A New Sensitive Bioassay for Determination of Microbially Available Phosphorus in Water

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The content of assimilable organic carbon has been proposed to control the growth of microbes in drinking water. However, recent results have shown that there are regions where it is predominantly phosphorus which determines the extent of microbial growth in drinking waters. Even a very low concentration of phosphorus (below 1 μg of P liter⁻¹) can promote extensive microbial growth. We present here a new sensitive method to determine microbially available phosphorus concentrations in water down to 0.08 μg of P liter⁻¹. The method is a bioassay in which the analysis of phosphorus in a water sample is based on maximum growth of *Pseudomonas fluorescens* P17 when the energy supply and inorganic nutrients, with the exception of phosphorus, do not limit bacterial growth. Maximum growth (CFU) in the water sample is related to the concentration of phosphorus with the factor $373,200 \pm 9,400$ CFU/ μg of $\text{PO}_4\text{-P}$. A linear relationship was found between cell growth and phosphorus concentration between 0.05 to 10 μg of $\text{PO}_4\text{-P}$ liter⁻¹. The content of microbially available phosphorus in Finnish drinking waters varied from 0.1 to 10.2 μg of P liter⁻¹ (median, 0.60 μg of P liter⁻¹).

The growth of heterotrophic microbes is a source of hygienic and aesthetic problems in drinking water distribution systems. Microbes can be inactivated in the water plant by disinfection, but microbial growth begins when the residual content of disinfectants, often chlorine, disappears in drinking water distribution systems (7). Disinfection with high doses of chlorine is undesirable, since it can lead to formation of mutagenic chlorinated by-products (1, 9, 18, 22). High doses of chlorine can also cause taste and odor problems in the drinking water. Therefore, microbial growth should be limited by eliminating their growth factors (7). The amount of organic carbon, especially the content of assimilable organic carbon (AOC), has been considered to be the main chemical factor controlling microbial growth in drinking water distribution systems (8, 21).

The content of assimilable organic carbon does not always correlate with the extent of heterotrophic microbial growth in distribution networks (3, 13). There are regions where microbial growth in drinking waters is regulated by the content of phosphorus, rather than organic carbon (11, 12, 19). For example, drinking waters in Finland contain high contents of organic carbon (20, 23) and assimilable organic carbon (AOC) (13). In these drinking waters, microbial growth is typically limited by phosphorus; similar results were also obtained in Japan (19). Therefore, even a very minor change in the phosphorus concentration can have a major influence on the growth of microbes (12). Phosphorus concentrations in Finnish drinking waters are generally low, being less than 2 μg of total P liter⁻¹, but are still high enough to allow remarkable microbial growth (12).

There are sensitive chemical methods for analyzing total phosphorus or phosphate. By the automatic ascorbic acid method, $\text{PO}_4\text{-P}$ can be analyzed down to 1 μg liter⁻¹ (4). With capillary electrophoresis detection, the limit for phosphate is 10

μg of $\text{PO}_4\text{-P}$ liter⁻¹ in clean lake water (16). The most sensitive chemical method, the magnesium-induced coprecipitation procedure has sensitivity down to 31 ng of P liter⁻¹ (6). However, all of the chemical methods, even sensitive ones, are unable to show the fractions of phosphorus supporting microbial growth. For limnic ecosystems, there are methods for analyzing the fraction of phosphorus available for algal growth (2). In this paper, we present a new method for determination of submicrogram concentrations of microbially available phosphorus (MAP) in water. This bacterial bioassay was used to analyze the concentrations of MAP in several drinking waters in Finland.

MATERIALS AND METHODS

Glassware. All pieces of glassware (Pasteur pipettes, tubes, Erlenmeyer flasks with glass stoppers) and plastic pipette tips were first washed with phosphate-free detergent (Deconex; Borer Chemie AG, Zuchwil, Switzerland), immersed in 2% HCl solution for 2 h, and then rinsed with deionized water (Millipore, Molsheim, France). Finally, clean glassware was heated for 8 h at 250°C.

Inocula. *Pseudomonas fluorescens* P17, biotype 7.2 (ATCC 49642), was used in the bioassay. Strain P17 has phosphatase activity. This was tested by a fluorometric method with 4-methylumbelliferylphosphate- Na_2 salt (Fluka) as a substrate according to the method of Miettinen et al. (10). Bacteria were cultured and stored in spring water (Fons; Valio, Ltd., Kouvolaa, Finland). This spring water contains 38.6 mg of Ca liter⁻¹, 4.0 mg of Mg liter⁻¹, and 1.8 mg of K liter⁻¹. An organic carbon source, CH_3COONa , was added to attain a final concentration of 1,000 μg of C liter⁻¹. The cultures were stored at +4°C.

