

Distribution and abundance of aerobic anoxygenic phototrophic bacteria in the tropical coastal waters of Gunungkidul, Yogyakarta, Indonesia

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Abstract. Aprilyanto V, Sembiring L, Djohan TS. 2020. Distribution and abundance of aerobic anoxygenic phototrophic bacteria in the tropical coastal waters of Gunungkidul, Yogyakarta, Indonesia. *Biodiversitas* 21: 5506-5513. Aerobic anoxygenic phototroph (AAP) is a community of bacteria capable of performing anoxygenic photosynthesis in the presence of oxygen. AAP abundance in most oceanic regions signals its ecological importance in marine microbial loop. This study was conducted to reveal the distribution and abundance of AAP in Siung coast, Gunungkidul, Yogyakarta, Indonesia. Total bacterioplankton and AAP were enumerated using acridine orange direct count (AODC) and real-time quantitative PCR (qPCR), respectively. Several water physicochemical parameters as well as dissolved nitrate, ammonium, phosphate, and sulfate were also measured. The results showed that total bacterioplankton was distributed thoroughly in the water column with abundance ranging from 2.5×10^4 to 3.5×10^4 cells/mL. Within this total bacterioplankton, AAP ranged from 3.83×10^2 – 7.48×10^2 cells/mL, comprising about 1% to ~2.5%. Inorganic nutrient concentration, mainly nitrate and phosphate were thought to be the regulating factors to the distribution and abundance of both communities. In conclusion, AAP comprises a relatively low portion of the total bacterioplankton community in the tropical coastal water in Gunungkidul, Yogyakarta.

Keywords: Bacterioplankton, AAP bacteria, microbial loop, qPCR

Abbreviations: AAP: Aerobic anoxygenic phototrophic; AODC: Acridine orange direct count; bchl-a: Bacteriochlorophyll-a; qPCR: quantitative polymerase chain reaction

INTRODUCTION

Aerobic anoxygenic phototroph (AAP) is a group of bacteria capable to perform phototrophy while still relying on organic matter as the main carbon source (Yurkov 2006; Ferrera et al. 2011, 2017; Tang et al. 2011; Kirchman and Hanson 2013). Similar to their purple bacteria counterpart, AAP bacteria utilize photosystem-II which contains bacteriochlorophyll-a (bchl-a) to conduct cyclic photophosphorylation (Zheng et al. 2013). However, unlike the rest of purple bacteria, phototrophy in AAP bacteria could only operate in aerobic conditions due to their electron acceptor difference. The main electron acceptor in AAP bacteria is unique and present in reduced state during anaerobic conditions and therefore could not accept the electrons (Nagashima et al. 2014). Oxygen molecules are required to re-oxidize this reduced electron acceptor and maintain the process of photophosphorylation.

Most of the species in AAP bacteria are classified as a member of Alphaproteobacteria, while the others are in the Beta- and Gammaproteobacteria (Csotonyi et al. 2011; Boeuf et al. 2014). The most notable genera of marine AAP bacteria are *Erythrobacter* (Shiba and Simidu 1982) and *Roseobacter* (Shiba et al. 1991). Currently, 39 and 6 species are known as members of genera *Erythrobacter* (*Erb*) and *Roseobacter* (*Rsb*), respectively (Parte 2014, 2018).

However, not all of those species possess photosystem-II photosynthetic apparatus, hence not all of them belong to AAP bacteria. Species such as *Roseobacter ponti* (Jung et al. 2017), *Roseobacter cerasinus* (Muramatsu et al. 2020), *Erythrobacter aquimixticola* (Park et al. 2017), *Erythrobacter arachoides* (Xing et al. 2017), *Erythrobacter insulae* (Park et al. 2020) and *Erythrobacter atlanticus* (Zhuang et al. 2015) are several species from both genera which do not possess phototrophic trait and hence are suggester for genera reclassification (Hördt et al. 2020; Xu et al. 2020)

