MICROBIAL ECOLOGY

Microb Ecol (1999) 38:201–214
DOI: 10.1007/s002489900172

Localization and Identification of Populations of Phosphatase-Active Bacterial Cells Associated with Activated Sludge Flocs

F. Van Ommen Kloeke, G.G. Geesey

Department of Microbiology and Center for Biofilm Engineering, Montana State University, Bozeman, MT 59717-3980, USA

Received: 17 March 1999; Accepted: 9 June 1999

Abstract

The majority of phosphatase (PO_4ase) activity detected in fresh aerobic activated sludge from a municipal wastewater treatment plant was associated with suspended floc material. PO_4ase activity appeared to be localized in discrete bacteria-containing areas of the floc matrix based on the distribution of nucleic acid–stained cells and precipitated fluorescent crystals produced as a result of reaction of the enzyme(s) with the artificial substrate ELF™-PO_4. Of the total floc-associated bacterial cells that stained positive with the nucleic acid–binding fluorochrome acridine orange (AO), 8.8 ± 1.2% displayed PO_4ase activity based on the proximity of AO-stained cells to precipitated ELF crystals. Using a 16S rRNA oligonucleotide probe specific for the cytophaga–flavobacteria group, it was determined that 17–20% of the floc-associated bacteria that probed positive also displayed PO_4ase activity. Furthermore, 35–45% of the ELF fluorescence was associated with bacterial cells that probed positive for the cytophaga–flavobacteria group. The results suggest that the cytophaga–flavobacteria, as a group, is important in mediating the liberation of inorganic orthophosphate (P_i) from phosphomonoesters of detrital organic phosphate (organic-P) in the aerobic activated sludge process of wastewater treatment.

Introduction

Phosphorus (P) removal from various types of wastewater has become an important consideration in wastewater treatment. Whereas a conventional activated sludge process may reduce the P content of the effluent to 60–66% of that present in the influent, enhanced biological P removal processes can achieve a 90–95% P reduction [42]. Certain key steps in the biochemical pathway of enhanced biological P removal are now known, but the microorganisms responsible for the process have not yet been identified [43]. Furthermore, many of the basic biochemical mechanisms of biological P removal have not yet been elucidated [9].

Activated sludge P exists in a variety of forms. Fuhs and Chen [16] determined that 31% of the total P existed as

Correspondence to: G.G. Geesey; E-mail: gill_g@erc.montana.edu

nucleotide-P, lipid-P, nucleic acid-P, and protein-P. This contribution of organic-P to total P does not include that associated with the trichloroacetic acid–insoluble fraction (additional 13%). Kulaev and Vagabov [25] described the acid insoluble fraction as poly-P associated with the cell surface, whereas Rao et al. [34] indicated that this fraction contains organic-P in the form of RNA and protein. These various forms of organic-P are distributed between living microbial biomass and nonliving detritus. Inorganic-P as various dissociation products of phosphoric acid or orthophosphate (P_i) and polyphosphate (poly-P_i) contribute 14% and 32–44%, respectively, of the total P [16].

While considerable attention has focused on identification of those populations of bacteria that accumulate poly-P_i, little effort has been directed toward the microbial processing of detrital organic-P or P regeneration. P regeneration has been defined as the hydrolysis of P_i from organic or other complex P compounds, soluble or particulate, in which the hydrolyzed P_i is released outside the cell [3]. Little is known of the processes or the microbial populations that depolymerize high molecular weight detrital organic-P compounds in wastewater, or those that hydrolyze the resulting phosphomonoesters to the organic monomers and P_i: the degradation products of detrital organic-P that are readily taken up and metabolized by bacterial cells. These processes, however, may influence overall P removal from the system.

P_i release from organic matter occurs via different classes of enzymes, depending on the nature of the organic-P molecule. Phosphatase enzymes (phosphomonoesterase or phosphomonoesterhydrolase) (E.C. 1.3.3) hydrolyze the P–O bond of phosphomonoesters [11], whereas nuclease enzymes (phosphodiesterase) hydrolyze the P–O bond of phosphodiesters [23]. Phosphatase (PO_4ase) enzymes are further subdivided into alkaline and acid PO_4ases, depending on the pH for optimum activity. However, the pH ranges over which activity occurs may overlap, particularly at neutral pH. Alkaline PO_4ases are induced at low external P_i concentrations and localized at or near the cell surface, where they hydrolyze phosphomonoesters, including esters of primary and secondary alcohols, sugar alcohols, cyclic alcohols, phenols, and amines, in the surrounding environment to provide an alternative source of P_i for the cell [10, 23]. However, alkaline PO_4ases may also be induced under P_i-sufficient but carbon-limited conditions to meet other metabolic needs of bacterial cells [46]. Acid PO_4ases have pH optima in the 4–6 range, are considered to be active in internal cell metabolism, and are not repressed by P_i [23, 35, 36].

To date, there is only limited information on PO_4ase activity in activated sludge [28] and virtually no knowledge of the types and distribution of microorganisms responsible for this activity. A better understanding of the mechanisms and microbial populations responsible for detrital organic-P regeneration in the activated sludge step of wastewater treatment should offer a more thorough understanding of the enhanced biological P removal process, and possibly lead to even higher removal efficiencies or better control over the process. In this paper we identify the activated sludge fraction responsible for PO_4ase activity, the distribution of enzyme activity within activated sludge flocs, the proportion of floc-associated bacterial cells contributing to the enzyme activity, and the identity and physiological state of a significant portion of the bacteria responsible for the activity.

Materials and Methods

Collection and Processing of Activated Sludge

Activated sludge was collected from the primary aeration tanks of the Bozeman Municipal Wastewater Treatment Facility and immediately transported to the laboratory. The pH was determined, the inorganic phosphate concentration assayed by the method of Penney [32], a subsample stored briefly (<4 h) at 5°C for later determination of PO_4ase activity in whole activated sludge ("as-collected"), and the remaining sample separated into subsamples and phases as described below for subsequent determination of PO_4ase activity (Fig. 1).

Replicate subsamples to be used for determination of abiotic contributions to the fluorescence reaction of the PO_4ase assays (see following sections) were autoclave sterilized for 20 min at 121°C, then cooled to 20°C prior to assaying for PO_4ase activity. Autoclaving irreversibly inactivates the PO_4ase-producing bacteria as well as any PO_4ase enzyme present in the sample, allowing determination of the contribution of fluorescence via nonbiological reactions. Replicate subsamples of "as-collected" (not autoclaved) activated sludge to be used for determination of biotic PO_4ase activity were separated into a solids phase and bulk aqueous phase by centrifugation at 5°C at 27,000 × *g* for 15 min. The supernatant (bulk aqueous phase) was decanted and the pellet (solids phase) gently resuspended to original volume using 100 mM HEPES buffer (pH 7.0). The subsamples and phases of subsamples were then immediately assayed for PO_4ase activity.

PO_4ase Assay (Soluble Product)

The PO_4ase enzyme activity of the activated sludge subsamples and subsample phases was assayed using the soluble, artificial, fluorogenic enzyme substrate methylumbelliferyl phosphate (MUFP)

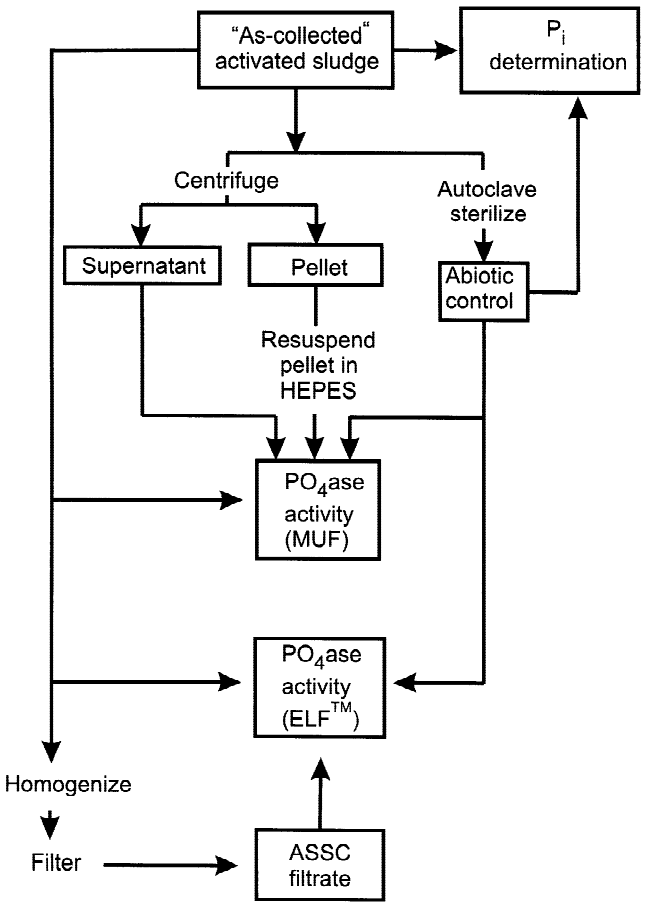

Fig. 1. Flow chart showing processing of activated sludge.

