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Evaluation of nutrient limitation in aquatic ecosystems with nitrogen fixing bacteria

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Abstract: There has always been a great need for simple and accurate bioassays for evaluating nutrient limitation in aquatic ecosystems. Whereas organic carbon is usually considered to be the limiting nutrient for microbial growth in many aquatic ecosystems, there are, however, many water sources that are limited by phosphorus or nitrogen. A method named "nitrogen fixing bacterial growth potential" (NFBGP) test, which is based on pre-culturing of autochthonous (target) microorganisms was described. The method was applied to evaluate phosphorus or nitrogen nutrient limitation in lake and sewage water samples using an isolate of the nitrogen fixing bacterium, *Azorhizobium* sp. WS6. The results corresponded well to those from the traditional algal growth potential (AGP) test and the bacterial regrowth potential (BRP) test, suggesting that the NFBGP test is a useful supplementary method for evaluating the limiting nutrient, especially phosphorus, in an aquatic environment.
Keywords: environment monitoring; nitrogen fixing bacterium; limiting nutrient; phosphorus; growth potential

Introduction

Growth potential tests have been widely used for evaluating the limiting nutrients of aquatic ecosystems (Mallin *et al.*, 2004). For example, an algal growth potential (AGP) test is usually performed to forecast production of a lake or an ocean ecosystem (Maestrini *et al.*, 1998), and a bacterial regrowth potential (BRP) test is frequently applied to evaluate the effects of management techniques in drinking water distribution systems (Sathasivan and Ohgaki, 1999). With the increasing attention focused on environmental protection, the determination of limiting nutrients has also become necessary in sewage disposal plants and bioremediation sites in order to remove pollutants more effectively (Zuo *et al.*, 2000).

Although several biomonitoring techniques have been established for assessing the limiting nutrients in environments (Lyngby *et al.*, 1999; Alden *et al.*, 2001), each one has its own drawbacks in certain aspects. For example, in the AGP test, a species of alga (*Selenastrum capricornutum*, *Chlorella vulgaris* or other species) is recommended for inoculating the water sample. Such an alga may not be the dominant one in the tested aquatic ecosystem. Moreover, the constitution and richness of the algal community vary with temporal and spatial variation. The results of an AGP test, with only one inoculated algal strain cannot therefore, be expected to reflect the real nutrient state of an aquatic environment. As for the BRP test, there are various kinds of bacteria that are able to grow in a water distribution system (Tokajian *et al.*, 2005), making it impossible to precisely assess the limiting nutrient with only one pure inoculum, because such an inoculum may not be adapted to live in oligotrophic drinking water. Additionally, the mixed autochthonous inocula make enumeration difficult because of their morphological and physiological diversification (Bussmann *et al.*, 2001).

Experiments have shown that the significance of nutrients as limiting factors for microbial growth cannot be assessed from water chemical data and traditional chlorophyll or productivity measurements (Lyngby *et al.*, 1999). A simple and more accurate biomonitoring method is therefore needed. Since nitrogen and phosphorus are the two main nutrients necessary for microbial growth, and either of them may be the limiting factor in an aquatic ecosystem (Carlsson and Caron, 2001). We described in this paper a novel biomonitoring method, the nitrogen fixing bacterial growth potential (NFBGP) test. With an isolate of the nitrogen fixing bacterium, *Azorhizobium* sp. WS6, which propagates rapidly in a nutrient balanced medium and grows slowly in a phosphorus deficient environment, the NFBGP test was applied in a eutrophic lake sample, a drinking water sample and 2 simulated industrial sewage samples. The results were compared to those obtained from the AGP and BRP tests. The close correlation suggests that the NFBGP test can provide a favourable alternative to the two traditional methods.

1 Materials and methods

1.1 Screening for the nitrogen fixing bacterium

The nitrogen fixing bacterium used in this test was isolated from a natural freshwater ecosystem with Winogradsky nitrogen-free medium (glucose 2%, KH_2PO_4 0.02%, $MgSO_4 \cdot 7H_2O$ 0.02%, NaCl 0.02%, $CaCO_3$ 0.01%, $FeSO_4 \cdot 7H_2O$ 5 mg/L, $Na_2MoO_4 \cdot 2H_2O$ 5 mg/L, $MnSO_4 \cdot 4H_2O$ 5 mg/L, washing agar 2.0%, pH