The ecological role of marine AAP bacteria was not really known until 2000, when it was discovered that photoheterotrophic ability might contribute to their survival in nutrient-poor marine environments, such as open ocean (Kolber et al. 2000; Kirchman 2018). Marine AAP bacteria abundance was firstly reported in 2001 where it contributes to 10% of total bacterioplankton in Pacific Ocean, implying its significant contribution towards global carbon cycle (Kolber et al. 2001; Ritchie and Johnson 2012). However, not all studies regarding AAP abundance were in agreement with it. Similar studies conducted on the off coast of Southern California and Mediterranean sea using real-time quantitative PCR (qPCR) reported that AAP comprises only 1% of total bacterioplankton (Schwalbach and Fuhrman 2005; Lamy et al. 2011; Graham et al. 2018). More studies of AAP bacteria across the oceans revealed a

wide-ranging abundance, comprising of 1-16% in Atlantic (Cottrell et al. 2006; Sieracki et al. 2006), <5% in North Pacific (Cottrell et al. 2006), 24% in South Pacific (Lami et al. 2007), 10-14% in Arctic (Boeuf et al. 2013), 1-8% in Antarctic (Kirchman et al. 2014), 1-24% in Adriatic Sea (Tomaš et al. 2019), as well as 5-25% in Delaware estuary (Stegman et al. 2014). With such distribution and abundance data of AAP bacteria, it can be said that this community is distributed globally in the marine waters and made up a significant portion of total bacteria in the euphotic zone. These measurements on AAP abundance, however, had been conducted primarily on subtropical and temperate seas with some occasion in polar seas, but relatively lacking in tropical seas such as Indian ocean. Therefore, data concerning distribution and abundance of AAP bacteria in the tropical seas were scarce and so far, there had been no reports concerning the Indonesian tropical sea.

Siung is one of the southern coasts of Java located in Gunungkidul, Yogyakarta. In this study, we used real-time quantitative PCR analysis to assess the distribution and abundance of AAP bacteria in the water column of Siung coast. These data could provide insight in the ecological importance of AAP bacteria in the microbial loop as well as providing baseline data concerning the distribution and abundance of AAP bacteria in Indonesian sea.

MATERIALS AND METHODS

Study site

Siung coast is one of many coasts in Gunungkidul area of southern Java island, Indonesia. It lies next to Nglambor coast and can be reached from the nearest town, Wonosari. Geographically Siung coast is located in the equatorial zone and experiences only dry and wet season alternating every six months. Just like all coasts in southern Java,

Siung coast is a part of shelf sea ecosystem (Spalding et al. 2007). Samples were collected from the coastal zone of Siung ($8^{\circ}11'05.98''E$; $110^{\circ}40'47.09''S$) about 1 kilometer from the coastline during early dry season in May 2012 (Figure 1). The maximum and Secchi depths of the study site were 22 and 7 meters, respectively. Four determined sampling points were 0, 4, 6, and 20 meters, representing surface, subsurface, light compensation, and near-bottom depths, respectively. Water samples were collected from four depths: 0, 4, 6, and 20 meters in the water column using modified 2-liter-Van Dorn water sampler with five replicates. The samples were stored in the 600 mL sterile dark polystyrene bottles. Fifty mL of these samples were stored in polypropylene tubes and formaldehyde 37% was added into each tube until reaching 1% of the final concentration. Another 200 mL from each water sample was directly filtered through 0.2 μm -pore-size polycarbonate filters. All the filters were then stored in bead tubes and kept at -20°C until DNA extraction. Filtrate from replicates of each depth was composited, stored into sterile dark polystyrene bottles, and kept in -20°C until further analyses.

Physicochemical parameters

Along with the sampling from each depth, several physicochemical parameters were also taken. The parameters were comprised of water temperature, Secchi depth, salinity, alkalinity, pH, and dissolved oxygen. Inorganic nutrients comprising of nitrate, ammonium, phosphate, and sulfate were also measured from water samples. Measurement of dissolved oxygen was conducted using Winkler method, while concentration of inorganic nutrients in the water was measured using spectro-photometric methods, comprising ascorbic acid reduction for nitrate, phosphomolybdate complex formation for phosphate, and atomic absorption spectroscopy for iron (Rand et al. 1976).

