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Stimulation of Regeneration of Inorganic Nitrogen and Phosphorus in Surface and Bottom Waters of a Eutrophic Inlet by Adding Effective Bacteria

ARUN B. PATEL[1], KIMIO FUKAMI[2]*, ABDUL KARIM[2] and TOSHITAKA NISHIJIMA[2]

[1] *Environmental Management Division, National Research Institute of Aquaculture, Nansei, Mie 516–0193, Japan*
[2] *LAQUES, Kochi University, Otsu 200 Monobe, Nankoku, Kochi 783–8502, Japan*

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Two promising bacterial strains, one isolated from the sediment surface of the study site (9410-O, mesophilic) and the other isolated from the Antarctic (AN-1, psychrotolerant) were used to enhance inorganic nitrogen (DIN) and phosphorus (DIP) regeneration in surface and bottom waters from a eutrophic coastal inlet in different seasons. Unfiltered waters with or without added bacteria were incubated in the dark at in situ water temperatures and at 10°C for 10–15 days, and the accumulation of DIN and DIP was monitored. Bacteria were always added as resuspensions prepared in filtered (0.2 μm) in situ waters. Strain 9410-O stimulated DIN and DIP net regeneration by ca. 2-6-fold in surface water, and 3-25-fold in bottom water at relatively high in situ/incubation temperatures viz. 23 and 26°C. However, it caused little enhancement of nutrient regeneration at 10°C. On the other hand, strain AN-1 stimulated net DIN and DIP regeneration in waters at both relatively high (23.5°C; 2-4-fold) and low temperatures (10–12°C; ca. 2-10-fold). Live cells of either strain yielded remarkably higher DIN releases than the same volume of killed counterparts indicating that the activities of the added bacteria rather than their utilization as organic matter were primarily responsible for additional nutrient regeneration.

Key words: mesophilic, psychrotolerant, eutrophic coastal inlet, inorganic nitrogen and phosphorus regeneration, bacterial resuspension

A major portion of feed input in intensive fish and shrimp culture facilities is finally discharged into the environment without any treatment[3,17,33]. The resulting hyper organic enrichment of the water column and underlying sediments of recipient ecosystems[16,18] is considered one of the most important factors affecting further development and sustainability of the aquaculture industry[24]. In order to ensure a sustainable and environmentally friendly aquaculture, it is important that efforts are made to facilitate the decomposition and utilization of these allochthonous organic materials under conditions close to those in situ.

Recent advances in microbial ecology and biotechnology have made possible the on-site treatment of wastes even in relatively large areas of water[19,23], and the biological treatment of wastes from intensive fish culture is considered feasible[29]. In fact, several commercial products of viable bacteria are now being used to control water quality in aquacultural facilities, however, not many scientific studies to validate their effectiveness in situ have been reported[22]. Such a lukewarm response is presumably due to the consideration that ambient microorganisms are able to degrade labile organic matter on their own. But, considering that intensive farming in enclosed or semi-enclosed spaces causes organic matter loading at much faster rates than those at which ambient heterotrophic bacteria can decompose and utilize or recycle, there is a pressing need for biotechnological intervention[6], especially in situations where the activities of indigenous bacteria are limited by prevailing ecological variables[26,27].

* Corresponding author; E-mail: fukami@cc.kochi-u.ac.jp, Tel: +81–88–864–5152, Fax: +81–88–864–5197

We envisaged accelerating the mineralization of organic matter in unamended surface and bottom waters from a eutrophic coastal inlet which is heavily utilized for intensive fish culture in cages by adding some promising bacterial strains at in situ temperatures. In the present paper, we report a series of representative experiments describing the effects of the addition of two bacterial strains namely 9410-O, a mesophilic; and AN-1, a psychrotolerant, on the regeneration of inorganic nitrogen (DIN) and phosphorous (DIP) in surface and bottom waters of the Uranouchi Inlet (Japan) in different seasons and/or different incubation temperatures.

Material and Methods

Study site and sample collection

Uranouchi Inlet is a semi-enclosed and a eutrophic coastal ecosystem which witnesses only limited water exchange and a prominent density stratification[9,11,26]. The sampling station of Mitsumatsu is located in the central part of the inlet (average depth is ca. 16–17 m) which is heavily utilized for intensive fish culture in cages. A detailed description of the study site and sampling station may be found in other published works[9,11,26,27].

Water samples from depths of 2 m (surface) and 16 m (bottom) were collected using a 5 L Niskin water sampler, and were gently transferred into 10 L carboys via a silicon tube. The carboys were filled completely without leaving any air space. The samples were carried back to the laboratory within 1–2 hours of collection at ambient atmospheric temperature under semi dark condition.

