Application of Microautoradiography to the Study of Substrate Uptake by Filamentous Microorganisms in Activated Sludge

KJÆR ANDREASEN¹ AND PER HALKJÆR NIELSEN^{2*}

Krøger A/S, DK-2860 Søborg,¹ and Environmental Engineering Laboratory,
Aalborg University, DK-9000 Aalborg,² Denmark

Received 3 April 1996/Accepted 30 June 1997

Excessive growth of filamentous microorganisms in activated-sludge treatment plants is a major operational problem which causes poor settlement of activated sludge. An enhanced understanding of the factors controlling growth of different filamentous microorganisms is necessary in order to establish more successful control strategies. In the present study, the in situ substrate uptake was investigated by means of microautoradiography. It was demonstrated that the uptake of labeled organic substrates by the filamentous microorganisms, during short-term incubation, could be detected by microautoradiography. Viability and respiratory activity of the filaments were also detected by reduction of CTC (5-cyano-2,3-ditolyl tetrazolium chloride) and by incorporation of [³H]thymidine. Gram, Neisser, and fluorescence staining techniques were used for the localization and identification of the filaments. Activated-sludge samples from five wastewater treatment plants with bulking problems due to filamentous microorganisms were investigated. *Microthrix parvicella*, *Nostocoida limicola*, and Eikelboom's type 0041 and type 021N were investigated for their ability to take up organic substrates. A panel of six substrates, i.e., [¹⁴C]acetate, [³H]glucose, [¹⁴C]ethanol, [³H]glycine, [³H]leucine, and [³H]oleic acid, was tested. The uptake response was found to be very specific not only between the different filamentous types but also among filaments of the same type from different treatment plants. Interestingly, *M. parvicella* consistently took up only oleic acid among the tested substrates. It is concluded that microautoradiography is a useful method for investigation of in situ substrate uptake by filamentous microorganisms in activated sludge.

Excessive growth of filamentous microorganisms in activated sludge (bulking), which reduces the settling properties of the sludge, remains a major operational problem for wastewater treatment plants (WTPs). When present in high numbers, filamentous microorganisms hinder sludge floc separation in the final clarifier, resulting in a reduction in effluent quality. While the process of activated-sludge bulking has been actively investigated for over a quarter of a century, control of the growth of some filamentous types still remains problematic. An important step in the study of bulking came with the development of the classification system of Eikelboom for the filamentous microorganisms present in sludge (6, 7). This microscopy-based scheme characterized in excess of 20 different organisms or filament types. Some of these microorganisms have since been examined in monoculture (18, 22), and based upon physiological characteristics and experience from full-scale treatment plants, strategies for their control in activated-sludge treatment plants have been successfully developed (12, 21).

Several nations have incorporated biological nitrogen and phosphorus removal into their wastewater treatment upgrading programs. These changes, however, appear to correlate with an increased bulking problem (13, 21). Control of the problem organisms necessitates further research to gain a comprehensive understanding of those factors which may limit or enhance the growth. Compounding the difficulties associated with retarding filamentous growth is that information about growth factors for pure cultures (reviewed by Wanner [27] and Jenkins [12]) is not readily transferred to full-scale operations. While on-line monitoring systems for measurement of oxygen, ammonia, nitrate, and phosphorus have provided detailed site-

specific information under various process conditions, limited information concerning utilization of different organic substrates and electron acceptors by filamentous organisms, under actual process conditions, is available. Such information would be of significant value for developing or improving control strategies. The lack of such detail is mainly due to methodological problems associated with in situ analysis of substrate uptake. One technique which might provide novel insights into the generation of such data with activated sludge is microautoradiography.

Microautoradiography has been successfully applied to investigate microbial growth and substrate competition in systems other than activated sludge (4, 5, 8). Carman (4) used microautoradiography to investigate the uptake of radioactive tracers by a natural assemblage of sedimentary microorganisms. He demonstrated that [³H]acetate and [³H]thymidine were taken up only by heterotrophic bacteria and that [¹⁴C]bicarbonate was taken up only by microalgae. Microautoradiography has also been used in the study of nutrient competition between leaf epiphytes and plant pathogens (5), as well as in a comparative study of photoheterotrophy, chemoheterotrophy, and photolithotrophy in a eutrophic reservoir and an oligotrophic lake (8). In the present study, microautoradiographic methods were applied to study the uptake of labeled organic substrates by several filamentous microorganisms under different conditions of incubation. The applicability of the method to the study of activated sludge was also evaluated.