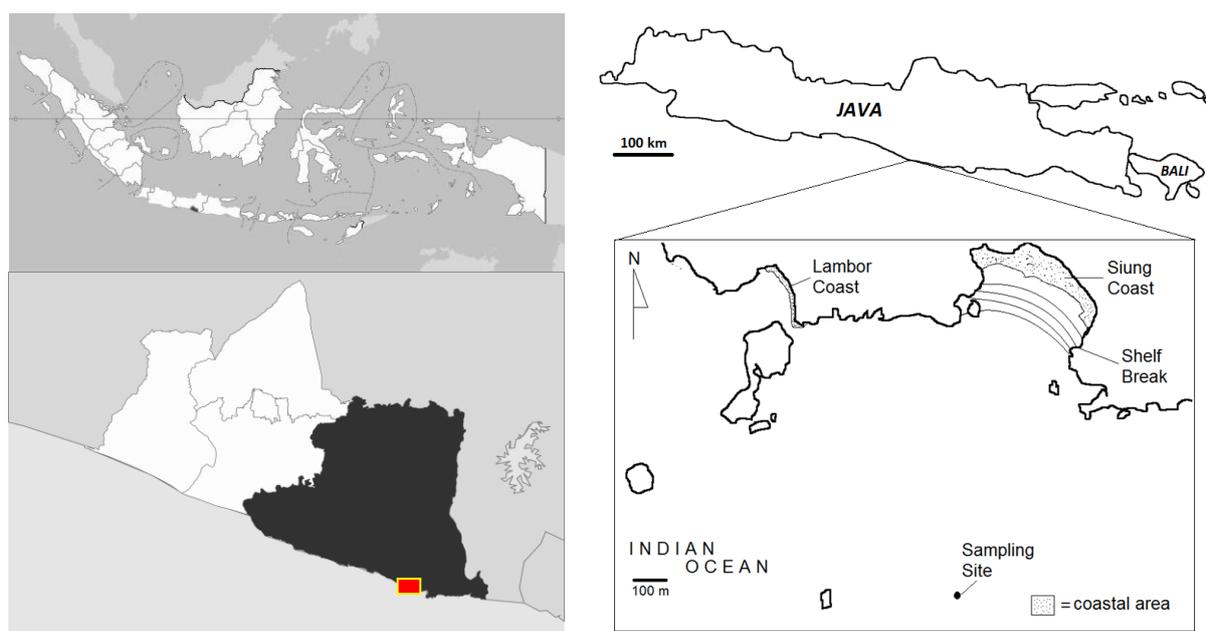


Figure 1. Sampling site at Siung coastal zone, Gunungkidul District, Yogyakarta, Indonesia

All the nutrient concentration measurements were taken at the Laboratory of Chemistry, Faculty of Mathematics and Sciences, Universitas Gadjah Mada, Yogyakarta, and Health and Calibration Laboratory Center (Balai Laboratorium Kesehatan dan Kalibrasi), Yogyakarta.

Enumeration of total bacterioplankton

Total bacterioplankton is enumerated using acridine orange direct count (AODC) technique with some modifications (Ruyitno 1988). A 2.29 mL of acridine orange 1% solution was added to 5 mL water from each sample and incubated for at least 48 hours. Each sample was filtered through 0.2- μ m-pore-size black polycarbonate filter (Millipore). After filtering, each filter was placed on top of a glass slide, stacked by cover glass, and given a drop of immersion oil on the top of the deck glass. Enumeration was conducted on sums of ten random field of views and the number of bacterioplankton were calculated by the following formula:

$$cell/ml = n \times \frac{A_1}{A_2} \times \frac{1}{V}$$

Where; n : cell average count; A_1 : area of the filter paper in contact with water sample (490.87 mm²); A_2 : microscope field of view area at 1000x magnification (0.02405 mm²); V : volume filtered water sample. One-tail LSD using Agricolae (R-package) was conducted to test the significance of total bacteria differences among depths.

Enumeration of AAP bacteria

AAP bacteria were enumerated by real-time quantitative PCR (qPCR) on extracted total DNA. Extraction of total DNA was conducted using bead-beating approach towards polycarbonate filters using Power Water DNA extraction kit (Qiagen, Germany). The extracted total DNA (Table S1) was then quantified for its *pufM* gene by quantitative real-time PCR (qPCR; BioRad CFX 96) using primer pairs *pufM*(F) 5'-CCATSGTCCAGCGCCAGAA-3' and *pufM*(R) 5'-TACGGSAACCTGTWCTAC-3' (Achenbach et al. 2001; Bèjà et al. 2002). The PCR composition proceeds as follows: 12,5 μ L SsoFast™ Evagreen® Supermix (Bio-Rad, USA); 11 μ L PCR grade H₂O; 0,25 μ L of each primer (40 μ M), and 1 μ L DNA template (2,5 ng/ μ L). The following PCR thermal profile is 3' in 95°C followed by 50 cycles of 30s in 60°C. The resulting C_q value from each sample was then converted into initial *pufM* template number (C_0) using the standard curve from Du et al. (Du et al. 2006) according to the formula:

