FISHERIES SCIENCE 2003; **69**: 1146–1157

Enhancement of inorganic nutrient regeneration in a eutrophic sediment–bottom water complex system by adding effective indigenous bacteria

M ABDUL KARIM,[1] KIMIO FUKAMI[1]* AND ARUN B PATEL[2]

[1]*Laboratory of Aquatic Environmental Science (LAQUES), Faculty of Agriculture, Kochi University, Nankoku, Kochi 783-8502 and* [2]*Core Research Laboratory, Mie Prefecture Shima Branch Office, Ugata, Mie 517-0501, Japan*

ABSTRACT: To enhance heterotrophic activities for decomposing organic matter in a eutrophic bottom environment, two promising bacterial strains isolated from the study site, named *Enterobacter* sp. 9410-O and *Pseudomonas* sp. W-4 were introduced by absorbance onto porous substrates in the sediment-bottom water complex system. Strain 9410-O grew well at >20°C, considered as mesophilic, while the other strain W-4 grew at a wide rage of temperatures from 5°C to 30°C and was psychrotolerant. Addition of 9410-O stimulated net regeneration of inorganic nitrogen (DIN) and inorganic phosphorus (DIP) from the sediment by approximately four–fivefold at a high temperature of 26°C, but some stimulation was noticed at the lower temperature of 13°C. However, strain W-4 stimulated DIN and DIP net regeneration both at low (13°C; approx. threefold) and high temperatures (26°C; approx. four–fivefold). These results suggest that the application of such effective bacteria as described here would be promising for the stimulation of self-purification in the field of eutrophic bottom environments.

KEY WORDS: **bottom environment, DIN and DIP regeneration, eutrophic coastal inlet, indigenous bacteria, porous substrates.**

INTRODUCTION

Intensive aquaculture practices in the coastal area are characterized by high nutrient inputs, mostly in the form of high protein content food and fish juveniles, to the fish farm since the aquaculture industry began. The small portion of the total nutrients input is recovered as the harvest of cultured organisms, while a large portion of organic materials, mainly in the form of excess feed and feces, becomes the waste and is discharged into the environment without any treatment.[1–5] Briggs and Funge-Smith estimated the amount of added nitrogen and phosphorus to the intensive shrimp ponds through feed and fertilizers as being 95% and 71% of total amount of nitrogen and phosphorus in the natural environment, respectively, while harvested shrimp accounted for only 24% nitrogen and 13% of phosphorus loaded into the pond.[6] Thus, the portion of nutrients in the feed consumed by shrimp and converted to shrimp flesh is relatively small and a greater portion is wasted in the water column. The nutrient wastes are delivered into the surrounding environment, which as a result quite often exceeds the holding capacity of the recipient ecosystems[7] and consequently results in hypernutrification of the water column and underlying sediment.[4,8–10] As a result, a serious ecological and economical impact is observed on the recipient ecosystem,[11,12] which changes the characteristics of the living flora and fauna at or near the sediment water interface.[13–16] Fish-farm wastes deposited on the sea bottom may lead to organic and nutrient enrichment of sediment, an increase in sediment oxygen demand, anoxic conditions, and production of toxic gases (e.g. methane and H_2S), which adversely affect benthic organisms.[17–19]

To ensure eco-friendly aquaculture, it is very important that this organic matter of allochthonous origin must be decomposed, and utilized or recycled. Numerous strategies have been developed to mitigate the environmental impacts of cage fish farm activities. Microbial ecology and biotechnologies have advanced to the point that commercial products and technologies are available to treat large areas of water bodies and land by enhancing the population densities of some selective microbial species or promoting their biochemical activ-

*Corresponding author: Tel: 81-88-864-5152.
Fax: 81-88-864-5197. Email: fukami@cc.kochi-u.ac.jp
Received 31 March 2003. Accepted 14 July 2003.

ities.[20–23] The biological treatment of effluent from intensive fish cage farming has been considered recently using specialized microorganisms.[24]

Aerobic heterotrophic bacteria decompose organic matter in the water column and sediment surface in aquaculture pond systems.[25–29] But the intensive farming in enclosed or semi-enclosed spaces in a coastal region causes the organic matter to load at much faster rates than can be decomposed, utilized or recycled by ambient heterotrophic microorganisms. Therefore, the need for biotechnological intervention arises,[30] particularly in situations where the indigenous bacterial activities are limited by prevailing ecological variables.[31,32] Several commercial products of effective bacterial strains of exogenous origin are now being used to control the water quality in aquaculture facilities but there is not much scientific validation of their effectiveness for *in situ* conditions.[29]

The previous study was conducted with isolated bacteria of exogenous origin and introduced the bacterial cells as a re-suspension directly into the experimental system.[33] By considering the bottom environments of the study site, that method may not be effective. Furthermore, bacteria of exogenous origin may be inactive with indigenous microorganisms or dominate or interfere with their ecosystem. In the present study we tried to determine the best way to enhance the decomposition rate of organic matter in a sediment–bottom water complex of a eutrophic coastal inlet, by using some effective indigenous bacterial strains under *in situ* conditions. Here, we describe a series of experiments in which we investigate the effect of adding two bacterial strains, namely *Enterobacter* sp. 9410-O (mesophilic) and *Pseudomonas* sp. W-4 (psychrotolerant), on the net release of inorganic nitrogen (DIN) and inorganic phosphorus (DIP) by decomposition of organic wastes in a sediment–bottom water complex system in different seasons.

MATERIALS AND METHODS

Study site and sampling

Sampling was carried out at the small and shallow eutrophic Uranouchi Inlet in Kochi Prefecture, Japan.[34,35] The inlet is characterized by a great deal of intensive fish rearing cages, limited water exchange, and pronounced density stratification in summer with oxygen-rich surface water and oxygen-deficient bottom water.[31] Serious algal blooms have also been reported in that inlet.[34] The sampling station is located at the central part of the inlet where the average depth is approximately 16–17 m, close to one of the cage farms. A description in detail of the study site and sampling station may be found in other published papers.[31,32,34,35]

Water samples were taken from 16 m depths using a 5 L Niskin water sampler, and were gently transferred into 10 L carboys via a silicon tube, during winter and summer seasons at several occasions in 2002. The carboys were filled completely without leaving any air space. Sediment samples were collected at the same time from the study area by using an Ekman–Birge grab sediment sampler. Both water and sediment samples were carried back to the laboratory within 1–2 h of sampling under ambient atmospheric temperature. Special care was taken to avoid contamination of the atmospheric air during sample handling and transportation.