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7.0—7.2, prepared with distilled water). The isolated bacterium was purified, and the cells were disrupted with freeze thawing method and its partial 16S rDNA was amplified by a polymerase chain reaction (PCR) using 341F and 907R as primers (Teske *et al.*, 1996). The resulting PCR product was analyzed by electrophoresis in 1.0% agarose gel and purified with Qiaquick PCR purification kit column (Qiagen, Germany). The sequence was determined in a MegaBACE 1000 DNA automated sequencer with 907R as sequencing primer, and similarity search was performed by comparing the determined sequence to other sequences in National Center for Biotechnology Information (NCBI) databases by blast software (http://www.ncbi.nlm.nih.gov). A phylogenetic tree was generated using DNAMAN package (Lynnon Biosoft, Quebec, Canada). The determined partial 16S rDNA sequence has been deposited in European Molecular Biology Laboratory (EMBL) Nucleotide Sequence Database under accession number AJ616732.

1.2 Description of the NFBGP test

The NFBGP test involves four steps: preculturing, filtration, inoculation and growth potential measurement. The collected sample was precultivated at optimal conditions of the predominant indigenous microorganism until its maximum growth. Then the primary microbial biomass was filtered away with 0.45 μm acetate cellulose filter, and 48.5 ml filtrate (or supernatant) was put into 250 ml flask each (all of the tests were in triplicate). 1 ml 10% glucose and 0.5 ml sterilized nutrient salt ($MgSO_4 \cdot 7H_2O$ 2%, NaCl 2%, $CaCO_3$ 1%, $FeSO_4 \cdot 7H_2O$ 500 mg/L, $Na_2MoO_4 \cdot 2H_2O$ 500 mg/L, $MnSO_4 \cdot 4H_2O$ 500 mg/L) were added to each flask, and the nitrogen fixing bacterium was inoculated. The flasks were incubated at 30℃, with 2 times shaking (1 min each, by hand) every day for bacterial suspension and oxygen solution, and bacterial cells were enumerated after every 24 h.

1.3 Growth conditions for the nitrogen fixing bacterium

The optimum growth temperature and pH of the selected nitrogen fixing bacterium were examined with Winogradsky nitrogen-free medium. The growth yields were evaluated by measuring bacterial biomass after 72 h culturing. To determine the optimum temperature, flasks with media at pH 7.0 were cultivated at 20, 25, 30, 35 and 40℃. To determine the optimum pH, flasks with media at pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10 were cultivated at 30℃. The salt-resisting tests of the selected nitrogen fixing bacterium were performed at 30℃ and pH 7.0 in Winogradsky nitrogen-free medium with 1%—3% of NaCl. Bacterial biomass was determined after every 12 h until 72 h.

1.4 Effect of N and P concentration on the growth of the selected nitrogen fixing bacterium

Growth curves of selected nitrogen fixing bacterium were determined under different concentrations of nitrogen and phosphorus. 50 ml Winogradsky medium without nitrogen (N) and phosphorus (P) was poured into each flask, and $(NH_4)_2SO_4$ or KH_2PO_4 added to make the N concentration 0, 0.5, 2.5 mg/L and P concentration 0, 0.25, 0.5, 1.0 mg/L, respectively. After inoculating with the selected nitrogen fixing bacterium (to 10^3 cells/ml), the flasks were incubated at 30℃, with 2 times shaking every day, and the bacterial cell numbers were enumerated after every 12 h until 72 h.

1.5 Application of NFBGP test

The NFBGP test was applied to a lake water sample, a drinking water sample and 2 artificial foul water samples. The lake water sample, obtained from a eutrophic lake, was continuously cultivated at 30℃ in the light chamber (2200 lx, with shaking twice a day) for 48 h. The NFBGP test was performed on its filtrate. At the same time, the AGP test with *Chlorella vulgaris* was carried out as a control.

The drinking water sample, obtained from a laboratory water faucet, was vacuum pumped to volatilize the dissolved chlorine and then cultivated at 30℃ for 48 h with occasional shaking. After filtration, the NFBGP test was performed on the filtrate. The control BRP test was carried out according to the method of Sathasivan and Ohgaki (1999), which comprised of 3 groups inoculated with *Escherichia coli*, *Staphylococcus aureus* and the autochthonous bacteria.

The industrial sewage was simulated with artificial foul water samples, which composed of pond water with different concentrations of phenol (100, 250, 500, 750 and 1000 mg/L) or $CuSO_4$ (10, 50, 100, 200 and 500 mg/L Cu^{2+}).