$$AAPB/ml = \frac{C_0 \times \frac{DNA_A}{DNA_B} \times V_{DNA}}{V_{water}}$$

Where; C_0 : initial template number, DNAA: stock DNA concentration (ng/ μ L), B: DNA amount used in qPCR (ng), C: total volume of extracted DNA (μ L), V: total vol of filtered water (mL). The corresponding 1:1 ratio of C_0 :AAP was used to convert the initial template number to the number of AAP bacteria cells (Schwalbach and Fuhrman 2005).

RESULTS AND DISCUSSION

Distribution and abundance of total bacterioplankton

Siung coast contains total bacterioplankton ranging from 2.5x10⁴ to 3.5x10⁴ cells/mL seawater. The distribution of bacterioplankton tends to be high (3.55x10⁶ cells/mL) on the surface (0 m), then declines at 4 m depth and then increase again near above the light compensation depth zone (Figure 2.A). Going further down to 20 m depth, the number of total bacterioplankton is decreasing towards 2.5x10⁴ cells/mL (Figure 2.A). Analysis of variance towards total bacterioplankton counts showed that the numbers from one depth to another did not differ significantly (Table S2). This observation might be attributed to the mixing of the water column.

Distribution and abundance of AAP bacteria

The abundance of AAP bacteria in this study was determined using the copy number of *pufM*, a gene expressing photosystem-II protein subunit which is a signature of AAP bacteria community. Although homologous gene was also present in other purple bacteria not belonging to AAP bacteria, the occurrence of this gene in the oxygenated water column is unique to the latter community. The abundance of AAP bacteria measured was relatively small, ranging from 300 to 400 cells/mL of seawater corresponding to 1-2% of total bacterioplankton (Figure 2.B-C). In terms of distribution, AAP bacteria were distributed quite similar to the total bacterioplankton by being more abundant on the surface water and decreasing along with the depth. However, near the light compensation depth zone, the number of AAP bacteria got higher compared to other depths. Similar to the total bacterioplankton, the variance of AAP bacteria counts among depths differed insignificantly (Table S3). This might also indicate that the same mixing causes the homogenous count of AAP bacteria in the water column.

Water physicochemical parameters

Similar to both total bacterioplankton and AAP bacteria, most water physicochemical parameters did not show significant differences between one depth to another (Figure 3; Table S4). This further confirms the occurrence of water column mixing on the coast of Siung. Dissolved oxygen, temperature, salinity, and pH showed steady levels with almost no value fluctuations. An interesting thing to note is that the parameter of inorganic nutrients, comprising nitrate, ammonium, phosphate, and sulfate displays little fluctuations, particularly between 4 to 6-meter depths. Nitrate, ammonium, and phosphate were slightly depleted at these depths indicating microbial utilization of these nutrients. The significantly high sulfate concentration might indicate a high surface run-off at the time of sampling. Since all of the measured water physicochemical parameters were distributed homogeneously, it is quite difficult to draw relations between these parameters to total bacterioplankton or AAP bacteria.