On each sampling occasion, physicochemical variables including temperature, DO, pH, salinity and redox potential in the water column were measured in situ using a CTD system of Ocean Seven (Idronaut, model no. 301 S). In addition, chlorophyll a concentrations by a spectrophotometric method[32] and bacterial abundances by direct counts using epifluorescence microscopy after staining with DAPI (4',6-diamidino-2-phenylindole dihydrochloride)[8,28] in surface and bottom water subsamples were estimated in the laboratory.

Bacterial selection, growth, and proteolytic activity

We screened 10 potential strains from a large number of stock of bacterial strains available at our laboratory through visual inspection of turbidity in sterile liquid FeTY growth medium[12] at various temperatures ranging 5–25°C under asepectic conditions. We then evaluated the actual growth performances of these potential strains at temperatures ranging 0–30°C by incubating in liquid FeTY and taking subsamples of growing cells aseptically under a clean bench at different time intervals. The number of cells in the subsamples was counted as direct counts using epifluorescence microscopy after staining with DAPI as referenced above.

Proteolytic activities of these strains were estimated semi-quantitatively by assaying the growing bacterial cells on 1% casein agar plates and observing the area of the clear zone[7]. Based on these two parameters, we primarily selected two bacterial strains with distinctly different temperature-growth responses, a mesophilic 9410-O and a psychrotolerant AN-1 as potentially useful candidates, considering that bacterial activities in the water column of the study site are limited in summer as well as in winter. The strain 9410-O was isolated from the upper layer of sediment of the study site in October when proteolytic activities in the sediment peaked. This strain exhibited remarkably high growth rates between 20–30°C but did not grow so well at relatively lower temperatures (≤7°C) (Fig. 1). As concerns the proteolytic activity of 9410-O, a similar trend was apparent viz. the area of the clear zone was considerably high at 20–30°C but quite low at temperatures ≤10°C (data not shown). On the other hand, strain AN-1 that was isolated from the Antarctic[31], exhibited remarkably high growth (Fig. 2) and proteolytic activity (data not shown) at rather a broad range of temperatures (6–30°C).

Preparation of bacterial resuspensions

Suspensions of growing cells in liquid FeTY were obtained by incubating bacterial strains in liquid FeTY asepti-

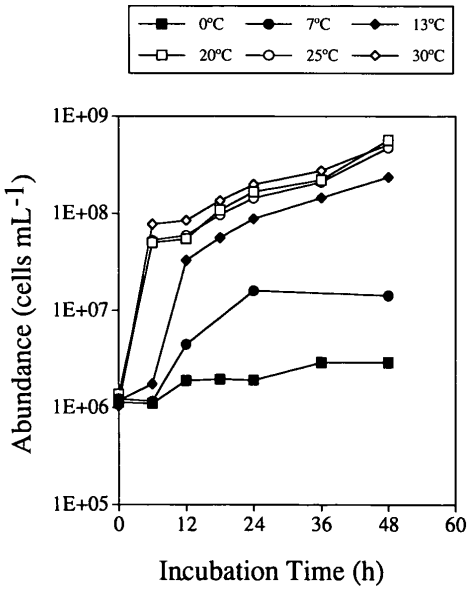

Fig. 1. Growth of strain 9410-O in liquid FeTY medium under various temperature conditions.

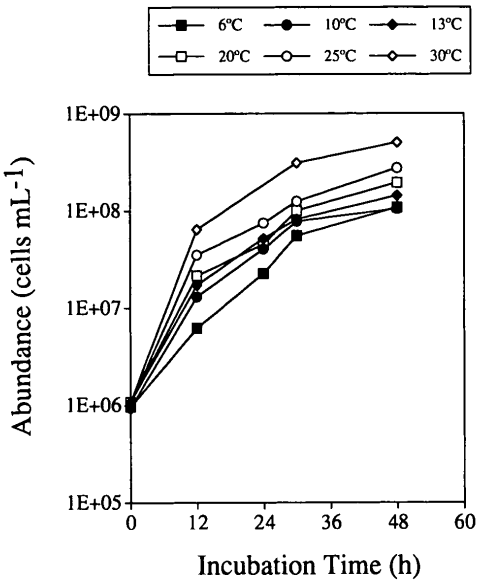

Fig. 2. Growth of strain AN-1 in liquid FeTY medium under various temperature conditions.

cally (inoculum size; 1 mL in 200 mL) at the optimum growth temperature of 25°C for ca. 24–30 h. Bacteria were added into experimental waters as resuspensions of bacterial cells. For preparing the resuspensions, FeTY suspensions of growing cells were centrifuged for 10 minutes at 10,000 rpm at 10°C (Himac CR 21E, Hitachi); the supernatant was discarded and bacterial cells obtained in the form of a pellet at the sidewalls of centrifuge tubes were gently rinsed at least three times and subsequently resuspended in filtered (0.2 μm) in situ waters. Resupensions were collected in an autoclaved glass bottle. Part of resuspension was preserved with filtered (0.2 μm) formalin (final concentration 2%; v/v) to estimate the numbers of added bacteria and part was simply frozen for nutrient analyses.