Standardization. Phosphorus standards were made in acid-washed Erlenmeyer flasks in deionized water (Millipore). Six milliliters of inorganic nutrient solution was added to the 94-ml water sample. Nutrients were added to ensure that only the phosphorus of the inorganic nutrients is limiting for growth. Addition of inorganic salts to the deionized water ensured the same electric conductivity level (ca. 100 $\mu\text{S}/\text{cm}$) as in drinking water in general. The salt addition prevents osmotic shock and a possible destruction of bacterial cells in standard water. The salt solution consisted of $(\text{NH}_4)_2\text{NO}_3$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, KCl, and NaCl (Merck). After addition of salt solution, the standard water had 15 mg of N liter⁻¹, 0.6 mg of Mg liter⁻¹, 1.6 mg of Ca liter⁻¹, 3.2 mg of K liter⁻¹, 2.4 mg of Na liter⁻¹, and 8.9 mg of Cl liter⁻¹. In order to ensure that standard water is not carbon limited, CH_3COONa was added to reach final concentration of 2,000 μg of C liter⁻¹.

Standardization was made with addition of different amounts of phosphorus (Na_2HPO_4 ; Merck) into the standard (described above) water. The concentrations of phosphorus ranged from 0.05 to 10 μg of $\text{PO}_4\text{-P}$ liter⁻¹ (see Fig. 2). Standardization was repeated with four standard series with different phosphorus

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TABLE 1. Water treatment techniques and concentrations of microbially available phosphorus in drinking waters of 10 different waterworks ($n = 1$)^a

Water-works	Raw water	Chemical treatment	Disinfection	MAP concn ($\mu\text{g liter}^{-1}$)
A	GW	pH	No disinfection	1.47
B	GW	pH	Hypochlorite	2.35
C	GW	—	No disinfection	10.2
D	ARG	SSF, pH	UV radiation	1.24
E	ARG	SSF, pH	No disinfection	0.10
F	ARG	SSF, pH	No disinfection	0.62
G	ARG	CC, FL, RSF, pH	Hypochlorite	0.30
H	SW	CC, RSF, O ₃ , pH	Chloramine	0.20
I	SW	CC, FL, RSF, O ₃ , GAC, pH	UV radiation + chloramine	0.58
J	SW	CC, FL, RSF, pH	Hypochlorite	0.10

^a GW, groundwater; ARG, artificial recharged groundwater; SW, surface water; CC, chemical coagulation; FL, flotation; SSF, slow sand filtration; RSF, rapid sand filtration; GAC, granular activated carbon filtration; O₃, ozonation; pH, pH adjustments; —, no treatment.

concentrations. Every standard set contained three to six different concentrations of phosphorus and a blank sample.

After addition of nutrients and carbon, standards were pasteurized at 60°C in a water bath for 35 min. After cooling, the samples were inoculated with *P. fluorescens* P17. The cell density of *P. fluorescens* P17 in inoculated samples was approximately 1,000 CFU/ml. Inoculated water samples were incubated at 15°C to obtain the maximum cell numbers at the PO₄-P concentrations tested. The bacterial cells in water were enumerated daily by spread plating on R2A agar (17). The plates were incubated at room temperature ($22 \pm 2^\circ\text{C}$) for 3 days before enumeration.

A linear regression model was calculated by using the maximum growth of *P. fluorescens* (plate counts in CFU per milliliter) at different phosphorus concentrations. The model was calculated by using combined results of four experiments. Calculations and drawings of figures were done with the Microcal Origin 4.1 program.

Analyses for MAP. Water samples were taken into acid-washed glass flasks and kept at +4°C before analysis. The analysis was started within 24 h after sampling. Residual chlorine in water samples (100 ml) was removed with the addition of 50 μl of 0.02 M sodium thiosulfate. Inorganic salts and organic carbon (see the standardization procedure described above) were added to the samples to ensure that inorganic nutrients (except phosphorus) or organic carbon did not restrict microbial growth. The final concentrations of added nutrients in samples were 250 μg of N liter⁻¹, 53 μg of K liter⁻¹, 10 μg of Mg liter⁻¹, 27 μg of Ca liter⁻¹, 40 μg of Na liter⁻¹, and 149 μg of Cl liter⁻¹. Sodium acetate was added as described above. After addition of nutrients and thiosulfate, samples were pasteurized and inoculated with *P. fluorescens* P17.

Water samples were incubated at 15°C. The growth of bacteria in water samples was enumerated after 4, 5, 6, 7, and 8 days from the inoculation by spread plating on R2A agar (17). Spread-plated R2A agar was incubated for 3 days at room temperature ($22 \pm 2^\circ\text{C}$) before enumeration. The maximum plate counts of *P. fluorescens* P17 were transformed with a conversion factor (from standardization) into the amount of phosphorus.

Water samples. Drinking water samples were collected from three groundwater works, four artificial groundwater works, and three surface waterworks in Finland (Table 1). Water samples were analyzed for MAP.

