

Wide-ranging abundances of aerobic anoxygenic phototrophic bacteria in the world ocean revealed by epifluorescence microscopy and quantitative PCR

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Abstract

Reports that aerobic anoxygenic phototrophic bacteria (AAnPB) containing bacteriochlorophyll *a* (BChl*a*) constitute 10% or more of total bacteria cell counts in seawater prompted us to quantify AAnPB abundances in local waters 15 km off the coast of Southern California and at 18 sites around the globe using infrared epifluorescent microscopy (IREM) and quantitative polymerase chain reaction (QPCR). IREM-estimated AAnPB comprised $1.66\% \pm 0.55\%$ (mean \pm SD) of total bacterial counts in the euphotic zone between April and September 2001 off Southern California. IREM counts of AAnPB need to be interpreted with caution due to detection sensitivity concerns and overlap between chlorophyll *a* and bacteriochlorophyll *a* autofluorescence at wavelengths >850 nm. QPCR-estimated AAnPB were $1.17\% \pm 1.56\%$ of total bacteria counts at 5-m depth between January 2001 and July 2003 off the coast of Southern California (assuming one *pufM* gene cell⁻¹ and 2.5 fg total DNA cell⁻¹). Global QPCR surveys of AAnPB conducted in tropical to polar waters yielded low estimates ($<2.5\%$ of total bacteria counts) of AAnPB in most marine environments surveyed, but estimates $>10\%$ were observed in estuarine waters of Long Island Sound and Chesapeake Bay. AAnPB abundances are lower in most marine systems than previously reported from the Eastern Pacific and demonstrate that AAnPB abundances are more variable than previously realized. Our results do not support the notion AAnPB are more abundant in oligotrophic environments and, in fact, suggest the opposite. These results raise questions about where aerobic anoxygenic photosynthesis is advantageous in marine ecosystems.

Aerobic anoxygenic phototrophic bacteria (AAnPB), which contain the pigment bacteriochlorophyll *a* (BChl*a*), were discovered in organic-rich marine environments 25 yr ago (Shiba et al. 1979) and since have been found in a variety of environments (Rathgeber et al. 2004). AAnPB have a polyphyletic distribution (Stackebrandt et al. 1996; Giovannoni and Rappe 2000) and cultivated isolates augment a heterotrophic metabolism with light-derived energy (Yurkov and Beatty 1998). Given the majority of the ocean is highly dilute in organic matter, it was initially hypothesized phototrophs would gain the largest ecological advantage and thus be more abundant in the open ocean. However, reports of undetectable amounts of BChl*a* pigment in the Sargasso Sea by Mullins et al. (1995) suggested AAnPB are not common in open ocean environments. These early results are in sharp contrast with more recent, surprising results by Kolber et al. (2000, 2001), who reported AAnPB comprise at least 11% of total bacterial abundances in the upper open ocean. Genetic surveys for *pufLM* genes by Beja et al. (2002) revealed a highly diverse AAnPB community in the open

ocean, and the discovery of proteorhodopsin (Beja et al. 2000) suggests non-chlorophyll *a* phototrophy may be a more common life history strategy among marine bacteria than previously realized. The results of Kolber et al. (2000, 2001) and Beja et al. (2002) combined raise new questions about the abundance and global distribution of AAnPB in the marine environment.

To date, estimates of the quantitative significance of AAnPB in seawater have been obtained using various forms of pigment analysis. Kolber et al. (2000, 2001) measured transient fluorescence kinetics via infrared (IR) fast repetition rate fluorometry (Kolber et al. 1998) to infer a globally averaged BChl*a*:Chl*a* ratio that may be as high as 5–10%. However, the BChl*a*:Chl*a* ratio in the Southern California Current measured directly via reverse-phase high performance liquid chromatography ranged between 0.5% and 1.1% (Goericke 2002). These conflicting results highlight the need for further estimates of AAnPB abundances in seawater. Direct microscopy estimates of AAnPB cell abundances currently rely on a form of pigment analysis as well. Kolber et al. (2001) developed IR epifluorescent microscopy (IREM) to detect BChl*a* pigment in bacteria cells and directly estimated AAnPB constitute greater than 10% of total bacterial cell counts off the coast of Oregon (48°N, 128°W). The high abundances of AAnPB reported by Kolber et al. (2001) but low BChl*a*:Chl*a* ratios reported by Goericke (2002) prompted us to collect IREM measurements of AAnPB in local waters off the coast of Southern California.

In an effort to independently corroborate IREM estimates of AAnPB abundances, we also developed a sensitive quantitative polymerase chain reaction (QPCR) assay that is capable of quantifying *pufM*-containing AAnPB independent of intracellular pigment concentrations. The *pufM* gene is

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part of a contiguous, 45-kilobase superoperon that encodes a pigment-binding protein subunit of the reaction center complex (Alberti et al. 1995). The *pufM* gene is shared among all purple anoxygenic bacteria as well as *Chloroflexus* sp. (Karr et al. 2003) and can be located on the bacterial chromosome or on extrachromosomal elements (Pradella et al. 2004). In this study, we assume the presence of a *pufM* gene indicates the potential to carry out aerobic anoxygenic photosynthesis. Finally, in an effort to understand the global distribution of AAnPB, we used QPCR to quantify AAnPB abundances in archived bacterial community DNA samples that had been collected over the past 14 yr from various locations around the world.