Bacterial enumeration and biomass determination was done by spreading 50 μl of the culture solution on a slide to form a 2.5×5.5 cm^2 rectangle. After simple staining with ammonium oxalate crystal violet solution, bacterial cells in 5 representative fields (0.25×10^{-4} cm^2 each) were enumerated under the microscope by oil immersion, and the biomass (cells/ml) calculated. To evaluate the difference between two treatments, average values were calculated and U test was carried out.

2 Results and discussion

2.1 Screening for nitrogen fixing bacteria and preliminary characterization of the selected strain

Eighteen strains with nitrogen fixing ability were isolated and purified on Winogradsky nitrogen-free agar. Strain A produced a red pigment, and its growth could be conveniently determined by colorimetry. Strain B and C had relatively large cell volumes, and

could be easily enumerated under the microscope. The 3 strains were therefore chosen as test strains for the NFBGP method. However, further experiments revealed that strain A grew slowly in Winogradsky liquid medium with the optical density of its culture solution changing insignificantly within the first 4 d. Strain B grew fast but in its stationary phase, secreted much slime and a lot of cells clump together, making enumeration difficult. Strain C had no such disadvantages, and was therefore selected as the test strain. Molecular characterization of strain C showed that this isolate closely matches *Azorhizobium caulinodans* (96.94% identity with 1.09% gap), a nitrogen fixing bacterium usually found in root nodules. It is therefore, preliminarily designated as *Azorhizobium* sp. WS6. Phylogenetic affiliation of this isolate with other bacteria indicates that it belongs to the division of α-Proteobacteria (Fig.1).

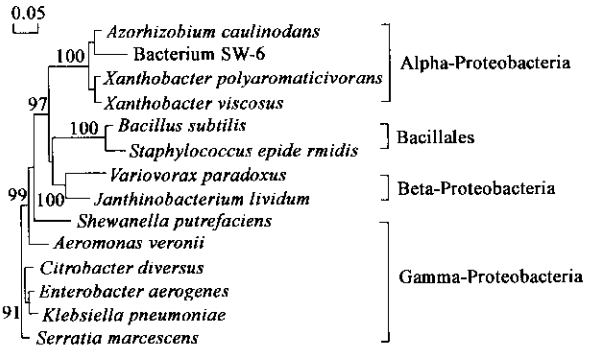

Fig.1 Phylogenetic affiliation of bacterium SW-6 as revealed by comparative analysis of partial 16S rDNA sequences and those stored in public nucleotide databases

The sequences were aligned and the phylogenetic tree was constructed by DNAMAN package. Division level groupings are bracketed at the right of the figure. The scale-bar represents 5% estimated sequence divergence. Numbers indicate bootstrap confidence values as the percentage of 100 bootstrap replications

2.2 Growth temperature and pH of *Azorhizobium* sp. WS6

The optimum growth tests of *Azorhizobium* sp. WS6 showed that its optimum growth pH is 7.0 and its optimum growth temperature is 30℃, but it grew well at pH 6.0—9.0 and at 25—35℃ (Fig.2). Although an aerobic bacterium, it grew better at lower dissolved oxygen concentration. A static culture with 2 times shaking (one minute each, by hand) every day grew better than a rotary culture shaken at a higher rate (180 r/min).

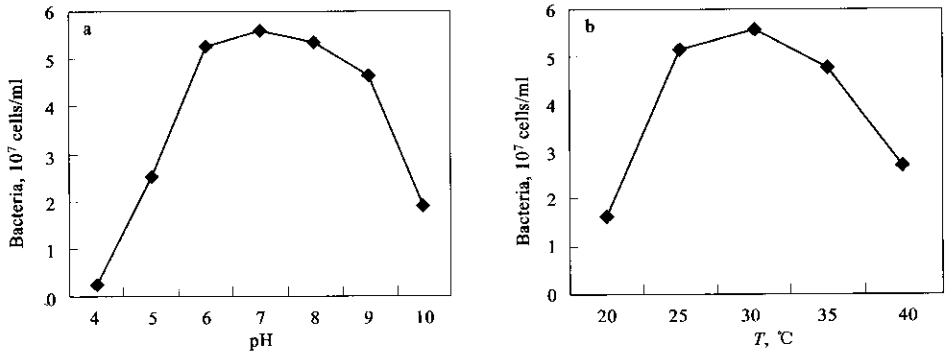

Fig.2 Effect of pH(a) and temperature(b) on growth of *Azorhizobium* sp. WS6

The salt-resisting test showed that *Azorhizobium* sp. WS6 grow slowly at salt concentration reaching 3% (Fig.3), suggesting that this nitrogen fixing bacterium, isolated from fresh water, is not adapted to grow in an ocean ecosystem. It is therefore necessary to screen for a salt resisting nitrogen fixing bacterium to evaluate the limiting nutrient of marine water samples with the NFBGP test.