Bacterial abundance

Bacterial abundances were monitored by epifluorescence microscopy after staining with 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) for 20 min at 4°C.[36,37] For the sediment samples, the bacteria were dispersed from their sites of attachment by sonicating in ultrasonic cleaner (930 W) for 30 min with tetra-sodium pyrophosphate ($Na_4P_2O_7$; final concentration, 0.01 M),[38–40] and then stained with DAPI (5 μg/mL) for 20 min at 4°C.

Bacterial isolation, growth and proteolysis activity

The study site has a high dissolved oxygen content in winter but the microbial activity was low due to the low temperature. Therefore, to isolate effective bacterial strains of indigenous origin that have high activities in the cold conditions (psychrophiles[41]), water and sediment samples were collected during the winter season (temperature approx. 12°C). After appropriate decimal dilution, water or sediment samples were spread onto FeTY (trypticase peptone 0.5 g, yeast extract 0.05 g, ferric citrate 0.01 g, in 80% aged seawater) agar plates[42] and incubated at 4°C. Fast-growing bacterial colonies were tentatively selected and isolated and purified for further study. The growth curves of each bacterial strain under different temperatures were monitored by epifluorescence microscopy as aforementioned.

To detect the protein hydrolyzing abilities, bacterial strains were cultured overnight in FeTY semi-solid medium and inoculated onto double-layered 1% casein agar plates and incubated for 4 days at different temperatures (10–30°C), and zones of hydrolysis (clear zones) were observed every day.[43] The presence of protease was estimated by mea-

suring the diameter of the zones of hydrolysis (clear zone) of each bacterial strain. Effective bacterial strains were selected by proteolytic (casein hydrolysis) activity test and temperature-dependent (5–30°C) growth curves.

Preparation of bacterial re-suspension

Two bacterial strains, 9410-O and W-4, were selected and used for the experiment. Both bacterial strains were grown for 48 h into FeTY broth medium at 20°C and after sufficient growth of bacterial culture, cells were rinsed by centrifuge at 10°C for 10 min at 12 000 ×*g*. (Himac CR 21E; Hitachi, Ibaraki, Hitachi Naka City, Takeda, Japan) at least five times in order to remove associated nutrients. After re-suspension with filtered (0.22 μm) bottom sample seawater by pipetting, 4 mL of re-suspension was absorbed onto 10 g of porous substrates (Neo-coal; Toyodenka Kougyo Kabushiki Kaisha, Ogimachi, Japan; compositions are wood charcoal and ceramic, diameter: approx. 10 mm) and used as inocula. Some of the bacterial cell suspension (5 mL) was used for counting densities of bacteria added to the system.

Incubation experiment

Two incubation experiments were conducted with *in situ* sediment–water complex systems collected on 15 February and 3 August in 2002. The sediment–water complex was prepared by adding 50 g of well-mixed sediment to a 1 L glass bottle, into which 1 L of bottom water was poured slowly, taking special care to avoid pronounced mixing with sediment. These samples were transferred to a water bath at *in situ* temperature and incubated in darkness overnight to minimize disturbance effects.[44,45] The following day 10 g of porous substrate containing bacteria were added on the sediment surface of the treatment bottles. A bottle of sediment–bottom water complex to which was added only the porous substrates (10 g) without the bacteria, was used as a control and was incubated together with bottles to which bacteria had been added. The experimental systems were incubated in a water bath at ambient temperature and winter (10°C) or summer (26°C) temperatures were simulated, separately, for 10–12 days without any shaking.

All the treatments were employed at least in duplicate. To minimize atmospheric oxygen contamination, floating plastic balls (diameter: 10 mm) were introduced into each incubating bottle and the water surface was covered with them.

Subsampling procedure

During incubation, 25 mL of subsamples were taken from 2 cm above the sediment surface at the beginning of the incubation as 0 day and at regular time intervals by using a pipette aseptically from each bottle without any disturbance of the system. These collected samples were filtered onto pre-combusted (450°C for 4 h) and pre-rinsed (by double distilled water) GF/F filters (Whatman, Maidstone, England). The filtrate was preserved at –25°C until undergoing analysis. We monitored initial and final dissolved oxygen (DO) concentration at 2 cm above the sediment surface by DO meter, YSI (Model no. 85/10 FT; YSI, Yellow Springs, OH, USA) before taking subsamples without any shaking.

Inorganic nutrients analysis

The concentrations of inorganic nitrogen (DIN: ammonium, nitrate and nitrite) and phosphate (DIP) were measure by automatic analyzer (Bran+Luebbe TRAACS 800; Sendagaya, Shibuya, Tokyo, Japan) by using preserved subsamples.

Identification of the bacterial strain

For identification of bacterial strains 9410-O and W-4, morphological and physiological characteristics were monitored following *Bergey's Manual of Determinative Bacteriology*.[46]

RESULTS

Growth curves of bacteria

The strain 9410-O was isolated from the upper layer of sediment of the study site in October.[33] This strain exhibited remarkably high growth rates between 20°C and 30°C but did not grow well at relatively low temperatures (≤7°C), and was considered to be mesophilic.[33] The strain W-4 was also isolated from the bottom water of the same place during winter. The growth curve of W-4 strain is presented in Fig. 1. As is evident, W-4 had high growth rates at high temperature (20–30°C) but it also had relatively good growth at lower temperatures (5°C). Therefore, W-4 can be considered to be 'psychrotolerant'. In regards to proteolytic activities, both 9410-O and W-4 possessed considerably high activities at high temperatures (20°C and 30°C; Fig. 2a,b), but at low temperatures (e.g. 10°C) only the W-4 strain had higher activities (Fig. 2b).

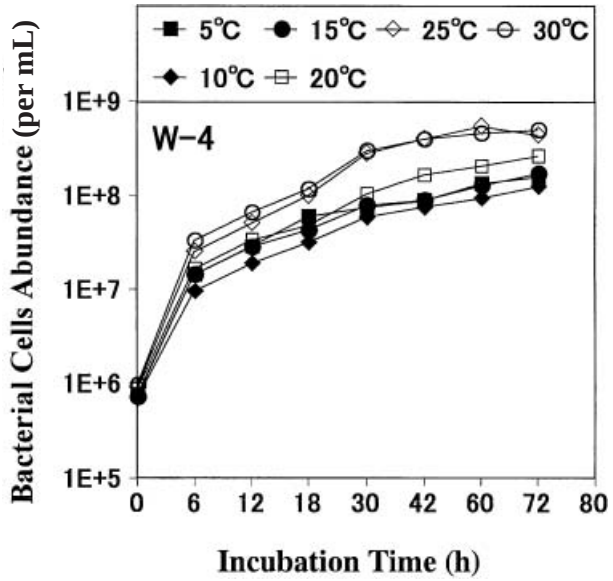

Fig. 1 Growth curve of W-4 bacterial strain in FeTY broth medium at different temperatures ranged from 5°C to 30°C.

