

Abundance, Depth Distribution, and Composition of Aerobic Bacteriochlorophyll *a*-Producing Bacteria in Four Basins of the Central Baltic Sea^{∇†}

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The abundance, vertical distribution, and diversity of aerobic anoxygenic phototrophic bacteria (AAP) were studied at four basins of the Baltic Sea. AAP were enumerated by infrared epifluorescence microscopy, and their diversity was analyzed by using *pufM* gene clone libraries. In addition, numbers of CFU containing the *pufM* gene were determined, and representative strains were isolated. Both approaches indicated that AAP reached maximal abundance in the euphotic zone. Maximal AAP abundance was 2.5×10^5 cells ml⁻¹ (11% of total prokaryotes) or 1.0×10^3 CFU ml⁻¹ (9 to 10% of total CFU). Environmental *pufM* clone sequences were grouped into 11 operational taxonomic units phylogenetically related to cultivated members of the *Alpha*-, *Beta*-, and *Gammaproteobacteria*. In spite of varying *pufM* compositions, five clones were present in all libraries. Of these, *Jannaschia*-related clones were always found in relative abundances representing 25 to 30% of the total AAP clones. The abundances of the other clones varied. Clones potentially affiliated with typical freshwater *Betaproteobacteria* sequences were present at three Baltic Sea stations, whereas clones grouping with *Loktanella* represented 40% of the total cell numbers in the Gotland Basin. For three alphaproteobacterial clones, probable *pufM* phylogenetic relationships were supported by 16S rRNA gene analyses of Baltic AAP isolates, which showed nearly identical *pufM* sequences. Our data indicate that the studied AAP assemblages represented a mixture of marine and freshwater taxa, thus characterizing the Baltic Sea as a “melting pot” of abundant, polyphyletic aerobic photoheterotrophic bacteria.

Aerobic bacteriochlorophyll *a*-producing bacteria, or so-called aerobic anoxygenic phototrophic bacteria (AAP), are strict aerobes, carrying out a photoheterotrophic metabolism. They require organic substrates for growth, but they can supplement a significant portion of their metabolic requirements by light-derived energy (8, 16, 26, 31, 39). AAP were discovered in the Bay of Tokyo by Shiba et al. (34) in 1970s. Later, their wide distribution in marine environments was documented by infrared (IR) kinetic fluorometry (16, 18), IR epifluorescence microscopy (IREM) (9, 14, 19, 23, 33) and pigment analyses (12), as well as cultivation-based (1, 15, 38) and genetic (3, 5, 13, 28, 41) studies. The planktonic bacterial assemblages are known to contain various AAP groups belonging to *Alpha*-, *Beta*-, and *Gammaproteobacteria* (41). Microscopic analyses have shown that AAP abundances vary in different environments from 0 to 24% of total prokaryotes (9, 21, 33).

The ecological role of aerobic anoxygenic phototrophy is still unclear. A recent study suggested that AAP significantly contribute to bacterial production in oligotrophic oceans (17). Their ability to use light energy in addition to respiration could provide them an advantage over their heterotrophic bacterial

relatives (2). In contrast, Schwalbach et al. (32) found no effect of light on microbial community structure. Clearly, more data on AAP abundance and activity are required.

The Baltic Sea is a brackish, rather heterogeneous, enclosed-shelf sea system (27) harboring periodic anoxic conditions in its depths. During early summer three strata are formed with a stable thermocline around 20 m and a permanent halocline at 50 to 60 m. The presence and concentration of AAP in these surface waters of the central Baltic has been documented in two studies. Using IR kinetic fluorometry, bacteriochlorophyll *a* concentrations of between 8 and 50 ng liter⁻¹ were detected in samples in the late summer of 2003 (18). IREM analyses demonstrated seasonal changes in AAP abundance ranging from 0 to 12% of total prokaryotes (23). The highest cell counts were detected between April and September. Using IREM coupled with fluorescence in situ hybridization, it was found that the Baltic Sea AAP community consisted of *Alpha*- and *Gammaproteobacteria*. However, representative studies of AAP depth distribution and a reliable phylogenetic identification of such bacteria are still lacking. In the present study AAP abundances and depth distributions in four different basins of the central Baltic Sea were investigated by using IREM coupled with analysis of AAP diversity using environmental *pufM* gene clone libraries and cultivation-dependent methods.