Incubation experiment and nutrient analyses

Unamended surface and bottom water samples from carboys were gently distributed into HCl-washed (2N; 1–2 days) 1L glass bottles, separately (1L in each bottle). Incubation was started by adding 10 mL of resuspension of either 9410-O or AN-1 into half of the series of glass bottles for both surface and bottom waters. The remaining half of the bottles that contained only unamended surface or bottom waters were also incubated simultaneously and treated as a control, for the respective waters. Incubation was always conducted in the dark at a constant temperature corresponding to the in situ water temperature at the time of sampling for 10–15 days without any shaking. In August and November, waters with or without bacteria additions were simultaneously incubated at 10°C, a winter simulated temperature. Treatments were employed at least in duplicate. To minimize the atmospheric oxygen contamination, we covered the air-water interface of each glass bottle with floating plastic balls (diameter ca. 2 cm) as a simple and practical rather than ideal approach. As will be described later in the text, covering did not completely stop the oxygen transfer between the water surface and atmosphere. Nevertheless, our experience with water-sediment complexes had indicated that doing so does reduce the oxygen dissolution.

In order to know the total amount of nutrient that was being added in the form of cell resuspensions, we estimated total N and P concentrations in the resuspension of 9410-0 and AN-1 on 02 July 1999 and 04 November 1999, respectively. Furthermore, in later part of the study, we autoclaved part of the re-suspension (121°C for 20 minute) and added 10 mL of killed cells into control bottles against 10 mL of live cell resuspension in treatment bottles. This was done to ascertain whether enhancement of the DIN and DIP regeneration was due to the activity of the added bacteria or due to the degradation of the added bacterial cells. In these experiments, we enriched experimental waters with a grounded common commercial fish feed procured from the "Laboratory of fish nutrition of Kochi University, Japan" (0.08 g fresh weight; protein content ca. 30% on dry matter basis and moisture content ca. 10%), the rationale of which will be discussed later in the "discussion" section.

The accumulation of dissolved inorganic nitrogen (DIN: NH_4^+, NO_2^- and NO_3^-), and phosphorous (DIP) in waters during incubation was monitored by taking subsamples of 25 mL from each incubation bottle at the beginning (after employing the treatment) and at regular intervals. The subsamples were passed through a pre-combusted GF/F (450°C for 3 h) and filtrates were stored at –25°C until nutrient analyses were made. An automatic analyzer (Bran+Luebbe TRAACS 800) was used to analyze NH_4^+ by the phenol hypocholrite method, NO_2^- and NO_3^- by the cadmium reduction method and DIP by ascorbic acid method[1]. The total nitrogen and phosphorus concentrations in resuspensions were measured by oxidizing the samples with $K_2S_2O_8$ and measuring the resultant NO_3[25] and phosphate[21]. We also measured initial and final dissolved oxygen concentrations (DO) in water samples by using Sibata-DU-1 DO probes.

Results

The physico-chemical and biological variables including

Table 1. Ecological variables of in situ surface and bottom waters and numbers of added bacterial cells and their ratio to natural bacterial abundances on different sampling/incubation occasions.

Sampling Date	Depth	Temperature (°C)	DO (mg L^{-1})	Salinity (psu)	Chl-a (μg L^{-1})	Bact. Abund. (10^6 cells mL^{-1})	Added Bact. (10^5 cells mL^{-1})	Added bacteria: Natural abudance (%)
02 Jul 1999	2 m	24.5	7.53	27.65	15.4	3.6	7.7	21.4
	16 m	23.2	0.80	32.58	0.5	2.2	7.7	35.0
20 Aug 1999	2 m	26.1	6.93	17.76	3.2	5.0	7.9	15.8
	16 m	26.0	0.14	30.62	0.5	3.0	7.9	26.3
4 Nov 1999	2 m	20.5	5.83	29.41	5.9	3.4	8.6	21.5
	16 m	23.6	2.24	32.16	1.3	3.2	8.6	25.3
11 Feb 2000	2 m	12.3	7.25	33.31	1.4	2.2	8.4	38.1
	16 m	12.6	6.91	33.47	1.5	2.0	8.4	42.0

temperature, dissolved oxygen, salinity, chlorophyll-a and natural bacterial abundances, and number of added bacteria and their ratio to the natural population in both, surface and bottom waters on different occasions are summarized in Table 1. In general, these variables indicated that the water column was strongly stratified during July-August, while it was partially or well mixed in November and February. Usually, the chlorophyll a concentration of surface water was higher than that of bottom water. Natural bacterial abundances ranged 2–5×10^6 cells mL^{-1}, while the total number of added bacteria ranged 7.7–9.6×10^5 cells mL^{-1}. Thus, the ratio of added bacteria to the natural bacterial abundances varied from 16 to 42%.