MATERIALS AND METHODS

Activated sludge. Activated-sludge samples were obtained from four Danish WTPs and one Norwegian WTP with nitrogen and phosphorus removal. WTP 1 is a municipal plant with biological nitrogen and phosphorus removal. Bulking problems are caused mainly by *Microthrix parvicella* and *Nostocoida limicola*. WTP 2 is a municipal plant with biological nitrogen removal and simultaneous precipitation of phosphorus with iron. It has minor bulking problems caused by *M. parvicella* and type 0041. WTP 3 is a municipal pilot plant with biological

* Corresponding author. Mailing address: Environmental Engineering Laboratory, Aalborg University, Sohngaardsholmsvej 57, DK-9000 Aalborg, Denmark. Fax: 45-98-142555. E-mail: i5phn@civil.auc.dk.

nitrogen removal; bulking problems are caused by *N. limicola*. WTP 4 is a municipal plant with biological nitrogen removal and simultaneous precipitation of phosphorus with iron; it has bulking problems caused mainly by type 021N. WTP 5 is an industrial plant treating dairy wastewater with biological nitrogen and phosphorus removal. Bulking problems are caused by type 021N.

Filamentous microorganisms were characterized according to the procedure described by Eikelboom and van Buijsen (7). The filamentous populations at the treatment plants were characterized in the actual samples used for the microautoradiographic tests. In addition, during surveying programs, the filamentous populations at the plants were monitored regularly (2, 13).

Samples were taken from aeration tanks or from the return sludge line in order to obtain nitrate-free samples for anaerobic incubations. Activated-sludge samples were incubated with the radioactive organic substrate within 6 h after sampling, except for WTP 3 sludge, which was incubated 24 h after sampling. Before incubation, sludge samples were diluted to approximately 1.5 g of dry matter (suspended solids [SS]) per liter with water obtained from filtration (Whatman GF/C glass microfibre filter; pore size, 1 μ m) of a part of the activated-sludge sample. For anaerobic incubations, sludge was diluted with nitrate-free filtered water from the activated sludge. The nitrate concentration was checked with a nitrate test kit (Hach model N1-12) (0 to 0.5 mg of $\text{NO}_3\text{-N}$ /liter measured as nitrite after reduction with cadmium).

Incubation. The general pattern of substrate uptake in the sludges was investigated under aerobic conditions. A panel of six different organic substrates was tested. Two-milliliter samples of sludge suspensions were transferred to 9-ml glass vials. After addition of radioactive substrates, the samples were incubated in the dark for 3 h at 20 to 22°C and gently shaken. To test for optimal incubation time, sampling was performed after 2 min and after 0.5, 1, 3, 4, and, in some experiments, 24 h. After incubation, the samples were treated as described below.

The effect of various electron acceptors on uptake of oleic acid was investigated for activated sludge with *M. parvicella* and *N. limicola*. Incubation was performed in parallel under anaerobic conditions without nitrate present, under anaerobic conditions with nitrate present (denitrifying conditions), and under aerobic conditions with oxygen and nitrate present. The nitrate was added to a final concentration of 2 mM. Prior to addition of the substrate, the anaerobic vials were sealed with thick gas-tight rubber stoppers. Vials were then flushed with oxygen-free nitrogen gas to remove oxygen and further preincubated for 1 h to ensure complete oxygen removal. After preincubation, oxygen-free radioactive oleic acid was injected through the rubber stopper with a syringe. The incubation time was 3 h.

Reproducibility was ascertained by including two vials incubated under identical conditions. No differences in results between duplicates were ever recorded. Furthermore, incubation of three sludge samples taken from WTP 1 over several weeks gave identical results.

As a control for possible adsorption phenomena, a pasteurized sludge sample (70°C for 10 min) was incubated with the radioactive substrate in parallel with the normal samples. This was done for all combinations of substrates and sludges.

Preparation of samples for autoradiography. After incubation, samples were withdrawn from the vials with a syringe and immediately transferred to a centrifuge tube containing 5 ml of a 4% formaldehyde solution prepared in filtered effluent from the WTP (Whatman GF/C glass microfibre filter; pore size, 1 μ m). This stopped the metabolic activity, and tests showed that the formaldehyde had no effect on the subsequent procedure. To remove excess soluble radioactive substrate, the activated sludge was washed three times with 5 ml of filtered effluent without formaldehyde. Effluent from the treatment plant was used to prevent possible damage to the bacteria and flocs due to rapid changes in the ionic concentration and composition. The washing procedure consisted of centrifugation at $2,600 \times g$ for 10 min, removal of the supernatant, addition of a further 5 ml of filtered effluent, and mixing. Finally, the pellet was resuspended in filtered effluent to approximately 0.3 ml. One drop of sample was transferred to each of several standard microscope slides, and they were allowed to dry at ambient temperature.