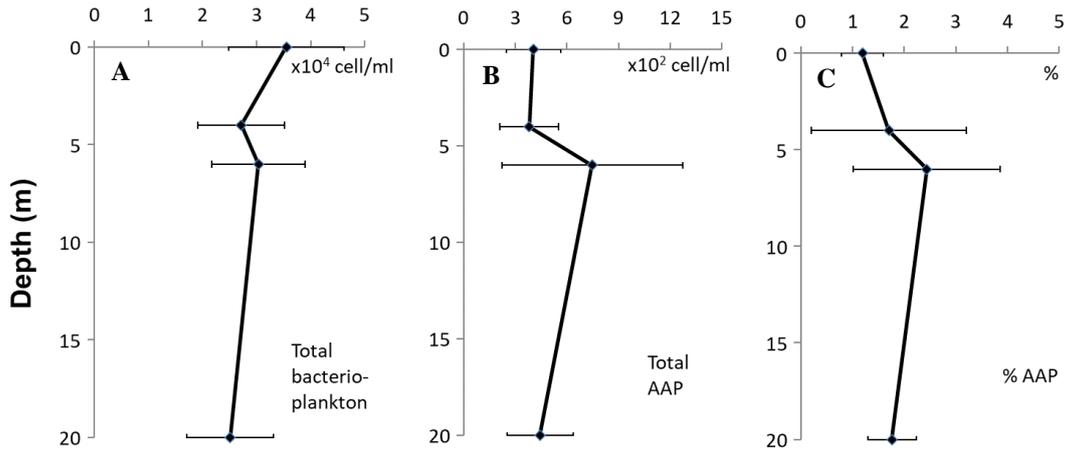


Figure 2. Distribution of total bacterioplankton (A), AAP bacteria (B) and percentage of AAP to total bacterioplankton (C) in Siung coastal water column at 0, 4, 6, and 20 meters