(Sigma Chemical Co., St. Louis, MO). The reaction mixture consisted of 0.05 ml of activated sludge fraction, 0.2 ml of 100 mM HEPES buffer (pH 7.0), 0.74 ml of autoclaved Milli-Q water, and 0.01 ml of a stock solution of MUFP to achieve a final MUFP concentration of 100 μM or 500 μM in the assay mixture. The reaction was carried out in 1.5-ml microcentrifuge tubes. The tubes were incubated for various times at 20°C in the dark before the reaction was stopped by centrifugation at 27,000 × *g* for 5 min at 4°C. The 20°C incubation temperature was the upper temperature limit (14–20°C) measured in the aeration basin over a 1-year period. A 0.8-ml volume of the supernatant was transferred to a clean tube, 0.2 ml of 10 M NaCl/Glycine buffer (pH 9.5) was added to increase fluorescence efficiency, and the samples were stored at −40°C (maximum of 2 weeks) until fluorescence was measured. Storage at this temperature does not affect the level of fluorescence of the ELF after thawing [13].

Immediately prior to measurement of fluorescence, samples were thawed and a 25-μl sample volume introduced into the analysis chamber of a HP 1045A fluorescence detector. The fluorescence intensity of the sample was determined at an excitation wavelength of 360 nm and emission wavelength of 430 nm and converted to MUF concentration using a standard curve relating fluorescence intensity to MUF concentration over a range of 50–1500 nM. Each sample was assayed in triplicate.

PO_4ase Assay (Insoluble Product)

PO_4ase activity associated with autoclave-sterilized and "as-collected" activated sludge floc material, prepared as described above (Fig. 1), was localized using the artificial, fluorogenic substrate ELF™-PO_4 (ELF-P) (Molecular Probes, Eugene, OR). This substrate is converted to a water-insoluble, crystalline, fluorescent product at the site of enzymatic hydrolysis, thus reporting the location of active enzyme when viewed by fluorescence microscopy [27].

A 5-μl volume of 5 mM ELF-P was added to four 1-ml subsamples of freshly collected activated sludge (biotic samples) and to two 1-ml subsamples of freshly collected activated sludge that was subjected to autoclave sterilization (abiotic controls) to achieve a final ELF-P concentration of 25 μM. The subsamples were allowed to incubate in the dark at 20°C for 60 min. Four 0.1-ml volumes of each subsample were then spotted onto four separate microscope slides. Solids were visualized by phase contrast microscopy using an Olympus B-max microscope. The precipitated ELF crystals were visualized by epifluorescence microscopy using an Olympus U-MWU filter (excitation 330–385 nm, 400 nm dichroic mirror, 420 nm barrier filter). ELF crystals produced a green/yellow fluorescence in areas where PO_4ase activity had occurred. Six phase contrast and epifluorescence images were acquired from each slide with a digital, color, Peltier-cooled, charge coupled device (CCCD) camera (Optronics Engineering, Goleta, CA). With increased exposure, crystal fluorescence changes from green to yellow in the captured image. Phase contrast and epifluorescent images of the same field of view were superimposed using Image-Pro Plus, version 3.01, image analysis software (Media Cybernetics, Silver Spring, MD).

Preliminary studies were carried out with an ASSC preparation (see below) in which the density of discrete ELF crystals was followed microscopically over increasing time of ELF-P exposure. A 60-min incubation period was selected on the basis that, although the size of crystals continued to increase beyond the 60-min incubation, the density of crystals achieved a maximum within this period of incubation.

PO_4ase activity was also evaluated in activated sludge floc material that was cut into thin sections. After incubation of duplicate samples of freshly collected activated sludge in the presence of ELF-P as described earlier, the mixture was centrifuged at 1,000 × *g* to pellet the suspended solids fraction, the supernatant was decanted, the pellet was mixed with a small amount of OCT embedding compound (Sakura FineTec U.S.A. Inc., Torrance, CA), and the mixture frozen on dry ice. The frozen mixture was cut into four sections according to the procedure of Yu et al. [48] using a Reichert-Jung Cryocut, Model 1800, Leica cryostat. The four frozen sections of each sample were transferred to glass microscope slides where they were thawed, dried, and then stained with a 0.1% aqueous acridine orange (AO) solution for 10 min to aid in microscopic visualization of the floc material. After staining, the slides were rinsed with deionized water and examined by epifluorescence microscopy using the Olympus U-MWIBA and U-MWU filter sets to visualize AO-reactive material and ELF crystals, respectively. Six images were acquired from each section and superimposed as described above using Image-Pro Plus software.

Detection of PO_4ase-Producing Bacterial Cells in Homogenized Activated Sludge

A sample of freshly collected activated sludge was homogenized at 2,500 rpm for 2 min in a tissue homogenizer with a glass-impregnated Teflon pestle and glass tube mortar to disperse bacterial cells associated with the solids fraction. The homogenate was filtered through a polycarbonate membrane (5.0-μm pore size; Corning, Inc., Acton, MA) to remove large aggregates of material. The filtrate (ASSC) containing individual bacterial cells was then assayed for PO_4ase activity as follows. HEPES buffer was added to the ASSC to achieve a final buffer concentration of 20 mM. Duplicate 1-ml samples of HEPES-buffered ASSC were incubated with ELF™-P at a final concentration of 25 μM for periods ranging from 2 to 120 min at 20°C in the dark. A subsample from each was then filtered onto a blackened polycarbonate membrane (0.45-μm pore size; Corning, Inc., Acton, MA). The bacterial cells retained on the surface of the membrane were stained with a 0.1% aqueous AO solution for 30 s, then rinsed with filter-sterilized, autoclave-sterilized deionized (DI) water to remove weakly bound AO. The filter was air-dried, then mounted on a glass microscope slide, and examined by epifluorescence microscopy using the filter sets described above to distinguish between AO and ELF fluorescence. Separate images of AO-stained bacteria and ELF crystals, collected from the same field of view with the CCCD camera, were digitally superimposed. The fraction of all AO-stained bacterial cells that were associated with ELF crystals in five different fields of view on each membrane was determined using Image-Pro Plus image analysis software.

Detection and Enumeration of Protozoans in Activated Sludge

A subsample of the homogenized activated sludge was stained with an aqueous solution of 4′,6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co., St. Louis, MO) for 20 min (final concentration of DAPI, 10 μg ml^{-1}). The homogenate was serially diluted in sterile deionized water and filtered through a polycarbonate membrane (5.0-μm pore size; Corning, Inc., Acton, MA). The membrane was air dried, and then transferred to a glass microscope slide containing a drop of immersion oil. A drop of immersion oil was placed on the surface of the membrane, and a microscope cover slip placed on top of the membrane. DAPI-stained protozoans trapped on the surface of the filter were visualized by epifluorescence microscopy using an Olympus U-NUA filter combination at a magnification of 400×. Protozoan density was based on manual counts obtained from 87 fields of view on a single membrane through which 8 ml of a 1/100 dilution of a single sample of activated sludge had been filtered.

Assessment of Physiological Status of Bacterial Cells Associated with Flocs

Physiological status of individual bacterial cells associated with activated sludge flocs was determined using the Live/Dead kit (Molecular Probes) using the manufacturer's recommended protocol. The Live/Dead kit utilizes a combination of fluorescent stains that reports the presence/absence of a membrane potential in a bacterial cell [21]. Membrane-permeable SYTO9 is taken up by all cells, regardless of the existence of a membrane potential, and binds intracellular nucleic acids. Propidium iodide (PI) is taken up only by membrane-compromised, inactive (nonviable) cells and binds their DNA. When excited at 470 nm, SYTO9-stained cells fluoresce green with an emission maximum at 535 nm, whereas nonviable, PI-stained cells fluoresce red with an emission maximum around 625 nm upon excitation at 530 nm. When applied together, PI stains only the nonviable cells, allowing SYTO9 to reveal the viable cells in the population. This differential staining technique was carried out on diluted ASSC preparations of a freshly collected sample of activated sludge.