Samples without added radioactivity were always prepared as controls to check for chemography.

Organic substrates tested. The following radioactive substrates were tested: $[2\text{-}^{14}\text{C}]\text{acetic acid}$ (sodium salt) with a specific radioactivity of 57 mCi/mmol, $\text{D-}[6\text{-}^3\text{H}]\text{glucose}$ with a specific activity of 44 Ci/mmol, $[1\text{-}^{14}\text{C}]\text{ethanol}$ with a specific activity of 3.5 mCi/mmol, $[^3\text{H}]\text{glycine}$ with a specific activity of 20 Ci/mmol, $[^3\text{H}]\text{leucine}$ with a specific activity of 50 Ci/mmol, and $[^3\text{H}]\text{oleic acid}$ with a specific activity of 7.6 Ci/mmol. The tracers were obtained from Amersham International plc., Amersham, United Kingdom, except for the ethanol, which was obtained from NEN Life Science Products, Boston, Mass. The specific activity of stock solutions, except for ethanol and oleic acid, was adjusted to 25 mCi/mmol by adding unlabeled compounds. $[^{14}\text{C}]\text{acetic acid}$ was adjusted with 5 mM sodium acetate, $[^3\text{H}]\text{glucose}$ was adjusted with 5 mM D-glucose, $[^3\text{H}]\text{glycine}$ was adjusted with 5 mM glycine, and $[^3\text{H}]\text{leucine}$ was adjusted with 5 mM L-leucine. $[^{14}\text{C}]\text{ethanol}$ had a specific activity of 3.5 mCi/mmol without adjustment. $[^3\text{H}]\text{oleic acid}$ was supplied in toluene; the latter was removed by evaporation, and oleic acid was redissolved in 0.01 N sodium hydroxide. The specific activity of oleic acid was adjusted to 60 mCi/mmol with 1 mM oleic acid (sodium salt). The pH was adjusted to 8 with 0.01 N HCl. Unlabeled compounds were

pure grade chemicals from Sigma. Stock solutions of substrates used for anaerobic incubations were stored in serum vials with thick butyl rubber stoppers. The oxygen was removed from these stock solutions by evacuating and refilling the vials four times with oxygen-free nitrogen gas. Approximately 0.5 ml of the radioactive stock solution was added to the incubation vial; radioactivity was 3 to 10 $\mu\text{Ci}/\text{mg}$ of SS. The amount of organic substrate added to each vial was 0.4 to 2.9 μmol , corresponding to a concentration of 0.2 to 1.4 mM and a chemical oxygen demand of 25 to 140 mg/liter. The pH was between 7 and 8 in the incubated activated sludge upon addition of the radioactive substrate from stock solutions.

Investigation of activity and viability. If no uptake of labeled substrate could be detected, it was crucial to investigate if this was due to physiologically inactive filaments. In this study, reduction of CTC (5-cyano-2,3-ditolyl tetrazolium chloride) and uptake of $[^3\text{H}]\text{-labeled thymidine}$ were used as indicators for viability and activity. CTC was obtained from Polyscience Europe Inc., Eppelheim, Germany. Diluted sludge was incubated aerobically with 5 mM CTC for 3 h, and formation of intracellular fluorescent crystals indicated respiratory activity (9). $[\text{methyl-}^3\text{H}]\text{thymidine}$ with a specific activity of 25 Ci/mmol was obtained from Amersham International plc. It was added to diluted sludge samples to a final concentration of 1 μM , resulting in an added activity of 15 $\mu\text{Ci}/\text{mg}$ of SS. The samples were incubated at 20 to 22°C for 3 h, and after incubation they were treated and investigated like the other labeled samples. A concentration of 10 to 50 nM is usually recommended for the study of microbial growth (3), but due to the high biomass concentration and a need for a certain activity for the detection with autoradiography, a higher thymidine concentration was chosen.

Autoradiographic procedure. The autoradiographic procedure followed the general procedures for autoradiography with liquid emulsions described by various authors (1, 15, 17). No precoating of the slides or heat fixing of the biomass was used. Two different autoradiographic emulsions from Amersham International plc. were tested: LM-1, designed for light microscopy, and EM-1, designed for high-resolution autoradiography by light or electron microscopy. The emulsion was melted in a water bath at 43°C in a darkroom. After the slides had been dipped in a dipping vessel containing the emulsion, the emulsion was wiped away from the back side of the slides with absorbent tissue. The slides were placed horizontally on an iced metal plate for 10 min to solidify the emulsion. They dried slowly at ambient temperature for 1 to 2 h, depending on the thickness of the emulsion layer. Finally, the slides were placed in a black sealed plastic slide box with a bag of silica gel and kept at 4°C during exposure of the autoradiographic emulsion.