By considering growth curve and proteolytic activities, bacterial strains of 9410-O and W-4 were tentatively selected for further study.

Identification of 9410-O and W-4 strains

For identification of the strains 9410-O and W-4, morphological characteristics of the bacterium, such as cell shape, colony characteristics, pigmentation were investigated. Different physiological characteristics such as Gram reaction, motility test by flagella stain, catalase test, oxidase test, indole formation, citrate utilization, Voges Proskauer (VP) test, methyl red (MR) test, oxidation fermentation test, acid from D-glucose, L-arabinose and lactose were also monitored.

The strain 9410-O was Gram-negative, motile by peritrichous flagella, catalase-positive, oxidase-negative, formation of indole-positive, utilization of citrate-positive, VP test-positive, MR test-negative, it had a fermentative type of pathway; and acids from D-glucose, L-arabinose and lactose were positive. By consideration of the aforementioned physiological characteristics we tentatively identified the strain 9410-O as *Enterobacter* sp. (Table 1).

As regards strain W-4, it was Gram-negative, motile by polar flagella, catalase-positive, oxidase-positive, formation of indole-negative, utilization of citrate-positive, VP test-positive, MR test-negative, it had an oxidative type of pathway; acids from D-glucose and L-arabinose were positive, and acid from lactose was negative. We tentatively identified the strain W-4 as *Pseudomonas* sp. by consideration of physiological characteristics of that strain (Table 2).

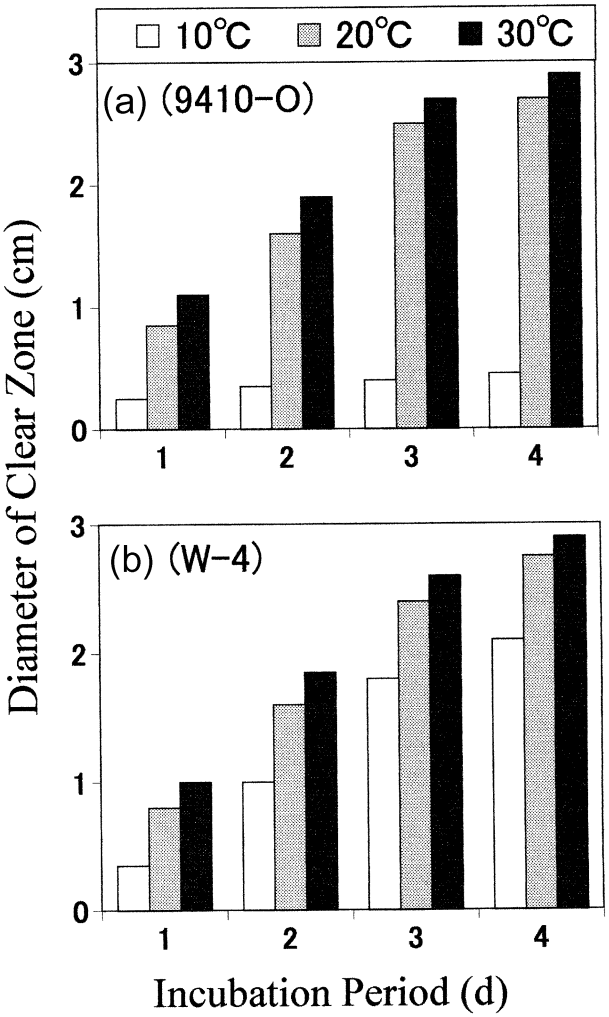

Fig. 2 Proteolysis activity test (by casein hydrolysis) of bacterial strains (9410-O and W-4) by measuring the diameter of the clear zone of hydrolyzed casein at different temperatures.

Net release of DIN and DIP, and effects of bacterial addition

Two experiments were carried out using *Enterobacter* sp. 9410-O and *Pseudomonas* sp. W-4 strains. The final cell densities of added bacterial strains of *Enterobacter* sp. 9410-O and *Pseudomonas* sp. W-4, and their ratio of natural bacterial populations during two incubation experiments are presented in Table 3. As such, during the experiment with

Table 1 Physiological characteristics of bacterial strain 9410-O

Characteristics	Results
Gram reaction	–
Cell shape	Short rods
Colony	Typically circular, low convex and smooth with entire edges
Colony color	White
Motility in liquid media	Motile
Flagellar arrangement	Peritrichous
Fluorescent pigment	–
Catalase	+
Oxidase	–
Indole production (Kovac's method)	+
Citrate utilization	+
Voges Proskauer reaction	+
Methyl red test	–
Oxidation fermentation (Hugh and Leifson)	Fermentative
Acid from D-glucose	+
Acid from L-arabinose	+
Acid from lactose	+

Table 2 Physiological characteristics of bacterial strain W-4

Characteristics	Results
Gram reaction	–
Cell shape	Short rods
Colony	Typically circular, convex and smooth with entire edges
Colony color	White
Motility in liquid media	Motile
Flagellar arrangement	(polar flagella)
Fluorescent pigment	–
Catalase	+
Oxidase	+
Indole production (Kovac's method)	–
Citrate utilization	+
Voges Proskauer reaction	+
Methyl red test	–
Oxidation fermentation (Hugh and Leifson)	Oxidative
Acid from D-glucose	+ (gas)
Acid from L-arabinose	+
Acid from lactose	–

Table 3 No. added bacterial cells and their ratio to natural bacterial abundances on different sampling/incubation occasions

Sampling date	Bacterial strain	Bacterial abundance in bottom water ($\times 10^6$ cells/ mL)	Total bacterial abundance in sediment ($\times 10^{10}$ cells)	Total added bacterial density ($\times 10^{10}$ cells)	Added bacteria: natural abundance (%)
15 February 2002	9410-O	2.0	45.5	10.0	22.0
	W-4	2.1	45.0	10.4	23.1
3 August 2002	9410-O	3.0	49.0	13.2	26.9
	W-4	3.1	48.5	14.0	28.9

samples collected in 15 February 2002, added bacterial abundances of strains *Enterobacter* sp. 9410-O and *Pseudomonas* sp. W-4 were 22.0% and 23.1%, respectively (Table 3). And in the experiment with samples collected on 3 August 2002, added bacterial abundances of strains 9410-O and W-4 were 26.9% and 28.9%, respectively (Table 3).