Methods

Sample collection—Samples for abundance measurements: Monthly samples were collected from 5-m depth at the San Pedro Channel Ocean Time Series (SPOTS) station located off the coast of Southern California (33°33'N, 118°24'W) between January 2001 and July 2003. All samples were collected using Niskin bottles and subsequently transferred to duplicate 20-liter polycarbonate carboys that had been rinsed once with 1.2 N HCl and three times with small amounts of collected seawater prior to being filled. From April through September 2002, a 50-ml sample was collected from 5 m and chlorophyll maximum depth and preserved with 1% (final vol:vol) formalin for enumeration of AAnPB via IREM. In May 2002, the abundance of AAnPB was also recorded at discrete depths to 500 m via IREM.

Bacterial community DNA for use in QPCR was obtained by prefiltering 20 liters of seawater through a 142-mm 1.2- μ m (nominal pore size) A/E glass fiber filter (Pall Gelman) and subsequently filtered onto a 142-mm 0.22- μ m Durapore filter (Millipore) in series. Durapore filters were frozen at -80°C until the DNA could be extracted using the hot sodium dodecyl sulfate (SDS): phenol:chloroform: isoamyl alcohol, ethanol precipitation extraction protocol initially described by Fuhrman et al. (1988).

Archived bacterial community DNA samples collected between 1990 and 2003 from various sites around the globe were used in QPCR assays of partial *pufM* genes to quantify *pufM*-containing AAnPB at these locations. Surface-water samples were collected from each site using either Niskin bottles or acid-washed buckets and passed through 1.2- μ m (nominal pore size) A/E glass fiber filters and onto a 0.22- μ m Durapore filter as described above. Durapore filters were frozen at -80°C until DNA could be extracted using the hot SDS, phenol:chloroform: isoamyl alcohol, ethanol precipitation extraction protocol initially described by Fuhrman et al. (1988). Global DNA samples have been stored at -80°C following extraction.

Abundance measurements—Preparation of DAPI slides for IREM: Formalin-preserved seawater samples that had been stored at 4°C in the dark until the time of slide preparation (60 min or less) were used to enumerate total bacterial and AAnPB abundances. Duplicate filters from each sample were prepared by filtering between 10 and 20 ml of

preserved seawater through a 0.2- μ m black, polycarbonate Nucleopore filter using a gentle vacuum (≤ 15 cm Hg) until approximately 0.5 ml of seawater remained above the filter. At that time, the vacuum was removed and all cells were stained by the addition of 40 μ l of a 0.1 mg ml $^{-1}$ 4',6-diamidino-2-phenylindole (DAPI) solution (Sigma) to the remaining 0.5-ml sample. Samples were incubated with DAPI for 5 min in the dark, after which time a vacuum was re-applied and the remaining 0.5 ml of water was drawn through the filter. To mount the filters, a drop of immersion oil (Olympus, Fisher #Z-81012) was placed onto a glass slide and flattened by placing a coverslip on top of the immersion oil. The coverslip was then slid off the slide, leaving a smooth layer of immersion oil onto which the filter was placed and subsequently covered with the coverslip. Smoothing the immersion oil prior to mounting resulted in significantly less plane variation in the mounted filter when viewed at high magnification and greatly facilitated the imaging and counting of AAnPB via IREM.

Total bacteria enumeration: Total bacterial counts were determined manually on an Olympus BX60 microscope equipped with an Olympus Planapochromat $\times 100$ lens and $\times 10$ eyepiece by exciting DAPI-stained cells with ultraviolet (UV) light (excitation, 330–390 nm; blue emission, 440–490 nm; beam splitter, 400–430 nm) generated from a 150-W xenon lamp (Opti-Quip). A xenon lamp rather than a mercury lamp was used as a light source because its more even spectral pattern reduced fading during image acquisition of BChla-containing bacteria via IREM.

AAnPB enumeration via IREM: AAnPB containing BChla were enumerated on the same DAPI slides used to quantify total bacteria abundances using a slight modification of the protocol described by Kolber et al. (2001). Because BChla fluoresces at wavelengths invisible to the human eye (>850 nm), AAnPB were enumerated from images captured using an IR-sensitive camera. Three separate images of the same field of view were collected for each data point using a microscope equipped with an Olympus MagnaFire CCD camera remotely operated in turbo mode by MagnaFire software (Olympus). A field of view was initially excited using UV light (as above) and, upon focusing, an image was taken of the entire DAPI-stained community using a 2-s exposure (Fig. 1A). Following acquisition of an image containing all bacterial cells, the cyanobacteria present in the same field of view were excited using blue light (excitation, 450–480 nm; yellow-red emission, >515 nm; beam splitter, 500 nm) and a second image, containing only cyanobacteria autofluorescence, was acquired with another 2-s exposure of the CCD camera (Fig. 1B). Finally, AAnPB carotenoids in the same field of view were excited in a third exposure, using blue-white excitation (excitation, 400–550 nm; IR emission, >850 nm; beam splitter, 620 nm) and a final image (IR image) was acquired using a 1-min 11-s exposure (Fig. 1C). Optical filters were obtained from Chroma Technology and are based on specifications originally described in Kolber et al. (2001).