After an exposure time of 2 to 10 days, the slides were developed by a standard photographic procedure. The slides were first placed in a common slide rack normally used for staining purposes, which allowed the processing of batches of 20 slides. The developer was Kodak D19 (40 g/liter of deionized water), and the stop solution was 0.5% acetic acid in deionized water. The fixation solution was 30% (wt/vol) sodium thiosulfate in deionized water. The slide rack was placed in the developer for 1 to 3 min, transferred to the stop solution for 1 min, and finally placed in the fixation solution for 4 min. It was washed in very slowly running tap water for 10 min and placed in deionized water twice for 2 min. Slides were allowed to air dry. For a detailed introduction to the theoretical background of autoradiography, the excellent work of Rogers (17) is recommended.

Microscopy techniques. Slides were inspected by various light microscopy techniques at magnifications of $\times 400$, $\times 500$, and $\times 1,000$. A positive uptake of radiolabeled substrate by filamentous bacteria was clearly seen as a dense band of silver grains on the top of the filaments. It was subjectively characterized as weak or strong labeling. Gram and Neisser stains were applied as poststainings after processing of the slides in order to identify the different filamentous microorganisms according to the Eikelboom types (7). Fluorescence staining with acridine orange (AO) and DAPI (4',6-diamidino-2-phenylidole) (19) was used to separate the autoradiographic information from that needed for localization and identification of the filamentous microorganisms. Normally, AO and DAPI stains are applied in aqueous solutions, but in this study they were applied not only as prestains after fixation with formaldehyde but also directly as poststains on the dry processed slides. Preliminary tests, where the normal AO and DAPI procedures in aqueous solution were compared with a direct staining of dry samples, with and without the autoradiographic emulsion, showed that similar stainings of the filaments were obtained by the different procedures.

RESULTS

General experience with the autoradiographic method. Both the ^{14}C - and ^3H -labeled substrates gave a good response on the autoradiographic emulsion. The activated-sludge flocs, in particular, were strongly stained with silver (Ag) grains, but the uptake of substrate by the various filaments also could be clearly observed (Fig. 1C to H). For the thicker-filament types, e.g., type 021N, it was sometimes possible to detect single cells within the filaments not taking up substrate (Fig. 1H).

The influence of incubation time on the staining intensity was investigated in several experiments, but usually no strong

FIG. 1. Autoradiographic images of activated sludge incubated with labeled substrates under aerobic conditions. (A) Pasteurized control incubated with [^{3} H]glucose. No uptake in filaments or flocs and a low background level are observed. (B) Pasteurized activated sludge incubated with [^{3} H]oleic acid. A higher background level caused by some nonspecific adsorption is observed. (C) *M. parvicella* (Mp) from WTP 1, with uptake of [^{3} H]oleic acid. (D) Type 0041 from WTP 2, with uptake of [^{3} H]glucose. (E) *N. limicola* (NI) from WTP 1, with uptake of [^{3} H]glucose (*M. parvicella* negative). (F) *N. limicola* from WTP 1, with uptake of [^{3} H]glycine. (G) Type 021N from WTP 4, with uptake of [^{3} H]glucose. (H) Example of inactive cells in filaments of type 021N incubated with glucose. Bars, 10 μm. Combined light and fluorescence microscopy of DAPI (A to G)- and AO (H)-stained samples is shown.

influence was observed (data not shown). When the filaments were incubated with the radioactive organic substrate, the uptake was often very rapid (within a few minutes), and in only a few cases was any further change in the staining intensity observed during the normal incubation period of 3 h.

A fairly large amount of radioactive tracer was used in the incubations, and the uptake of labeled organic substrates by the filamentous bacteria could easily be distinguished from the background. An increased background level could be observed with increased uptake of labeled substrate, which could indicate a certain delocalization of the labeled compounds in connection with the autoradiographic procedure. The unlabeled controls gave very low background levels of silver grains, showing that there were no problems with chemography. The pasteurized controls also gave very low background levels (Fig. 1A), except for oleic acid. Oleic acid showed some adsorption, and a slight nonspecific silver staining of filaments and floc-forming bacteria could be detected (Fig. 1B). However, an active uptake of labeled oleic acid could easily be distinguished from the adsorption (Fig. 1C).