When samples were collected on 15 February 2002 and incubated at *in situ* temperature of 13°C, the net releases of DIN and DIP in control counter-

parts (without adding bacteria) were 12.7 μM and 1.30 μM, respectively. Even when the 9410-O strain was added to the system, the effects were almost negligible (Fig. 3c,d). However, when the sample was incubated at a simulated summer temperature of 26°C, although the net increase of DIN in the control was 61.7 μM and that of DIP was 2.82 μM, strain 9410-O stimulated net releases of DIN and DIP of 239 μM and 14.9 μM, respectively (Fig. 3a,b). Simultaneously, we conducted an experiment with another bacterial strain (W-4) by incubating at the same *in situ* temperature (13°C) and simulated summer temperature (26°C). When incubated at an *in situ* temperature of 13°C, the net releases of DIN and DIP were stimulated to 52.7 μM and 4.18 μM, respectively (Fig. 4c,d) by adding the W-4 strain. At the high temperature of 26°C, the net increases in DIN and DIP were also accelerated to 261 μM and 11.9 μM, respectively, by adding W-4 (Fig. 4a,b).

In experiments with summer samples (3 August 2002) that were incubated at the ambient temperature of 25.5°C, the net release of DIN and DIP in the 9410-O-supplemented sediment–water complex system were prominently higher compared to the control. Net releases of 274 μM DIN and 15.4 μM DIP in the 9410-O-supplemented system are shown in comparison with 75.0 μM of DIN and 2.91 μM of DIP in the control, respectively (Fig. 5a,b). A parallel experiment with 9410-O was conducted at a simulated winter temperature of 10°C and no effective enhancement was noticed (Fig. 5c,d). Furthermore, the same experiment conducted at an ambient temperature of 25.5°C with addition of the W-4 strain showed that net releases of DIN and DIP were also stimulated to 282 μM and 16.1 μM, respectively (Fig. 6a,b). During an experiment performed with W-4 and incubated at a simulated winter temperature of 10°C, although the net releases of DIN and DIP in the control were very low at 13.3 μM and 2.51 μM, respectively (Fig. 6c,d), the addition of W-4 stimulated the release to 124 μM in DIN and 15.2 μM in DIP (Fig. 6c,d).

Mean DO concentration at the beginning and termination of incubation at *in situ* and simulated

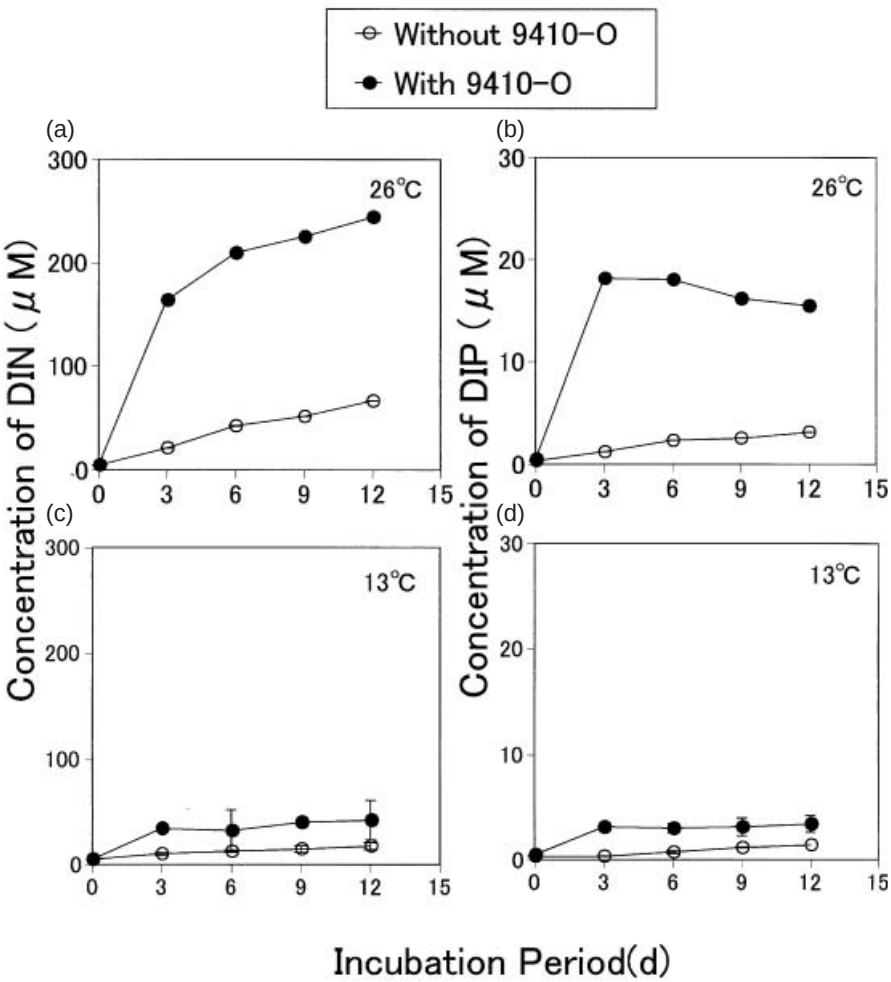

Fig. 3 Changes in concentration (μM) of dissolved inorganic nitrogen (DIN), and dissolved inorganic phosphorus (DIP) during incubation of sediment–bottom water complex system collected on 15 February 2002 (●) with or (○) without 9410-O. Incubation was carried out at (a,b) a summer simulation temperature of 26°C; and (c,d) an *in situ* temperature of 13°C. Error bars represent mean ± 1 SD ($n = 2$). (No error bar means SD is smaller than symbol.)