2.3 Effect of N and P concentration on the growth of *Azorhizobium* sp. WS6

Azorhizobium sp. WS6 propagated well in the phosphorus-limiting medium for the first 24 h (Fig.4a, b). This can be attributed to the small amount of phosphorus brought in with the inoculum. The bacterium absorbs trace phosphorus depending on the nitrogen concentration in the medium. In the case of higher N/P ratio, the bacterium absorbs phosphorus more completely (Fig.4b). In a nitrogen free

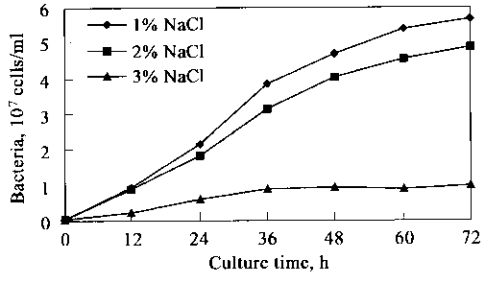

Fig.3 Effect of NaCl concentration on the growth of *Azorhizobium* sp. WS6

environment, the bacterial biomass increased with the rise of phosphorus concentration (Fig.4c). The correlation coefficient at 12, 24, 48 and 72 h reached 0.85, 0.81, 0.87 and 0.92, respectively ($\alpha_{0.05}$=0.878). Under the conditions of exogeneous addition of both $(NH_4)_2SO_4$ and KH_2PO_4, the bacterium grew rapidly,

but when phosphorus concentration reached 1 mg/L, the growth rate changed little with the rise of nitrogen concentration (Fig.4b). These observations suggest that with supplying enough carbon and phosphorus source, the bacterium could fix atmospheric nitrogen almost as effectively as it absorbed ammonium.

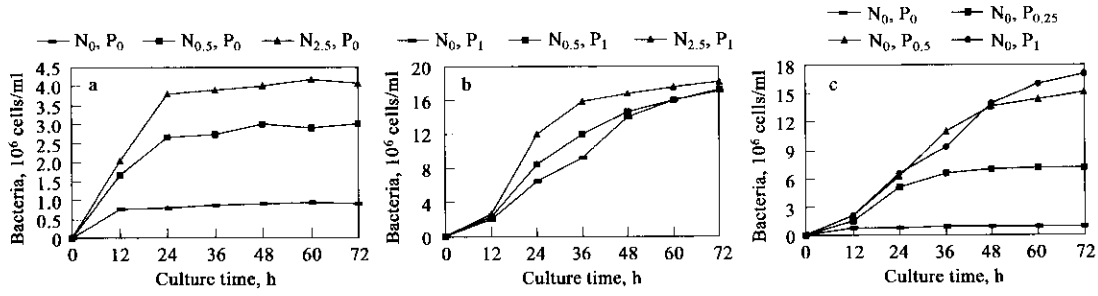

Fig.4 Effect of nitrogen and phosphorus concentrations on the growth of *Azorhizobium* sp. WS6
N_0: without nitrogen; $N_{0.5}$: 0.5 mg/L nitrogen; $N_{2.5}$: 2.5 mg/L nitrogen; P_0: without phosphorus; $P_{0.25}$: 0.25 mg/L phosphorus; $P_{0.5}$: 0.5 mg/L phosphorus; P_1: 1 mg/L phosphorus