Three μl of staining solution was mixed with duplicate 1-ml volumes of the diluted ASSC preparation from an activated sludge sample and allowed to react with the bacterial cells for 10 min in the dark. The staining solution consisted of equal volumes of a 20 mM PI solution in dimethyl sulfoxide (DMSO) and a 3.34 mM SYTO 9 solution in DMSO. Each stained cell suspension was then filtered through a 0.45-μm pore size polycarbonate membrane. The cells retained on the membrane surface were washed with filter-sterilized, autoclave-sterilized DI water to remove unreacted stain.

To assess the fraction of the total bacterial cell population that stained with PI and SYTO9, the cells collected on each membrane were subsequently stained with 1 ml of an autoclave- and filter-sterilized, 100 mg L^{-1} aqueous solution (deionized water) of DAPI for 20 min. Each membrane was rinsed with filter-sterilized, autoclave-sterilized DI water to remove unreacted DAPI, air dried, and then transferred to a glass microscope slide containing a drop of immersion oil. A drop of immersion oil was also placed on the surface of each membrane, and a microscope cover slip placed on top of the membrane before microscopic examination of the stained bacteria on the membrane surface. PI- and SYTO9-stained cells were visualized by epifluorescence microscopy using Olympus filter sets U-MWIG and U-MWIBA, respectively. DAPI-stained cells were visualized using an Olympus U-NUA filter combination. Images of PI/SYTO9 and DAPI stained cells were captured from six different areas of each membrane and analyzed using the camera and image analysis software described above.

To verify that the differential staining protocol accurately reported viability of bacterial cells associated with the activated sludge flocs, duplicate samples of an ASSC preparation were treated with paraformaldehyde and ethanol to destroy cell membrane potentials prior to staining with SYTO9 and PI. An appropriate dilution of duplicate ASSC preparations in PBS was subjected to one of the following treatments: (1) staining with PI/SYTO9 for 10 min as described above; (2) fixation with freshly prepared 4% paraformaldehyde solution followed by rinsing twice with filter- and autoclave-sterilized DI water, and staining with PI/SYTO9 as above; (3) fixation with 4% paraformaldehyde, rinsing twice with filter-sterilized, autoclave-sterilized DI water, followed by 50% ethanol fixation, and then staining with PI/SYTO9 as above. Each preparation was examined microscopically as described above. PI/SYTO9 images were captured from five different areas of each membrane

with the CCCD camera and processed with the Image-Pro Plus image analysis software described above to determine the efficiency with which PI and SYTO9 reported cell viability.

Association of PO_4ase Activity with SYTO9- and PI-Stained Bacteria Recovered from Activated Sludge Floc

The contribution of PO_4ase activity from viable and nonviable bacteria associated with activated sludge flocs was evaluated by exposing duplicate samples of an ASSC preparation from a single sample of activated sludge to ELF-P prior to staining with PI and SYTO9. The protocols used were the same as those described above for independent localization of PO_4ase activity and assessment of cell viability in ASSC preparations. Separate images of the same field of view were acquired using Olympus filter sets U-MWIG, U-MWIBA, and U-MWU for PI, SYTO9, and ELF fluorescence, respectively, from five different fields of view. Images of PI- and SYTO9-stained cells and ELF crystals from the same field of view were then superimposed using the Image-Pro Plus image analysis software. The distribution of ELF crystals relative to the positions of PI- or SYTO9-stained bacterial cells was then determined.

Semiquantitative assessment of the amount of PO_4ase activity associated with viable and nonviable bacterial cells was achieved by first determining the locations of all the ELF crystals within different fields of view of the membrane surface that displayed a brightness from crystal fluorescence above a threshold value. Then, the area encompassed by the fluorescence was determined for each ELF crystal using the Image-Pro Plus image analysis program software. Finally, images of SYTO9- and PI-stained bacteria and ELF crystals from the corresponding fields of view were superimposed, and the areas of ELF crystal fluorescence containing either SYTO9- or PI-stained bacterial cells identified and integrated over the five fields examined for each replicate sample using the Image-Pro Plus image analysis program software. The fraction of the total area contributed by ELF crystal fluorescence that was represented by the sum of the areas defining discrete crystals that also contained SYTO9- or PI-stained bacterial cells was then determined.

Assessment of Activity of Bacterial Cells Associated with Activated Sludge Flocs

Microautoradiography (MAR) was used to assess activity of bacteria associated with activated sludge flocs. MAR was carried out according to the method of Tabor and Niehof [41]. Duplicate samples of ASSC prepared from a freshly collected sample of activated sludge were incubated in the presence of 7 nM ^{3}H-leucine (specific activity, 1 μCi ml^{-1} in 5 ml total volume) for 4 h. Leucine uptake by the bacteria was stopped by the addition of 2 ml of ice cold 12% trichloroacetic acid (final concentration 5%). The reaction mixture was filtered through a blackened polycarbonate membrane (0.2-μm pore size) and rinsed three times with 5 ml of cold 5% trichloroacetic acid, followed by eight rinses with 5 ml of PBS buffer (pH 7.2) containing 0.13 g L^{-1} of cold carrier leucine. The membrane was air dried, after which the surface containing the bacteria was laid face-down on the surface of a glass microscope slide coated with Kodak NTB2 emulsion and incubated in that position for 3 days at 9°C, in the dark, in the presence of desiccant. After exposure, the emulsion was developed in Kodak D-19 developer and fixed in sodium thiosulfate solution. The cells embedded in the emulsion were stained by immersing the slide in a 5 μg ml^{-1} DAPI solution for 30 min; then the slide was rinsed in a series of citrate buffer solutions at pH 6.6, 5.0, and 4.0 to reduce background fluorescence. The slide was soaked in a 1% glycerol solution and air dried. Then the membrane was peeled away from the slide leaving the cells embedded in the emulsion on the slide ready to view microscopically.

DAPI-stained, emulsion-embedded bacterial cells were visualized by epifluorescence microscopy. Silver grains on the slide were visualized using transmitted light. An epifluorescence and transmitted light image was collected from each of five different fields of view on each slide with the CCCD digital camera and stored electronically. Corresponding fluorescent and transmitted light images were superimposed using the Image-Pro Plus image analysis software in order to determine which bacterial cells gave rise to silver grains. Stained bacterial cells juxtaposed to silver grains indicated active cells, whereas cells distal to silver grains were considered inactive. Nonspecific adsorption of ^{3}H-leucine to the cells and emulsion surface was determined using a sample containing cells killed prior to incubation in the presence of ^{3}H-leucine. Nonradioactive development of silver grains on the emulsion-coated slide was assessed using duplicated samples of activated sludge floc material, which received no ^{3}H-leucine. Background silver grain densities were determined from silver grain counts obtained from five different areas of each slide.

Isolation of PO_4ase-Positive Bacteria from Activated Sludge

PO_4ase-positive clones were isolated from a homogenized sample of activated sludge using the method of Van Ommen Kloeke et al. [44]. Briefly, a sample of ASSC was serially diluted in sterile PBS, and each dilution plated in duplicate on agar medium containing 1/10-strength Luria–Bertani (LB) medium supplemented with 2-(5′-chloro-2′-phosphoryloxphenyl)-4-(3H)-quinazolinone (CPQP), and incubated for 4 days at 20°C. Colonies formed within that period of time that displayed an intense green fluorescence upon illumination with UV light (Chroma-Vue Transilluminator, Model TM-20, UVP, Inc., San Gabriel, CA) were selected for further characterization.

Characterization of PO_4ase-Positive Bacterial Isolates

The DNA was isolated from pure cultures of cells derived from four morphologically distinct fluorescent colonies recovered on the plates above. The region of the 16S rRNA gene corresponding to the intervening sequence between nucleotides 1055 and 1406 of the *Escherichia coli* gene was amplified by the polymerase chain reaction (PCR) using primer set 1056F/1392R. The PCR products were sent to Macromolecular Resources (Colorado State University, Fort Collins, CO) for sequencing. The resulting sequences were com-

pared with other known sequences in the Ribosomal Database Project using Similarity Rank version 2.5 to obtain similarity rankings [29].