For water samples collected in the month of July and incubated at 23°C, we obtained net increases of 23.2 μM DIN and 1.38 μM DIP in 9410-O-supplemented surface water as against 13.9 μM DIN and 0.67 μM DIP in the unamended counterpart (Fig. 3A and B). Similarly, we obtained net increases of 4.9 μM DIN and 0.55 μM DIP in 9410-O-supplemented bottom water compared to only ca. 0.2 μM DIN and 0.04 μM DIP in the unamended counterparts, respectively (Fig. 3C and D). Thus, addition of 9410-O apparently stimulated the release of DIN and DIP by ca. 2 fold in surface water and 10–25 fold in bottom water. On another occasion in the month of August, 9410-O stimulated net DIN regeneration by 4-6-fold in both surface (Fig. 4A) and bottom waters (Fig. 4C). However, in a parallel incubation with the same waters at a winter simulated temperature of 10°C, 9410-O caused little enhancement of net DIN regeneration (Fig. 4B and D). Similarly, 9410-O stimulated net DIP regeneration to a greater extent at 26°C (Fig. 5A and C) but had little effect at 10°C (Figs. 5B and D).

On the other hand, addition of strain AN-1 into waters collected in November enhanced the net DIN (Fig. 6) and DIP (Fig. 7) release not only at a relatively higher incubation temperature viz. 23.5°C but also at 10°C. For that matter, AN-1 stimulated the net DIN increase by >2-fold in surface water (Fig. 6A) and ca. 4-fold in bottom water (Fig. 6C) at 23.5°C, and by ca. 2-fold in surface water (Fig. 6B) and 7-fold in bottom water (Fig. 6D) at 10°C in a simultaneously run incubation. Likewise, AN-1 enhanced the net DIP regeneration in surface water by ca. 4-fold (Fig. 7A) and in bottom water by ca. 6-fold (Fig. 7C) at 23.5°C, and by ca.7-fold in surface (Fig. 7B) and by >5-fold (Fig. 7D) in bottom water at 10°C. AN-1 also stimulated DIN (Fig. 8A

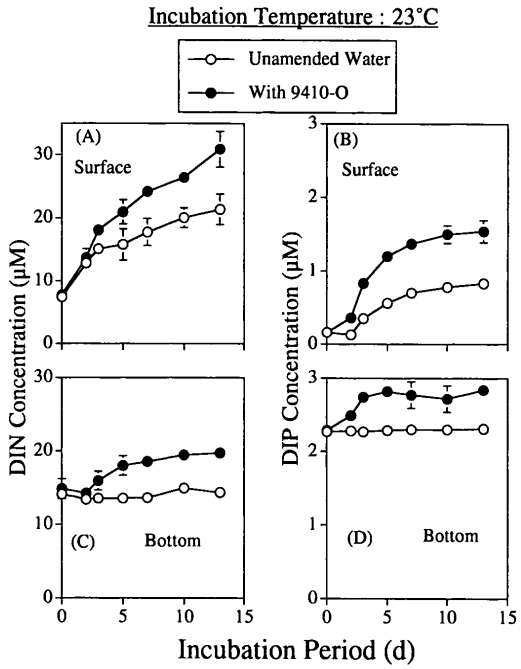

Fig. 3. Changes in concentrations (μM) of dissolved inorganic nitrogen (DIN), and dissolved inorganic phosphate (DIP) during incubation of unamended or 9410-O supplemented surface (A and B), and bottom (C and D) waters collected on 2 July 1999. Error bar is ±1 standard deviation (SD) (no error bar means SD is smaller than symbol).

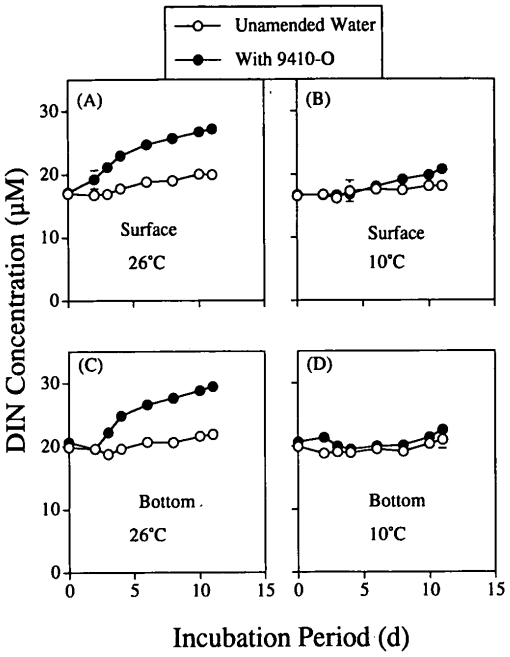

Fig. 4. Changes in concentration (μM) of DIN during incubation of unamended or 9410-O supplemented surface (A and B), and bottom (C and D) waters collected on 19 August 1999. Incubation temperature is indicated. Error bar is ±1 SD (no error bar means SD is smaller than symbol).

