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Effect of Phosphorus on Survival of *Escherichia coli* in Drinking Water Biofilms[▽]

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The effect of phosphorus addition on survival of *Escherichia coli* in an experimental drinking water distribution system was investigated. Higher phosphorus concentrations prolonged the survival of culturable *E. coli* in water and biofilms. Although phosphorus addition did not affect viable but not culturable (VBNC) *E. coli* in biofilms, these structures could act as a reservoir of VBNC forms of *E. coli* in drinking water distribution systems.

Pathogens may enter the distribution system either through the source water or at any point within the distribution system (16). In the network enteric microorganisms, such as *Escherichia coli*, may survive and even exhibit metabolic activity in biofilms on the surfaces of pipes and reservoirs (22, 5). This phenomenon compromises the use of *E. coli* as a reliable indicator for fecal pollution. Due to its very low infection dose the accumulation and subsequent release of pathogenic *E. coli* from a biofilm to the water phase are increasing the health risk of tap water consumption. The survival and culturability of *E. coli* in water distribution networks are dependent on many environmental factors, including the disinfectant type and dose (14), the presence of predators (20), the pipe material, the temperature (21), the amount of corrosion products (4), the iron (1), heavy metal, and oxygen concentrations (18), and the water saturation (7). However, the role of nutrients, which in drinking water are normally present at low concentrations, in the survival of *E. coli* is not fully understood. Phosphorus (P) is an important nutrient and part of biomolecules in bacterial cells (e.g., DNA, polyphosphates, phospholipids, and ATP). In some drinking waters P regulates bacterial growth (12); thus, removal of this nutrient during water treatment (e.g., during chemical coagulation) may lower the bacterial numbers in the water and biofilms (9). P may also influence many mechanisms of *E. coli* survival, including transport of nutrients into the cell, biofilm formation, and motility. At concentrations below 5 μg liter^{-1} the mechanisms of nutrient uptake and energy conservation in *E. coli* change (3). Thus, reducing the P concentration below this level may decrease the potential for *E. coli* survival in drinking water distribution systems. However, the same effect may be obtained by increasing the levels of P (the growth-limiting nutrient) because this may enhance antagonism reactions by the faster-growing indigenous microbial population (2). The aim of this study was to evaluate the effect of P on survival of *E. coli* in drinking water distribution networks. Two forms of *E. coli*, culturable and viable but not culturable (VBNC), were investigated.

A model biofilm reactor (Fig. 1), a Propella reactor (Xenard, Mechanique de Precision, Seichamps, France) with a distribution pipe that was 100 mm in diameter and 500 mm long, was used to simulate a drinking water distribution system. The inner surface of the pipe was made of high-density polyethylene. The reactor had a volume of 2.23 liters and a high-density polyethylene pipe surface area of 1,604 cm^2. It was continuously supplied with tap water from a local drinking water network (surface water after chemical coagulation, followed by biofiltration) at a flow rate of 186 ml h^{-1}. The water velocity, 0.2 m s^{-1}, was controlled with a marine propeller, which

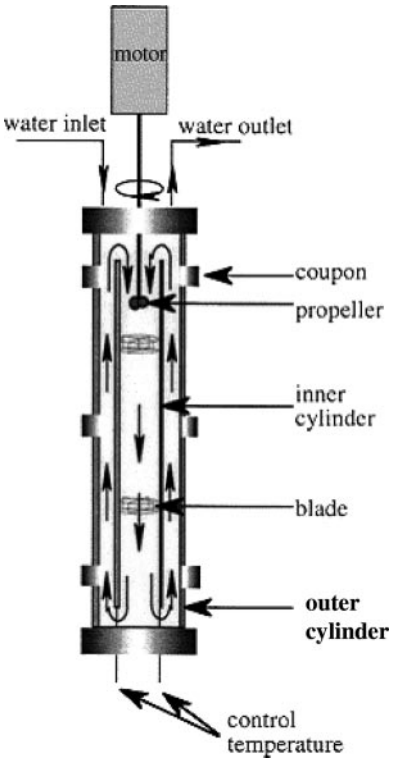

FIG. 1. Cross-sectional view of the Propella reactor.