RESULTS

Standardization. Maximum cell count was generally reached after an incubation time of 4 to 6 days (Fig. 1).

There was a linear relationship between maximum cell count of *P. fluorescens* P17 and phosphorus concentration in the range 0.05 to 10 μg of PO₄-P liter⁻¹ (Fig. 2). Above 10 μg of PO₄-P liter⁻¹ there was no longer any linear relationship between the phosphorus concentration and bacterial growth (data not shown). Microbial counts in blank water samples varied from 2.8×10^4 to 7×10^4 CFU/ml. The arithmetical mean of microbial counts in blank water was 4.7×10^4 CFU/ml. The detection limit of the bioassay was 0.08 μg of PO₄-P liter⁻¹ (Fig. 2) and was calculated by the mathematical technique of Hubaux and Vos (5).

The yield factor was derived from the slope of the line when

FIG. 1. Growth of *P. fluorescens* P17 with different phosphorus concentrations (<0.05 to $2 \mu\text{g}$ of PO₄-P liter⁻¹) in one standardization test. The maximum cell counts used for the determination of the conversion factor are shown by arrows.

cell growth was plotted against PO₄-P concentration. The yield factor was $373,200 \pm 9,400$ CFU/ μg of PO₄-P.

MAP in Finnish drinking waters. The MAP analysis gave a positive result in every drinking water sample studied. The concentration of MAP varied from 0.1 to 10.2 μg PO₄-P liter⁻¹ (Table 1) and was highest in drinking water produced in groundwaterworks. In drinking waters purified from surface waters, or produced in artificial recharge plants with slow sand or bank filtration, the concentration of MAP was invariably below 1 μg liter⁻¹ (Table 1).

DISCUSSION

It is often necessary to analyze microbially available phosphorus, since extremely small changes in the concentration of phosphorus can have profound effects on microbial growth. With phosphorus standardization, 1 μg of PO₄-P corresponded

FIG. 2. Relationship between maximum colony counts of *P. fluorescens* P17 and concentration of phosphorus. The regression lines and confidence limits are based on four separate standardization experiments. The inset figure shows regression line and confidence limits with phosphorus concentrations from 0 to 0.5 μg liter⁻¹; X_D is the detection limit (7). The regression equation there is $y = 378,800 (\pm 35,500)x + 53,800 (\pm 7,700)$; $n = 14$. The regression equation with all phosphorus concentrations is $y = 373,200 (\pm 9,400)x - 10,000 (\pm 31,700)$; $n = 25$. x is measured in micrograms of P per liter, and y is measured in CFU per milliliter. Standard errors are in parentheses.

to 3.73×10^8 CFU of *P. fluorescens*, while 1 µg of AOC (acetate) results in 2.04×10^6 CFU of *P. fluorescens* (14). Thus 1 µg of phosphorus accounts for 180 times higher cell counts than 1 µg of acetate carbon. This is also the basis for the high sensitivity of the present MAP bioassay. Our results showed that the bioassay can be applied for assaying very low phosphorus concentrations. There was a linear response from 0.05 up to 10 µg of $\text{PO}_4\text{-P}$ liter⁻¹. This range was adequate, because in drinking waters, the maximum microbial growth occurs in the range of 5 to 10 µg of $\text{PO}_4\text{-P}$ liter⁻¹ (12, 19).

There are also some sensitive chemical methods for analyzing very low concentrations of phosphorus in waters (4, 6), but the chemical analyses do not represent the amount of phosphorus available for microbial growth. The present MAP analysis is sensitive enough to also show low concentrations of phosphorus supporting microbial growth. The standardization of the MAP analyses is based on inorganic phosphate. Because *P. fluorescens* P17 has phosphatase activity, the MAP analyzed from water samples also includes organic phosphorus compounds. Presently, we do not know the ratio of microbially available inorganic phosphorus to microbially available organic phosphorus in drinking water.

There already exist water purification techniques that effectively can remove phosphorus from water. The generally used method of chemical coagulation with polyaluminum chloride effectively removes phosphorus (15). Our study substantiates these results; the MAP concentration was low in the chemically treated waters. However, artificially recharged groundwaters also contained low levels of MAP, suggesting that biological filtration can also remove phosphorus. Surprisingly, untreated groundwater contained the highest levels of MAP. This might increase the risk for microbial regrowth in distribution systems using untreated groundwater, especially if drinking water contains a high concentration of organic carbon and is not initially disinfected. Drinking water produced from groundwater is usually not disinfected in Finland. Therefore, it might be necessary to reduce the phosphorus concentration in plants distributing groundwater.

This novel bioassay offers a useful tool when the efficiency of the present drinking water purification technology is being evaluated or new technologies for drinking water processes are being developed in order to improve the chemical and microbiological quality of drinking water.

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