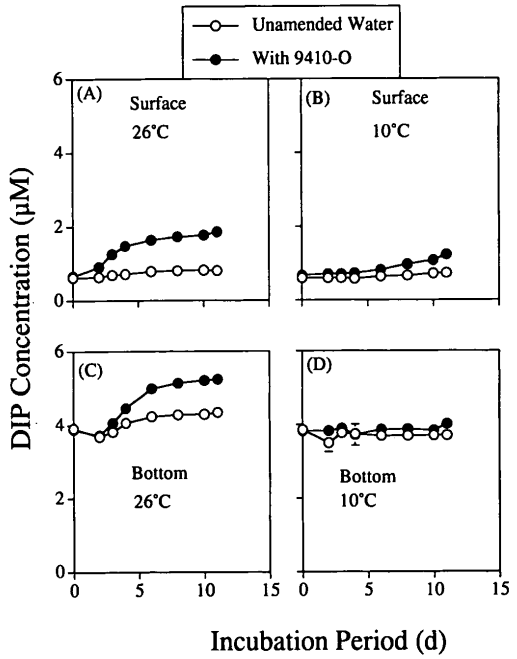

Fig. 5. Changes in concentration (μM) of DIP during incubation of unamended or 9410-O supplemented surface (A and B), and bottom (C and D) waters collected on 19 August 1999. Incubation temperatures are indicated. Error bar is ±1 SD (no error bar means SD is smaller than symbol).

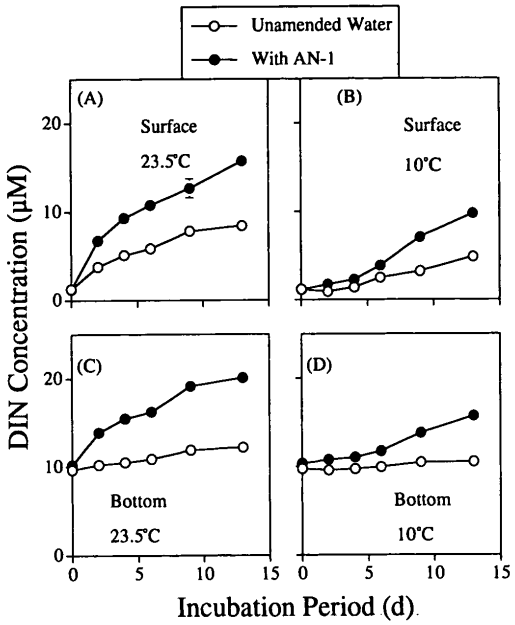

Fig. 6. Changes in concentration (μM) of DIN during incubation of unamended or AN-1 supplemented surface (A and B), and bottom (C and D) waters collected on 04 November 1999. Incubation temperatures are indicated. Error bar is ±1 SD (no error bar means SD is smaller than symbol).

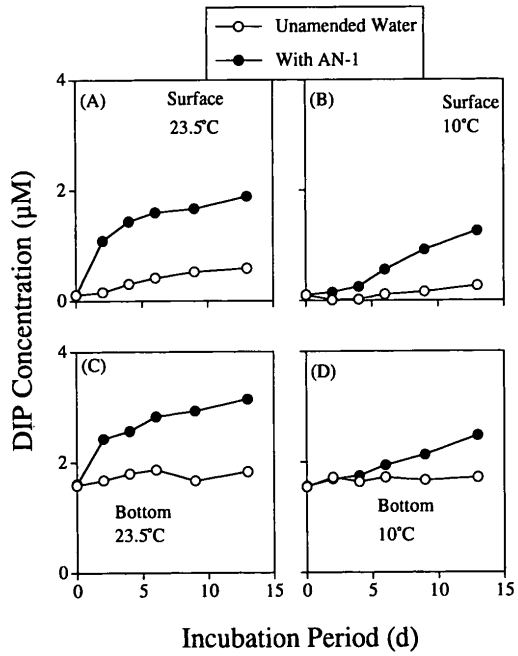

Fig. 7. Changes in concentration (μM) of DIP during incubation of unamended or AN-1 supplemented surface (A and B), and bottom (C and D) waters collected on 04 November 1999. Incubation temperatures are indicated. Error bar is ±1 SD (no error bar means SD is smaller than symbol).