MATERIALS AND METHODS

Sampling. Samples were obtained from onboard the R/V *Professor Albrecht Penck* during cruise 40/06/16 in 2006 to the Arkona Basin (station 113, 7 July;

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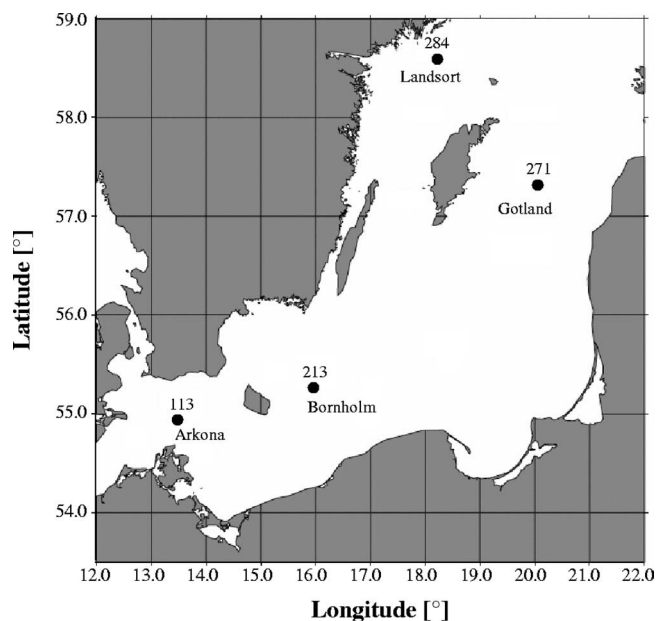


FIG. 1. Map of the central Baltic Sea and positions of the sampling stations.

54°55.42'N; 13°30.17'E), Bornholm Basin (station 213, 8 July; 55°14.76'N; 15°59.22'E), Gotland Basin (station 271, 10 July; 57°19.82'N; 20°02.82'E), and Landsort Deep (station 284, 11 July; 58°34.95'N; 18°14.62'E) (Fig. 1). Water samples from different depths were collected in 5-liter Free-Flow bottles (Hydrobios) attached to a polyvinyl chloride-coated stainless steel rosette equipped with conductivity, temperature, and depth sondes (SBE 911plus; Seabird), which included an electrode for measuring oxygen. For microscopy, water samples were fixed with 2% formaldehyde (0.2- μ m pore size, prefiltered), gently mixed, and incubated at 4°C.

Preparation of cultivation-based samples occurred during cruise 44/03/08 (9 August 2003) to the eastern Gotland Basin (station 271) onboard the R/V *Alexander von Humboldt*.

Microscopic quantification of AAP. Determination of the total cell numbers (TCN) and determination of AAP was done by IREM analyses as described by Mašín et al. (23).

Environmental *pufM* gene clone library construction and RFLP. Samples obtained at depths of maximal AAP concentration in the Arkona Basin (0.5 m), Bornholm Basin (4.0 m), Gotland Basin (9.0 m), and Landsort Deep (3.0 m) were filtered immediately onto Nuclepore filters (pore size, 0.2 μ m) and stored frozen at -80°C. In the laboratory, nucleic acids were extracted according to the procedure described by Weinbauer et al. (37). The *pufM* gene was amplified by using the *pufL* (forward, 5'-CTKTTGACTTCTGGGTSGG-3') and *pufM*_uniR (reverse, 5'-YCCATNGTCCANCKCCARAA-3') primer system (40), which included parts of the *pufL* and nearly the entire *pufM* gene. PCR mixtures contained 1 \times JumpStart REDTaq ReadyMix reaction mix (Sigma), 100 nM concentrations of the forward and reverse primers, and usually 10 ng of template. The reactions were incubated in a MyCycler (Bio-Rad) with initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 60 s, followed in turn by one 72°C step for 10 min. PCR products were purified (MiniElute PCR purification kit; Qiagen) and cloned in competent *E. coli* JM109 cells according to the manufacturer's instructions for the pGEM-T Easy vector system (Promega). Vector-specific primers T7 and SP6 were used to amplify PCR products for restriction fragment length polymorphism (RFLP) analyses and sequencing. The restriction enzymes MspI and HinfI were used to digest and visualize the unpurified PCR products as described previously (4). Operational taxonomic units (OTUs) were grouped by identical RFLP patterns, and representative fragments were sequenced by using the reverse primer *pufM*_uniR (40) by MWG Biotech AG (Martinsried, Germany).