The LM-1 and EM-1 emulsions were compared in 12 sets of slides, and LM-1 was found to be more sensitive than EM-1 (data not shown). EM-1 gave a slightly better resolution due to the smaller silver halide complexes in this emulsion (0.2 μm for the LM-1 emulsion and 0.13 μm for the EM-1 emulsion). Both emulsions had good adhesion to the dried activated-sludge samples at the slides, but great care should be taken not to disturb or detach the emulsion from the slides during photographic processing.

Histochemical stainings. Stainings were necessary to locate and identify the different filamentous microorganisms according to the Eikelboom system. Both the traditional Gram and Neisser stains could be applied as poststains after the processing of the autoradiographic emulsion. Gram staining of some of the filamentous bacteria was slightly changed, and the result of the staining should not be used for classification purposes without consideration. On the other hand, this simple staining technique gave a good basic histological staining of the filaments. Therefore, when these slides were compared with the fresh samples together with Gram and Neisser stainings prepared in the normal way, a classification according to the Eikelboom system could be made without problems.

Gram staining, and to a certain extent also Neisser staining, reduced the clarity of the autoradiographic images. Fluorescence staining techniques were therefore applied as an alternative. Both AO and DAPI gave good results, especially when a combination of transmitted-light microscopy and fluorescence microscopy was used. The DAPI stain was preferred, as this dye could be used simultaneously with the CTC staining. DAPI could be used as either a pre- or a poststain.

Uptake of different organic substrates. A panel of six organic substrates was evaluated for the substrate uptake pattern in activated-sludge samples from five different WTPs. Most of the substrates were expected to be taken up by many of the microorganisms in the sludge. All substrates were taken up by some floc-forming microorganisms, resulting in a general labeling of the flocs. Especially for acetate and glucose, a strong labeling could be observed, while the labeling with glycine and leucine was much weaker but still clear.

For the filamentous microorganisms, the uptake pattern was more complex, and clear differences between different filament types were observed. Furthermore, among the same filament type there were some differences in uptake patterns between WTPs. The results are summarized in Table 1.

M. parvicella was present in WTPs 1 and 2. In both cases it was strongly labeled with oleic acid (Fig. 1C), but none of the

TABLE 1. Response to radiolabeled organic substrates, CTC reduction, and thymidine incorporation by different filamentous microorganisms in activated sludge under aerobic conditions

Substrate or indicator	Labeling, reduction, or incorporation ^a by:							
	<i>M. parvicella</i>		<i>N. limicola</i>		Type 0041		Type 021N	
	WTP 1	WTP 2	WTP 1	WTP 3	WTP 2	WTP 4	WTP 4	WTP 5
Acetate	—	—	+	—	—	—	++	++
Glucose	—	—	++	++	++	++	++	—
Ethanol	ND	—	ND	ND	—	—	—	ND
Glycine	—	—	+	—	—	—	+	—
Leucine	—	—	+	—	+	—	+	—
Oleic acid	++	++	++	++	+	—	—	+
CTC	+	+	++	++	++	++	+	+
Thymidine	—	—	+/-	+/-	+/-	ND	ND	—

^a —, no reaction; +, weak reaction; ++, strong reaction; +/-, reaction of only a minor part of the filaments present; ND, not determined.

simple substrates were taken up even after 24 h of incubation. *N. limicola* was present in WTPs 1 and 3, where it was strongly labeled with glucose (Fig. 1E) and oleic acid. In WTP 1, weak labeling with acetate, glycine (Fig. 1F), and leucine was observed. Type 0041 was present in WTPs 2 and 4, where it was strongly labeled with glucose (Fig. 1D), and in WTP 2, weak labeling with leucine and oleic acid was observed. Type 021N was present in WTPs 4 and 5. In WTP 4 it was strongly labeled with both acetate and glucose (Fig. 1G) and weakly labeled with glycine and leucine. In WTP 5 it was strongly labeled with acetate but weakly labeled with oleic acid.

Investigation of viability and activity. All filament types in the investigated sludge samples showed respiratory activity as indicated by reduction of CTC (Table 1). In each sludge sample, however, some filaments were not active, but usually this was only a minor fraction. Major differences in intensity among different filament types were found. The intracellular crystals from CTC reduction in *M. parvicella* appeared to be small with a low fluorescence intensity (Fig. 2A), while the other filament types showed a higher intensity. Type 021N normally showed intracellular crystals with regular intervals of two to six cells (Fig. 2B). For type 021N the CTC reduction pattern did not correspond to the pattern of uptake of radiolabeled substrates, as the filaments in most cases were uniformly labeled with silver grains along the filaments, as shown in Fig. 1G.