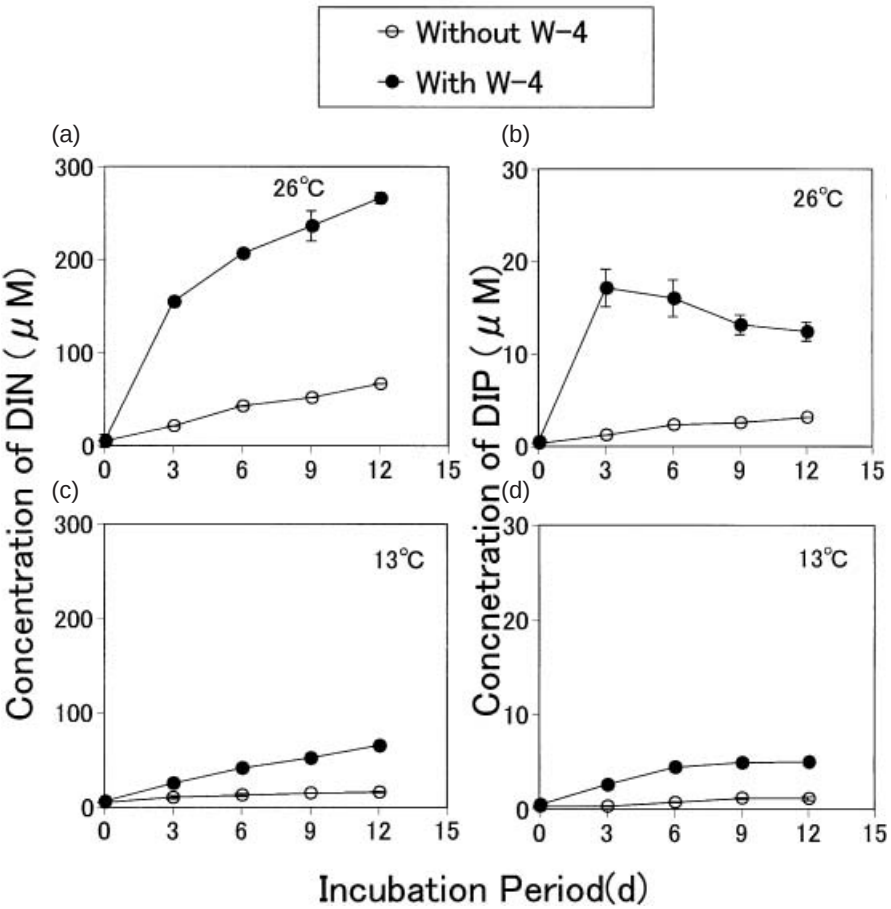

Fig. 4 Changes in concentration (μM) of dissolved inorganic nitrogen (DIN), and dissolved inorganic phosphorus (DIP) during incubation of sediment–bottom water complex system collected on 15 February 2002 (●) with or (○) without W-4. Incubation was carried out at (a,b) a summer simulation temperature of 26°C; and (c,d) an *in situ* temperature of 13°C. Error bars represent mean ± 1 SD ($n = 2$). (No error bar means SD is smaller than symbol.)

winter or summer temperatures are presented in Table 4. As is evident, the DO concentration at the termination was significantly lower than at the start of incubation, especially in the case where high temperature DO concentration was decreased from the second day of incubation (data not shown).

DISCUSSION

Semi-enclosed coastal areas such as our study site, in particular, are vulnerable considering their limited water exchange; and accumulation of wastes in the water and sediment takes place because the site is inhabited by a great number of intensive fish rearing cages but has limited water exchange.[35] The 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 toxic H_2S concentrations in summer, especially in bottom water.[31] Inducing the growth of particular bacteria could facilitate the biodegradation process of organic wastes even in large areas of water.[22,23,47,48] But almost all of the bioremediative efforts have been targeted at recalcitrant, xenobiotic compounds and heavy metals or oily sludge-contaminated areas. In the present study we tried to apply this method in a eutrophic ecosystem and monitored the effects of adding bacterial strain(s) to enhance the heterotrophic activities, by 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.[31,32] The indigenous mesophilic strain *Enterobacter* sp. 9410-O was isolated from the surface sediment of the study site in October when proteolysis activity in sediment was at its peak.[32] It exhibited high growth rates and proteolytic activities at temperatures ranging from 20°C to 30°C (Fig. 2a).[33] The study site has a high dissolved oxygen content in winter but due to low temperature the microbial activity was very low. Therefore, use of a psychrophilic or psychrotolerant bacteria that can be active at low temperatures may be a possible way to enhance heterotrophic activities during the winter season.[41] Strain *Pseudomonas* sp. W-4, which is psychrotolerant, was isolated from bottom water of the study

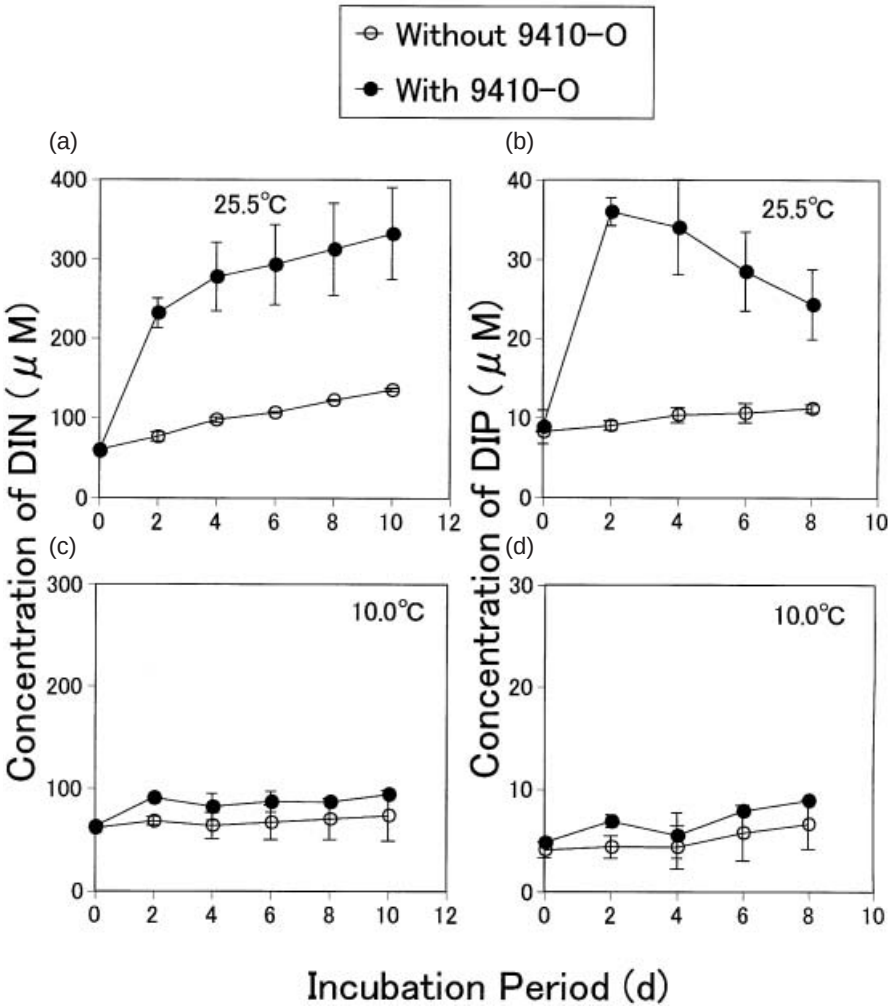

Fig. 5 Changes in concentration (μM) of dissolved inorganic nitrogen (DIN), and dissolved inorganic phosphorus (DIP) during incubation of sediment–bottom water complex system collected on 3 August 2002 (●) with or (○) without 9410-O. Incubation was carried out at (a,b) an *in situ* temperature of 25.5°C; and (c,d) a winter simulation temperature of 10°C. Error bars represent mean ± 1 SD ($n = 2$). (No error bar means SD is smaller than symbol.)

site in the coldest season (December) and had high growth rates and proteolytic activities even at low temperatures such as 10°C (Figs 1,2b). Because one bacterial strain may not be effective throughout the year, we therefore used two bacterial strains and hoped that they would be capable of possessing high metabolic activities in different seasons.