Generally, between 10 and 15 sets (Fig. 1) of images were acquired from each DAPI-stained Nucleopore filter, with du-



Fig. 1. Representative set of images captured via IREM from DAPI-stained slides when excited using (A) UV light, blue emission, (B) blue light, yellow-red emission, and (C) blue-white light, infrared emission. BChla cells were determined to be cells present in images A and C but not present in image B. Note the majority of the cells in image C are cyanobacteria that were also observed in image B. Cyanobacteria do not contain BChla. Photos are shown as a negative image for clarity; scale bar indicates 10 μm .

plicate Nuclepore filters being imaged per sample. Best results were obtained when samples were imaged immediately following slide preparation. Given the long exposure times required to image BChla-containing cells, imaging was not practical while at sea. Following image acquisition, contrast and brightness of images was manipulated using ImagePro software (version 4.5.1) and AAnPB were counted manually. AAnPB were considered to be cells observed in the infrared image (Fig. 1C) but not in the image of yellow-red emission (Fig. 1B). The yellow-red emission image was used to correct for cyanobacteria autofluorescence observed in the IR images (Fig. 1C).

AAnPB enumeration via QPCR: QPCR was used to quantify partial *pufM* sequences in DNA collected from the San Pedro Ocean Time Series (SPOTS) station off the coast of California and from archived global DNA samples. Gene abundances were determined in triplicate 25- μl QPCR reactions that contained the following: 12.5 μl 2 \times SYBR Green I Supermix (BioRad 2 \times SYBR Green I Supermix contains 100 mmol L⁻¹ KCl, 40 mmol L⁻¹ Tris-HCl, pH 8.4, 0.4 mmol L⁻¹ each dNTP, 50 U ml⁻¹ iTaq DNA polymerase, 6 mmol L⁻¹ MgCl₂, SYBR Green I, 20 nmol L⁻¹ fluorescein and proprietary stabilizers), 11 μl of 0.02-filtered deionized water, 1 μl of DNA stock (DNA stocks: 2.5 ng μl^{-1}) and 0.25 μl of each primer (40 μmol L⁻¹ stock). The 156 base-pair (bp) partial *pufM* target was amplified using forward (5'-CCATSGTCCAGCGCCAGAA-3') (T_m , 62.0°C) and reverse (5'-TACGGSAACTGTWCTAC-3') (T_m , 54.0°C) primers originally described in Beja et al. (2002) and Achenbach et al. (2001). In addition, a forward (5'-TATAAYC-CATTTCAAYGC-3') (T_m , 51.0°C) and reverse (5'-GCRAAC-CACCAAGCCCA-3') (T_m , 61.0°C) primer were designed that amplified a 228-bp portion of the *pufM* genes reported by Venter et al. (2004) (Fig. 2, Institute for Biological Energy Alternatives (IBEA) clones). Negative controls contained 12 μl of 0.02-filtered deionized water and no template DNA. Standards in QPCR reactions were serial dilutions of a pGem-TEasy plasmid (Invitrogen) that contained a representative partial *pufM* sequence isolated from local waters (clone SPOTS1, GenBank accession #AY579991; Fig. 2; see below for cloning method). QPCR assays were run on a Stratagene Mx3000P thermocycler in 96-well plates using

the following thermal profile: 3 min at 95°C followed by 50 cycles of 30 s at 95°C and 30 s at 60°C. Individual well fluorescence values were measured at the end of each thermal cycle for a total of 50 optical measurements per well and 150 optical measurements per sample.

The number of *pufM* copies in an environmental DNA sample was measured via QPCR by comparing unknown environmental samples to a serial dilution of a known number of *pufM* copies. To estimate the percent AAnPB, we assumed one copy of the gene cell⁻¹ and therefore divided the estimated number of *pufM* copies in a sample as measured by QPCR by the total number of cells in the initial template DNA, multiplied by 100. The total number of cells in the initial template DNA was determined by dividing the mass of the initial template DNA by an assumed average DNA cell⁻¹ concentration of 2.5×10^{-15} g DNA cell⁻¹ (Fuhrman and Azam 1982; Button and Robertson 2001).

Calibration of QPCR: The sensitivity, efficiency, and accuracy of QPCR was calibrated by amplifying known amounts of extracted *Erythrobacter longus* (ATCC #33941) or *Roseobacter litoralis* DNA and comparing the resulting QPCR estimates of *pufM* abundance in the initial template to the known number of *E. longus* or *R. litoralis* in the initial template. To determine if the DNA extraction method employed in this study inhibited QPCR reactions, QPCR reactions containing positive-control standards were amplified with or without various amounts of extracted environmental DNA. The difference between reactions without extracted environmental DNA and those with environmental DNA was attributed to inhibition by the extracted environmental DNA.

Cloning and sequencing—A small number of partial *pufM* sequences were cloned and sequenced to verify proper amplification of the targeted *pufM* genes. Briefly, DNA samples from SPOTS were amplified in 50- μl reactions that contained (final concentrations) 1 \times Promega PCR Buffer, 1.5 mmol L⁻¹ MgCl₂, 40 pmol of each primer (listed above), 250 μmol each dNTP, 2.5 U Taq polymerase (Promega), and 2.5 ng template DNA. PCR products were amplified using cycle parameters identical to the QPCR parameters, in a Perkin-Elmer Cetus GeneAmp 9600 thermal cycler, and subsequently ligated into a pGem-T Easy vector. The vector was