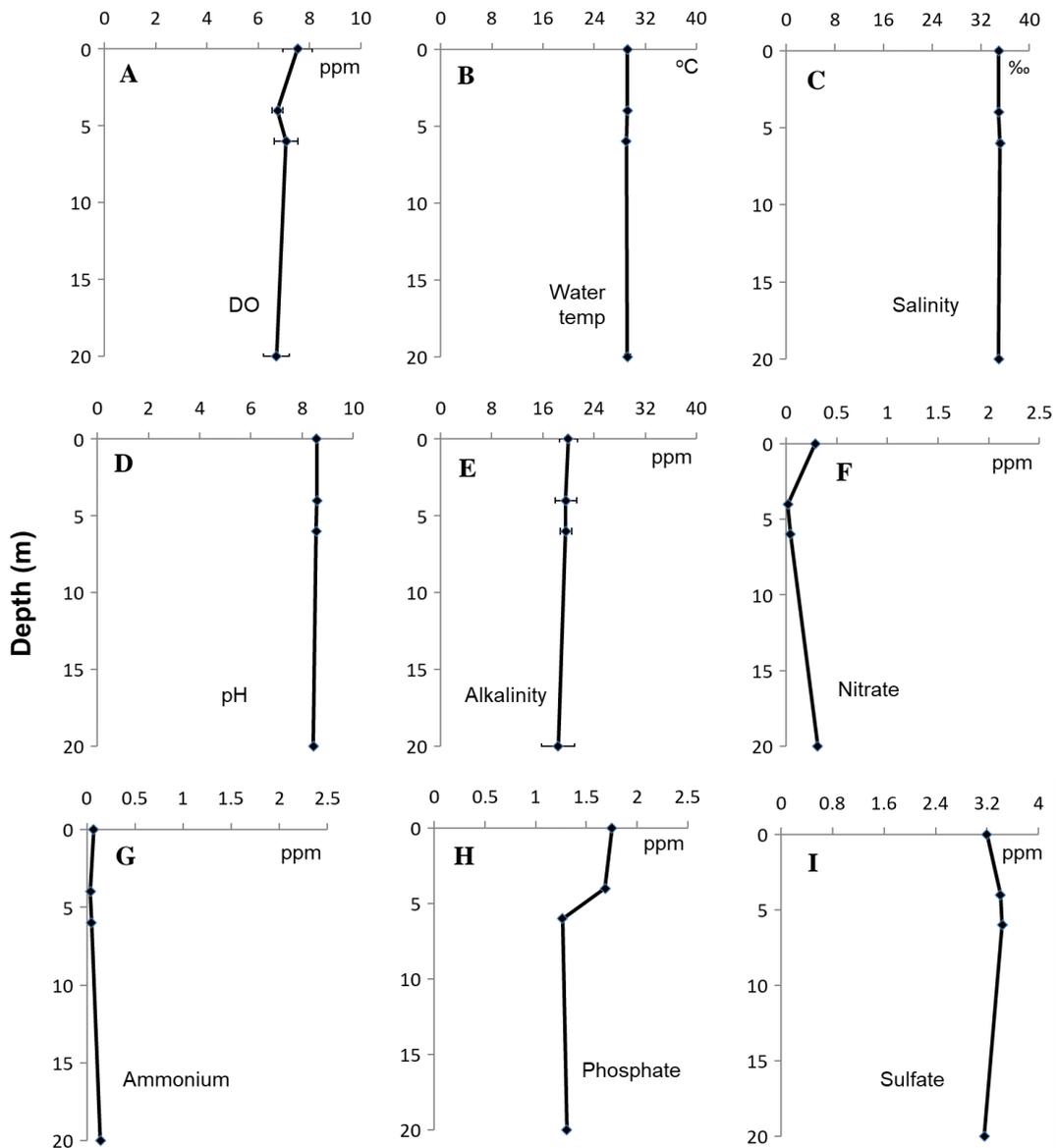


Figure 3. Distribution of water physicochemical parameters of Siung coastal zone water column comprising: dissolved oxygen (DO) (A), temperature (B), salinity (C), pH (D), alkalinity (E), nitrate (F), ammonium (G), phosphate (H) and sulfate (I). All parameters were measured from water column at 0, 4, 6, and 20-meter depths

Discussion

The percentage of AAP bacteria which only comprised 1–2.5% of total bacterioplankton in Siung water was considered low compared to similar data in other oceans. This percentage was similar to the low-end value of Northwest Atlantic (Sieracki et al. 2006) and North Pacific (Cottrell et al. 2006) oceans as well as Mediterranean sea (Hojerová et al. 2011). These similarities might imply that the distribution and abundance of both total bacterioplankton and AAP bacteria communities might be regulated by the same factors. Low water temperature or spring bloom is unlikely to be the causing factor since Siung water is located in the equator. Other factors such as shelf sea nature and its location facing Indian ocean make the water column in Siung coast undergoes vertical mixing at its upper layer (Purba et al. 2018). Such mixing is likely to create homogenous water temperature and nutrient content along the water column. Therefore, nutrient availability and predation are the most likely factor causing the low abundance of AAP bacteria in Siung coastal water.

Based on the requirement of carbon source, AAP bacteria are similar to heterotrophic bacteria. Therefore, their distribution and abundance tend to follow the fluctuations of dissolved organic matter (DOM) produced by phytoplankton. In turn, the distribution and abundance of phytoplankton are affected by inorganic nutrients such as nitrate and phosphate (Bradley et al. 2010a,b; Longnecker et al. 2010; Voss et al. 2011; Björkman et al. 2012; Lin et al. 2016; Reed et al. 2016). High nitrate and phosphate concentration but low $\text{NO}_3^-/\text{PO}_4^{3-}$ ratio in the water column is thought to be the factor causing both low AAP and bacterioplankton abundances (Weber and Deutsch 2010; Halm et al. 2012). This ratio, which is ten times lower than in the Pacific (Weber and Deutsch 2010), clear pictures that Siung water is NO_3^- -limited.

Predation by nanoplanktons is likely to decrease the abundance of AAP bacteria even further in addition to affecting its distribution (Sanders and Gast 2012; Hisatugo et al. 2014; Batani et al. 2016). Studies on grazing of AAP bacteria by various nanoflagellate and nanociliate in marine and freshwater ecosystems had shown a higher preference towards AAP bacteria compared to other bacterial communities (Unrein et al. 2014; Garcia-Chaves et al. 2015; Rocke et al. 2015). The process of capturing, ingesting, and metabolizing the bacterial prey are entirely aerobic which consumes dissolved oxygen. This might indicate that the low count of AAP bacteria along with low DO level at 4m depth is indicating AAP bacteria predation by nanoplankton. The predator preference for AAP bacteria might be related to cell biovolume difference. AAP bacteria possess larger cell biovolume compared to other bacterioplankton (Sieracki et al. 2006; Lami et al. 2007) and this might be selectively preferred by nanoplankton community (Garcia-Chaves et al. 2015, 2016). More studies are needed to elucidate this top-down regulation of AAP bacteria and its implication to the overall microbial loop and its connection to the larger trophic food web on Siung coast.