After adding the 9410-O strain, net amounts and rates of DIN and DIP regeneration in overlying water column from sediment were markedly enhanced in the system collected in February and incubated at a summer simulated temperature of 26°C (Fig. 3a,b). However, when we conducted parallel incubation at the *in situ* temperature of 13°C, enhancements in DIN and DIP releases were quite limited (Fig. 3c,d). Therefore, results indicate that the strain 9410-O was most effective at high temperatures or in a summer situation, which is consistent with the growth characteristics and proteolytic activities of this strain (Fig. 2A).[33] In contrast to this strain, addition of W-4 strain to the same system collected in February enhanced the releasing rates of DIN and DIP incubated not only at an ambient lower temperature of 13°C (Fig. 4c,d), but also at a simulated higher summer temperature of 26°C (Fig. 4a,b). Such results are pertinent to the growth rate and proteolytic activities of this strain (Figs 1,2b).

During summer experiments in August, addition of 9410-O also stimulated the release rate of DIN and DIP when incubated at an ambient summer temperature of 25.5°C, but no enhancements were noticed for the winter simulated temperature of 10°C (Fig. 5). However, addition of W-4 strain enhanced the release rate of DIN and DIP both at the ambient 25.5°C temperature and simulated winter temperature of 10°C (Fig. 6). The effect of adding bacteria to the sediment–bottom water complex system has been evaluated by comparison with the control counterparts (bacteria not added). An interesting point is that addition of psychrotolerant *Pseudomonas* sp. W-4 in winter and *Enterobacter* sp. 9410-O in summer produced net releases of DIN and DIP that were

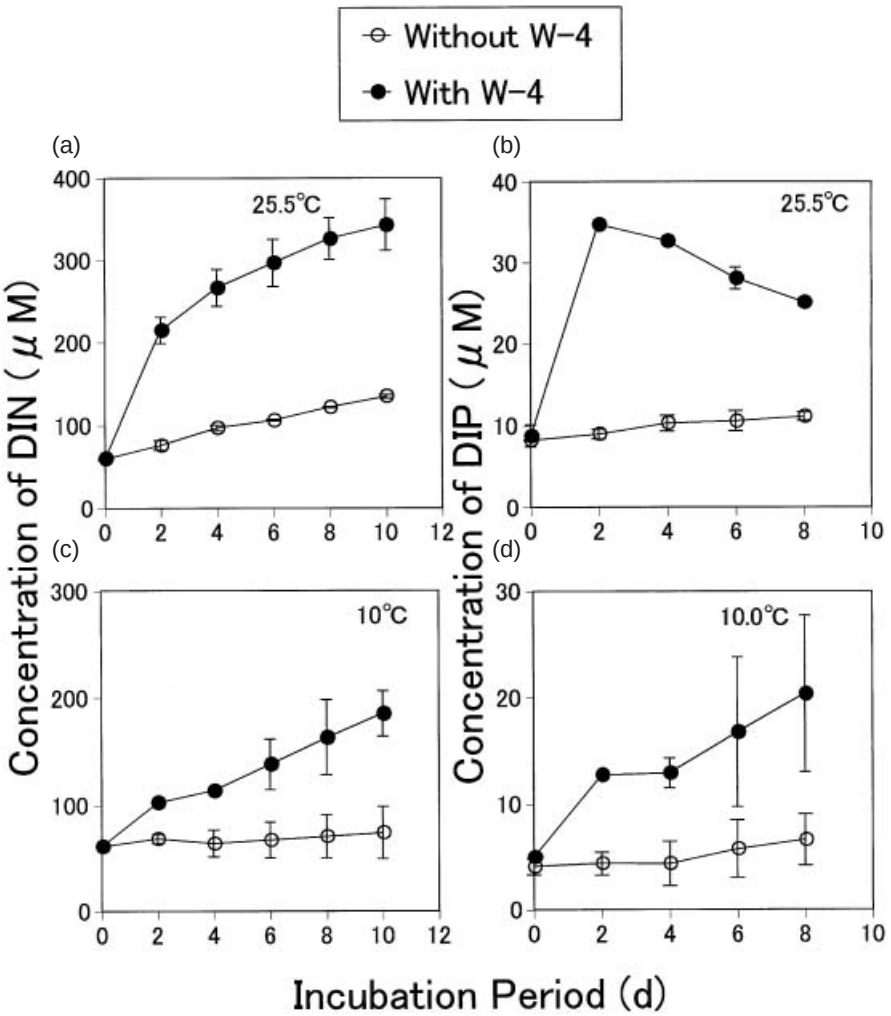

Fig. 6 Changes in concentration (μM) of dissolved inorganic nitrogen (DIN), and dissolved inorganic phosphorus (DIP) during incubation of sediment–bottom water complex system collected on 3 August 2002 (●) with or (○) without W-4. Incubation was carried out at (a,b) an *in situ* temperature of 25.5°C; and (c,d) a winter simulation temperature of 10°C. Error bars represent mean ± 1 SD ($n = 2$). (No error bar means SD is smaller than symbol.)

Table 4 Changes in concentration of DO at the beginning and end of incubation for different sampling/incubation combinations

		Concentration of DO							
		Without bacteria		With bacteria†		Without bacteria‡		With bacteria‡	
Sampling date	Bacterial strain	Beginning (mg/L)	End (mg/L)	Beginning (mg/L)	End (mg/L)	Beginning (mg/L)	End (mg/L)	Beginning (mg/L)	End (mg/L)
15 February 2002§	9410-O	5.5	3.5	5.6	3.4	5.4	1.9	5.4	1.9
	W-4	5.6	3.4	5.5	3.0	5.5	2.0	5.5	1.6
3 August 2002¶	9410-O	2.0	1.1	2.2	0.2	1.9	1.6	1.8	1.5
	W-4	1.8	0.9	2.1	0.3	1.8	1.5	2.0	1.1

†Incubated at *in situ* temperature on different sampling occasions; ‡incubated at simulated summer or winter temperature on different sampling occasions.
§Incubated *in situ* temperature was 13°C and simulated temperature was 26°C; ¶incubated *in situ* temperature was 25.5°C and simulated temperature was 10°C.
DO, dissolved oxygen.

almost four–fivefold higher compared with the control, even though heterotrophic activities (regeneration rate) in the control were relatively higher in summer than in the winter season. Furthermore, net releases of inorganic nutrients (both of DIN and DIP) after addition of the W-4 strain were higher in the experiment conducted in summer sediment sample than in winter sample, due to availability of labile organic matter in summer, respectively (Figs 4c,d,6c,d).