Contribution of the Cytophaga–Flavobacteria Group to the Activated Sludge Bacterial Community

The contribution of members of the cytophaga–flavobacteria group to the activated sludge bacterial community was determined by fluorescent *in situ* hybridization (FISH) and DAPI staining of the ASSC preparation. The cytophaga–flavobacteria group-specific oligonucleotide probe CF319a described by Manz et al. [30] was synthesized by Macromolecular Resources (Colorado State University, Fort Collins, CO). This probe targets a site at the 319–336 position of the rRNA (*Escherichia coli* numbering), according to Brosius et al. [8], in approximately 80% of the strains clustering in the cytophaga–flavobacteria group that have been screened [30]. Fluorescein was conjugated to the 5′ end of the oligonucleotide probe and the conjugated product purified using polyacrylamide gel electrophoresis.

Duplicate samples of an ASSC preparation from a sample of freshly collected activated sludge were fixed with 4% paraformaldehyde for 10 min, rinsed twice with filter-sterilized, autoclave-sterilized DI water, and then spotted on a microscope slide. An ethanol fixation step was omitted because of the solubility of ELF crystals in this solvent (see following section). Oligonucleotide probe was prepared according to the protocol of Manz et al. [30]. The hybridization reaction was carried out at 46°C for 2 h, the slides containing the cells were washed twice with DI water at 46°C and then co-stained with DAPI as described above to visualize all DNA-containing cells. Five images were captured from each slide and processed using the camera and image analysis software described above.

To determine efficiency of the FISH protocol, replicate samples of the ASSC preparation were first probed with *Bacteria* probe Eub338 conjugated to rhodamine, then co-stained with DAPI according to the procedures described above. The reverse sequence (anti-Eub338) probe was used as a negative control for both Eub338 and CF319a probes. The number of cells that reacted positively with the probes was compared with the number of DAPI-stained cells in the same field of view. The data for five different fields of view of each slide were then combined.

Determination of PO_4ase Activity Associated with Cytophaga–Flavobacteria Group in Activated Sludge

Duplicate samples of an ASSC preparation from a single sample of freshly collected activated sludge were incubated in the presence of ELF-P for 60 min at 20°C, the maximum annual temperature of the aeration basin at the treatment plant, as described above. A drop of the sample was spotted onto a clean microscope slide and air-dried. The slide was flooded with a 4% paraformaldehyde solution, then gently rinsed with autoclave- and filter-sterilized deionized water, then probed by FISH as described above with the following modification. Because of the solubility of ELF crystals in ethanol, it was necessary to delete the ethanol post-fixation step of the FISH protocol. Deletion of this step did not appear to affect the efficiency of the FISH reaction using the Eub338 probe with the ASSC samples, based on comparison with subsamples receiving the ethanol post-fixation treatment. Each slide was examined by epifluorescence microscopy using the U-MWIBA filter set to visualize the bacterial cells that hybridized with the fluorescein-labeled CF319a probe and the U-MWU filter to visualize the ELF crystals. Images of the same field of view collected with the two filter sets were digitally acquired with the CCCD camera and superimposed on one another using Image-Pro Plus image analysis software. Superimposed images from five different areas of each slide were used to determine the fraction of the total bacteria hybridizing with the CF319a probe, which also displayed ELF crystal fluorescence. Image analysis was also used to assess the fraction of the total area of ELF fluorescence derived from bacterial cells that hybridized with the CF319a probe using the approach described above.

Table 1. PO_4ase activity of various sludge fractions (μmol MUFP cleaved L^{-1} h^{-1})[a]

	MUFP concentration (μM)	
Fraction	100	500
"As collected"	8.7	15.2
Suspended solids phase	7.9	18.4
Bulk aqueous phase	ND[b]	0.1

[a] Activities were based on the rate of increase in MUF fluorescence over the 4-h period in which fluorescence intensity increased linearly with time. Data represent mean values obtained from triplicate assays for duplicate subsamples obtained from two samples collected at different times ($n = 6$).
[b] No data.

Results

Distribution of PO_4ase Activity in Activated Sludge

Freshly collected, aerobic activated sludge from the Bozeman, MT, wastewater treatment plant contained 7–14 mg L^{-1} of P_i, depending on the time of sample collection. It exhibited a pH of 7.0 and an annual temperature range of 14–20°C. All PO_4ase assays were thus carried out at pH 7.0 and 20°C to reflect *in situ* conditions. Under these conditions, PO_4ase activity was confined to the suspended solids phase of the activated sludge based on relative fluorescence intensity of the water-soluble MUF cleavage product in the solids and bulk aqueous phases (Table 1). The bulk aqueous phase contained less than 1% of the total PO_4ase activity in the "as collected" sample at two different sampling times. Subsamples of activated sludge that were subjected to autoclave sterilization prior to addition of the MUFP yielded no

