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Stimulation of Inorganic Nutrient Regeneration in Sediment Water Interface by *Pseudomonas perfectomarina* During Winter

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Abstract: *Pseudomonas perfectomarina* isolated from a eutrophic environment and introduced into it to stimulate inorganic nutrient regeneration and decomposing organic matter. The bacteria were introduced as cell suspension after absorbing with porous substrates. *Pseudomonas perfectomarina* stimulated net regeneration of dissolved inorganic nitrogen and inorganic phosphorus in the sediment water interface by more than ten folds at temperatures of 13°C, 14°C and 10°C, respectively. These results suggest that the application of *Pseudomonas perfectomarina* would be promising for the stimulation of nutrient regeneration of eutrophic bottom environment.

Key words: *Pseudomonas perfectomarina* · Sediment water interface · Inorg nitrogen · Inorg Phosphorus

INTRODUCTION

Intensive aquaculture practices have grown in the last decades at unprecedented rate, are characterized by high nutrient inputs mostly in the form of high protein content feed. The growth in aquaculture has led to an increase in the use of feeds applied to water for improving production. However, only a small portion of the total nutrient input is recovered as the harvest of cultured organism and as high as 70-75% of nitrogen and phosphorus content of the feed become the waste [1, 2]. Traditionally, the nutrient wastes, mainly in the form of ammonia, nitrite, nitrate, phosphorus and organic matter have been delivered into the surrounding environments without giving little thought to their holding capacity and consequently leading to the hypernutrification of their water column and sediment which in turn has been found to have serious ecological and economical impacts on the recipient ecosystems [3-5]. This organic enrichment has also caused lowered productivity in farms and increased mortality of the cultivated fish as a result of the conditions developed in the underlying sediments [6, 7].

Ammonia is excreted through the gills is the most toxic form of nitrogen when in the un-ionized form. Naturally occurring bacteria convert ammonia into less toxic forms that are utilized by plants and algae for growth. Water-column nutrient recycling by bacteria and other heterotrophic organisms is a dominant process supplying nutrients to phytoplankton in photic zones of both marine and fresh water ecosystems. The contribution of bacteria in nutrient regeneration depends on the chemical composition and supply rates of available bacterial substrates, temperature and food-web structure and dynamics [8, 9]. Dissolved organic nitrogen (DON) is actively released during photosynthesis and that low-molecular-weight compounds such as dissolved free amino acids are major substrates for bacteria [10]. Trace metals have a high affinity for adsorption and combination with organic material and will be accumulated in the sediment. Phosphorus is found in fish feeds and is broken down into a more useable form (phosphate) through decomposition. In most cases phosphorus and nitrogen contribute to eutrophication in a watershed by promoting growth of algae or plants. Watershed resource manager focused on reducing the amounts of phosphorus and nitrogen in a watershed when attempting to improve water quality. The major problem is to accelerate this metabolism sufficiently to cope with the rate at which the contaminant is delivered to the environment.

The study site Uranouchi Inlet, Kochi prefecture, Japan is highly eutrophic due to unlimited practices of fish cages with high protein nutrient input. In summer season ambient microorganisms stimulate the heterotropic

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activities in the Inlet while, in winter ambient microorganisms couldn't show their activities of decomposing organic matter particularly of the sediment properly due to the low temperature in winter even though water column was mixed and had sufficient DO [11]. To stimulate the heterotrophic activities during winter, using bacterial strain that's active even at low temperature may be a possible way. Considering that, we tried to isolate promising bacteria that can be active at winter low temperature ca. 10°C [12, 13].

The Objectives of the Present Study Are:

- Isolation of indigenous bacterial strains possessing high metabolic activities at low temperature.
- Stimulation of inorganic nutrient regeneration during winter at low temperature.

MATERIALS AND METHODS

Study Site and Sample Collection: Sampling station is a eutrophic area practicing intensive fish farming in cages. The sampling point was in vicinity of one of the farms. The average depth near the sampling point was 16-17 meter. Water samples were collected from bottom (16 m depth) environment with a 5-liter Niskin water sampler and kept into 12-liter polypropylene plastic pots. Sediment samples were also collected at the same time from the study site by using Ekman-Birge grab sediment sampler from surface sediment (0-1 cm depth) and kept into icebox. Samples were carried back to the laboratory within 2-3 hours of sampling under cooling condition.