It should also be considered that the stimulation of DIN and DIP compared to control counterparts was four–fivefold higher, while the percentage bacterial addition was 20–30%. Gardner *et al.* reported that increased ammonium regeneration rates corresponded to an increased addition of bacteria into unfiltered seawater and assumed that this was caused directly by bacteria; no elucidation was provided.[49] If so, it could be argued that added bacterial cells might be used as a labile organic matter source leading to increased inorganic nitrogen concentration. In order to determine whether active bacteria or degraded cells were responsible for the increased nutrient regeneration, we used killed bacterial cells of the same amount in the control bottles as was used in the test bottles by absorbing onto porous substrates as a negative control. Results showed that live bacterial cells produced a higher increase in inorganic nutrient in comparison to dead bacterial cells (data not shown).

We considered that the enhancement of the inorganic nutrient (DIN and DIP) regeneration into the overlying water column should be increased by the bacterial decomposition of organic matter of the sediment. So far, in the present study we tried to enhance the decomposition of organic matter by addition of effective indigenous bacteria. Numerous approaches have been developed to mitigate a high concentration of inorganic nutrients from an overlying water column, as such (i) to enhance the primary production by cultivating seaweeds or (ii) to take up the increasing nutrients by culturing sessile microalgae. It is necessary to conduct further study at the next stage regarding the removal of enhanced inorganic nutrients from the overlying water column.

Previous studies conducted in regards to alleviating the effect of pollution by using microorganisms of exogenous origin that were introduced as cell suspensions.[33] However, most of the efforts have been directed towards achieving the degradation of anthropogenic contaminants. But there is no scientific validation that those bacterial strains would be effective in the field of eutrophic bottom environments.[29] If we consider eutrophic bottom environments as the actual field, then addition of bacterial cell suspensions would not be effective because cells would be killed or diluted by native bacteria. Furthermore, bacteria added from an exogenous origin might be inactive or persist against and/or dominate the indigenous microorganisms and can have adverse effects on the ecosystem. As noted, our present results do not give any quantitative estimate of the kinetics of the decomposition of organic matter to DIN and DIP in sediment–bottom water systems in nature. However, we emphasize that for the ecosystem of the actual field of study we used indigenous bacterial strains, which were added to the system by absorbance with porous substrates. Results of the present study showed that bacteria of indigenous origin stimulate the decomposition of organic matter in the sediment of eutrophic environments. We suggest that the bacteria used in the present study would be effective in bottom environments in the field, and interaction with indigenous microorganisms and relevant ecosystem would be negligible or minimum. Further study is needed to evaluate the effects of these bacteria in the field of eutrophic bottom environments with natural ecosystems.

ACKNOWLEDGMENTS

The authors thank Professor Dr T Nishijima and Dr M Adachi for their valuable suggestions and help related to the study, present and previous students of the Laboratory of Aquatic Environmental Science (LAQUES) and the staff of the Marine Biological Research Center Usa (Japan) for their help during sampling and/or chemical or biological analysis. This study was supported in part by a grant-in-aid (Bio Renaissance Program) from the Ministry of Agriculture, Forestry and Fisheries (Japan) (BRP-98-II-B-1).

REFERENCES

1. Hans A, Enell M. Discharges of nutrients from Swedish fish farming to adjacent sea areas. *Ambio* 1990; **19**: 28–35.
2. Beveridge MCM, Phillips MJ, Clarke RM. A quantitative and qualitative assessment of wastes from aquatic animal production. In: Brune DE, Tomasso JR (eds). *Aquaculture and Water Quality*. World Aquaculture Society, Baton Rouge, LA. 1991; 506–533.
3. Kupka-Hansen P, Pittman K, Ervik A. Organic waste from marine fish farms: effects on the seabed. In: Mäkinen T (ed). Marine aquaculture and environment. *Nord* 1991; **22**: 105–119.
4. Hall POJ, Holby O, Kollberg S, Samuelsson M. Chemical fluxes and mass balances in a marine fish cage farm. IV. Nitrogen. *Mar. Ecol. Prog. Ser.* 1992; **89**: 81–91.
5. Johnsen RI, Grahl-Nielsen O, Lunestad BT. Environmental distribution of organic waste from a marine fish farm. *Aquaculture* 1993; **118**: 229–244.
6. Briggs MR, Funge-Smith SJ. A nutrient budget of some intensive marine shrimp ponds in Thailand. *Aquacult. Fish. Manage.* 1994; **25**: 789–811.
7. Rosenthal H. Aquaculture and the environment. *World Aquacult.* 1994; **25**: 4–11.
8. Gowen RJ, Bradbury NB, Brown JR. The use of simple models in assessing two of the interactions between fish farming