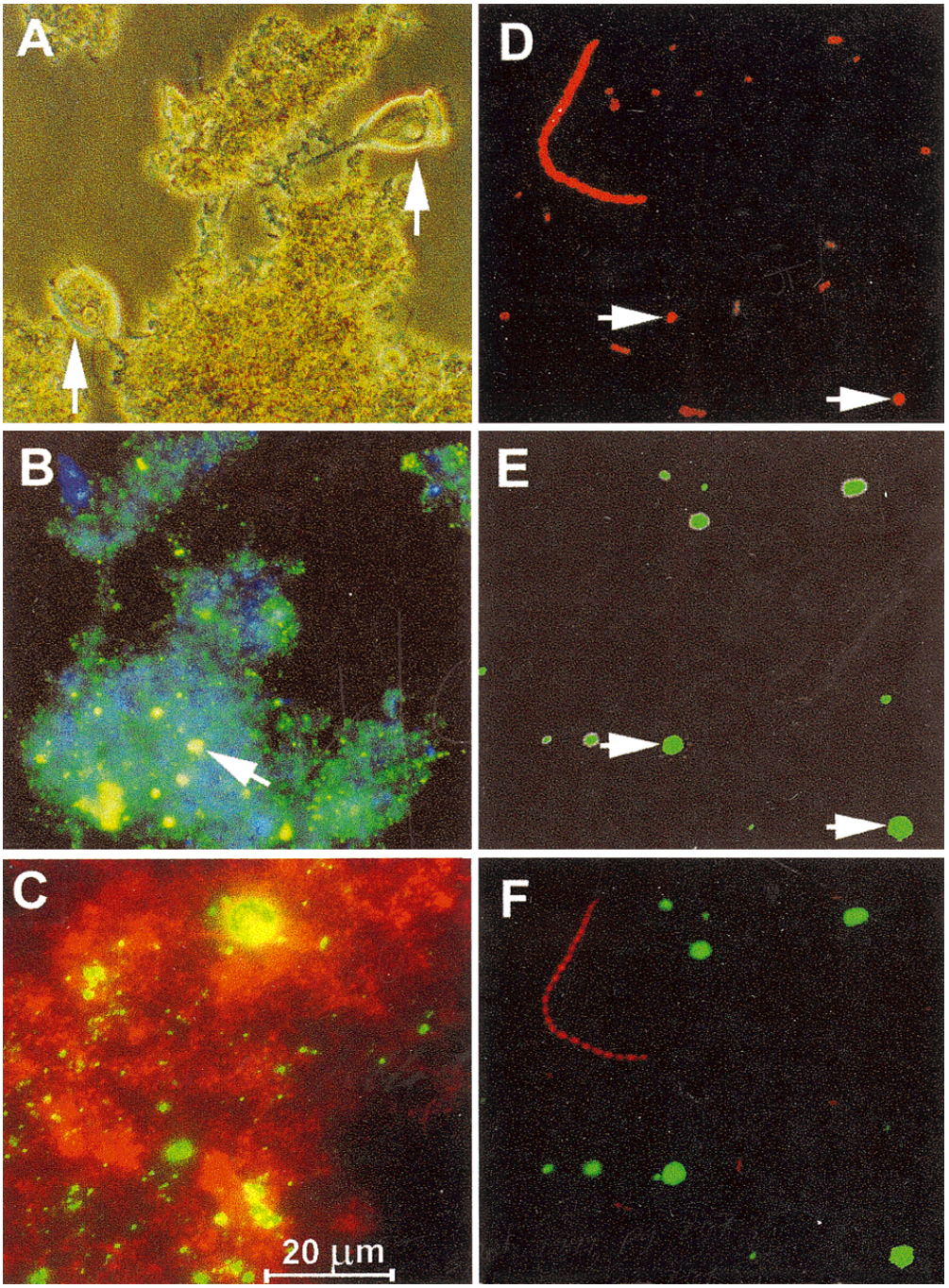

Fig. 2. (A) Phase contrast photomicrograph image of activated sludge floc material and associated microorganisms. Arrows locate protozoa grazing on the floc particles. Bar = 50 μm. (B) Epifluorescence photomicrograph image of same field of view as A above of whole floc material revealing the areas of intense PO_4ase activity (yellow spots where ELF crystals have precipitated, arrow) after incubating in the presence of ELF-P. (C) Epifluorescence photomicrograph image of thin section of a floc particle stained red-orange with AO revealing discrete regions within the floc displaying PO_4ase activity (green spots) as a result of incubation in the presence of ELF-P. Bar = 20 μm. (D) Epifluorescence photomicrograph image of ACCS preparation of floc bacteria using filter combination that reveals cells which react with CF319a oligonucleotide probe (red). (E) Epifluorescence photomicrograph image of same field of view as D above using filter combination that resolves crystals of ELF (green spots) indicating areas of PO_4ase activity. (F) Merged images of D and E above, revealing the subpopulation of cells that react positively with the CF319a and also display PO_4ase activity.

detectable fluorescent MUF product. These results indicate that MUF formation did not occur via abiotic reactions.

The suspended solids phase consisted of flocs of aggregated polymeric material, heavily colonized by a diversity of microorganisms (Fig. 2A). When enzyme activity was assayed using the fluorogenic, artificial substrate ELF-P, the distribution of the water-insoluble, yellow fluorescent product of the enzyme reaction appeared to be concentrated in discrete areas of floc material associated with the suspended solids phase of the activated sludge (Fig. 2B, arrow). The areas of PO_4ase activity (crystal size) varied from 4 to 40 μm in diameter when viewed microscopically at high magnification. A diffuse blue and green fluorescence was also observed throughout the floc material. The blue fluorescence may be due to adsorption of unreacted ELF-P to floc material. The green fluorescence may have been due either to a low level of active cell-free enzyme dispersed throughout the floc or to mixing of the yellow fluorescence signal emanating

from areas of intense enzyme activity with the background blue fluorescence of the unreacted ELF-P. Time course studies using ASSC cell preparations of an activated sludge sample indicated that an ELF-P incubation period of 60 min allowed the development of the maximum density of ELF crystals before increasing crystal size and fluorescence intensity caused the fluorescent image of adjacent crystals to merge into a single fluorescent object. Sixty-min incubations were therefore used for all subsequent ELF-P PO_4ase assays.

When the flocs were incubated in the presence of the ELF-P substrate, and then viewed as 5-μm thin sections after embedding and cryosectioning, the diffuse green fluorescence seen in the whole floc preparations in Fig. 2B was resolved as small crystal deposits from highly localized PO_4ase activity in the AO-stained floc matrix (Fig. 2C). Close inspection of the stained thin sections suggested that PO_4ase activity was associated with some of the bacterial cells in the floc material.

Subsamples of activated sludge that were autoclave sterilized prior to incubation in the presence of ELF-P yielded no ELF crystals in unembedded or embedded and sectioned preparations upon examination by fluorescence microscopy. These results suggest that, like MUF, the ELF crystals had not formed through an abiotic reaction.

Protozoans were present at a density of 2,237 ml^{-1} activated sludge, based on counts obtained from a single sample. Few protozoans, however, displayed ELF fluorescence (Fig. 2A), suggesting that they were not responsible for the observed PO_4ase activity in the activated sludge.

Characterization of Bacterial Cells in Homogenized Activated Sludge

When floc material was homogenized to disperse bacterial cells (ASSC preparation) and the individual cells exposed to ELF-P, 8.8 ± 1.2% of the total bacterial cells that subsequently stained with AO had ELF crystal deposits associated with them (Table 2). Similarly, 5.6 ± 0.9% of the dispersed floc bacteria that were visualized by DAPI-staining were associated with ELF crystal deposits (Table 2). PO_4ase activity in the floc was thus contributed by a small fraction of the floc-associated bacterial population.

Bacteria cells in ASSC preparations were subjected to differential staining with SYTO9 and PI after incubation in the presence of ELF-P to relate PO_4ase activity to cell viability. Together, the SYTO9- and PI-stained bacteria accounted for 93 ± 11% of the total bacterial population associated with the ASSC preparation, based on DAPI staining (Table 2). In two separate ASSC preparations of floc material from freshly collected activated sludge, SYTO9 reacted positively with 35 ± 2% and 45 ± 2% of the total DAPI-stained bacterial population, respectively (Table 2). Of the bacterial cells that stained green with SYTO9, 10.3 ± 2% also displayed ELF crystal fluorescence (Table 2). In contrast, only 1.5 ± 0.5% of the cells that stained with PI displayed ELF crystal fluorescence (Table 2).

To ensure that PI staining was providing an accurate assessment of nonviability of bacterial cells in the homogenized activated sludge population, freshly prepared ASSC was fixed in 4% paraformaldehyde and 50% ethanol prior to staining with SYTO9 and PI and examined microscopically. Following treatment with paraformaldehyde, 95% of the cells stained red with PI; only 5% stained green with SYTO9. When the ASSC preparation was treated with paraformaldehyde and ethanol, 99% of the cells stained red with PI; only 1% of the cells stained green with SYTO9. The ASSC preparation used above to evaluate the efficacy of PI staining, contained a bacterial population, 35% of which stained green with SYTO9 and 65% of which stained red with PI when no chemical treatment was used to destroy cell mem-

Table 2. Portion of bacteria associated with activated sludge floc material displaying various physiological characteristics

Parameter	Percentage
AO-stained cells in ASSC displaying PO_4ase activity	8.8 ± 1.2
DAPI-stained cells in ASSC displaying PO_4ase activity	5.6 ± 0.9
DAPI-stained cells in ASSC displaying leucine uptake	36 ± 8
DAPI-stained cells in ASSC that stained positive with SYTO9 and PI	93 ± 11
DAPI-stained cells in ASSC displaying viability based on SYTO9 staining	
Sample 1	35 ± 2
Sample 2	45 ± 2
SYTO9-stained cells in ASSC displaying PO_4ase activity	10.3 ± 2
PI-stained cells in ASSC displaying PO_4ase activity	1.5 ± 0.5
Portion of PO_4ase activity associated with total cells in ASSC contributed by viable, SYTO9-stained cells	82.4 ± 3.7
Portion of PO_4ase activity associated with total cells in ASSC contributed by nonviable, PI-stained cells	5.8 ± 2.9
Portion of DAPI-stained cells in ASSC that probed positive with CF319a	9.6 ± 2
Portion of total cells in ASSC displaying PO_4ase activity that reacted positively with cytophaga-flavobacteria group-specific probe	
Sample 1	17
Sample 2	20
Portion of total PO_4ase activity associated with bacteria in ASSC preparation that was contributed by cells of cytophaga–flavobacteria group	
Sample 1	35
Sample 2	45

brane potential. Thus, PI staining appears to report cell nonviability in the activated sludge floc bacterial population.

When bacterial cells in the ASSC preparation were assayed for their ability to take up the amino acid leucine using MAR, 36 ± 8% of the DAPI-stained bacterial cell population had silver grains associated with them (Table 2). These results and those above indicating that 35–45% of the cells stained green with SYTO9 suggest that one-third to one-half of the bacterial cells associated with activated sludge flocs are viable and that the majority of viable cells are metabolically active.

ELF crystals associated with SYTO9-stained viable cells were larger in size, based on area of brightness from fluorescence, than those associated with PI-stained nonviable cells. Of the total area of fluorescence produced by all crystals evaluated in ASSC preparations, 82.4 ± 3.7% was contributed by crystals associated with SYTO9-stained bacterial cells, while only 5.8 ± 2.9% was contributed by crystals associated with PI-stained cells (Table 2). The remaining ELF crystal fluorescence could not be affiliated with any identifiable objects associated with the floc. Thus, the floc-derived PO_4ase activity was contributed predominantly by viable bacterial cells.