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TABLE 1. Chemical and biological characteristics of inlet and outlet water and biofilms in Propella reactors before introduction of *E. coli*

Position	Parameter (units)	Reactor(s)	Mean	SD	No. of determinations
Inlet	Dissolved O_2 concn (mg liter^{-1})	A and B	22.8	2.9	20
	UV absorbance at 254 nm		0.045	0.007	20
	NH_4 concn (mg liter^{-1})		0.07[a]	0.02	18
	NO_3 concn (mg liter^{-1})		2.0	0.3	10
	Conductivity (μs cm^{-1})		295	98	10
	Total organic carbon concn (mg liter^{-1})		5.00	1.43	18
	Total chlorine concn (mg liter^{-1})		<0.1		1
	ATP concn (relative light units)		210	40	3
	pH		7.3	0	3
	Heterophilic plate count on day 7 (CFU ml^{-1})		4,502	8,759	2
	Temp (°C)	A	15.5	0.8	120
		B	14.8	0.9	120
	P_{total} (mg liter^{-1})	A	<0.01	0	6
		B	0.031	0.005	6
	Microbially available phosphorus concn (μg liter^{-1})	A	0.86	0.3	4
		B	11.15		1
Outlet	ATP concn (relative light units)	A	2,342	344	3
		B	2,707	1,520	3
Biofilm	Total bacterial no. (cells cm^{-1})	A	1.4E+7	6.9E+6	3
		B	1.1E+7	6.8E+6	3
	ATP concn (relative light units)	A	1,614	448	3
		B	2,356	195	3

[a] Value below detection level.

pushed the water through an inner pipe, providing a flux parallel to the pipe wall. The temperature was maintained around 15°C (Table 1). The biofilm on the pipe wall was studied using 15 stainless steel coupons (1.7 cm^2) which were inserted into the inner surface of the pipe. *E. coli* ATCC 25922 was subcultured overnight on R2A medium (Lab M, International Diagnostics Group, plc, United Kingdom) at 36°C. A bacterial suspension was prepared in sterile phosphate-buffered saline (130 mM NaCl, 7 mM N_2HPO_4, 3 mM NaH_2PO_4; pH 7.2) and centrifuged (3,000 rpm; Nüvefuge CN 090; Nüve, Ankara, Turkey) for 10 min at 20°C. The pellet was washed twice in phosphate-buffered saline to limit carbon and phosphorus contamination from the culture medium and then starved by incubation in sterile drinking water for 12 h at 20°C. The number of *E. coli* cells in suspension was determined using an epifluorescence microscope (Leica DM LB; Leica Microsystems GmbH, Wetzlar, Germany) equipped with a 50-W mercury lamp at a magnification of ×1,000 after staining with DAPI (4′,6′-diamidino-2-phenylindole). The investigation was performed in duplicate with two identical Propella reactors (reactors A and B) at a temperature of 15°C. Before the experiment we ensured that bacterial growth was reproducible in both reactors and that the reactors were not releasing bacterial nutrients. The systems were not modified during the experiment. The natural microbial flora that was present in the drinking water colonized the inner surface and contributed to the formation of a biofilm. Weekly, water was obtained from the inlets and outlets of the reactors and biofilm coupons were sampled to monitor the biofilm formation in the reactors and to control biofilm development. In reactor B, H_3PO_4 was continuously added to maintain the P concentration at about 20 μg liter^{-1} during the entire experiment. After 2 weeks, the two experimental systems were colonized with bacteria at similar concentrations (Table 1). Then 10 ml of an *E. coli* suspension was added to each reactor over a period of 2 h to obtain final concentrations of 3×10^7 and 4×10^7 cells cm^{-2} (total *E. coli*) or 2×10^7 and 3×10^7 cells cm^{-2} (culturable *E. coli*) in the water and biofilm, respectively. The concentration of inoculated culturable cells was determined by multiplying the total number of *E. coli* cells by the experimentally obtained Colisure/DAPI ratio for overnight cultured and washed *E. coli* cells. The two reactor systems were analyzed 24, 48, 96, 144, 240, and 408 h after inoculation. At each time, outlet water samples (12 ml) and three coupons were collected. For the biofilm analysis, stainless steel coupons were aseptically re-

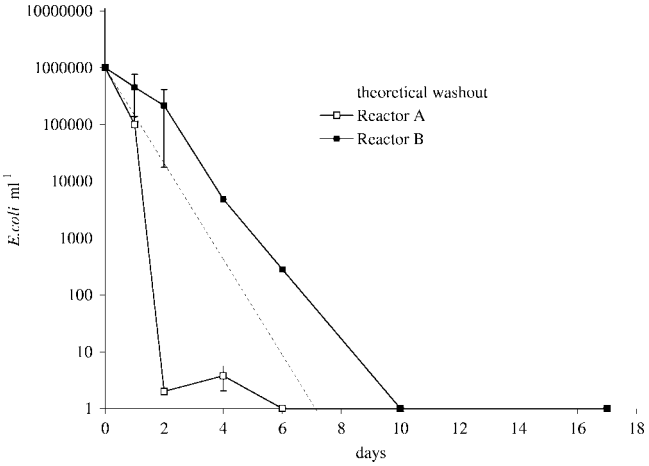

FIG. 2. *E. coli* bacteria in outlet water of Propella reactors A and B (with 20 μg liter^{-1} phosphorus added). *E. coli* numbers were determined with the Colisure method.