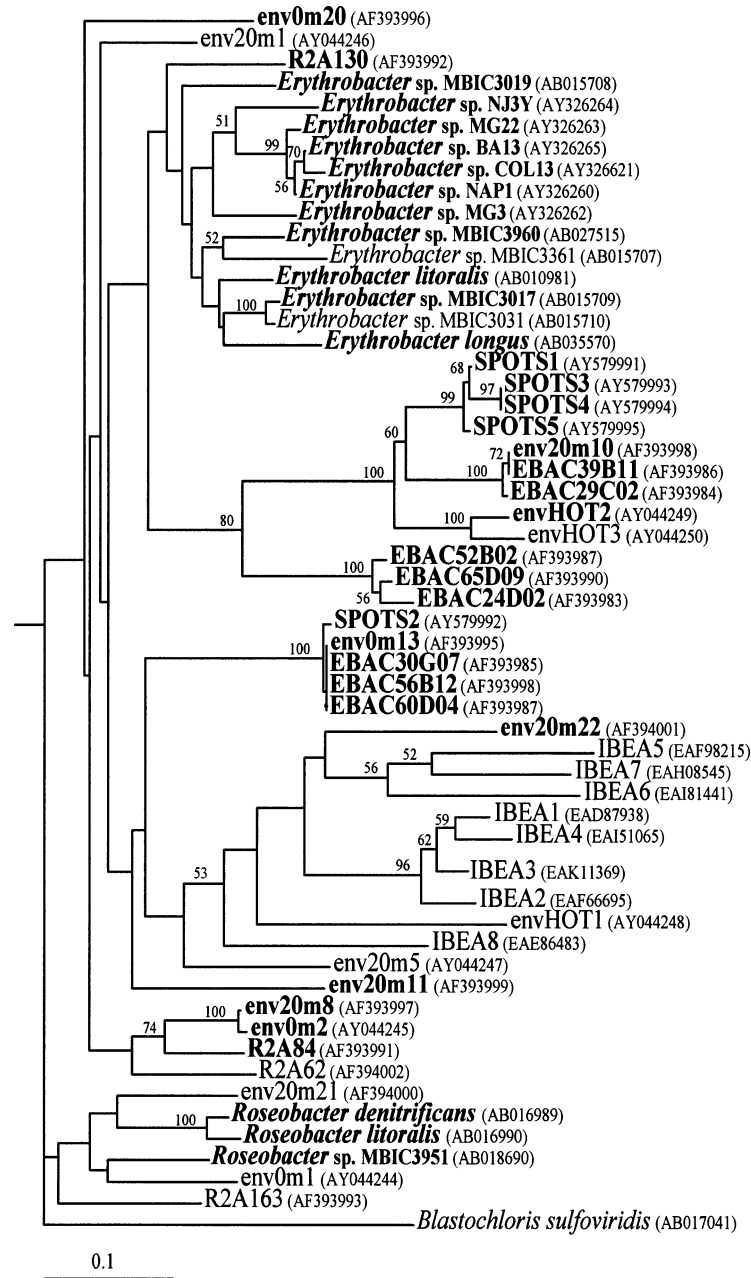


Fig. 2. Neighbor-joining trees of predicted *pufM* DNA sequences (122 nucleotides) obtained from environmental samples. Tree construction and bootstrapping ($n = 100$) were performed in PHYLIP. Bootstrap values >50 are shown at nodes. SPOTS clones were isolated in this study from the San Pedro Channel Ocean Time Series Station off the coast of Southern California. Dilutions of SPOTS1 were used as standards in QPCR reactions. Bolded sequences and all IBEA clones listed would be amplified (one mismatch maximum excluding mismatches at position 1, with the *pufM* primers used in the QPCR survey).

then used to transform competent JM109 *Escherichia coli* cells (Promega) and transformed *E. coli* cells were grown in 5 ml of Luria-Bertani (LB) broth overnight. Plasmids were obtained using QIAprep Spin Miniprep Kit (Qiagen, #27106) and sequenced using the ABI BigDye Terminator v1.1 Cycle Sequencing Kit and BigDye v1.1/3.1 Sequencing Buffer (5 \times) on an ABI 377XL Sequencer. Resulting sequences were aligned with representative *pufM* sequences

available in GenBank using BioEdit Software (Hall 1999). Neighbor-joining tree construction and bootstrap analyses ($n = 100$) were conducted in PHYLIP (Fig. 2). Sequences obtained in this study were deposited in GenBank with accession numbers AY579991–AY579995.

Melt curve analysis—In addition to sequencing partial *pufM* PCR products, the dissociation curve from each QPCR

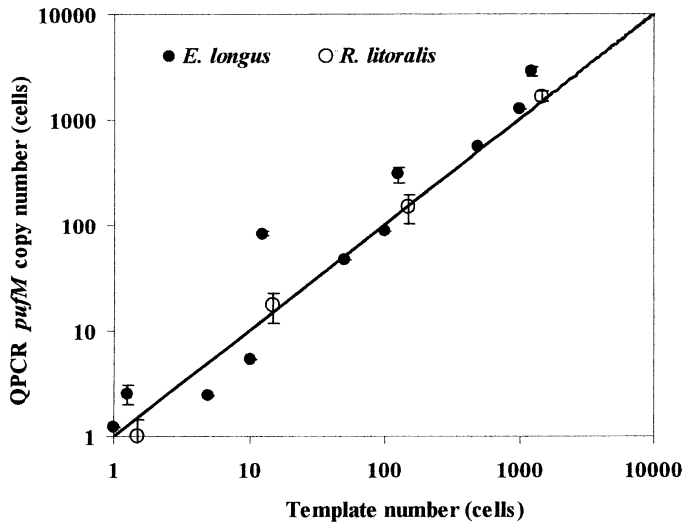


Fig. 3. QPCR estimates of cultivated *Erythrobacter longus* or *Roseobacter litoralis* cell abundance via *pufM* genes using the same standards used for field samples, plotted versus cell abundance calculated from DNA concentrations exactly as we do for field samples. Error bars are plus or minus one standard deviation. The black trend line indicates a perfect 1:1 relationship. Note the slopes of 1.02 (*E. longus* data, $r^2 = 0.95$) and 1.11 (*R. litoralis* data, $r^2 = 0.96$) represent near-perfect prediction capability.