The CTC reduction was usually investigated by using slides without the autoradiographic emulsion, because the silver grain covered and masked the fluorescence signal from the CTC crystals; thus, a direct correlation between activity and substrate uptake was difficult to obtain. Simultaneous investigation of CTC response and substrate uptake on the same slide was possible only in cases where the filament was not too strongly labeled with the tested substrates.

Thymidine incorporation was tested for only some of the sludges (Table 1). Incorporation could be detected for many floc-forming bacteria and for some *N. limicola* and type 0041 filaments but not for *M. parvicella* and type 021N.

Effect of electron acceptor conditions. Uptake of oleic acid under aerobic and anaerobic conditions was tested for *M. parvicella* and *N. limicola* in activated sludge from WTP 1. Both filament types took up oleic acid under aerobic and anaerobic conditions (data not shown). The uptake of oleic acid under anaerobic conditions was lower than that observed under aerobic conditions but was still clear. The presence of nitrate did not influence the uptake under anaerobic conditions.

FIG. 2. (A) CTC reduction by *M. parvicella*. (B) CTC reduction by type 021N. (C) Thymidine incorporation in type 0041 (*M. parvicella* [Mp] negative). (D) Thymidine incorporation in *N. limicola* (NI). Bars, 10 μm. (A and B) Fluorescence microscopy; (C and D) combined light and fluorescence microscopy of DAPI-stained samples.

DISCUSSION

The results presented here demonstrate that microautoradiography may be employed to examine substrate uptake by activated-sludge filamentous microorganisms under conditions mimicking the applied process conditions in the treatment plants. The method had a low background level, a positive uptake of radiolabeled substrates by filamentous microorganisms was very easy to see, and for most substrates tested there were no problems with chemography or adsorption. The non-specific adsorption of the hydrophobic compound oleic acid shows, however, that controls always must be included. Good resolution was obtained with both ^{14}C - and ^3H -labeled substrates. In theory, a higher resolution could be obtained with the ^3H due to the lower energy of the β -particles (15). In this study, however, no large difference between the ^{14}C - and ^3H -labeled substrates could be observed. The difference in sensitivity and resolution obtained with the two autoradiographic emulsions LM-1 and EM-1 indicates that care should be taken in emulsion selection for specific studies. From the results presented here, and under the conditions stated, LM-1 expressed higher sensitivity.

Results pertaining to substrate uptake patterns by the filamentous microorganisms are mainly qualitative. The method requires further refinement for acquisition of reliable quantitative and kinetic information. A reduction in the added radioactivity may improve the resolution in time series and enlarge the differences found under different substrate and redox conditions. It is also important to note that the incubation methods used were designed to test the immediate uptake response to the substrate added and not to give information about the capability of microorganisms to adapt to the substrate. Moreover, uptake illustrates only the presence and ac-

tivity of a transport system for the substrate added and not whether bacteria are able to respire and grow under the given conditions or whether the substrate was transformed into storage products.

M. parvicella is by far the most common filamentous microorganism in nutrient removal plants, and it often causes severe bulking and foaming problems (13, 21). Only limited information about the physiology of this filament exists, and effective control strategies have not been established. It has previously been studied in pure culture by Slijkhuys (18), where oleic acid was identified as one of very few organic substrates utilized by this organism. Unfortunately, this strain is not available any more, and the generality of the original results has been questioned by various researchers due to experiences gained from full-scale operation of WTPs (10, 21). Based on these experiences, it has been suggested that *M. parvicella* also should be able to utilize more simple substrates such as ethanol. However, our results support those of Slijkhuys, in that oleic acid is a suitable substrate for *M. parvicella* and simple organic compounds are not. Moreover, our results strongly suggest that *M. parvicella* was able to take up oleic acid also under anaerobic conditions. Whether it can grow under anaerobic or denitrifying conditions is still uncertain, as the uptake of labeled substrate was lower than that observed under aerobic conditions.

N. limicola can also be found in high numbers in some nutrient removal plants. This filament showed a high uptake of glucose and oleic acid and, at one of the plants, a minor uptake of acetate, glycine, and leucine. These observations are more or less in accordance with studies of organisms in pure culture by Novak and Brown (14). They found good growth on a variety of carbohydrates, some alcohols, and, interestingly, also on Tween 80 (polyoxyethylenesorbitan mono-oleate). Growth

on acetate, citrate, and succinate was poor. Novak and Brown (14) demonstrated a capability of *N. limicola* to ferment carbohydrates under anaerobic conditions. We did not test the uptake of glucose under anaerobic conditions, but the observed anaerobic uptake of oleic acid supports their findings that *N. limicola* may be active under anaerobic conditions.