moved from the sampling devices and put in 25 ml of sterile ultrapure water (Elga PureLab Ultra; Veolia Water Ltd., United Kingdom). Adherent cells were removed by gentle sonication (ColeParmer) for 2 min at 20 μA and 22 KHz. Heterotrophic plate counts in water and biofilm samples were estimated by the spread plate method (17), where samples were spread on R2A agar plates and incubated for 7 days at 22 ± 2°C before the CFU were counted. The total bacterial number was determined using epifluorescence microscopy and DAPI staining. At least 300 cells were counted, showing that the coefficient of variation of the bacterial number between the counted fields was less than 30%. The Image Pro Plus 4.5.1 software (Media Cybernetic, Inc., Silver Spring, MD) was used for image processing. The ATP concentration was determined with a luciferin-luciferase assay (Pi-102 luminometer; Hygiena International Limited). The number of metabolically active *E. coli* cells was determined by direct viable counting (DVC) (8) in combination with fluorescence in situ hybridization (FISH). The biofilm suspension was resuscitated in 0.5× R2A medium containing 10 μg ml^{-1} pipemidic acid {8-ethyl-5,8-dihydro-5-oxo-2-(1-piperazinyl)-pyrido[2,3-*d*]pyrimidine-6-carboxylic acid} for 8 h at 20°C. The antibiotic stopped cell proliferation, and in the presence of nutrients metabolically active cells became elongated. Then 30% formamide was added at a final concentration of 3 to 4%. Cells were concentrated on 25-mm-diameter 0.2-μm-pore-size filters (Anodisc; Whatman plc.), followed by FISH with the *E. coli* 16 rRNA-specific peptide nucleotide acid probe 5′ TCA ATG AGC AAA GGT 3′ (15) labeled with the cyanine dye Cy3 (excitation wavelength, 550 nm; emission wavelength, 570 nm) and flanked with solubility enhancers (Applied Biosystems). Samples were covered with a hybridization buffer (50 mM Tris-HCl buffer [pH 7.5], 50% dextran sulfate, 10% [wt/vol] 0.1 mM NaCl, 30% [vol/vol] formamide, 30% [vol/vol] tetrasodium pyrophosphate, 0.2% [wt/vol] polyvinylpyrrolidone, 0.2% [wt/vol] Ficoll 400, 5 mM Na_2EDTA, 0.1% [vol/vol] Triton X-100) containing 200 nM probe and incubated for 90 min at 57°C, followed by incubation in washing buffer (5 mM Tris, 15 mM NaCl, 1% Triton X-100; pH 10) for 30 min at 57°C. Hybridized cells whose size had increased at least 1.5-fold were assumed to be metabolically active and were counted with an epifluorescence microscope. The bacterial numbers during DVC incubation increased approximately 10-fold. In parallel, culturable *E. coli* cells in outlet water (washout) and in sonicated biofilm samples were quantified using the Colisure method (IDEXX Laboratories, Inc., United States) (6). At least three dilutions were prepared for each sample to obtain the optimal bacterial number for analyses. Unless stated otherwise, general water quality parameters were determined with standard methods. The concentration of potentially assimilable organic carbon was determined as described by Miettinen et al. (13). The concentration of microbially available phosphorus, the fraction of total P which supports bacterial growth, was determined with a bioassay developed by Lehtola et al. (10).

The results showed that the chemical and bacteriological characteristics of water (total organic carbon concentrations, pH, total chlorine concentration, and bacterial counts) in both reactors were relatively stable during the entire investigation, and the only significant difference was a difference in P concentration. The microbially available phosphorus concentration was less than 1 μg $liter^{-1}$ in reactor A (with no P added) and was about 10 times higher in reactor B (with P added). According to ATP and total bacterial count measurements (Table 1), addition of P did not significantly increase the bacterial numbers in biofilms. The possible reason for this is explained elsewhere (19). The concentration of culturable *E. coli* (determined with the Colisure method) in the outlet water from reactor A decreased rapidly after inoculation, and after 6 days no culturable *E. coli* was detected in the outflow from the reactor (Fig. 2). Addition of P in reactor B increased the number of culturable *E. coli* cells in water at the outlet, and no culturable cells were detected after 10 days. The total number of *E. coli* cells (determined with the DVC-FISH procedure) (Fig. 3) in the biofilms of both reactors did not change significantly during the entire experiment (Fig. 4), whereas the concentration of culturable *E. coli* in the biofilms decreased rapidly. Addition of P in the reactor prolonged the survival of culturable *E. coli* cells in biofilms by extending the period of bacterial complete washout from 4 to more than 10 days.