reaction was examined to further ensure proper target sequence amplification and to investigate the diversity of partial *pufM* genes obtained from environmental samples. Dissociation data were obtained using a Stratagene MX3000P QPCR thermal cycler and Stratagene MX3000P software version 2.0. Briefly, dissociation curves were obtained by initially heating QPCR products to 95°C for 1 min, the temperature was then dropped to 60°C and gradually increased at a rate of 0.5°C cycle⁻¹ up to 95°C for a total of 70 cycles. Individual well fluorescence was measured optically following each cycle. Triplicate fluorescence measurements per sample per cycle were pooled and an average fluorescence was obtained for each sample per cycle. These values were then used to construct the overall melt curve. After collection of melt curves, the maximum dissociation values were plotted with error bars that indicate the region of the melt curve that is greater than half the maximum dissociation temperature. Thus, wider error bars indicate broader melt curves and were interpreted to represent greater levels of diversity within a sample.

Results

QPCR calibration—Strong and precise linear relationships, with slopes of 1.02 and 1.11, were found between *pufM* gene-copy number determined via QPCR and standardized concentrations of *E. longus* or *R. litoralis* cells, respectively (Fig. 3). This is virtually identical to the expected slope of 1.0 indicated in Fig. 3. Importantly, this calibration used a natural SPOTS clone as standard, not a cloned *E. longus* or *R. litoralis* gene. Nearly identical results for *E. longus* data were obtained using either of the two most phylogenetically distant SPOTS clones as standards (data not

shown). The average efficiency of QPCR amplifications was $92.6\% \pm 4.29\%$ (mean \pm SD) and the average slope of the standard curve (threshold cycle vs. log of standard copy number) was -3.51 ± 0.2 . QPCR is capable of detecting the equivalent of 1 cell ml⁻¹ in the extracted DNA. Primers employed in this study would prime approximately 85% of the known *pufM* sequences collected from marine bacteria when up to one mismatch was allowed within the primer sequence (Fig. 2). PCR-inhibition tests indicate QPCR reactions were inhibited by $16.1\% \pm 7.1\%$, and there was no significant relationship ($r^2 = 0.002$) between the age of a DNA sample and percent AAnPB.

Enumeration of AAnPB in seawater—Monthly counts of AAnPB obtained via IREM at 5 m and the chlorophyll *a* maximum depths between April and September 2002 were low (mean \pm SD: $1.66\% \pm 0.55\%$, $n = 5$) after correcting for cyanobacteria autofluorescence in the IR images (Fig. 4A). Had cyanobacteria autofluorescence in IR images not been corrected for, AAnPB abundances would have appeared to comprise $7.9\% \pm 3.6\%$ of the microbial community. Abundances of BChl_a-containing bacteria in the San Pedro Channel were consistently low while Chl_a concentrations ranged between 0.18 and 1.42 $\mu\text{g Chl}_a \text{L}^{-1}$ during the period of sampling. Measurements of AAnPB with depth in the San Pedro Channel in May 2002 estimated AAnPB comprised $0.68\% \pm 0.04\%$ of the bacterial community at the surface, increasing to a subsurface maximum of $3.09\% \pm 0.16\%$ at 5 m, after which counts steadily decreased to levels below detection at 100-m depth (Fig. 4B). No BChl_a-containing bacteria were observed below 100-m depth, which is approximately the yearly maximum depth of the euphotic zone in the San Pedro Channel.

It was noted during IREM imaging that cultures of the cyanobacteria *Prochlorococcus marinus* str. NATl2A, grown under a 14:10 light:dark cycle (LD) at 21°C in PRO-99 medium, were occasionally not detectable in yellow-red emission images captured with short camera exposures but were detectable in IR images acquired with longer camera exposure. We also observed *P. marinus* str. NATl2A, which were detectable in yellow-red emission images but faded below detection during the long exposure required to capture IR images of AAnPB (data not shown). Similarly, fading during IR-image acquisition was also a concern with *E. longus* cells grown in 14:10 LD of ambient light.

QPCR assays of partial *pufM* genes on samples collected from 5-m depth in the San Pedro Channel between January 2001 and July 2003 estimated AAnPB abundances on average comprised $1.17\% \pm 1.56\%$ of the bacterial community, and calculated percentages ranged between 0.03% and 6.23% of total community counts (Fig. 5A). Dissociation temperatures of dominant QPCR products varied from month to month and more than one peak or broad humps (presumably representing increased *pufM* gene sequence diversity) were observed in the melting curve plots in some months (Fig. 5B). SPOTS1 clones used as standards in QPCR reactions dissociated at 89.5°C, but dissociation temperatures from natural mixed communities ranged between 83.0°C and 92.0°C for all *pufM* sequences (Figs. 5B, 6). Spurious products presumed to be primer dimers dissociated