2.4 Application of NFBGP method

2.4.1 Applied to eutrophic lake water

It is always necessary to assess the limiting nutrient for algal growth in a eutrophic aquatic ecosystem (Perkins and Underwood, 2000). Table 1 shows the results of a NFBGP test with a lake water sample. *Azorhizobium* sp. WS6 propagated well at the filtrate of pre-culture (condition 1) even after 24 h, suggesting that the sample may not be phosphorus limiting. Similarly, the biomass of filtrate with nitrogen addition (condition 2) at 24 h is more than that of filtrate with phosphorus addition (condition 3). Statistical analysis show that the difference in cell numbers between condition 1 and 3 ($u_{1,3}$=0.56) was smaller than that between 1 and 2 ($u_{1,2}$=9.86) at 24 h ($u_{0.05}$=1.645), but was almost the same at 72 h ($u_{1,3}$= 4.31 and $u_{1,2}$=4.27), demonstrating further that the water sample was nitrogen limiting. Under condition 1 (without addition of N and P), *Azorhizobium* sp. WS6 propagated by absorbing nitrogen and phosphorus dissolved in the filtrate. After about 24 h growth, the dissolved nitrogen was depleted, while at that point dissolved phosphorus was still high, therefore the bacterium could grow continuously by fixing atmospheric nitrogen. After about 72 h culturing, phosphorus was also used up, and bacterial biomass could not increase anymore. Under condition 2, the bacterium grew fast during the first 24 h by absorbing ammonium salt, accumulated large numbers of cell and depleted inorganic phosphorus, making further growth difficult. Under condition 3, the additional phosphorus prolonged the propagating period, and therefore the final biomass was higher than that under condition 1 and 2. The corresponding AGP test (condition 4, 5 and 6) also indicate that the lake water was nitrogen limiting.

A further test of the NFBGP method was carried out using artificial eutrophic water samples made by addition of various amounts of nitrogen and phosphorus into the lake water (Table 1). The results showed that to a eutrophic water sample with high concentration of nitrogen and phosphorus, a long pre-culture time was necessary, and two turns of pre-culture, that is, inoculating the ingenuous lake microorganisms into the first turn of filtrate, would be better (data not shown).

Table 1 NFBGP test with *Azorhizobium* sp. WS6 and AGP test with *Chlorella vulgaris* in a eutrophic lake water (10^4 cells/ml)

Group	Inoculum	Addition, mg/L	0 h	24 h*	72 h*	120 h*
1	*Azorhizobium*	----	0.1	185±10	320±15	341±22
2	*Azorhizobium*	2.5 N	0.1	305±19	365±11	372±21
3	*Azorhizobium*	0.15 P	0.1	192±13	370±14	397±12
4	*Chlorella*	----	0.5	8.6±0.3	15.7±3.0	16.0±2.9
5	*Chlorella*	2.5 N	0.5	10.2±1.3	25.4±3.1	29.1±2.5
6	*Chlorella*	0.15 P	0.5	9.4±0.6	16.8±1.0	18.0±1.3

Note: * Mean±*SD*; N is nitrogen; P is phosphorus

2.4.2 Applied to drinking water

Although organic carbon is usually the limiting nutrient for microbial growth in many countries, there are still many drinking water sources limited by phosphorus or nitrogen (Miettinen *et al.*, 1997). The NFBGP test will be useful for such samples.

Fig.5 shows that in the filtrate of an autochthonous bacterial culture solution, the nitrogen fixing bacteria could only propagate for about 24 h. Comparing this with the results presented in Fig.4, we came to the preliminary conclusion that phosphorus limited bacterial growth in the drinking water sample. Parallel tests with the addition of nitrogen (2.5 mg/L) and phosphorus (0.15 mg/L) also demonstrated that phosphorus was the limiting nutrient.

It is generally thought that the yields of heterotrophic bacteria would increase significantly with addition of the limiting nutrient. The results in Table 2 show that bacterial biomass with addition of phosphorus was more than that with addition of nitrogen in all three controlled tests (with *E. coli*, *S.*

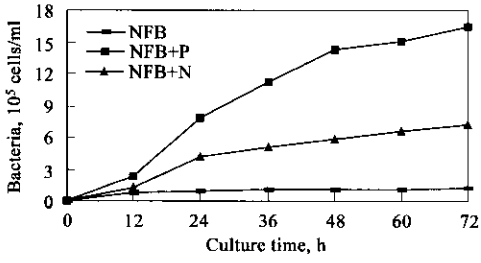

Fig.5 The NFBGP test of drinking water
NFB: NFBGP test without phosphorus and nitrogen; NFB+P: NFBGP test with 0.15 mg/L phosphorus; NFB+N: NFBGP test with 2.5 mg/L nitrogen

aureus and autochthonous bacteria), demonstrating that phosphorus influenced bacterial growth more significantly than nitrogen.