In summary, this study showed that a higher concentration of P in water increased the cultivability of *E. coli* in biofilms of water distribution systems. It is known that P is effectively removed during conventional water treatment processes by the chemical coagulation method. Therefore, it is more likely that water supply systems which use this water treatment technology provide less favorable conditions for the survival of *E. coli* and perhaps also for

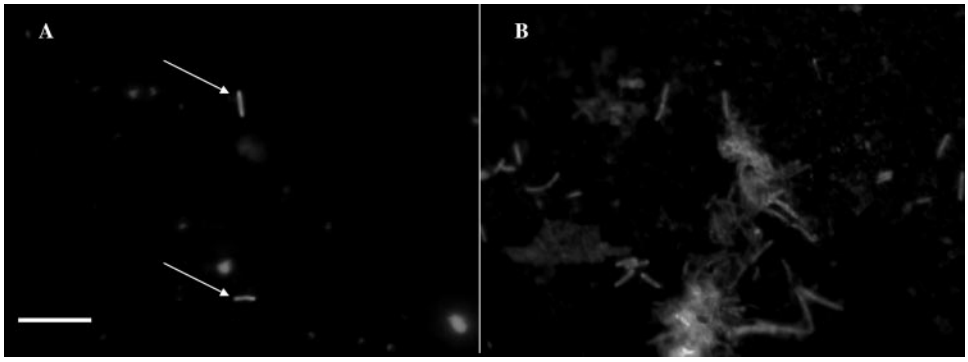

FIG. 3. (A) Metabolically active *E. coli* (arrows) as determined with DVC-FISH. (B) All bacteria as determined with DAPI (after the DVC-FISH procedure) in a sonicated biofilm sample. Bar = 10 μm.

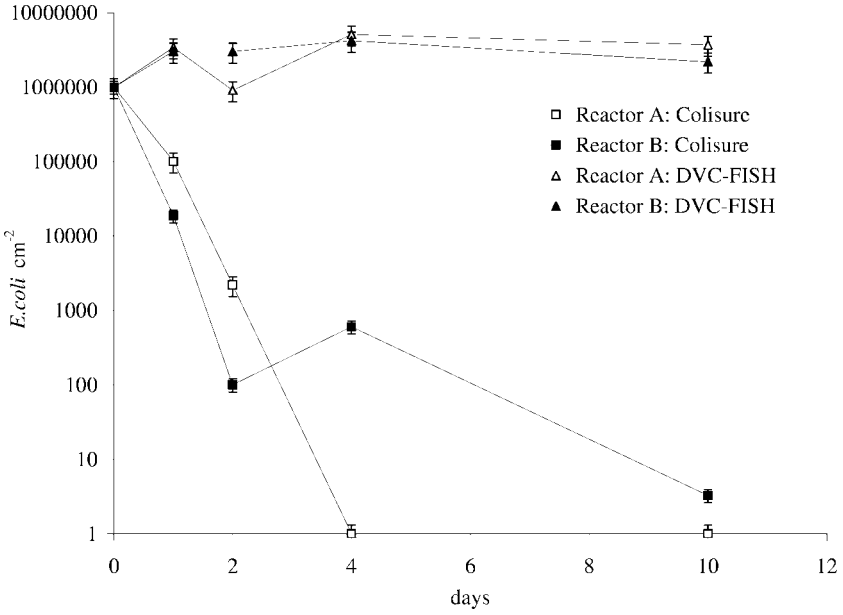

FIG. 4. *E. coli* in the biofilms of Propella reactors A and B (with 20 μg liter^{-1} phosphorus added). *E. coli* numbers were determined with the Colisure method and the DVC-FISH method.

the occurrence of coliforms. It is also known that an important reason for the occurrence of coliforms in a water supply is the high concentration of iron which has been released from corroded cast iron pipes (1). Our findings suggest that the use of phosphate-based corrosion inhibition should be critically evaluated as it may introduce P into the water, thus creating more favorable conditions for the survival of enteric bacteria. This study also showed that a biofilm serves as a reservoir for *E. coli*, where it remains in the VBNC form. Although the bacteria are no longer capable of growing on conventional bacteriological media, they can conserve pathogenic factors and genes (11); thus, we suggest that more attention should be paid to analyses of VBNC forms of *E. coli* in water distribution networks.

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