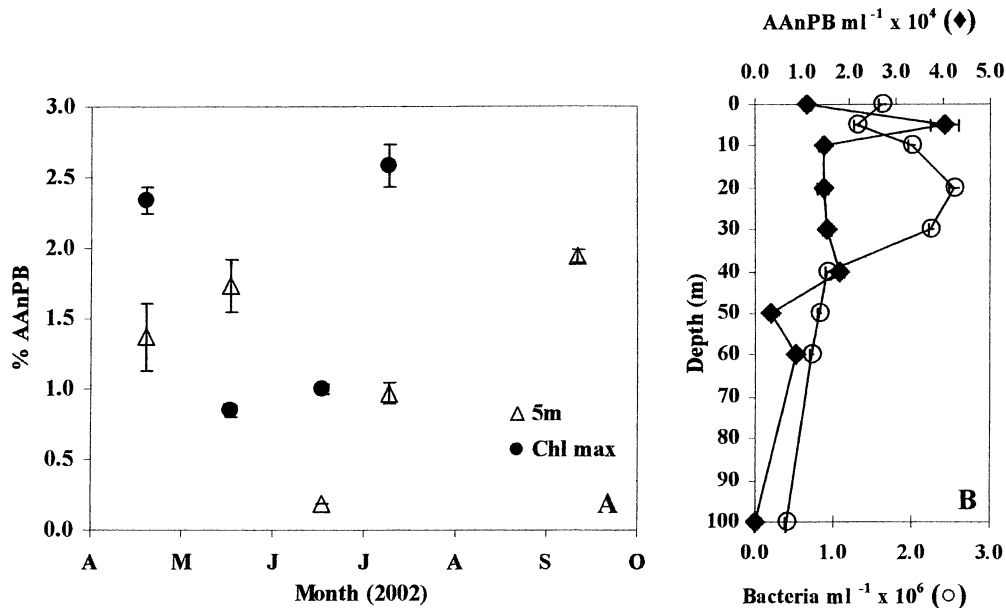


Fig. 4. (A) Percent AAnPB (mean \pm SD) determined using IREM at 5 m and the chlorophyll maximum depth (April 27, 5 m; May, 27.7 m; June, 34.9 m; July, 24.2 m) at the San Pedro Ocean Time Series Station. (B) Cell counts (mean \pm SD) of AAnPB and total bacterial cells in May 2002. AAnPB estimates were obtained from IREM images corrected for cyanobacteria autofluorescence. Total bacteria cell counts were obtained via DAPI staining. See text for details.

at 77°C, thus allowing for identification of primer dimers that occasionally amplified in negative controls. Positive primer dimer amplification and positive target amplification did not occur simultaneously within a single QPCR reaction.

Global distribution of AAnPB—Non-IBEA-related *pufM* genes were detected at all locations sampled; however, IBEA-like genes were not detected at three locations: Crane Neck (CN), Bermuda (BM), and Fiji (FJ). QPCR assays with globally collected DNA samples revealed low abundances (2.28% or less) of *pufM*-containing AAnPB at all oceanic locations sampled (Table 1). However, the estuarine environments of Long Island Sound and Chesapeake Bay contained nearly 11% and 19% of *pufM*-containing bacteria, respectively (Table 1). AAnPB abundances were consistently low (<0.1%) in Antarctic samples: Deception Island (DI), Nelson Island (NI), Weddell Sea (WS), and Gerlache Strait (GS), while percentages at tropical locations (Phillippines (PH), Wistari Reef (WR), Fiji (FJ), Noamea Lagoon (NL)) were only slightly higher (<1%). AAnPB abundance ranged from 0.12% to 1.42% in temperate locations (Bermuda (BM), CalCOFI station 87.110 (CC), SPOTS Station (SP), Villefranche (VR), Ferry Reach (FR)). Interestingly, the polar waters of the Norwegian Sea had the highest abundance of AAnPB of all oceanic samples. Dissociation data revealed variable levels of *pufM* diversity, with tropical locations relatively more diverse compared with other environments (Fig. 6). *PufM* diversity did not correlate with *pufM* gene abundance ($r^2 = 0.0001$); however, *pufM* genes collected from similar environments appear to have similar dissociation temperature maxima (Fig. 6).

Discussion

Both IREM and QPCR estimates of AAnPB independently suggest AAnPB generally constitute a minor component of the bacterioplankton community in the marine locations studied. Estimates of AAnPB via IREM determined AAnPB comprised a small (mean \pm SD: 1.66% \pm 0.55%) percentage of the bacterial community at 5 m and chlorophyll *a* maximum depths in the San Pedro Channel between April and September 2002. BChl*a*-containing bacteria would have erroneously seemed to have comprised nearly 8% of total bacterial abundances if cyanobacteria appearing in the IR images had not been subtracted. Enumeration of AAnPB via QPCR resulted in similar but slightly lower estimate of AAnPB (mean \pm SD: 1.17% \pm 1.56%) at the SPOTS station off the coast of California. The results from these independent methods combined suggest aerobic, anoxygenic photosynthesis is not a common life-history strategy among marine bacterioplankton in the Southern California Coastal System and are consistent with results reported by Goericke (2002).

It is known AAnPB and cyanobacteria are capable of regulating intracellular pigment concentrations based on environmental cues and at times appear to downregulate intracellular pigment concentrations below IREM detection limits. However, cyanobacteria were consistently detected in our IR images and we believe this may have led to an overestimate of AAnPB via IREM by Kolber et al. (2001), who did not report subtracting cyanobacteria from their IREM counts. We also noted *Prochlorococcus* is extremely difficult to enumerate via microscopy and at times a subset of the