Type 0041 is very common in Danish nutrient removal plants (2, 13). This type showed good uptake only of glucose and, in one of the two samples, minor uptake of leucine and oleic acid. This filamentous microorganism has not been examined in pure culture due to difficulties encountered in its isolation, which result from a substantial attached growth of unicellular or short-chained microorganisms. The attachment may also disturb the autoradiographic images, such that considerable care must be exercised in their evaluation.

Type 021N is rarely seen in high numbers in nutrient removal plants, but it can cause severe bulking problems in conventional WTPs without nutrient removal (21). The type 021N filament was strongly labeled with both acetate and glucose in WTP 4, whereas it was strongly labeled only with acetate in WTP 5. For the other test substrates, differences in the substrate uptake pattern between type 021N filaments from the two sludges were detected. This existence of several types or strains of type 021N with different growth capabilities is in agreement with the results presented by Williams and Unz (23). They demonstrated that their isolated filamentous microorganisms, in general, had a very specific use of organic substrates, not only between related species but also among different strains within the same species. However, all strains of type 021N tested by Williams and Unz (23) were able to utilize both acetate and glucose, in contrast to our observation. In accordance with our observation, they also found that only some strains of type 21N grew on leucine and ethanol.

Here the limitation of Eikelboom's classification system becomes obvious. Type 021N is a highly variable organism with only a few distinctive characteristics, so it is unknown whether the filaments in the two treatment plants were adapted to different substrates or whether they could be different species or strains. This study, therefore, points to a need for better taxonomic characterization and identification, for which the development and application of gene probes would be a significant advance (20).

Type 021N can use reduced sulfur compounds as an energy source (22, 23). In this study, no sulfur granules were observed in the cells of type 021N, neither in the full scale plants nor in connection with the experiments, which could have indicated an influence of reduced sulfur compounds on the uptake of carbon compounds.

The use of CTC reduction to assess viability of the microorganisms was found to be helpful if filaments failed to take up the tested substrates. However, since at least one substrate always showed positive uptake, thereby indicating activity of the filaments, this viability test was not critical in this study. However, in general it is recommended to test the filaments for metabolic activity in sludge samples before incubations with radiotracers are initiated. It is also noteworthy that uptake of [³H]labeled thymidine was not observed in all types of filamentous microorganisms under the tested conditions, although the organisms were viable as indicated by uptake of radiolabeled substrates and reduction of CTC. Since the reasons underlying this phenomenon remain to be elucidated, this method is not recommended.

The results clearly illustrate that filamentous microorganisms causing bulking can be very specialized in their substrate uptake and utilize only a single or very few organic substrates. Detailed information about the organic substrates (and elec-

tron acceptors) used by the different filamentous microorganisms responsible for bulking is therefore essential in order to improve control strategies. This study also shows that a more complete understanding about the presence of specific substrates in raw wastewater is necessary. These substrates may be produced by floc-forming bacteria in the sludge as well, but presently nothing is known about such interspecies interactions in activated sludge. Characterization of raw wastewater is based mainly on chemical oxygen demand and nutrient measurements, but these alone are not sufficient for a deeper understanding of bulking problems. The concentration of the tested organic substrate in the wastewater at the different treatment plants was not measured. Acetic acid is generally present in most wastewater (11), such that most activated sludges would be expected to be adapted to this substrate. The levels of glucose, ethanol, and amino acids in normal wastewater are undoubtedly low, except when they are discharged by specific industries or added as organic substrates for denitrification. Only very limited information on the levels of oleic acid and other long-chain fatty acids in wastewater is available. Raunkjær et al. (16) found that a substantial part (31%) of organic matter in wastewater belongs to the lipid fraction. *M. parvicella* and, to a certain extent, *N. limicola* seem to depend on long-chain fatty acids for growth. Hence, a better understanding of the degradation of the lipid fraction in activated sludge is likely to be very important in order to understand why *M. parvicella* prevails, especially in nutrient removal activated-sludge systems.

Our results show that the autoradiographic method provides very detailed information on which organic compounds and, to a certain extent, electron acceptors can be utilized by the different filaments in a specific sludge. The method is therefore very valuable as a supplement to physiological studies with pure cultures, where the validity of extrapolation of results to mixed cultures in activated sludge remains unknown.

ACKNOWLEDGMENTS

This study was supported by the Danish Academy of Technical Sciences ATV under an Industrial Ph.D. fellowship and by the European Commission under the ENVIRONMENT program.