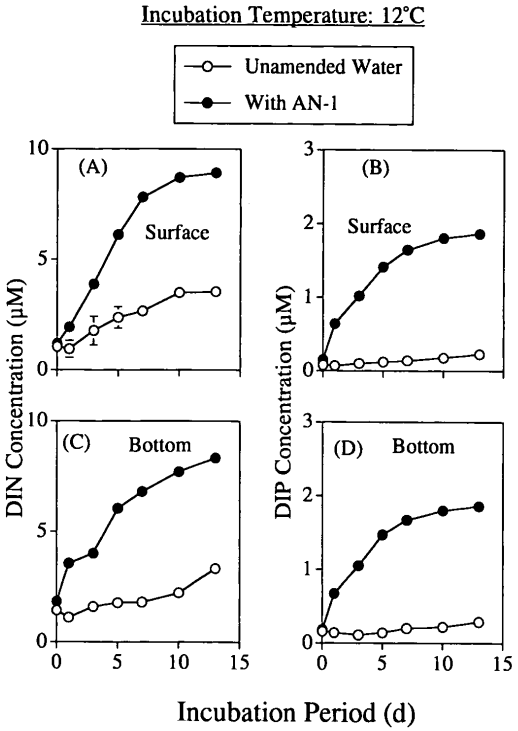

Fig. 8. Changes in concentrations (µM) of dissolved inorganic nitrogen (DIN), and dissolved inorganic phosphate (DIP) during incubation of unamended or AN-1 supplemented surface (A and B), and bottom (C and D) waters collected on 2 February 2000. Error bar is ±1 standard deviation (SD) (no error bar means SD is smaller than symbol).

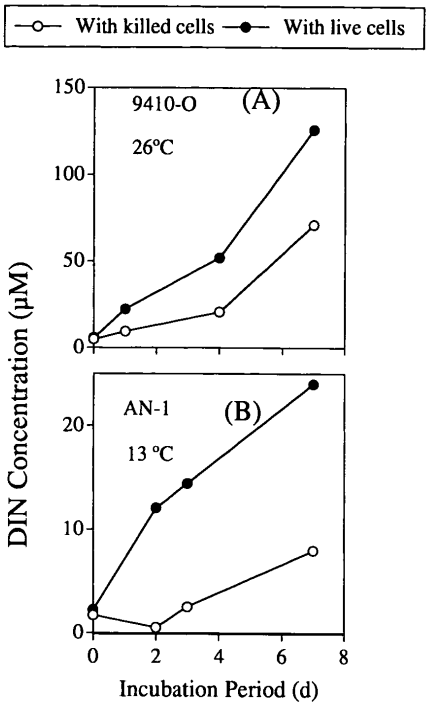

Fig. 9. Mean concentrations (µM) of DIN in surface water with live and killed cell resuspensions of 9410-O at 26°C (A), and AN-1 at 13°C (B).

Table 2. Dissolved oxygen concentrations in surface and bottom waters at the beginning and at the end of incubation at in situ temperatures on different occasions.

Date of Incubation	Water	Unamended Water		With Bacteria	
		Beginning (mg L^{-1})	End (mg L^{-1})	Beginning (mg L^{-1})	End (mg L^{-1})
02-07-1999	Surface	7.0	6.8	6.9	6.6
	Bottom	2.5	5.6	2.5	5.1
20-08-1999	Surface	6.8	8.4	6.8	7.6
	Bottom	2.9	5.6	2.9	5.7
04-11-1999	Surface	7.0	6.8	7.0	6.6
	Bottom	4.2	6.2	4.2	6.2
11-02-2000	Surface	5.8	5.9	5.8	5.6
	Bottom	5.8	5.8	5.8	5.5

and C) and DIP (Fig. 8B and D) regeneration prominently in water samples collected in mid-February when the water column temperature reached the minima (12°C) at the study site.

In additional experiments where we enriched experimental waters with powdered commercial feed and added the same volume (viz. 10 mL) of the resuspension of killed cells in control bottles as that of live cells in the treatment bottles, we obtained markedly high net regeneration for DIN with live 9410-0 or AN-1 cells (Fig. 9A and B) compared to controls, respectively. At 26°C, the net DIN increase in bottles with live 9410-O cells was ca. 126 µM in comparison to 71 µM with killed cells (9A). Thus, DIN regeneration in treatment bottles was >1.5-fold higher than the control value. Similarly, at 13°C, the net DIN regeneration in live AN-1-supplemented treatment bottles was ca. 4-fold higher than that of control bottles with killed cells (Fig. 9B).