Isolation and cultivation of AAP. Water samples from 13 different depths (6 to 180 m) at station 271 were taken in August 2003 (see Fig. S1 in the supplemental material). Dilutions (1 to 1:10,000) were prepared in 100- μ m-pore-size-filtered and autoclaved Baltic seawater. Volumes of 100 μ l were spread on Baltic sea-

water medium agar plates (three replicates) which had been prepared in Baltic seawater of 12‰ salinity amended with (per liter) 0.1 g NH₄-acetate, 0.05 g of K₂HPO₄, 0.05 g of KH₂PO₄, and 15 g of Bacto agar. CFU were determined after an 8-month incubation period at 10°C in the dark.

Nucleic acids of all colonies originating from one representative replicate plate out of three were extracted according to the method of Weinbauer et al. (37). *pufM* gene analyses, RFLP, and sequencing of representative OTUs were carried out as described for the environmental *pufM* gene. Dilution series were used to isolate all *pufM*-positive colonies on the Baltic seawater agar medium. These were then stored deep-frozen in glycerol (15% [vol/vol]) at -80°C.

Additional water samples were taken from the surface layer of different areas of the Baltic Sea, resulting in the isolation of strains B04, B09, and B11 on agar plates (15), and strains WM1 and WM2 using a two-step dilution technique with subsequent fluorescence screening (M. Koblížek et al., unpublished data). Additional information regarding the water samples for AAP cultures is summarized in Table 1.

Identification of AAP isolates. The full-length 16S rRNA gene was amplified from *pufM*-positive isolates by using the bacterial primers 27f (5'-AGAGTTG ATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3'). PCR mixtures contained 1 \times PCR buffer, 250 μ mol of each deoxynucleoside triphosphate, 150 nM concentrations of the forward and reverse primers, and 0.025 U of *Taq* polymerase (Fermentas) μ l⁻¹. PCR was done in a MyCycler (Bio-Rad) with initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 45°C for 45 s, and 72°C for 90 s, followed by one 72°C step for 2 min. PCR products were sequenced by MWG Biotech AG (Martinsried, Germany).

Phylogenetic analysis. Using ARB (<http://www.arb-home.de>), *pufM* sequences from the present study, together with sequences from public databases, were translated into amino acids and aligned. The protein alignment was used to realign nucleic acids, and phylogenetic trees were reconstructed based on sequences of approximately 400 nucleotides. These were reduced to unambiguously alignable positions by using group-specific filters. An evolutionary-distance dendrogram was constructed by using the Jukes-Cantor correction and neighbor-joining. The reliability of the branching patterns was evaluated by using 500 random bootstrap resamplings.

Statistical analyses. To investigate relationships of temperature and salinity to AAP, as well as of picocyanobacteria and heterotrophic prokaryotic cell numbers, Spearman rank correlations were used. Levels of significance were defined as $P \leq 0.05$ and $P \leq 0.001$.

Nucleotide sequence accession numbers. Partial *pufM* gene sequences were deposited in the GenBank database under accession numbers EU564436 (isolate B04), EU009365 (isolate B09), EU009366 (isolate B11), EU009373 (isolate WM1), EU026123 (isolate WM2), EU1680083 (isolate IOW23b), EU1680098 (isolate IOW11b), and EU1680084 to EU1680096 (*pufM* clones).

RESULTS

AAP cell distribution. AAP abundances were assessed in the major basins of the Arkona, Bornholm, Landsort, and Gotland basins. Depth profiles showed the typical summer stratification of the upper water column for each of the investigated sites (Fig. 2A to D). Salinity usually increased slightly (5 to 8‰) with depth, except for the Arkona Basin where salinity reached 17‰ at a depth of 40 m (Fig. 2A). The highest prokaryotic cell numbers were measured in the upper water column ranging from 2.1×10^6 (Fig. 2E) to 3.4×10^6 cells ml⁻¹ (Fig. 2F) but declined below the thermocline (Fig. 2E to H). Picocyanobacteria showed maximum abundance of 21.7% of the TCN in concentrations of up to 4.5×10^5 cells ml⁻¹ (Fig. 2G). The AAP cell numbers ranged from 4×10^4 cells ml⁻¹ up to a maximal level of 25×10^4 cells ml⁻¹ observed in the Bornholm Basin (Fig. 2F). In relative numbers AAP formed 2 to 11% of the total prokaryotes present in the euphotic zone. The highest numbers (5 to 11%) were detected at the upper 10 m at the Gotland Basin station (Fig. 2G). Zones for maximal cell numbers of AAP, picocyanobacteria, and TCN varied between the surface and depths of 5 to 17 m (Fig. 2E to G). TCN, picocyanobacteria, and AAP abundances at different depths were correlated in terms of salinity and temperature. Salinity did not