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Table S1. DNA concentration from the extracted water samples

Depth (m)	n	Abs280	Abs280/260	DNA conc (ng/uL)	Depth (m)	n	Abs280	Abs280/260	DNA conc (ng/uL)
0	n1	0.025	1.342	33.9	6	n1	0.019	1.285	34.8
	n2	0.017	1.115	28.2		n2	0.019	1.109	25.8
	n3	0.014	1.073	19.2		n3	0.016	1.190	26.7
	n4	0.018	1.123	22.4		n4	0.02	1.182	26.9
	n5	0.012	1.055	21.6		n5	0.014	1.078	22.5
4	n1	0.014	1.121	23.1	20	n1	0.017	1.115	23.8
	n2	0.015	1.109	22.8		n2	0.016	1.132	26.5
	n3	0.015	1.191	24.9		n3	0.014	1.089	22.8
	n4	0.016	1.130	24.1		n4	0.015	1.077	23.5
	n5	0.014	1.085	23.1		n5	0.015	1.111	26.1

Table S2. Total bacteria count using acridine orange direct count (AODC)

Depth (m)	Replicate	# bacteria in field of view										Total	Avg	# cell per mL water ¹	Avg per depth ²	SD per depth
		1	2	3	4	5	6	7	8	9	10					
0	n1	5	5	5	7	14	18	12	8	8	12	94	9.4	38348	35492 ^a	10642
0	n2	8	6	8	13	8	9	10	19	19	13	113	11.3	46099		
0	n3	3	4	3	7	11	10	17	11	11	22	99	9.9	40388		
0	n4	13	6	6	6	9	11	7	9	9	9	85	8.5	34676		
0	n5	6	2	3	2	3	3	7	4	8	6	44	4.4	17950		
4	n1	7	8	10	7	8	9	11	7	9	8	84	8.4	34268	27170 ^a	8018
4	n2	8	5	4	8	11	5	9	5	9	9	73	7.3	29781		
4	n3	7	13	5	5	8	9	9	8	10	7	81	8.1	33044		
4	n4	2	2	3	2	3	4	8	4	3	5	36	3.6	14686		
4	n5	3	4	3	7	5	6	8	9	6	8	59	5.9	24069		
6	n1	8	4	7	9	4	5	6	5	9	7	64	6.4	26109	30352 ^a	8618
6	n2	7	6	4	6	6	6	8	4	6	5	58	5.8	23661		
6	n3	3	7	8	2	2	4	16	7	5	7	61	6.1	24885		
6	n4	5	15	8	9	7	8	16	11	11	19	109	10.9	44467		
6	n5	10	8	9	7	9	10	6	7	6	8	80	8	32637		
20	n1	8	4	4	4	7	7	12	8	8	6	68	6.8	27741	25130 ^a	7992
20	n2	4	6	7	9	3	4	6	6	7	7	59	5.9	24069		
20	n3	8	7	8	8	12	11	8	9	9	11	91	9.1	37124		
20	n4	4	2	5	10	2	4	2	7	7	8	51	5.1	20806		
20	n5	3	2	5	5	6	4	3	4	3	4	39	3.9	15910		

Note: ¹based on the formula: $(n \times (A1/A2) \times (1/V))$, where n: average of cell count (column O); A1: area of filter paper in contact with sample (625 mm²); A2: field of view area at 1000x magnification (0.02405 mm²); V: volume of filtered water (mL). ²based on ANOVA conducted on agricolae R-package. Same letters indicate a non-significant difference ($p \leq 0.05$).