and the marine environment. In: De Pauw N, Jaspers E, Ackefors H, Wilkins N (eds). *Aquaculture: A Biotechnology in Progress*. European Aquaculture Society, Bredene, Belgium. 1989; 1071–1080.
9. Iwama GK. Interaction between aquaculture and the environment. *Crit. Rev. Environ. Control.* 1991; **21**: 177–216.
10. Silvert W. Assessing environmental impacts of finfish aquaculture in marine waters. *Aquaculture* 1992; **107**: 67–79.
11. Gowen RJ, Rosenthal H, Makinen T, Ezzi I. Environmental impact of aquaculture activities. In: de Pauw N, Wilkins N (eds). Aquaculture Europe 89: Business Joins Science. *Eur. Aquacult. Soc. Spec. Publ.* 1990; **12**: 1071–1080.
12. Gowen RJ, Weston DP, Ervik A. Aquaculture and benthic environment: A review. In: Cowey CB, Cho CY (eds). *Nutrition Strategies and Aquaculture Waste*. Department of Nutritional Sciences, University of Guelph, Ontario. 1991; 187–206.
13. Nishimura A. Effects of organic matters produced in fish farms on the growth of red tide algae *Gymnodinium* type 65 and *Chattonella antiqua*. *Bull. Plankton Soc. Jpn* 1982; **29**: 1–7.
14. Enell M, Lof J. Miljoeffekter av vattenbruk: sedimentation och narsaltbelastning from fishkasseodingar. *Vatten* 1983; **39**: 364–375.
15. Schwedler TE, Tucker CS. Empirical relationships between percent methemoglobin in channel catfish and dissolved nitrite and chloride in ponds. *Trans. Am. Fish. Soc.* 1983; **112**: 117–119.
16. Weston DP. Quantitative re-examination of macrobenthic community changes along an organic enrichment gradient. *Mar. Ecol. Prog. Ser.* 1990; **61**: 233–244.
17. Tucholski F, Eclawski I, Wijno T. Studies on removal of wastes produced during cage rearing of rainbow trout (*Salmo gairdneri*) in lakes. 1. Chemical composition of waste. *Rocz. Nauk. Roln* 1980; **82**: 17–30.
18. Hall P, Holby O. *Environmental Impact of a Marine Fish Cage Culture*, Report CM 1986/F: 46. Mariculture Committee, Sweden. 1986; 20.
19. Folke C, Kautsky N. The role of ecosystems for a sustainable development of aquaculture. *Ambio* 1989; **18**: 234–243.
20. Pomeroy LR. The ocean's food web, a changing paradigm. *Bioscience* 1974; **24**: 499–504.
21. Fuhrman J. Bacterioplankton roles in cycling of organic matter: the microbial food web. In: Falkowski PG, Woodhead AD (eds). *Primary Productivity and Biogeochemical Cycles in the Sea*. Plenum Press, New York. 1992; 361–383.
22. Moriarty DJW. Control of luminous *Vibrio* species in penaeid aquaculture ponds. *Aquaculture* 1998; **164**: 351–358.
23. Master ER, Mohn WW. Psychrotolerant bacteria isolated from arctic soil that degrade polychlorinated biphenyls at low temperatures. *Appl. Environ. Microbiol.* 1998; **64**: 4823–4829.
24. Rijn JV. The potential for integrated biological treatment systems in re-circulating fish culture: a review. *Aquaculture* 1996; **139**: 181–201.
25. Holmer M, Kristensen E. Impact of marine fish cage farming on metabolism and sulfate reduction of underlying sediments. *Mar. Ecol. Prog. Ser.* 1992; **80**: 191–201.
26. Holmer M, Kristensen E. Seasonality of sulfate reduction and pore water solutes in a marine fish farm sediment: the importance of temperature and sedimentary organic matter. *Biogeochemistry* 1996; **32**: 15–39.
27. Roos N, Eriksen JR. Sediment respiration and denitrification in integrated carp polyculture. *Aquaculture* 1995; **129**: 393.
28. Suplee MW, Cortner JB. Temporal changes in oxygen demand and bacterial sulfate reduction in inland shrimp ponds. *Aquaculture* 1996; **145**: 141–158.
29. Moriarty DJW. The role of microorganisms in aquaculture ponds. *Aquaculture* 1997; **151**: 333–349.
30. Cole J. Controlling environmental nitrogen through microbial metabolism. *Tibtechnical* 1993; **11**: 368–372.
31. Patel AB, Fukami K, Nishijima T. Regulation of seasonal variability of aminopeptidase activities in surface and bottom waters of Uranouchi Inlet, Japan. *Aquat. Microb. Ecol.* 2000; **21**: 139–149.
32. Patel AB, Fukami K, Nishijima T. Extracellular proteolytic activity in the surface sediment of a eutrophic inlet. *Microb. Environ.* 2001; **16**: 25–35.
33. Patel AB, Fukami K, Karim MA, Nishijima T. Stimulation of regeneration of inorganic nitrogen and phosphorus in surface and bottom waters of a eutrophic inlet by adding effective bacteria. *Microb. Environ.* 2002; **17**: 205–213.
34. Fukami K, Nishijima T, Murata H, Doi S, Hata Y. Distribution of bacteria influential on the development and the decay of *Gymnodinium nagasakiense* red tide and their effects on algal growth. *Nippon Suisan Gakkaishi* 1991; **57**: 2321–2326.
35. Fukami K, Murata N, Morio Y, Nishijima T. Distribution of heterotrophic nanoflagellates and their importance as the bacterial consumer in a eutrophic coastal seawater. *J. Oceanogr.* 1996; **52**: 399–407.
36. Porter KG, Feig YS. The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.* 1980; **25**: 943–948.
37. Fukami K, Meier B, Overbeck J. Vertical and temporal changes in bacterial production and its consumption by heterotrophic nanoflagellates in a north German eutrophic lake. *Arch. Hydrobiol.* 1991a; **122**: 129–145.
38. Velji MI, Albright LJ. Microscopic enumeration of attached marine bacteria of seawater, marine sediment, fecal matter, and kelp blade samples following pyrophosphate and ultrasound treatments. *Can. J. Microbiol.* 1986; **32**: 121–126.
39. Schallenberg M, Kalff J, Rasmussen JB. Solutions to problems in enumerating sediment bacteria by direct counts. *Appl. Environ. Microbiol.* 1989; **55**: 1214–1219.
40. Furtado ALdS, Casper P. Different methods for extracting bacteria from freshwater sediment and a simple method to measure bacterial production in sediment samples. *J. Microbiol. Methods* 2000; **41**: 249–257.
41. Fukami K, Nakajima R., Patel AB, Nishijima T. Bioremediation by using optical fibers and psychrophilic bacteria. MEDCOAST 99-EMECS 99 Joint Conference, Land–Ocean Interactions. In: Özhan E (ed). *Managing Coastal Ecosystems*. Antalya, Turkey. 1999; 9–13.
42. Fukami K, Nishijima T, Hata Y. Availability of deep seawater and effects of bacteria isolated from deep-sea water on the mass culture of food microflora *Chaetoceros ceratosporum*. *Nippon Suisan Gakkashi* 1992; **58**: 931–936.
43. Denkin SM, Nelson DR. Induction of protease activity in *Vibrio anguillarium* by gastrointestinal mucus. *Appl. Environ. Microbiol.* 1999; **65**: 3555–3560.

44. Novitsky JA. Heterotrophic activity throughout a vertical profile of seawater and sediment in Halifax Harbor, Canada. *Appl. Environ. Microb.* 1983; **45**: 1753–1760.
45. Wellsbury P, Rodney A, Herbert R, Parkes J. Bacterial activity and production in near-surface estuarine and freshwater sediments. *FEMS Microbiol. Ecol.* 1996; **19**: 203–214.
46. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (eds). *Bergey's Manual of Determinative Bacteriology*. Williams and Wilkins, Baltimore, Maryland, USA. 1994.
47. Rijn JV, Fonarev N, Berkowitz B. Anaerobic treatment of indusive fish culture effluents: digestion of fish feed and release of volatile fatty acids. *Aquaculture* 1995; **133**: 9–20.
48. Mishra S, Jyot J, Kuhad RC, Lal B. Evaluation of inoculum addition to stimulate in situ bioremediation of oily-sludge-contaminated soil. *Appl. Environ. Microbiol.* 2001; **67**: 1675–1681.
49. Gardner WS, Cavaletto JF, Cortner JB, Johnson JR. Effects of natural light on nitrogen cycling rates in the Mississippi river plume. *Limnol. Oceanogr.* 1997; **42**: 273–281.