Isolation and Selection of Bacteria: For isolation of effective bacteria that might be active at low temperature in winter, we collected bottom water and surface sediment sample from the study site during winter when temperature was ca. 10°C. After coming back to the laboratory, we inoculated 1 ml of bottom water into 10 ml of FeTY broth medium and incubated at 4°C. Simultaneously, we added 1 g of wet sediment into 10 ml of filter-sterilized bottom water. One ml of sediment-water suspension was inoculated into 10 ml of FeTY broth medium and incubated at 4°C. After one week of incubation, we transferred 1 ml of bacterial culture from each inoculated tube into new FeTY broth medium. It was continued until 4 times to enrich the psychrophilic/psychrotolerant bacteria. Finally, we took 0.1 ml of bacterial culture from each tube and inoculated onto FeTY agar plates and incubated at 4°C. One hundred and fifty fast growing bacterial strains were isolated from the bottom water of the study site in November 2000. Considering growth pattern and colony morphology 25 bacterial strains were primarily selected for further study.

The growth curves of *Pseudomonas perfectomarina* were prepared by direct count method using DAPI (4', 6-diamidino-2-phenylindole dihydrochloride) stain under epifluorescence microscopy [14, 15, 16].

Preparation of Sediment-Water System: All experiments were conducted into the 1 liter glass bottle, which was previously washed in 2N HCl for two days. Sediment-water system was prepared as follows:

Initially, 50 g (wet weight) of well-mixed surface (0-1 cm) sediment was added into the glass bottle by stainless steel spatula in aseptic condition and kept it for a while at low temperature in dark condition. After that, 1 liter of sample water was slowly added onto the sediment to avoid the profuse mixture of the sediment into the overlying water and kept it for 2 hours to minimize the disturbance effects [17, 18] and transparency of the overlying water of sediment-bottom water complex system could be assured by visual estimation [19].

Inocula Preparation and Incubation: Bacterial broth culture was centrifuged at 10,000 rpm (Himac CR 21E, Hitachi) for 10 minutes at 10°C. The supernatant was discarded and bacterial cells plate was gently rinsed with filter-sterilized (0.22 μm) sample water at least five times in order to remove associated nutrients and re-suspended with the same filtered water by pipetting. Three ml of bacterial cell re-suspension was used as inocula. Five ml was fixed with pre-filtered (0.22 μm) formaldehyde for counting the density of bacteria added. Natural bacterial cells abundance was also counted following the same way. Bacterial cells were inoculated into the treatment bottles absorbing with substrates. In the control bottles same amount of substrates were used.

The glass bottles were incubated in a dark condition *in situ* (in duplicate) at 13°C temperature during February 2002; at 14°C during March 2002 and at 10°C during January 2003 for 12 days without any shaking to estimate bacterial activity. To minimize the atmospheric oxygen contamination, floating plastic balls (10 mm diameter) were introduced into each incubating bottles and water surface was covered with them. Dissolved oxygen concentration was monitored by YSI, Model No. 85/10 FT, at every sub-sampling occasion before taking sub-samples without shacking.

Bacterial activities were evaluated by the changes of inorganic nutrient regeneration within the sediment-water interface during incubation. The fluxes (rate of nutrients regeneration) were calculated by considering the first slope of dissolved inorganic nitrogen (DIN) and inorganic phosphorus (DIP) released during incubation with or without adding bacteria.

Sub-Sampling and Nutrient Analysis: Just after adding bacteria 25 ml sub-samples were taken at the beginning of the incubation as 0-day. At 3 days interval 25 ml sub-samples were pipetted aseptically from 2 cm above the sediment surface of each bottle without any disturbance of the system and after filtration onto Whatman GF/F filters preserved at -25°C temperature. Concentrations of dissolved inorganic nitrogen and phosphorus in the sub-samples collected were measured by using automatic analyzer (Bran+Luebbe TRAACS 800).

RESULTS AND DISCUSSION

For stimulation of the nutrient regeneration in sediment water interface, selected bacterial strain of *Pseudomonas perfectomarina* was added. During the incubation of sediment-water system collected in February 21, 2002 and incubated at *in situ* 13°C, concentration of DIN and DIP released into the overlying water increased after addition of bacteria more than without addition (Fig. 1). In the results, the net releases of DIN and DIP in 12 days within sediment-water interface in a control parts were 7.11 μM and 0.32 μM, respectively, while in the system of adding of bacteria DIN and DIP increased 92.72 μM and 4.71 μM, respectively (Fig. 1). Total amount of added bacteria were 8.04 $\times 10^{10}$ cells that was 22.0% of the natural bacterial abundance (Fig. 4A). The DO concentration at the beginning of the experiment was 3.44 mg/l and at the end of the incubation was 1.60 mg/l (Fig. 5A).