TABLE 1. 16S rRNA gene sequence length, nucleotide accession number and closest phylogenetic neighbor, as well as the location, temperature, and sampling date of isolated Baltic AAP

Isolate	Location ^a	Latitude	Longitude	Date	Temp (°C)	16S rRNA sequence length (bp)	Nucleotide accession no.	Closest phylogenetic neighbor	Similarity (%) ^b
B04	Eastern Gotland Basin, station F1*	58°39'N	21°24'E	27 August 2003	16	666	EU564435	<i>Erythromicrobium ramosum</i> DSM 8510	99
B09	Gulf of Finland, station F4*	59°39'N	24°09'E	28 August 2003	17	1430	DQ648518	<i>Roseobacter</i> sp. strain BS90	98
B11	Northern Baltic Proper, station F5*	59°20'N	22°33'E	30 August 2003	16	1434	DQ659411	Marine alphaproteobacterium AS-11	98
WM1	Warnemünde beach	54°11'N	12°06'E	7 July 2006	19	1416	EF421433	<i>Loktanella vestfoldensis</i> LMG 22003 [†]	99
WM2	Warnemünde beach	54°11'N	12°06'E	7 July 2006	19	1421	EF044234	Uncultured <i>Rhodobacter</i> group bacterium clone LA4-B3	97
IOW 11b	Gotland Basin, station 271 [†]	58°00'N	19°54'E	9 August 2003	21	1344	EU168097	Marine bacterium BS110	96
IOW 23b	Gotland Basin, station 271 [†]	58°00'N	19°54'E	9 August 2003	21	1334	EU168099	<i>Loktanella vestfoldensis</i> LMG 22003 [†]	99

^a *, samples were obtained during the "Biooptics cruise" aboard R/V *Oceania* in August 2003, as described previously (19); †, samples were obtained onboard R/V *Alexander von Humboldt* during cruise 44/03/08.

^b As determined by the program BLAST.

significantly correlate with AAP abundance, but Spearman rank correlations between water temperature and heterotrophic bacteria (TCN minus cyanobacteria and AAP), picocyanobacteria, and AAP cell counts confirmed that, at a significance of $P \leq 0.05$, temperature significantly correlated with all three parameters. At a higher significance level ($P \leq 0.001$) only AAP cell numbers were influenced by temperature (see Table S1 in the supplemental material).

Environmental *pufM* gene diversity. Based on restriction patterns and partial *pufM* sequencing 274 *pufM* clones could be grouped into 11 different OTUs using RFLP analysis. These grouped together with cultivated members of the *Alpha*-, *Beta*-, and *Gammaproteobacteria* (Fig. 3). The *pufM* phylogenetic tree exhibited three principal clusters: OTUs similar to *Alpha*-3-*proteobacteria*, namely, *Loktanella vestfoldensis* (represented by clone 170), *Rhodobacter sphaeroides* (clones 173 and 417), *Jannaschia* sp. (clones 203 and 291), and *Roseobacter denitrificans* (clone 289), as well as OTUs from two other clusters which were related to *Alpha*-4-*proteobacteria* (clone 390) or *Gammaproteobacteria* (clone 466). Clone 433 was most similar to the *pufM* sequence of *Rubrivivax gelatinosus* (80% sequence similarity) according to BLAST. However, this betaproteobacterial relationship was not reflected in the phylogenetic tree where *Beta*- and *Gammaproteobacteria* clustered together (Fig. 3A). The phylogenetic affiliation of *pufM* clone 77 could not be determined.

Associations between seven to nine different OTUs could be formed for each of the basin-specific *pufM* clone libraries (Fig. 3B). Five OTUs were present at all stations (clones 170, 289, 291, 340, and 433). Of these, *Jannaschia*-related clone 291 accounted for 25 to 30% of the analyzed sequences, whereas the relative abundances of the other groups varied among the stations. High abundances of 20 to 30% were found for betaproteobacterial clone 433 in the Arkona, Bornholm, and Landsort libraries, as well as in the Gotland library for *Loktanella*-

related clone 170 (40%). The *pufM* sequences in Bornholm, Gotland, and Landsort libraries related to *Gammaproteobacteria* (clone 466) contributed less than 10%.