Mean DO concentrations at the start and termination of incubation at in situ temperatures are presented in Table 2. As is evident, the concentration at the commencement of incubation was significantly higher than the respective in situ concentrations, especially in the case of bottom waters in summer (compare Table 1 and Table 2). This is probably due to multiple transfers of waters viz. from water sampler to carboys, from carboys to the measuring cylinder, and finally from the measuring cylinder to bottles for incubation and handling during subsampling (bottles were gently shaken before subsamples for nutrient analyses were taken). The DO concentrations further increased during incubation indicating floating plastic balls did not completely stop the exchange of oxygen between the water surface and atmosphere. However, when feed enrichments were made, the

DO concentration was typically <1 mg L^{-1} from the second day (data not shown).

Discussion

The accumulation of organic matter is one of the most common water quality problems in intensive fish culture systems[24]. The problem of waste management has increased over the past decade due to a rapid expansion of intensive fish and shrimp farming practices[30]. Semi-enclosed coastal areas like the present study site, in particular, are vulnerable considering their limited water exchange. Patel *et al.*[26] demonstrated that degradation of polymeric nitrogen compounds in the water column of the study site was limited by low temperature when the water column was mixed (October-March), and by low dissolved oxygen or high inorganic nutrient concentrations in summer, especially in bottom water. Considering that the major pathway of utilization of organic nutrients by primary producers/phytoplankton in nature is through bacterial or free enzymatic mediated breakdown to easily exploitable inorganic components such as NH_4^+ and DIP[2], it is important that the decomposition of organic compounds is facilitated in situ. Such strategies may help in alleviating the accumulation of organic matter, which in turn will contain the eutrophication[6] and may be of special significance for point sources of eutrophication like aquacultural facilities, especially when activities of ambient bacteria get limited.

Master and Mohn[19] and Moriarty[23] have demonstrated that inducing the growth of particular microorganisms could facilitate the biodegradation of organic wastes or control pathogenic microorganisms even in large volumes of water. However, the majority of such bioremediative efforts have been targeted at the recalcitrant, and/or xenobiotic compounds[4,19,20]. The present study was undertaken to investigate if the decomposition of organic matter in the water column of a eutrophic ecosystem can be stimulated under conditions close to those in situ by employing effective bacteria. Considering that heterotrophic activities at the study site are limited in summer as well as in winter, and one limiting situation is characteristically different from another[26,27], we primarily targeted two categories of bacteria; one that exhibited a high growth rate and proteolytic activity at temperatures ranging 20–30°C and may survive hypoxic conditions (to be employed in summer), and the other possessing a remarkably high growth rate and high proteolytic activity at relatively low temperatures such as ca. 10°C (to be used in winter). The indigenous mesophilic strain 9410-O that was isolated from the surface sediment of the study site in autumn when proteolytic activities in sediment peaked[27] exhibited impressive growth rates (Fig. 1) and proteolytic activities at temperatures ranging 20–30°C. Since the sediment of the study site witnessed hypoxic conditions during summer, 9410-O was considered to survive low DO. On the other hand AN-1, a psychrotolerant, was isolated from the Antarctic[31], and exhibited remarkably high growth rate (Fig. 2) and proteolytic activities even at relatively low temperatures such as ca. 6°C.

As concerns the addition of bacteria into water samples, we always used resuspensions of rinsed growing cells in filtered (0.2 μm) in situ water that was prepared as described in the "materials and methods" section. This was done to minimize artifacts due to FeTY-associated nutrients. In addition, we took care that the numbers of added bacteria were lower than those of ambient bacteria. The ratio of added to natural bacteria ranged 16–42% (Table 1). It should be conceded that DO concentrations in water at the start of and during the incubation period were considerably higher than in situ concentrations, especially in the case of bottom water in summer (compare Table 1 and Table 2). Elevated DO concentrations may have stimulated the aerobic decomposition in general, and hence a direct extrapolation of these results to in situ conditions may not be possible. At the same time, however, it should also be noted that despite the increase in DO in bottom water from a quite early phase of incubation, nutrient regeneration remained limited compared to that in surface water.

Addition of the 9410-O resuspension markedly enhanced the DIN and DIP net regeneration and/or regeneration rates in both, surface and bottom waters collected in the months of July and August and incubated at in situ temperatures viz. 23 and 26°C (Figs. 3, 4A and C, 5A and C). However, when we added 9410-O to the water collected in August and conducted a parallel incubation at a winter-simulated temperature of 10°C, enhancements in DIN and DIP releases were quite limited (Figs. 5B and D, 6B and D). Thus, results indicated that the strain 9410-0 was mostly effective at high temperature/summer situation, which is consistent with the obtained growth characteristics and proteolytic activities of this strain. In contrast to this strain, Fukami *et al.*[10] had demonstrated that addition of an obligatory psychrophilic strain CA(20)14 that was isolated from the Antarctic[13], enhanced the DIN releasing rates in a water-sediment complex by >2-fold at 10°C, but apparently had no effect at the relatively high temperature of 25°C. On the other hand, we observed that the resuspension of AN-1 enhanced the net regeneration of DIN and DIP not only at the relatively higher ambient temperature of 23.5°C, but also at 10–12°C (Figs.