Table 2 Effect of nitrogen and phosphorus on the bacterial growth in drinking water (10^5 cells/ml)

Group	Phosphorus, 0.15 mg/L	Nitrogen, 2.5 mg/L	*S.aureus**	*E.coli**	Autochthonous bacteria*
1	-	-	2.85±0.61	2.36±0.42	0.47±0.13
2	+	-	3.95±0.52	3.29±0.62	1.04±0.26
3	-	+	3.22±0.38	2.28±0.55	0.79±0.15
4	+	+	4.06±0.64	3.75±0.48	1.11±0.23

Note: * Mean±*SD*, determined at 72 h culturing

Results from experiments with artificial water samples, made by adding different concentrations of nitrogen or phosphorus into the drinking water, show that with longer pre-culture of samples, the NFBGP test was still applicable (data not shown). However, the addition of too much nitrogen or phosphorus makes a water sample carbon limiting, resulting in a long propagation period of the nitrogen fixing bacterium in the NFBGP test.

2.4.3 Applied to artificial sewage water

To test the feasibility of applying the NFBGP test on industrial sewage samples, studies on simulated water samples with different concentrations of phenol and Cu^{2+} were performed. Results showed that *Azorhizobium* sp. WS6 was significantly inhibited (biomass was 90% lower than the control) at 500 mg/L phenol and 100 mg/L Cu^{2+}, suggesting that the test is not applicable to severely poisonous industrial sewage, but it is still useful for the remediation of slightly poisonous sewage. When the phenol decomposing bacterium *Pseudomonas* sp. was inoculated into the phenol polluted pond water (about 100 mg/L phenol), and pre-cultivated at 30℃ for 7 d, the NFBGP test results showed that the pond water was phosphorus limiting (Table 3). To remedy the polluted water more effectively, phosphorus must be added into such a water sample.

Assessment of nutrient availability or nutrient limitation is an important work in environmental

Table 3 NFBGP test on *Pseudomonas* sp. in pond water with phenol (10^5 cells/ml)

Culture time, h	0	12	24	72
NFB	0.01	0.45±0.12	1.22±0.15	2.02±0.18
NFB+0.15 mg/L P	0.01	1.13±0.17	3.16±0.54	6.09±0.22
NFB+2.5 mg/L N	0.01	0.72±0.38	1.67±0.36	2.70±0.32

Note: The same as Table 1

microbiology (Kontas *et al.*, 2004). However, specialists in different fields focus on different target microorganisms. For example, a limnologist is interested in the algal growth potential so as to forecast a water bloom correctly, while a sewage engineer often concentrates on the microorganisms having the ability of de-nitrification or de-phosphorus so that nutrient salts can be removed effectively, and an ecologist is frequently attracted by the microorganisms with bio-remedying function so that pollutants can be degraded successfully *in situ* (Liu *et al.*, 2004). However, in a natural aquatic ecosystem, there are various kinds of microorganisms that are able to change the existing forms of nitrogen or phosphorus (Jaspers *et al.*, 2001), making it difficult to accurately separate the bio-available nutrients from bio-unavailable ones. In other words, an incorrect conclusion may result from inoculating only one microorganism using traditional methods. Pre-culturing of autochthonous (or target) microorganisms to their maximum growth will make it possible to assess nutrient limitation with only one pure inoculum. With the propagation of autochthonous microorganisms, one nutrient is depleted and the microorganisms stop to grow. In the case where nitrogen is the limiting factor, nitrogen fixing bacteria can propagate by fixing the nitrogen in the air, while in the case where phosphorus is the limiting factor, the inoculated bacteria will not propagate further after phosphorus depletion. The NFBGP test developed in this study is based on such a principle of pre-culturing. For the lake and drinking water samples, autochthonous microorganisms were used, while for the sewage and bioremediation samples, corresponding repairing microbes were inoculated. After their maximum growth, the pre-culture was filtrated, and a certain kind of nitrogen fixing bacterium was inoculated into the filtrates. From its growth curve, the limiting nutrient can be easily deduced.

The NFBGP test can be applied directly to almost all water samples (drinking water, lake water and municipal sewage) since most aquatic ecosystems are pH neutral or near neutral. However, pretreatment by adjusting pH to 7.0 would make the results more accurate. The method is still applicable for industrial sewage samples, as long as they are not poisonous, and for the ocean water, if a salt-resisting nitrogen fixing bacterium is used.

3 Conclusions

Founding a universal method for monitoring nutrient limitation is a long-desired dream. The NFBGP test will make it reality. To the lake water samples and drinking water samples, autochthonous microorganisms were used. To the sewage samples and bioremediation samples, corresponding repairing microorganisms were inoculated. After the maximum growth, samples were filtrated, and nitrogen fixing bacterium was inoculated into the filtrates. From its growth curve, the limiting nutrient can be easily deduced.

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