AAP CFU. The depth profiles for temperature and oxygen recorded in August 2003 confirmed the typical summer stratification of the upper water column for the Gotland Basin (see Fig. S1 in the supplemental material). Highest TCN (8.9×10^6 cells ml⁻¹) and CFU numbers (1.2×10^4 colonies ml⁻¹) were found at a depth of 6 m. A total of 224 colonies from 13 different depths between 6 and 180 m were screened for the *pufM* gene. Only colonies of 1.0×10^3 and 5.0×10^2 CFU ml⁻¹ (9 to 10% of total CFU), originating from depths of 6 and 14 m, respectively, were *pufM* positive. Based on RFLP analyses, the *pufM*-positive colonies were grouped into two OTUs, represented by strains IOW11b and IOW23b (Table 1), which were phylogenetically located within the *Roseobacter* cluster (Table 1 and Fig. 3A).

Five more AAP strains were isolated from other areas of the Baltic Sea (Table 1). In accordance with their position in the phylogenetic *pufM* tree (Fig. 3A), their partial 16S rRNA gene strain sequences were most closely related to members of the *Alpha*-3- and *Alpha*-4-*proteobacteria* (Table 1). Interestingly, the *pufM* gene sequences of obtained isolates showed high similarities to environmental sequences. The *pufM* gene sequences of strain B04 and clone 390 and of strain WM2 and clone 203, as well as strains WM1, IOW 23b, and clone 170, were identical or nearly identical (Fig. 3A). No gamma- or betaproteobacterial AAP strains could be isolated.

DISCUSSION

The aim of this study was to identify the diversity and distribution of AAP in the deepest stations of four major Baltic basins. The Baltic Sea was chosen for AAP analyses because its distinct vertical and horizontal salinity gradients allow the co-

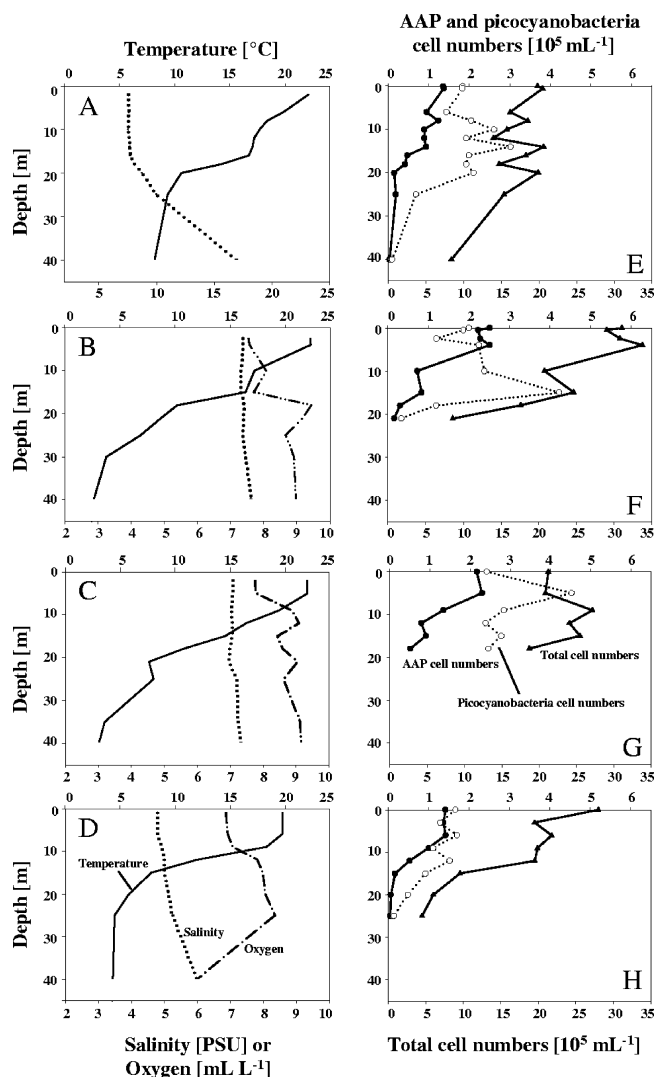


FIG. 2. Depth profiles throughout the Arkona Basin (A and E), Bornholm Basin (B and F), Gotland Basin (C and G), and Landsort Deep (D and H) in July 2006: temperature, oxygen, and salinity (A to D), as well as total, picocyanobacteria, and AAP cell numbers (E to H).

existence of freshwater and marine species (29). An increasing number of reports concerning the AAP presence in estuarine, coastal (33, 35), or fresh (24) waters formed by nonmarine species (41) suggested that these environments are inhabited by distinct AAP assemblages. In accordance with this, the diversity of *pufM* genes detected and high concentrations of AAP cells measured in the present study reflected specific brackish shelf conditions in the Baltic Sea.