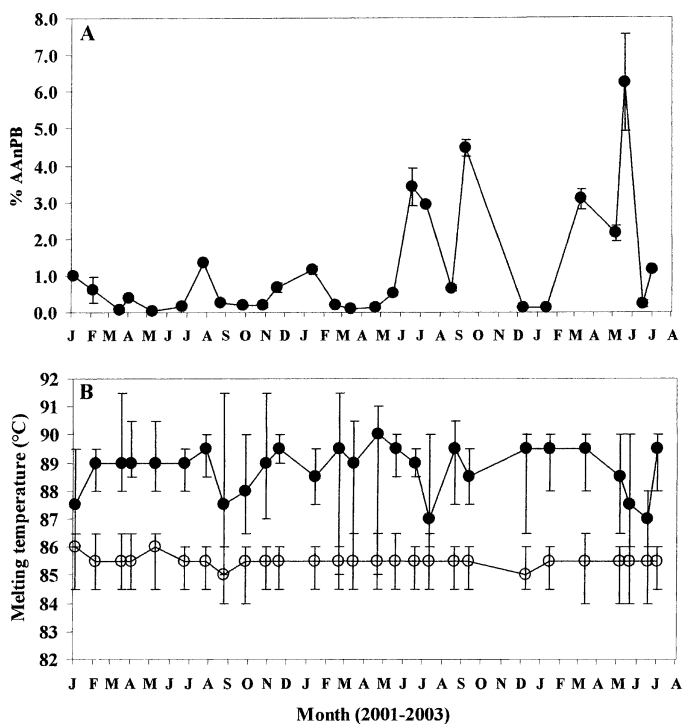


Fig. 5. (A) Average calculated percentage of AAnPB determined by QPCR of partial *pufM* genes. Percentages reflect a fraction of total bacterial DNA at 5-m depth in the San Pedro Channel. Error bars indicate plus or minus one standard deviation from the mean. (B) Diversity of community *pufM* genes amplified at 5-m depth at SPOTS station, as indicated by range of PCR product melting-temperature ranges. The diversity of IBEA-like genes is shown with open circles, all others are shown in black. The dominant (mode of the melt curve) dissociation temperature (°C) of *pufM* QPCR products is shown for each month. Error bars indicate temperatures where corrected raw fluorescence readings were greater than half the mode. Note error bars do not represent uncertainty in the measurement but rather the range of varied melting points among the amplified *pufM* PCR products. Thus, broader error bars indicate greater phenotypic diversity among amplified *pufM* sequences.

cyanobacteria were not detectable in yellow-red emission images due to short camera exposures but were visible in IR images captured with longer camera exposures. Therefore, cyanobacteria with low intracellular pigment concentrations may at times erroneously appear to be AAnPB even after subtraction of cyanobacteria. These two observations indicate IREM estimates of AAnPB may either over- or underestimate the relative amount of AAnPB in a mixed community and, thus, IREM counts need to be interpreted with caution.

The strong linear relationship observed between standardized *E. longus* or *R. litoralis* abundances and QPCR estimates of *pufM* copy number (Fig. 3) suggests QPCR assays of partial *pufM* genes accurately reflect the abundances of primed, *pufM*-containing AAnPB. We note the primers employed in this survey are not universal *pufM* primers and contain mismatches to currently known *pufM* sequences (Fig. 2). Significantly, though, the primers in this study have up to two mismatches to the currently known *pufM* sequences of *E. longus* and *R. litoralis* (one internal and one at the

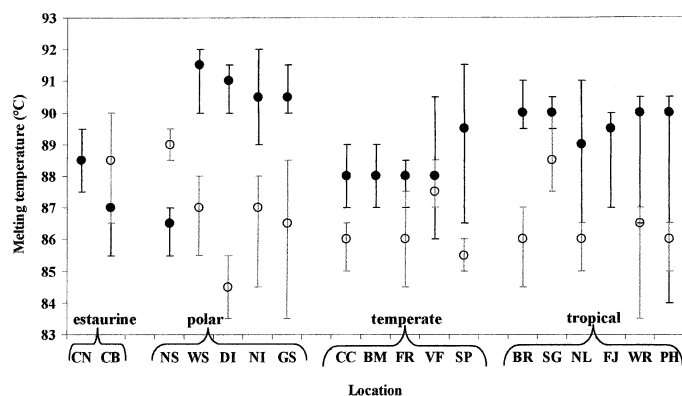


Fig. 6. Diversity of community *pufM* genes amplified from samples collected from widespread locations around the world, expressed as in Fig. 5B. The diversity of IBEA-like genes is shown with open circles, all others are shown in black. Location abbreviations as in Table 1. Samples from similar environments are grouped together. Note no IBEA-like *pufM* genes were obtained from CN, BM, or FJ.

5' end that should affect hybridization much less), yet they still gave accurate quantitative estimates of standardized *pufM* abundances. A search of GenBank would suggest our primers could potentially miss as many as 15% of the *pufM* genes present within a sample. However, this assumes all known *pufM* sequences are present and evenly distributed in every community, something that is rather unlikely. Nonetheless, the restricted scope of the primers employed in this study and minor amounts of PCR inhibition noted may account for why QPCR estimates were lower than IREM estimates of AAnPB abundances in coastal waters near Southern California. Despite inherent weaknesses of each method, both the original IREM method, similar to that used by Kolber et al. (2001), and an independent QPCR assay yielded low estimates of AAnPB abundances off the coast of Southern California.

