters off Oregon, USA, we found that total bacterial concentration positively correlated with temperature and *in situ* chlorophyll fluorescence. Significant positive correlation was observed between total bacterial concentrations and concentrations of PI-, SYTO®9- and 6CFDA-stained bacterioplankton (see below), suggesting that bacteria in each of these metabolic states contributed to increases in the total bacterial concentration.

BacLight™-stained bacteria

Most of the bacteria in both summer and winter transects of the Southern Ocean were stained by PI with the BacLight™, suggesting that they had compromised or 'leaky' cell membranes. Choi et al. (1996) reported staining of 65 to 93% of bacterioplankton PI and concluded that these were dead. However, estimates of the relative proportion of dead bacteria vary greatly. Some studies show that 20 to 80% of bacterioplankton in natural marine assemblages assimilate radiolabelled amino acids (Douglas et al. 1987, Karner & Fuhrman 1997). Others report that 68 to 98% of bacteria lack organised nucleoids (Zweifel & Hagström 1995), 42% of free-living bacteria have compromised cell membranes and 24% lack any internal structure (Heissenberger et al. 1996). In addition, concentrations of SYTO®9-stained bacteria were occasionally less than those of CTC- or 6CFDA-stained cells. This may be due to counting errors or PI-staining of dividing cells and/or cells with intact membranes due to physical, chemical or mechanical factors that could induce temporary permeabilisation to PI (e.g. Nebe-von-Caron et al. 1998, Howard-Jones et al. 2001). Methods that accurately determine concentrations of dead marine bacterioplankton have not, as yet, been resolved.

Temperature and *in situ* chlorophyll fluorescence correlated positively with the concentration of PI-stained bacteria in the Southern Ocean. The positive correlation between temperature and concentrations of PI-stained bacteria suggest that higher temperatures support higher concentrations of bacteria, most of which have relatively low metabolic rates. This increase in bacteria may be due to a number of factors, the most likely of which is nutrient limitation (Stevenson 1978, E. Sherr et al. 1999, Sieracki et al. 1999). The positive correlation between *in situ* chlorophyll fluorescence and concentrations of both active (see 'Active bacteria' below) and PI-stained bacteria indicates that it is unlikely that all PI-stained bacteria are dead. Instead, PI staining of cells may be due to the inherent spatial and temporal heterogeneity of nutrition for bacteria in the marine environment (Azam 1998)

Counter-intuitively, while environmental variables (temperature and salinity) were positively correlated with the concentration of bacteria that were impermeable to PI, these correlations were not significant. The reason(s) for this are uncertain but may be due to the removal of these bacteria by grazing and/or because only some SYTO®9-stained bacteria contributed to bacterial growth.

Active bacteria

The proportion of active bacterioplankton in the Southern Ocean was similar to those previously reported from elsewhere in the world's oceans (Rodriguez et al. 1992, Gasol et al. 1995, Choi et al. 1996, del Giorgio et al. 1996, Ullrich et al. 1996, Yamaguchi & Nasu 1997, Smith 1998, E. Sherr et al. 1999). Servais et al. (2001) found that CTC-stained bacteria were responsible for <60% of production by the bacterial community. In addition, many unstained bacteria become stained for metabolic activity under environmental conditions that are optimal for bacterial metabolism (Choi et al. 1999). While indicating that such stains do not detect all active bacteria, it does show that much of the bacterial production in natural marine communities is commonly attributable to <10% of the bacterial standing stock (e.g. Choi et al. 1996, Karner & Fuhrman 1997).

Like B. Sherr et al. (1999), we found that stains for bacterial metabolism gave a valuable and ecologically meaningful indication of the physiological state of natural bacterial communities. Sherr et al. (2001) found a correlation between chlorophyll concentration and the uptake of radiolabelled amino acids in the northeast Pacific Ocean off Oregon. We observed a significant positive correlation between *in situ* chlorophyll and the concentrations of 6CFDA-stained bacterioplankton in the Southern Ocean. Growth of bacterioplankton is strongly dependent on the concentration of dissolved organic matter (Kähler et al. 1997) and is enhanced by senescence and mortality of protists (Blackburn et al. 1998, Kirchman 1999). Thus, it is likely that increases in phytoplankton standing stock enhance the nutritional status of bacterioplankton, resulting in an increased concentration of active cells. However, unlike Sherr et al. (2001), we found no correlation between concentrations of 6CFDA-stained bacteria and temperature or salinity. This may be due to physiological differences between the study sites and/or intrinsic differences between bacteria that are stained by 6CFDA and those that are radiolabelled using tritiated leucine and thymidine. The absence of correlation between environmental variables and the concentration of CTC-stained bacteria in our study was likely due to the low number of samples stained.

Vertical profiles

The vertical distribution of SYTO®9-, PI- and 6CFDA-stained bacteria in profiles are preliminary, as only 2 profiles were performed in this study. At these sites, most of the SYTO®9-stained (PI-impermeable) bacteria were highly metabolically active. High concentrations of total, highly active, PI-impermeable bacteria persisted to depths well below the euphotic zone. This agrees with vertical distributions of bacterial production published by Moriarty et al. (1997).

Numerous authors report size-specific grazing of bacterivores, leading to preferential grazing of live, active and/or dividing bacteria (e.g. Monger & Landry 1992, Gasol et al. 1995, del Giorgio et al. 1996, Hahn & Höfle 2001). Grazing selectivity can reportedly result in active bacteria being grazed at rates around 4 times that of inactive cells (del Giorgio et al. 1996, Gasol & del Giorgio 2000). Our study showed that 20 to 30% of bacteria were impermeable to PI and around half these exhibited high levels of metabolic activity. If <30% of bacteria are responsible for much of the bacterial metabolism, including production, growth and division, our results suggest that the microbial loop may be largely supported by a relatively small proportion of the bacterial standing stock in the Southern Ocean. This would have major implications for nutrient availability, clearance rates and conversion efficiencies for bacterivores.

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