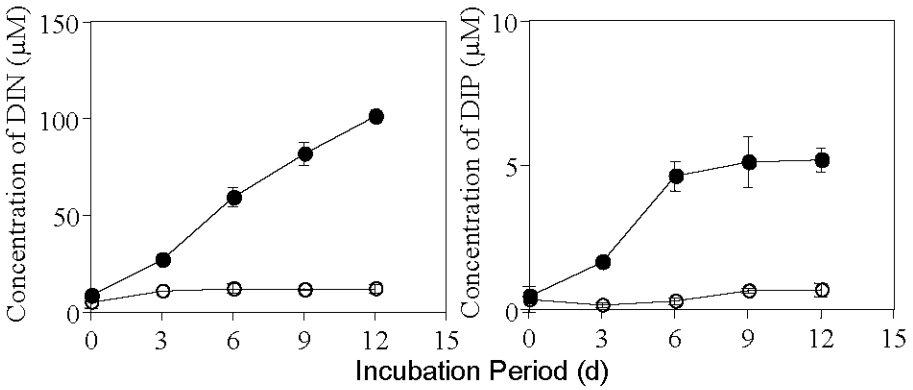

Fig. 1: Changes in concentration of inorganic nutrients by adding *Pseudomonas perfectomarina*. Incubation temperature was 13°C; sampling on February 21, 2002. Without *Pseudomonas perfectomarina* (-o-) and with *Pseudomonas perfectomarina* (-●-). Bars = standard deviation; n = 2.

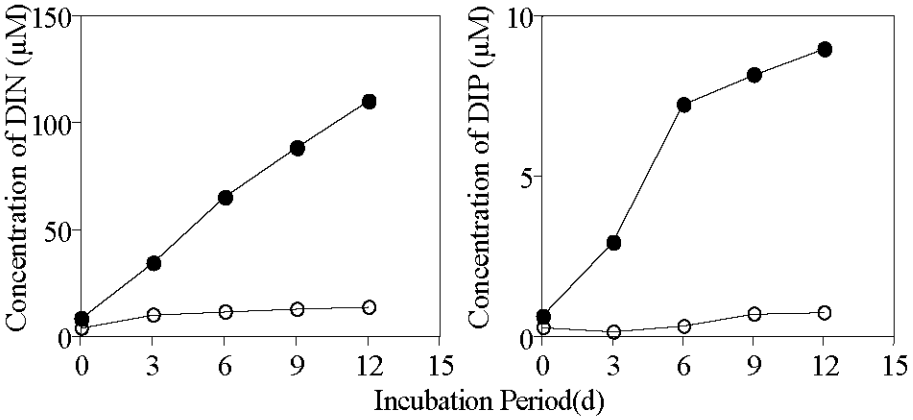

Fig. 2: Changes in concentration of inorganic nutrients by adding *Pseudomonas perfectomarina*. Incubation temperature was 14°C; sampling on March 15, 2002. Without *Pseudomonas perfectomarina* (-o-) and with *Pseudomonas perfectomarina* (-●-). Bars = standard deviation; n = 2

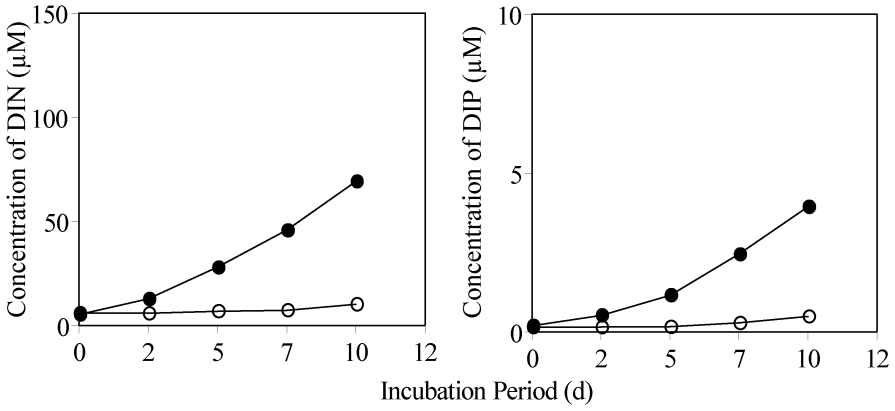

Fig. 3: Changes in concentration of inorganic nutrients by adding *Pseudomonas perfectomarina*. Incubation temperature was 10ºC; sampling on January 18, 2003. Without *Pseudomonas perfectomarina* (-○-) and with *Pseudomonas perfectomarina* (-●-). Bars = standard deviation; n = 2