Table S3. AAP bacteria count using real time quantitative PCR

Depth (m)	Replicate	Cq	Log C ₀ ¹	C ₀	DNA conc (ng/uL)	AAP bacteria (cell/mL) ²	Total bacteria (cell/mL) ³	% AAP
0	n1	35.3237	1.67808	47.6517	33.9	323	38348	0.84249
0	n2	33.7879	2.0196	104.616	28.2	590	46099	1.27992
0	n3	34.4729	1.86726	73.6655	19.2	283	40388	0.7004
0	n4	33.4091	2.10382	127.006	22.4	569	34676	1.64085
0	n5	34.7947	1.79571	62.4758	21.6	270	17950	1.50359
4	n1	34.9367	1.76413	58.0941	23.1	268	34268	0.78322
4	n2	34.5996	1.83909	69.0376	22.8	315	29781	1.0571
4	n3	33.9817	1.97649	94.731	24.9	472	33044	1.42765
4	n4	33.3266	2.12218	132.489	24.1	639	14686	4.3482
4	n5	35.3249	1.67781	47.6224	23.1	220	24069	0.91409
6	n1	33.1582	2.15962	144.418	34.8	1005	26109	3.84978
6	n2	33.1699	2.15701	143.552	25.8	741	23661	3.13052
6	n3	34.8571	1.78184	60.5117	26.7	323	24885	1.29849
6	n4	31.8928	2.441	276.058	26.9	1485	44467	3.33997
6	n5	35.5879	1.61932	41.6214	22.5	187	32637	0.57389
20	n1	33.1221	2.16765	147.112	23.8	700	27741	2.52425
20	n2	34.9468	1.76187	57.793	26.5	306	24069	1.27258
20	n3	33.3456	2.11794	131.202	22.8	598	37124	1.61158
20	n4	34.5575	1.84844	70.5414	23.5	332	20806	1.59352
20	n5	35.0230	1.74493	55.5817	26.1	290	15910	1.82358

Note: ¹based on linear regression equation $Cq = -4.497(\log C_0) + 48.87$; standards for linear regression were taken from Du et al. (2006). ²based on the formula: $(C_0 \times (A/B) \times C) / V$; where A: stock DNA conc, B: DNA amount used in qPCR, C: total vol of extracted DNA, V: total vol of filtered water. ³from Table S2.

Table S4. Physico-chemical parameters of the water column

Depth (m)	Repl-icate	Time	Temperature			Dissolved Oxygen				Salinity (%)			pH			Alkalinity (P)				Inorganic Nutrient (ppm)				
			Temp (C)	Avg	SD	Titr (mL)	DO (ppm)	Avg	SD	Salinity (%)	Avg	SD	pH	Avg	SD	Titr (mL)	Alk-P (ppm)	Avg	SD	NO ₃ ⁻	NH ₄ ⁺	PO ₄ ³⁻	SO ₄ ²⁻	Fe
0	n1	7:53	29.0	29.20	0.274	1.84	7.36	7.54	0.573	35.00	35.00	0.000	8.52	8.57	0.031	0.1	20	19.60	1.673	0.2900	0.0740	1.75383	3198.2	<0.003
	n2		29.5			1.73	6.92			35.00			8.56	0.11		22								
	n3		29.0			2.04	8.16			35.00			8.59	0.09		18								
	n4		29.0			2.03	8.12			35.00			8.59	0.1		20								
	n5		29.5			1.78	7.12			35.00			8.59	0.09		18								
4	n1	7:46	29.0	29.20	0.274	1.69	6.76	6.75	0.212	35.00	35.00	0.000	8.58	8.59	0.017	0.1	20	20.00	1.414	0.0210	0.0380	1.686	3406.9	<0.003
	n2		29.0			1.64	6.56			35.00			8.56	0.1		20								
	n3		29.5			1.75	7.00			35.00			8.59	0.09		18								
	n4		29.5			1.73	6.92			35.00			8.60	0.11		22								
	n5		29.0			1.63	6.52			35.00			8.60	0.1		20								
6	n1	7:35	29.0	29.00	0.000	1.83	7.32	7.09	0.456	35.00	35.20	0.447	8.58	8.56	0.027	0.1	20	19.60	0.894	0.0440	0.0490	1.26517	3436.4	<0.003
	n2		29.0			1.66	6.64			35.00			8.54	0.1		20								
	n3		29.0			1.69	6.76			36.00			8.59	0.1		20								
	n4		29.0			1.94	7.76			35.00			8.53	0.09		18								
	n5		29.0			1.74	6.96			35.00			8.54	0.1		20								
20	n1	7:26	30.0	29.20	0.447	1.54	6.16	6.71	0.500	35.00	35.00	0.000	8.35	8.45	0.081	0.07	14	18.40	2.608	0.3120	0.1440	1.30587	3156.3	<0.003
	n2		29.0			1.55	6.20			35.00			8.38	0.1		20								
	n3		29.0			1.72	6.88			35.00			8.48	0.09		18								
	n4		29.0			1.78	7.12			35.00			8.50	0.1		20								
	n5		29.0			1.8	7.20			35.00			8.54	0.1		20								