6–8). Such effects are consistent with the growth patterns (Fig. 1) and proteolytic activities (data not shown) this strain had exhibited at temperatures ranging 6–30°C. As far as the stimulation of DIN and DIP regeneration relative to unamended counterparts is concerned, we obtained more pronounced results for bottom water (up to ~25-folds) than for surface water (up to 7-folds). However, this should be viewed in light of the fact that net DIN and DIP regeneration in unamended bottom water was usually considerably lower than that in unamended surface water. As a matter of fact, net enhancement in terms of μM was usually higher in surface water (Figs. 3 and 6). Greater releases and prominent stimulations in surface water are reasonable considering that surface water usually possessed a higher biomass of suspended organic matter as evident by higher chlorophyll a concentrations. Chin-Leo and Benner[5] and Gardner *et al.*[14] have also observed markedly high nitrogen regeneration rates in surface compared to subsurface water. It should also be considered that the stimulations of DIN and DIP regeneration relative to unamended counterparts were as high as 2-25-fold, viz. 200–2500%, while the ratio of added bacteria to the ambient population was only ca. 16–42%.

Gardner *et al.*[15] also have demonstrated enhanced nitrogen regeneration due to addition of bacterial concentrates into unfiltered water, but though it was assumed that the increased NH_4^+ regeneration was caused directly by the added bacteria, no elucidation was provided, and it could be argued that added bacterial concentrates might have been used as easily labile organic matter leading to increased inorganic nutrient regeneration. To ascertain whether bacterial activity or the degradation of added bacteria was primarily responsible for the increased nutrient regeneration, we adopted two approaches; first, as indirect evidence, we estimated the total nitrogen and phosphorus concentrations being added in the form of live bacterial cells of 9410-O and AN-1. A total of ca. 4 μM N and a total of ca. 0.5–0.6 μM P were added into the treatment bottles. The fact that net regeneration was usually greater (ca. 6–9 μM DIN and ca. 1 μM DIP; Figs. 3–8) than the added N and P indicated that bacterial activity rather than degradation of the added bacteria by ambient bacteria was a more plausible reason for the increased net production. As a second approach, we added exactly the same volume (viz. 10 mL) of killed cells in control bottles as that of live cells in treatment bottles to obtain more direct and concrete evidence. As described in the materials and methods section, we enriched the water samples in these experiments with a common commercial fish feed. We did so to nullify the selective organic enrichment in control bottles due to addition of killed cells because we considered that killed/dead cells in control bottles would be readily available to indigenous bacteria for degradation and an additional release of about 4 μM DIN and a total of ca. 0.5–0.6 μM DIP could be expected just due to organic enrichment. In contrast, in treatment bottles, live bacterial cells could not be expected to be available to native bacteria as labile organic matter. By enriching the water with feed, we also eliminated the hypothetical possibility of a lack of labile organic matter limiting the nutrient regeneration that could lead to inconclusive results. We obtained remarkably high net DIN and DIP releases with live 9410-O (at 26°C) and AN-1 (at 13°C) cells compared to that with killed counterparts, respectively (Fig. 9A and B) clearly indicating that the activities of the added bacteria were primarily responsible for the enhanced nutrient regeneration. It should also be pointed out that we chose feed rather than simpler nitrogen-rich compounds like bacterial growth media such as FeTY for enriching the water considering that feeds are the most dominant form of organic input in intensive aquaculture.

Although it is beyond the scope of the present study to elaborate on the nature and type of interactions between added and ambient bacterial populations, it is important that we did not observe any marked differences in the quantitative composition of DIN (% contribution of NH_4^+, NO_2^- and NO_3^-) between control and bacteria supplemented treatments. Furthermore, it should also be conceded that the increase in dissolved oxygen at the beginning of as well as during the incubation might have had a stimulatory effect on aerobic degradation and hence the results of the present study can not be extrapolated to reflect the effectiveness of bacterial addition under in situ conditions. Nevertheless, results indicated the potential of using effective bacteria for enhancing the mineralization process in eutrophic waters during different seasons, especially for sources of eutrophication like small aquacultural facilities. Further study is needed to find the appropriate substrates for introducing bacteria in attached forms and to evaluate the effectiveness of other potential bacterial strains with more complex systems involving sediment.

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