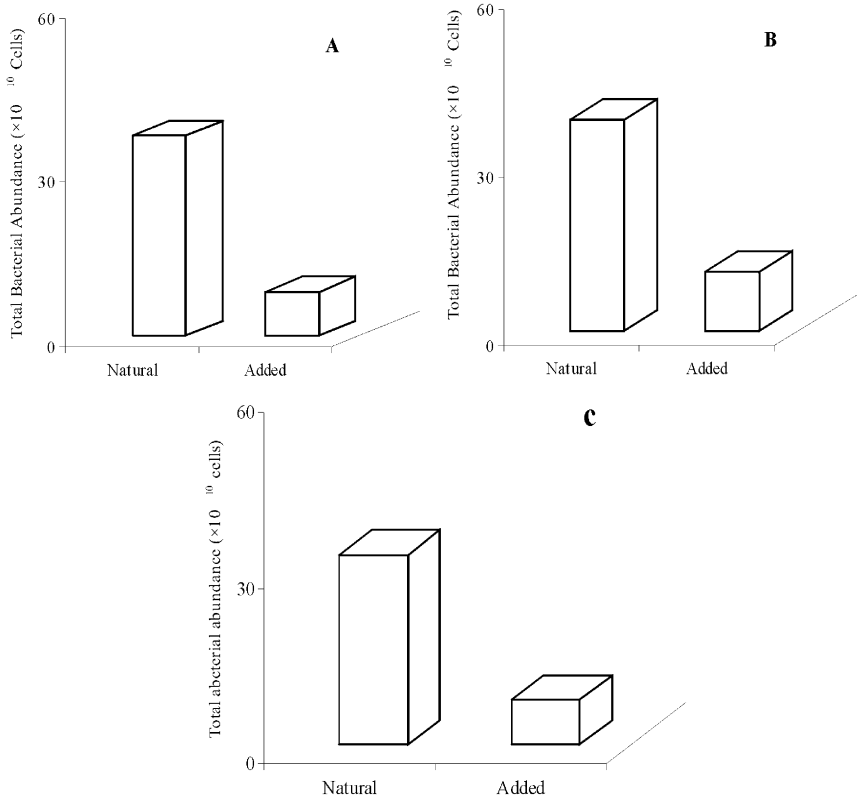

Fig. 4: Number of added bacterial cells and their ratio to natural bacterial abundances. Used bacteria was *Pseudomonas perfectomarina*. A: Incubation temperature was 13°C, sampling on February 21, 2002; B: Incubation temperature was 14°C, sampling on March 15, 2002 and C: Incubation temperature was 10°C, sampling on January 18, 2003

Result of the experiment with sample collected in March 15, 2002 incubated at *in situ* 14°C was illustrated in Fig. 2. The net releases of DIN and DIP supplemented with *Pseudomonas perfectomarina* were 101.61 µM and 8.32 µM and those of control were 9.72 µM and 0.46 µM, respectively (Fig. 2). Total amount of added bacteria were 10.5×10^{10} cells that was 28.0% of the natural bacterial abundance (Fig. 4B). The DO concentration at the beginning of the experiment was 5.00 mg/l and that at the end of incubation was 3.99 mg/l (Fig. 5B).