AAP diversity. The *Roseobacter* group plays an important role in diverse marine surface systems (7), forming the major part of AAP communities in the Mediterranean and the Red Sea (28). The *Roseobacter* group comprised a substantial portion of the bacterial community in the Skagerrak-Kattegat front as well (30), which is characterized by its surface brackish outflow from the Baltic Sea and deep saline inflow from the Skagerrak and North Sea. However, its occurrence in the central Baltic Sea was supported only by a few isolates, and con-

firmation by molecular methods is still lacking (6, 20). In the present study, potential members of the *Roseobacter* clade represented 50 to 90% of all clones investigated (Fig. 3). Three clones of this clade (Table 1) contained *pufM* sequences nearly identical to those of Baltic AAP isolates, enabling us to support the *pufM* phylogenetic relationships by 16S rRNA gene analyses (Fig. 3A).

The presence of genes grouping with *L. vestfoldensis* in all four basins (Fig. 3B) was remarkable and supported by independent isolation of two *Loktanella* strains (Table 1). Metagenomic data from the global ocean sampling expedition detected members of the *Loktanella* cluster in a sample of a suboxic-to-anoxic hypersaline lagoon, but not in oxic samples (41). In the central Baltic, *pufM* genes probably affiliated with *Loktanella* were especially abundant in the clone library from the Gotland Basin (Fig. 3B). The Gotland Basin is the largest basin area with anoxic bottom water; during the sampling the water column at station 271 was anoxic below 140 m (unpublished data). However, how local physicochemical conditions could influence the presence of the *Loktanella* cluster remains to be investigated.

Analogously, high abundances of potentially betaproteobacterial clones from the Arkona, Bornholm, and Landsort basins were found in the *pufM* clone libraries. Interesting is the presence of potential *Erythromicrobium* related genes (clone 390) in all of the studied libraries. *Erythromicrobium*- and *Erythrobacter*-related species are frequently found in cultivated material (15, 34, 39); however, they have not been found in genetic studies (3, 28, 41). Based on our analyses sequences clustering with *Erythromicrobium*/*Erythrobacter* accounted for 3 to 18% of Baltic *pufM* sequences, which is in fair agreement with previously published fluorescence in situ hybridization analyses (23). In the phylogenetic *pufM* tree clone 433 is positioned within the *Gamma*- and *Betaproteobacteria* (Fig. 3A). In general, the distinction between *Gamma*- and *Betaproteobacteria* is not simple. Indeed, recent principal-component analyses have shown that *Betaproteobacteria* could be a subgroup of much larger *Gammaproteobacteria* (11). Since BLAST analyses showed the closest similarity to the betaproteobacterium *Rubrivivax gelatinosus*, clone 433 was assumed to be of betaproteobacterial origin. *R. gelatinosus* is a freshwater bacterium that was first described in 1907 as "*Rhodocystis gelatinosa*" (25). Since the drainage area of the Baltic Sea is more than four times as large as the sea itself (10), the high abundances of freshwater-related betaproteobacterial *pufM* clones was expected.

Structure of bacterial communities based on phylogenetic relationships does not necessarily provide insight into their physiological properties and should be interpreted with caution. In addition, nucleic acid extraction, PCR, and cloning techniques are biased, which may have affected the determinations of *pufM* abundances. This might explain why more gammaproteobacterial AAP cells may have been detected by using fluorescence in situ hybridization (23) than in our study. Nevertheless, considering the whole *pufM*-based data set, the clones derived from the four basins were indicative of AAP assemblages formed by a mixture of marine and freshwater influences characteristic for brackish water environments such as the Baltic Sea. In summary, our study demonstrated a broad spectrum of AAP diversity, which is in contrast to the very low