Because of the small fraction of total bacterial cells in the activated sludge floc that displayed PO_4ase activity, a new, efficient screening method was used to detect PO_4ase-active clones cultivible on enrichment medium. When ASSC preparations were plated on 1/10-strength LB medium containing 1.5% agar and 2-(5′-chloro-2′-phosphoryloxyphenyl)-4-(3*H*)-quinazolinone (CPQP), a few of the many colonies that developed on the plates after 4 days of incubation at room temperature emitted a green fluorescence when viewed under UV light. The colonies remained fluorescent even after several transfers on this solid medium. When the DNA of cells from the fluorescent colonies was extracted and a segment of their 16S rDNA was amplified by PCR, sequenced, and compared with other 16S rDNA sequences in the Michigan State University Ribosomal RNA Database, several were found to group with the cytophaga–flavobacteria in the phylum cytophaga–flavobacter–bacteroides. Two isolates yielded similarity rankings of 0.756 and 0.610, respectively, with *Empedobacter brevis.* A third isolate yielded a similarity ranking of 0.603 with *Bergyella zoohelcum.* A fourth isolate yielded a similarity ranking of 0.478 with *Blastobacter natorius,* a member of the α-subclass of the Proteobacteria.

The cytophaga–flavobacteria group-specific rRNA probe CF319a reacted positively with the three PO_4ase-positive isolates recovered from the floc material whose rDNA sequence indicated relatedness to this group. The CF319a probe also produced a positive reaction with 9.6 ± 2% of the DAPI-stained bacteria associated with the ASSC preparations of homogenized floc material (Fig. 2D; Table 2). None of the bacterial isolates or the ASSC preparations reacted positively with the anti-Eub338 probe. The percentage of the cells that reacted positively with the Eub338 oligonucleotide probe for *Bacteria* was 95 ± 11% of those that stained with DAPI, indicating that the FISH technique was functioning as it should.

Of the bacteria in the homogenized floc displaying PO_4ase activity, based on ELF fluorescence, 17–20% reacted positively with the CF319a probe (Figs. 2E and F; Table 2). Of the total area of ELF fluorescence produced by all crystals evaluated in ASSC preparations of floc material, 35–45% was contributed by crystals associated with cells that stained positive with the CF319a probe (Table 2). These results indicate that the cytophaga–flavobacteria group is an important contributor of PO_4ase activity in activated sludge flocs.

Discussion

PO_4ase enzyme activity was detected in activated sludge using artificial, fluorogenic substrates. Essentially all PO_4ase activity associated with aerobic activated sludge at pH 7.0, when measured at 20°C, partitioned with the solids fraction based on the distribution of the enzyme hydrolysis product MUF. Another fluorogenic substrate, ELF-P, which formed the insoluble product ELF upon enzymatic hydrolysis of the phosphate ester bond, revealed that PO_4ase activity in aerobic activated sludge was associated with bacterial cells in the colloidal floc portion of the solids fraction. There was no evidence of MUF or ELF formation in autoclave-sterilized subsamples of activated sludge, indicating that they were not formed by abiotic reactions. Further evidence that the fluorescence reactions were biologically mediated was obtained by a linear relationship between the reciprocal of the fluorescence intensity and reciprocal of MUFP concentration (Lineweaver–Burke enzyme reaction plot) in "as-collected" activated sludge (data not shown).

In the present study, the ELF reaction was allowed to proceed for 60 min, based on preliminary studies with an ASSC preparation in which the density of discrete ELF crystals was followed over increasing time of ELF-P exposure. Since enzyme activity will vary depending on environmental conditions, the optimum incubation period should be determined for different types of samples.

Although PO_4ase enzyme activity has been detected previously in activated sludge systems, it had not been linked to any particular fraction prior to this study [28]. Based on the distribution and intensity of ELF crystal fluorescence, PO_4ase activity was primarily localized in the immediate vicinity of the bacterial cells rather than dispersed uniformly throughout the floc matrix. However, since ELF crystal formation requires an as yet unidentified threshold concentration of active enzyme, we cannot rule out the possibility of a significant but low (below threshold) concentration of active enzyme trapped in the floc matrix but free of the microbial cells in the floc. In other studies, Ammeman [2] reported that 15–73% of the particle phase–associated alkaline PO_4ase activity in the water column of the ocean was specifically associated with bacteria.

Regeneration of P_i from detrital organic-P has been shown to proceed faster and more completely in marine systems when ciliates and colorless flagellates are present than in the presence of bacteria alone [24]. Phagotrophic feeding by zooflagellates releases 60–80% more soluble reactive phosphorus (SRP) than that liberated by bacterial action alone [19]. As a result, protists have been assigned a more important role in P regeneration in the marine environment than originally thought [4]. However, it has been suggested that the elevated levels of SRP liberated from bacteria by protists may not be an accurate estimation of P_i release [15]. A portion of the SRP liberated by protists may be labile organic-P rather than P_i. Our results revealing little detectable PO_4ase activity associated with protists from activated sludge suggests these organisms are not as important as bacteria in P regeneration in P-enriched environments. That the majority of the PO_4ase activity was associated with bacterial-rich floc material is consistent with the idea that most of the biologically mediated P transformations in the activated sludge process of wastewater treatment are carried out by bacteria.

That most of the ELF crystals formed in the floc particles were positioned closely to bacterial cells suggests that the PO_4ase enzymes are bound to the cell envelope and/or present in the cell cytoplasm. Although MUFP is believed to be an artificial substrate for only ecto-PO_4ase enzymes such as alkaline PO_4ase [47], the permeability of the cell envelope of activated sludge bacteria to ELF-P has not yet been assessed. Thus, we are unable to determine at this time whether the observed crystal formation and fluorescence is the result of enzymatic hydrolysis of external detrital organic-P and/or intracellular P-containing metabolites of the cells.

Alkaline PO_4ase is known to hydrolyze inorganic poly-P_i [10], a P-containing storage product that is accumulated by a variety of activated sludge bacteria [5, 9, 16, 40, 45]. Alkaline PO_4ases have been implicated in P_i release from intact bacterial cells containing poly-P_i storage products in anaerobic lake sediment [17]. It has been proposed that alkaline PO_4ases, in conjunction with P_i membrane transport systems, enable cells to recover some of the P_i released during anaerobiosis.

The role of bacterial alkaline PO_4ases in poly-P_i processing in activated sludge is not presently known. Alkaline PO_4ases may be involved in the degradation of cell-surface localized poly-P_i, which represents a more transient but significant portion of the total poly-P_i accumulated by cells of *Acinetobacter Iwoffi* JW11 [20]. The enzyme is required for utilization of externally available poly-P_i by cells of *E. coli* [34]. Since poly-P_i formation and degradation exert significant control over enhanced P removal from wastewater [9, 42, 44], future studies to increase P removal efficiency should consider the role bacterial PO_4ases play in this regard.

The detection of PO_4ase activity in aerated activated sludge containing P_i concentrations of 7–14 mg L^{-1} at pH 7.0 in the present study is inconsistent with many studies indicating algal and bacterial enzyme inhibition by elevated P_i concentrations [12, 14, 35]. However, our results are consistent with the observations of Kuo and Blumenthal [26], Chrost and Albrecht [11], and Chrost and Overbeck [12] that alkaline PO_4ase synthesis in many bacteria, including aquatic bacteria, is consititutive and not inhibited by elevated P_i. Alkaline PO_4ase synthesis and activity in aquatic bacteria instead appear to be controlled by the levels of specific forms of external organic-P. Derepression of the enzyme in aquatic bacteria has been reported upon addition of external glucose 6-phosphate and adenosine triphosphate [10].

Nonspecific acid PO_4ase enzymes may also contribute to the observed PO_4ase activity in the activated sludge. Although they are typically involved in intracellular P transformations, Class A nonspecific acid PO_4ase enzymes of certain members of the family *Enterobacteriaceae* are only slightly inhibited by P_i concentrations of 100 mM and exhibit increased activity in the presence of 3′- and 5′-nucleoside monophosphates, nucleoside diphosphates, nucleoside triphosphates, hexose and pentose phosphates, α- and β-glycerol phosphates, and α-naphthyl phosphate, and pyrophosphate [36].