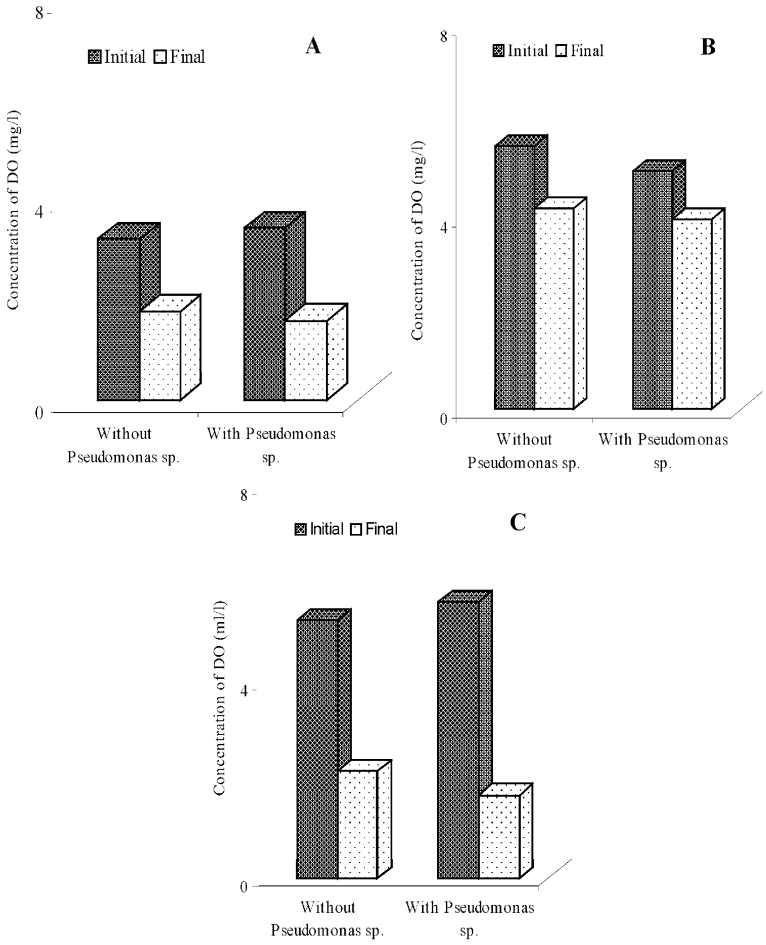

Fig. 5: Changes in concentration of dissolved oxygen (DO) at the initial and at the end of incubation on different sampling occasions. Used bacteria was *Pseudomonas perfectomarina*. A: Incubation temperature was 13°C, sampling on February 21, 2002, B: Incubation temperature was 14°C; sampling on March 15, 2002 and C: Incubation temperature was 10°C, sampling on January 18, 2003

Simultaneously, we conducted experiment with sample collected in January 18, 2003 at 10°C, heterotrophic activities also stimulated after addition of bacteria (Fig. 3). DIN concentration increased from 6.01 µM to 10.13 µM in control parts while after addition of bacteria it changed from 5.19 µM to 69.10 µM during incubation period of 10 days (Fig. 3). Accordingly, DIP concentration also increased from 0.14 µM to 0.49 µM without adding bacteria and after addition of bacteria it changed from 0.19 µM to 3.94 µM in 10 days of incubation. Net releases of DIN and DIP in control counterparts were 4.12 µM and 0.35 µM respectively, while after addition of bacteria it increased into 63.91 µM of DIN and 3.75 µM of DIP (Fig. 3). Total added bacterial abundance was 7.71×10^{10} cells those were 23.7% of the natural bacterial abundance (Fig. 4C). The DO concentration in the system with bacteria at the beginning of the experiment was at 5.63 mg/l and at the end of 10 days of incubation was at 1.69 mg/l (Fig. 5C).

The enhancement in the releasing rates of DIN and DIP after addition of *Pseudomonas perfectomarina* during experiment with samples collected in February 21, March 15 of 2002 and January 18 of 2003 are summarized in Fig. 6. During an experiment in February, the releasing rates of DIN and DIP were 0.53 ± 0.12 fM/d/cell and 0.009 ± 0.001 fM/d/cell respectively, while after adding bacteria it changed to 5.31 ± 0.36 fM/d/cell in DIN and 0.60 ± 0.09 fM/d/cell in DIP, respectively (Fig. 6A). In experiment with sample collected in March, releasing rates of DIN and DIP were 0.55 ± 0.14 fM/d/cell and 0.002 ± 0.003 fM/d/cell respective, after adding bacteria it changed to 6.30 ± 2.68 fM/d/cell and 1.04 ± 0.23 fM/d/cell in DIN and