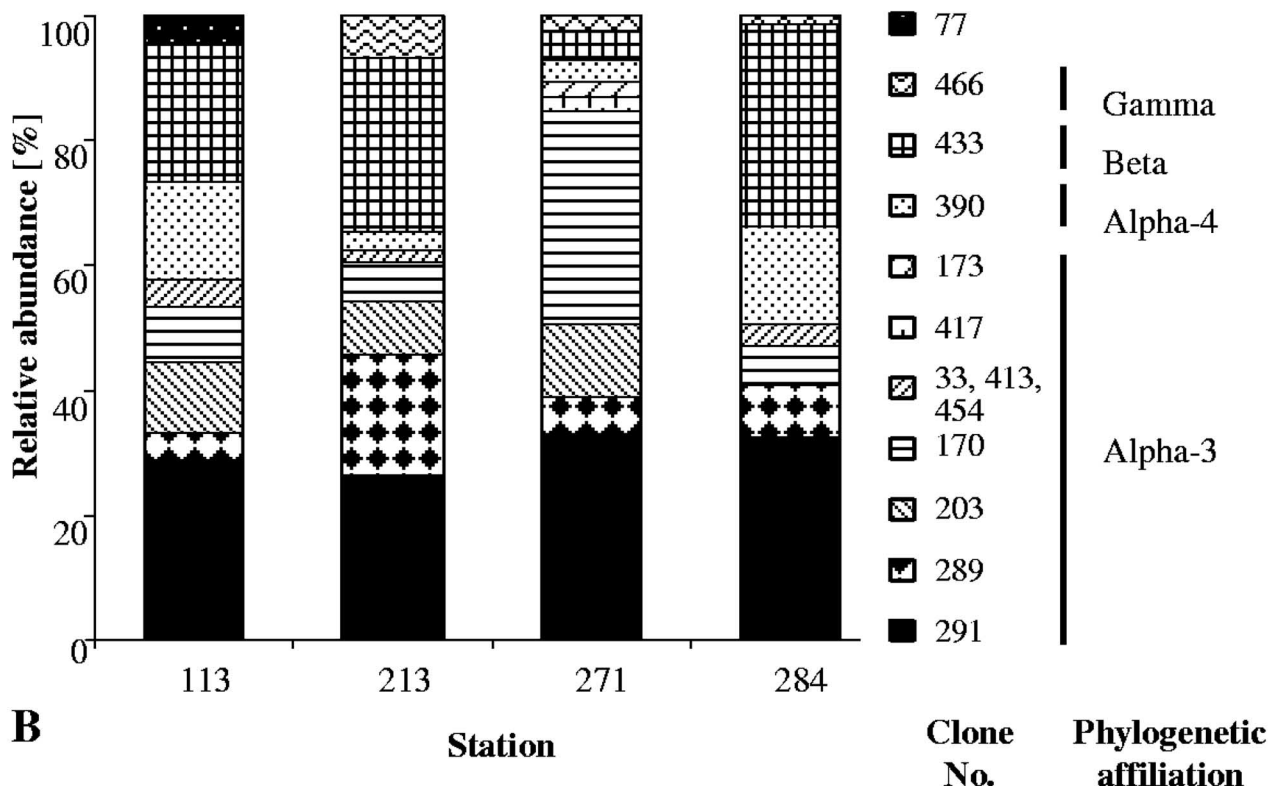
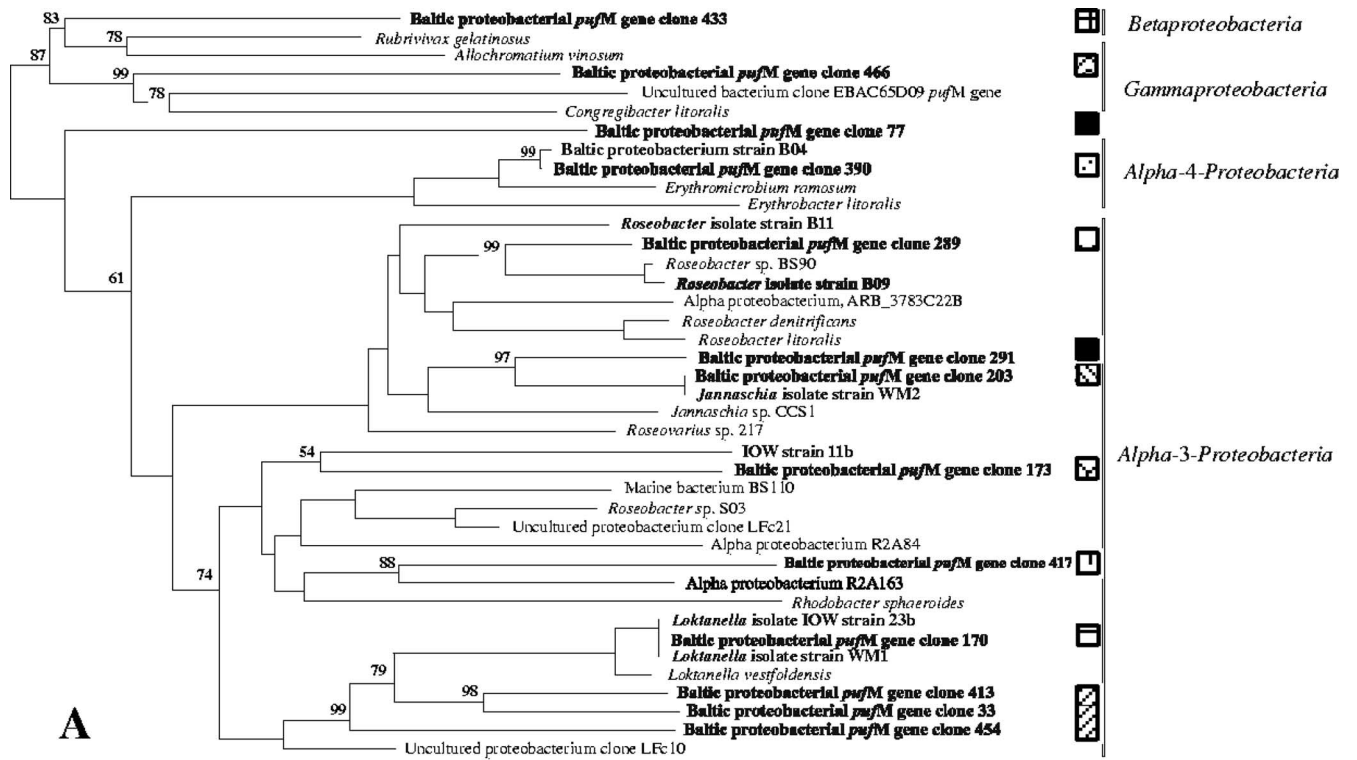