A survey of archived DNA samples collected from various locations around the globe yielded low estimates of AAnPB in marine environments ranging from tropical surface waters above coral reefs to Antarctic waters of the Southern Ocean, with the highest oceanic estimates (2.2%) occurring in the polar Norwegian Sea (Table 1). We speculate the slightly higher AAnPB abundances observed in the Norwegian Sea may be due to higher than average abundances of *Roseobacter* in this location because *Roseobacter* have been observed to be particularly abundant in other cold, northern, oceanic environments (Gonzalez et al. 2000; Zubkov et al. 2002). Our results do not support the notion AAnPB are more common in oligotrophic environments as has been previously suggested (Kolber et al. 2001; Beatty 2002). Rather, the highest percentages of AAnPB measured in this study were obtained from estuarine environments at Crane Neck in Long Island Sound (salinity 28) and Chesapeake Bay (salinity 10) (Table 1). Estuarine environments have high dissolved organic matter concentrations and are similar to the original environment from which *Erythrobaacter* sp. was isolated (Shiba et al. 1979). *Erythrobaacter* sp. have been shown to be more widely distributed than previ-

Table 1. Calculated estimates of AAnPB abundances from various sites around the world determined via QPCR of partial *pufM* genes.

Sample location	Location abbreviation	Latitude, longitude	Sample date	% AAnPB
Gerlache Strait, Antarctica	GS	67°2'S, 18°6'W	30 Dec 93	0.01±0.00
Deception Island, Antarctica	DI	62°35'S, 60°20'W	6 Jan 94	0.04±0.01
Nelson Island, Antarctica	NI	62°30'S, 58°30'W	8 Jan 94	0.05±0.01
Weddell Sea	WS	63°0'S, 54°0'W	3 Jan 94	0.06±0.01
Bermuda North Lagoon	BM	32°27'N, 64°45'W	29 May 90	0.12±0.02
Singapore	SG	1°6'N, 103°24'E	18 Sep 90	0.30±0.15
Noumea Lagoon, New Caledonia	NL	22°19'S, 166°24'E	30 Mar 98	0.51±0.31
Catalagan, Luzon Island, Philippines	PH	14°48'N, 121°12'E	1 Jul 94	0.71±0.21
Barbados (100 km North East)	BR	14°24'N, 58°14'W	5 Jun 94	0.72±0.05
Wistari Reed, Great Barrier Reef, Australia	WR	23°15'S, 151°33'E	16 Mar 99	0.73±0.93
CalCOFI station 87.110 (California, USA)	CC	31°19'N, 123°44'W	13 Apr 03	0.76±0.65
Suva Harbor, Fiji	FJ	18°9'S, 178°24'E	13 Apr 98	0.92±0.41
San Pedro Channel (California, USA) (2-y mean)	SP	33°33'N, 118°24'W	Jul 01–Aug 03	1.17±1.56
Villefranche, France	VF	43°31'N, 7°30'E	8 Jul 93	1.37±0.26
Ferry Reach, Bermuda	FR	32°22'N, 64°42'W	30 May 90	1.42±0.15
Norwegian Sea	NS	72°46'N, 7°15'W	3 Aug 96	2.28±0.21
Chesapeake Bay, near Potomac River (salinity = 10)	CB	38°3'N, 76°13'W	23 Aug 03	10.70±0.89
Crane Neck, Long Island Sound (New York, USA) (salinity = 28)	CN	40°58'N, 73°9'W	27 Aug 98	18.74±5.96

ously realized (Koblizek et al. 2003) and it may be the majority of the *pufM* genes amplified from the two estuarine locations in this study are related to *Erythrobacter*. Dissociation analysis of *pufM* QPCR products suggests the majority of the QPCR products from CN primarily dissociate at 88.5°C, similar to *E. longus pufM* (89.5°C). However, CB *pufM* genes were much more diverse than the CN *pufM* genes and the maximum dissociation temperature was lower (87°C) than that of an *E. longus* culture (89.5°C). We speculate high AAnPB abundances in estuarine environments may be due to transportation of AAnPB into the estuaries from adjacent freshwater sources; however, further efforts are needed to determine the source and phylogenetic affiliations of AAnPB in estuarine locations. Interestingly, the diversity of *pufM* genes via melt-curve analysis revealed similar environments appear to have similar dominant dissociation temperatures, suggesting an underlying biogeographic pattern to regional *pufM* distribution (Fig. 6).

Initial reports by Kolber et al. (2000, 2001) and isolation of *puf* genes from all global locations tested in this study and others (Beja et al. 2002; Allgaier et al. 2003) further suggest non-chlorophyll *a*-based phototrophs may be readily found among marine prokaryotes. These results combined with the discovery of numerous proteorhodopsin proteins (Beja et al. 2000; Venter et al. 2004) suggest augmenting a heterotrophic metabolism with light-derived energy is a common strategy for a portion of the bacterial community. However, contrary to results of Kolber et al. (2001), our surprisingly low estimates of AAnPB via the original IREM method used by Kolber et al. (2001) and independently by QPCR suggest AAnPB constitute a smaller percentage of the bacterial community in most marine locations than is currently believed. Our results also appear to be consistent with the results of Venter et al. (2004), given that an intensive BLASTp search of the whole-genome shotgun library

(WGSL) constructed from Sargasso Sea bacterioplankton yielded only 17 new *pufM* sequences out of 1.2 million genes. While it may be possible that highly divergent *pufM* homologs exist among the 1.2 million gene sequences currently annotated in the Sargasso Sea WGSL, by first approximation, the lack of readily identifiable *pufM* genes in the database of Venter et al. (2004) further suggests *pufM* genes, and the AAnPB that presumably possess them, are not as abundant in all marine environments as is currently proposed. Given the potentially large impact newly discovered bacterial phototrophs may have on marine carbon cycling and bacterial energetics, more work is needed to determine the ecological significance of these widespread but generally low-abundance photoheterotrophs capable of aerobic anoxygenic photosynthesis.

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