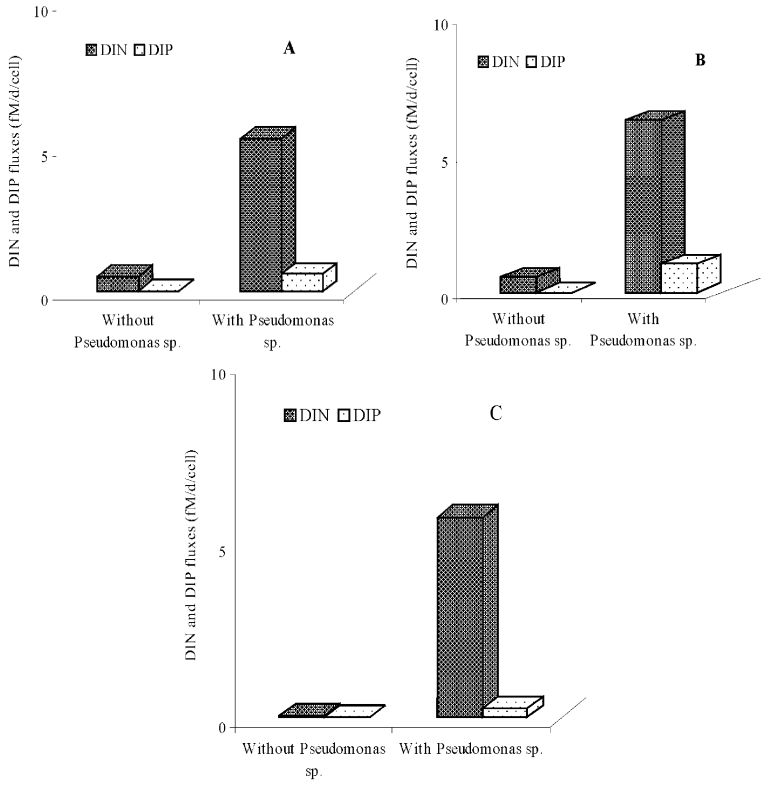

Fig. 6: Changes in releasing rate of DIN and DIP fluxes after addition of *Pseudomonas perfectomarina* at different *in situ* temperatures. A: Incubation temperature was 13°C, sampling on February 21, 2002; B: Incubation temperature was 14°C, sampling on March 15, 2002 and C: Incubation temperature was 10°C, sampling on January 18, 2003

DIP respectively (Fig. 6B). Such enhancement were also noticed with samples of January 18, 2003, after adding bacteria DIN changes from 0.05 ± 0.03 fM/d/cell to 5.69 ± 1.19 fM/d/cell and DIP changes from 0.001 ± 0.003 fM/d/cell to 0.25 ± 0.01 fM/d/cell, respectively in 10 days (Fig. 6C).

Increased ammonium regeneration rates corresponding to increased numbers of bacteria were assumed to be caused directly by bacteria, whereas the absence of increased ammonium regeneration rates with increased particle density was assumed to indicate that most of the ammonium regeneration was done by organisms [20]. The increased rates of ammonium regeneration with increased added bacterial concentrations that we observed at several stations may indicate that bacteria themselves directly regenerate ammonium [21] or may reflect the increased food supply made available to the bacterial grazers that would in turn cause regeneration rates to increase.

Soluble organic compounds attached to the plant and sediment surfaces are mainly degraded by bacteria aerobically. The oxygen needed to support the aerobic process is supplied directly from the atmosphere via diffusion through the sediment or water-atmosphere interface and by oxygen leakage from macrophytic roots [22].

The strain *Pseudomonas perfectomarina* enhanced net amounts and rates of inorganic nutrients (DIN and DIP) regeneration in overlying water column on the sample of February 15, 2002 when incubated at 13°C *in situ* (Fig. 1, 6A). During this experiment, added bacterial densities were 22% of the natural bacterial abundance (Fig. 4A). In the next experiment we observed the results consistent of the previous experiment that was conducted with samples collected in March 15, 2002 and incubated at 14°C *in situ* (Fig. 2, 6B) and added bacterial densities were 28% (Fig. 4B).

We conducted one experiment at the lowest temperature in winter season at 10°C in January 2003. Here after addition of bacteria, stimulation also occurred compared with control counterparts. An interesting point is that initial releasing rates of DIN and DIP were very low probably due to low temperature but possibly after acrotimization with temperature bacteria showed their proper activities (Fig. 3, 6C).

Results of the experiments conducted with strain *Pseudomonas perfectomarina* and incubated at different *in situ* and/ or simulated winter temperatures showed that this strain could be effective and promising for the stimulation of decomposing sediment organic matter at low temperature but high DO season of winter or winter simulated temperatures.

CONCLUSION

The degradation of polymeric nitrogen compounds in water column and underlying sediment in the inlet was limited mainly by low temperature in winter even though water column was mixed and had sufficient dissolved oxygen. The results from data analysis show that introduction of the strain *Pseudomonas perfectomarina* capable of high proteolytic activities may be one useful way to stimulate the nutrient regeneration rate into sediment-water interface. However, in order to have more certain answers on the assessment for eutrophication control purposes of the inlet, more sampling should be done from different sites and test results of the samples should be verified with different laboratories, while maintaining the same approach of analysis.

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