Some of the organic-P compounds identified above are likely to be part of the detrital organic-P pool of activated sludge and thus may control bacterial PO_4ase activity in the system. Optimization of system operating conditions to enhance (enzymatic) hydrolysis of detrital organic-P-containing compounds by floc-forming bacteria in the aerobic activated sludge should not only enhance P removal from the system, but also increase the nutrient/biomass ratio, which discourages growth of filamentous organisms and sludge bulking [42]. Stimulation of PO_4ase-active bacterial populations during the anaerobic step of the enhanced P removal process may provide additional utilizable reductants in the form of the organic residue of the hydrolyzed organic-P, and circumvent or reduce the need to add low molecular weight fatty acids or other substrates to maximize cellular poly-P_i degradation and P_i release.

PO_4ase activity has been associated with intact cells [23]. The PO_4ase activity reported in this study appeared to be associated primarily with the active bacterial fraction of the floc material. Approximately 82% of the ELF crystals reporting PO_4ase activity occurred around bacterial cells that fluoresced green with SYTO9 after co-staining with PI. SYTO9 staining appeared to be an accurate indicator of active or viable cells in the ASSC preparation since the portion of the total cell population that stained with SYTO9 was similar to that which transported the amino acid leucine based on independent MAR results. PI staining appeared to provide an accurate account of the inactive bacterial cell fraction based on the results of the chemically induced inactivation the cells in ASSC preparations prior to SYTO9/PI staining. That the majority (93%) of bacterial cells in the ASSC preparations had been accounted for as a result of SYTO9 and PI staining was verified by co-staining with DAPI. Thus, the majority of PO_4ase activity detected in the floc material was not derived from moribund or lysed cells, but rather from active cells, presumably capable of PO_4 uptake and incorporation into microbial biomass.

The utilization of a recently developed screening technique facilitated the detection of the few cultivable bacterial cells in ASSC preparations that exhibited PO_4ase activity. This provided the opportunity to obtain phylogenetic information on these bacterial cells. The few colony-forming units displaying PO_4ase activity among the hundreds of colony-forming units recovered on solid medium inoculated with ASSC preparations grouped with the cytophaga–flavobacteria based on 16S rDNA sequence data. Bacteria representing this group have been widely reported in activated sludge [7, 16, 18, 30, 31, 33]. However, their role in the activated sludge process, and in P transformations in particular, has not been elucidated until now.

When the PO_4ase-positive bacterial isolates, which grouped with the cytophaga–flavobacteria, were subjected to FISH using the CF319a oligonucleotide probe, shown previously to react selectively with approximately 80% of activated sludge isolates that fall within the cytophaga-flavobacteria cluster [30], all displayed a positive reaction with the probe. When the entire ASSC bacterial population was subjected to FISH, 17–20% of the bacteria that stained with DAPI reacted positively with the CF319a probe. These results are consistent with those obtained by Snaidr et al. [39], who reported that the cytophaga–flavobacteria group represented 12% of the DAPI-stained bacterial population of activated sludge. Similarly, Manz et al. [30] found that of the bacterial cells present in activated sludge from the Hirblingen and Berlin-Ruhleben wastewater treatment plants in Germany, the cytophaga–flavobacteria group contributed 10–12% of the cells that hybridized with the *Bacteria*-specific probe EUB338.

Probing by FISH to 16S rRNA in intact cells has been shown to detect the majority of targeted cells recovered from a variety of environments [1]. That 95 ± 11% of the DAPI-stained cells in the ASCS preparations probed positive for the *Bacteria*-specific probe EU338 indicates that the vast majority of activated sludge bacteria can be detected with rRNA-targeted probes. Others have reported activated sludge bacterial probing efficiencies ranging from 75 to 90% [31, 45]. Eliminating the EtOH step of the FISH protocol was necessary in order to prevent the ELF crystals from dissolving. Although elimination of the EtOH step seems to have little influence on the efficiency of hybridization of the EUB338 probe, other more specific probes suffer reductions in hybridization efficiency when this step is deleted. We do not know to what extent this applies to the specific probe used in this study.

Bacterial clones that cluster with the flexibacter–cytophaga–bacteriodes group have been reported to be more abundant in non-phosphate-removing sludge than in phosphate-removing sludge [7]. These investigators suggested that bacterial populations aligned with this phylogenetic group are opportunists that occupy a niche left vacant by phosphate-removing bacteria from other phylogenetic groups. However, the method used by these investigators to establish the abundance of clones that align with various phylogenetic groups does not evaluate the size of the popu-

lation contributed by each clone or phylogenetic group in the system. Thus, the approach does not assess the importance of those populations grouping with the flexibacter–cytophaga–bacteriodes relative to other populations in P transformations in the activated sludge process. Using the FISH probe methodology, it was found that the cytophaga–flavobacteria group contributed 17–20% of the total DAPI-staining population. By combining the FISH and PO_4ase-localization probe methodologies, it was possible to assess in a semiquantitative manner what portion of the total PO_4ase active, DAPI-stained ASSC bacterial cell population was contributed by bacterial cells that grouped with the cytophaga–flavobacteria. That 35–45% of the ELF crystals were located around CF319a probe-positive cells suggests that the cytophaga–flavobacteria as a group contributes a significant fraction of the total PO_4ase-active ASSC population.

The results also suggest that many of the PO_4ase-active bacteria in activated sludge resist cultivation on laboratory media. Although approximately 9% of the total AO- or DAPI-stained cells in the ACCS preparations displayed ELF fluorescence, less than 5% of the colony-forming units recovered on 1/10th strength LB agar displayed CPQ-fluorescence-based PO_4ase activity. These results are consistent with those of other studies, which suggest resistance to laboratory cultivation of activated sludge bacteria involved in other important activated sludge processes [6, 22, 37, 38]. The abundance of bacteria belonging to the cytophaga–flavobacterium cluster in the activated sludge process of a dairy wastewater plant was severely underestimated by cultivation methods [31].

In summary, a combination of FISH and specific enzyme activity localization probes has provided the opportunity to assign specific functions to specific phylogenetic groups of bacteria in an activated sludge system, independent of bacterial cultivibility. This approach offers the opportunity to monitor specific activities of specific bacterial populations in complex microbiological systems such as activated sludge, and the ability to define the influence of different operating or environmental conditions on specific processes such as enhanced P removal. That this can be achieved at the individual cell level offers the opportunity to evaluate intrapopulation heterogeneity in enzyme activity as well. It should be possible to synthesize and utilize a wide variety of precipitating, fluorogenic substrates for various enzymatic activities in a manner similar to that described here for PO_4ase. Use of such substrates in combination with phylogenetically based oligonucleotide probes should facilitate identification of the roles of each population in a microbial community, and thereby relate microbial population diversity, structure, and function in an ecosystem.

Acknowledgment

This research was supported by NSF grants OCE9720151 and EEC8907039.

References

1. Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA (1990) Combination of 16S rRNA-target oligonucleotide probes with flow cytometry for analyzing mixed populations. Appl Environ Microbiol 56:1919–1925
2. Ammerman JW (1991) Role of ecto-phosphohydrolases in phosphorus regeneration in estuarine and coastal ecosystems. In: Chrost RJ (ed) Microbial Enzymes in Aquatic Environments, Springer-Verlag, New York, pp 165–186
3. Ammerman JW, Azam F (1985) Bacterial 5′-nucleosidase in aquatic ecosystems: a novel mechanism of phosphorus regeneration. Science 227:1338–1340
4. Andersen OK, Goldman JC, Caron DA, Dennett MR (1986) Nutrient cycling in a microflagellate food chain: III. Phosphorus dynamics. Mar Ecol Prog Ser 32:47–55
5. Beacham AM, Seviour RJ, Lindrea KC (1992) Polyphosphate accumulating abilities of Acinetobacter isolates from a biological nutrient removal pilot plant. Wat Res 26:121–122
6. Biesterfeld S, Figueroa L, Hernandez M, Russell P (1998) Use of fluorescent oligonucleotide probes to characterize vertical population distributions of nitrifying bacteria in a full-scale nitrifying trickling filter. Paper presented at the IAWQ Conference on Microbial Ecology of Biofilms: Concepts, Tools and Applications, Lake Bluff, IL, October 8–10, 1998
7. Bond PL, Hugenholtz P, Keller J, Blackall LL (1995) Bacterial community structures of phosphate-removing and non-phosphate removing activated sludges from sequencing batch reactors. Appl Environ Microbiol 61:1910–1916
8. Brosius J, Dull TL, Sleeter DD, Noller HF (1981) Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli.* J Mol Biol 148:107–127
9. Christensson M, Blackall LL, Welander T (1998) Metabolic transformations and characterization of the sludge community in an enhanced biological phosphorus removal system. Appl Microbiol Biotechnol 49:226–234
10. Chrost RJ (1991) Environmental control of the synthesis and activity of aquatic microbial ectoenzymes. In: Chrost RJ (ed) Microbial Enzymes in Aquatic Environments. Springer-Verlag, New York, pp 29–59
11. Chrost RJ, Albrecht D (1986) A method for determining enzymatically hydrolyzable phosphate EHP in natural waters. Limnol Oceanogr 31:662–667
12. Chrost RJ, Overbeck J (1987) Kinetics of alkaline phosphatase