FIG. 3. (A) Unrooted tree showing the phylogenetic relationships of *pufM* gene sequences detected in the central Baltic Sea and their closest relatives. The tree was reconstructed by using the neighbor-joining method and is based on a comparison of approximately 400 nucleotides. Bootstrap values, expressed as a percentage of 500 replications, are given at branching points. Only values greater than 50% are shown. *Chloroflexus aurantiacus* was used as the outgroup. The *pufM* sequence of *L. vestfoldensis* DSM16212^T was obtained in the present study. Names in boldface indicate clones or isolates obtained from the present study. Patterns indicate representative clones that were retrieved throughout the stations. Bar, 10 substitutions per 100 nucleotides. (B) Relative abundance of different *pufM* genes in clone libraries, reflected by patterns identical to those from panel A. Clones derived from the depths of the maximal AAP concentration of the Arkona Basin (station 113), Bornholm Basin (station 213), Gotland Basin (station 271), and Landsort Deep (station 284).

diversity of AAP in eutrophic environments suggested by Jiao et al. (14).

AAP abundance. AAP cell concentrations of up to 11% of TCN were determined in 2006 by IREM (Fig. 2E to H), a level similar to those for the surface water in the summer of 2005 (23) and to the CFU of *pufM*-containing colonies compared to the total CFU (9 to 10%). This relatively high percentage is in agreement with other reports of high AAP abundances in coastal and shelf waters (35, 42).

IREM analyses showed the highest AAP cell numbers in the euphotic zone (upper 20 to 30 m of the water column) (Fig. 2E to H). Colonies containing *pufM* were only detected at comparable depths (Fig. S1 in the supplemental material), consistent with the phototrophic character of AAP. Light influence was already documented in different environments including the mid-Atlantic bight and the North Pacific gyre, where AAP cells were most abundant above a 1% light depth (9). In the Delaware and Chesapeake estuaries, AAP abundance was significantly correlated with light attenuation, as well as ammonium and nitrate concentrations (36), indicating that light is not the only factor influencing AAP assemblages. Thus, additional important ecological factors should also be considered. Analyses of AAP depth distributions in the Baltic showed a strong correlation between temperature and AAP abundance in the water column (Fig. 2). The highest AAP cell numbers were usually found at sites where temperatures were $\geq 20^{\circ}\text{C}$. Sieracki et al. (35) proposed that low temperatures (in this case, $<6^{\circ}\text{C}$) limited AAP abundances in the Northwest Atlantic, which would support our studies. On the other hand, temperature is linked to absorbed light energy, and it directly influences many other parameters. Thus, the extent of direct influence by temperature on AAP abundance in the central Baltic Sea during our sampling is unknown but should be taken into account in further analyses.

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