REFERENCES

1. Amersham International plc. 1989. Nuclear emulsions for high resolution microautoradiography. Product information and guidelines for the LM-1 and EM-1 emulsions. Amersham International plc., Buckinghamshire, England.
2. Andreasen, K., and L. Sigvardsen. 1996. Experiences with sludge settleability in different process alternatives for nutrient removal. *Water Sci. Technol.* 33:137-146.
3. Bell, R. T. 1993. Estimating production of heterotrophic bacterioplankton via incorporation of tritiated thymidine, p. 495-503. In P. Kemp, B. Sherr, E. Sherr, and J. Cole (ed.), *Aquatic microbial ecology*. Lewis Publishers, Boca Raton, Fla.
4. Carman, K. R. 1990. Radioactive labeling of a natural assemblage of marine sedimentary bacteria and microalgae for the trophic studies: an autoradiographic study. *Microb. Ecol.* 19:279-290.
5. Edwards, M. C., and J. P. Blakeman. 1984. An autoradiographic method for determining nutrient competition between leaf epiphytes and plant pathogens. *J. Microsc.* 33:205-212.
6. Eikelboom, D. H. 1975. Filamentous organisms observed in activated sludge. *Water Res.* 9:365-388.
7. Eikelboom, D. H., and H. J. J. van Buijsen. 1983. Microscopic sludge investigation manual. Report A94a. IMG-TNO, Delft, The Netherlands.
8. Ellis, B., and J. A. Stanford. 1982. Comparative photoheterotrophy, chemoheterotrophy and photolithotrophy in a eutrophic reservoir and an oligotrophic lake. *Limnol. Oceanogr.* 27:440-454.
9. Griebel, T., G. Schaule, J. Secker, and H.-C. Flemming. 1996. Bestimmung der stoffwechselaktiven bakterien im belebtschlamm, p. 155-168. In H. Lemer, T. Griebel, and H.-C. Flemming (ed.), *Ökologie der Abwasserorganismen*. Springer-Verlag, Berlin, Germany.
10. Holmström, H., J. Bosander, A. Dahlberg, A. Dillner, L. Flyborg, and K. Jokinen. 1996. Severe bulking and foaming at the Himmerfjärden WWTP. *Water Sci. Technol.* 33:127-135.

11. Hvitved-Jacobsen, T., K. Raunkjær, and P. H. Nielsen. 1995. Volatile fatty acids and sulfide in pressure mains. *Water Sci. Technol.* **31**:169–179.
12. Jenkins, D. 1992. Towards a comprehensive model of activated sludge bulking and foaming. *Water Sci. Technol.* **25**:215–230.
13. Kristensen, G. H., P. E. Jørgensen, and P. H. Nielsen. 1994. Settling characteristics of activated sludge plants with biological nutrient removal. *Water Sci. Technol.* **29**:157–165.
14. Novak, G., and G. D. Brown. 1990. Characterisation of *Nostocoida limicola* and its activity in activated sludge suspension. *Res. J. Wat. Pollut. Control Fed.* **62**:137–142.
15. Prescott, D. M. 1964. Autoradiography with liquid emulsions. *Methods Cell Physiol.* **1**:365–370.
16. Raunkjær, K., T. Hvitved-Jacobsen, and P. H. Nielsen. 1994. Measurement of pools of protein, carbohydrate and lipids in domestic wastewater. *Water Res.* **28**:251–262.
17. Rogers, A. W. 1977. *Techniques of autoradiography*. Elsevier/North-Holland Biochemical, New York, N.Y.
18. Slijkhuis, H. 1983. *Microthrix parvicella*, a filamentous bacterium isolated from activated sludge: cultivation in a chemically defined medium. *Appl. Environ. Microbiol.* **46**:832–839.
19. Suzuki, M. T., E. B. Sherr, and B. F. Sheer. 1993. Dapi direct counting underestimates bacterial abundance and average cell size compared to AO direct counting. *Limnol. Oceanogr.* **38**:1566–1570.
20. Wagner, M., R. Amann, P. Kämpfer, B. Assmus, A. Hartmann, P. Hutzler, N. Springer, and K.-H. Schleifer. 1994. Identification and *in situ* detection of Gram-negative filamentous bacteria in activated sludge. *System. Appl. Microbiol.* **17**:405–417.
21. Wanner, J. 1994. *Activated sludge bulking and foaming control*. Technomic Publishing Company, Inc., Lancaster, Pa.
22. Williams, T. M., and R. F. Unz. 1985. Filamentous sulfur bacteria of activated sludge: characterization of *Thiothrix*, *Beggiatoa*, and Eikelboom type 021N strains. *Appl. Environ. Microbiol.* **49**:887–898.
23. Williams, T. M., and R. F. Unz. 1989. The nutrition of *Thiothrix*, type 021N, *Beggiatoa* and *Leucothrix* strains. *Water Res.* **23**:15–22.