activity and phosphorus availability for phytoplankton and bacterioplankton in lake Plußsee (north German eutrophic lake). Microb Ecol 13:229–248
13. Chrost RJ, Velimirov B (1991) Measurement of enzyme kinetics in water samples: effect of freezing and soluble stabilizer. Mar Ecol Prog Ser 70:93–100
14. Cotner JB, Wetzel RG (1991) Bacterial phosphatases from different habitats in a small, hardwater lake. In: Chrost RJ (ed) Microbial Enzymes in Aquatic Environments. Springer-Verlag, New York, pp 187–205
15. Dolan JR (1997) Phosphorus and ammonia excretion by planktonic protists. Mar Geol 139:109–122
16. Fuhs GW, Chen M (1975) Microbiological basis of phosphate removal in the activated sludge process for the treatment of wastewater. Microb Ecol 2:119–138
17. Gachter R, Meyer JS, Mares A (1988) Contribution of bacteria to release and fixation of phosphorus in lake sediment. Limnol Oceanogr 33:1542–1558
18. Gude H (1980) Occurrence of Cytophagas in sewage plants. Appl Environ Microbiol 39:756–763
19. Gude H (1985) Influence of phagotrophic processes on the regeneration of nutrients in two-stage continuous culture systems. Microb Ecol 11:193–204
20. Halvorson HO, Suresh N, Roberts MF, Coccia M, Chikarmane HM (1987) Metabolically active surface polyphosphate pool in *Acinetobacter Iwoffi,* In: Torriani-Gorini A, Rothman FG, Silver S, Wright A, Yagil E (ed) Phosphate Metabolism and Cellular Regulation in Microorganisms. American Society for Microbiology, Washington DC, pp 220–224
21. Haugland RP (1996) Handbook of Fluorescent Probes and Research Chemicals. 6th ed. Molecular Probes Inc, Eugene OR, pp 372–373
22. Holben WE, Noto K, Sumino T, Suwa Y (1998) Molecular analysis of bacterial communities in a three-compartment granular activated sludge system indicates community-level control by incompatible nitrification processes. Appl Environ Microbiol 64:2528–2532
23. Jansson M, Olsson H, Pettersson K (1988) Phosphatases; origin, characteristics and function in lakes. Hydrobiol 170:157–175
24. Johannes RE (1965) Influence of marine protozoa on nutrient regeneration. Limnol Oceanogr 10:434–441
25. Kulaev IS, Vagabov VM (1983) Polyphosphate metabolism in micro-organisms. Adv Microb Physiol 24:83–171
26. Kuo M-H, Blumenthal HJ (1961) Absence of phosphatase repression by inorganic phosphate in some microorganisms. Nature 190:29–31
27. Larison KD, BreMiller R, Wells SK, Clements I, Haugland RP (1995) Use of a new fluorogenic phosphatase substrate in immunohistolochemical applications. J Histochem Cytochem 43:77–83
28. Lemmer H, Roth D, Schade M (1994) Population density and enzyme activities of heterotrophic bacteria in sewer biofilms and activated sludge. Wat Res 28:1341–1346
29. Maidak BL, Cole JR, Parker CTJr, Garrity GM, Larsen N, Li B, Lilburn TG, McCaughey MJ, Olsen GJ, Overbeek R, Pramanik S, Schmidt TM, Tiedje JM, Woese CR (1999) A new version of the RDP (Ribosomal Database Project). Nucleic Acids Res 27:171–173
30. Manz W, Amann R, Ludwig W, Vancanney M, Schleifer K-H (1996) Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga–flavobacter–bacteroides in the natural environment. Microbiol 142:1097–1106
31. Manz W, Wagner M, Amann R, Schleifer K-H (1994) *In situ* characterization of the microbial consortia active in two wastewater treatment plants. Wat Res 28:1715–1723
32. Penney CL (1976) A simple micro-assay for inorganic phosphate. Anal Chem. 75:201–210
33. Pike LF (1975) Aerobic bacteria. In: Curds CR, Hawkes HA (ed) Ecological Aspects of Used Water Treatment, Vol 1, Academic Press, London, pp 1–63
34. Rao NN, Roberts MF, Torriani A (1987) Polyphosphate accumulation and metabolism in *Escherichia coli.* In: Torriani-Gorini A, Rothman FG, Silver S, Wright A, Yagil E (ed) Phosphate Metabolism and Cellular Regulation in Microorganisms. American Society for Microbiology, Washington DC, pp 213–219
35. Reid TW, Wilson IB (1971) *E. coli* alkaline phosphatase. In: Boyer P (ed) The Enzymes. Academic Press, New York, pp 373–415
36. Rossolini GM, Schippa S, Ricco ML, Berlutti F, Macaskie LE, Thaller MC (1998) Bacterial nonspecific acid phosphohydrolases: physiology, evolution and use as tools in microbial biotechnology. Cell Mol Life Sci 54:833–850
37. Schramm A, deBeer D, Wagner M, Amann R (1998) Identification and activities *in situ* of *Nitrosospira* and *Nitrospira* spp. as dominant populations in a nitrifying fluidized bed reactor. Appl Environ Microbiol 64:3480–3485
38. Schramm A, Larson LH, Revsbech NP, Ramsing NB, Amann R, Schliefer K-H (1996) Structure and function of a nitrifying biofilm as determined by in situ hybridization and the use of microelectrodes. Appl Environ Microbiol 62:4641–4647
39. Snaidr J, Amann R, Huber I, Ludwig W, Schleifer K-H (1997) Phylogenetic analysis and in situ identification of bacteria in activated sludge. Appl Environ Microbiol 63:2884–2896
40. Streichan M, Golecki JR, Schon G (1990) Polyphosphate-accumulating bacteria from sewage plants with different processes for biological phosphorus removal. FEMS Microb Ecol 73:113–124
41. Tabor PS, Niehof RA (1985) Improved microautoradiographic method to determine individual microorganisms active in substrate uptake in natural waters. Appl Environ Microbiol 44:945–953
42. Toerien DF, Gerber A, Lotter LH, Cloete TE (1990) Enhanced biological phosphorus removal in activated sludge systems. Adv Microb Ecol 11:173–230
43. Van Niel EWJ, Appeldoom KJ, Zehnder AJB, Kortstee GJJ (1998) Inhibition of anaerobic phosphate release by nitric oxide in activated sludge. Appl Environ Microbiol 64:2925–2930

44. Van Ommen Kloeke F, Baty AM, Eastburn C, Diwu Z, Geesey GG. A novel method for screening bacterial colonies for phosphatase activity. J Microb Meth, in press
45. Wagner M, Erhart R, Manz W, Amann R, Lemmer H, Wedi D, Schleifer KH (1994) Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for *in situ* monitoring in activated sludge. Appl Environ Microbiol 60:792–800
46. Wilkins AS (1972) Physiological factors in the regulation of alkaline phosphatase synthesis in *Escherichia coli.* J Bacteriol 110:616–623
47. Wynne D, Kaplan B, Berman T (1991) Phosphatase activities in Lake Kinneret phytoplankton. In: Chrost RJ (ed) Microbial Enzymes in Aquatic Environments, Springer-Verlag, New York, pp 220–226
48. Yu FP, Callis GM, Stewart PS, Griebe T, McFeters GA (1994) Cryosectioning of biofilms for microscopic examination. Biofouling 8:85–91