Chlorine-Susceptible and Chlorine-Resistant Type 021N Bacteria Occurring in Bulking Activated Sludges

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Two filamentous bacteria causing bulking in two activated sludges were examined. Investigations using morphological features, staining techniques, and fluorescent in situ hybridization identified both filaments as type 021N. However, an examination of the effect of chlorine on the sludges revealed a chlorine-susceptible type 021N in one sludge and a chlorine-resistant type 021N in the other.

The performance of the activated sludge process is often determined by the gravity separation between the treated water and the sludge in the final clarifier. The proliferation of filamentous bacteria in the microbial community of the sludge, referred to as “filamentous bulking,” has often been reported to hamper this solid-liquid separation (7, 18). Filamentous bulking results in less dense and less settleable sludge flocs, and in severe cases the operation of the plant can be totally compromised. Addition of disinfectants (mostly chlorine based) to the sludge to selectively kill off the causative filamentous bacteria is often used in practice as a short-term and cost-effective solution (7). Jenkins et al. (7) presented sludge chlorination as a method of choice in the United States to combat filamentous bulking. They reported several case histories of successful control of bulking at full-scale treatment plants using chlorine and described the method as a “universal success” with any filamentous bacteria. Yet cases of partial success and cases of failure can be found in the literature. Lakay et al. (11) obtained only a partial elimination of Microthrix parvicella bacteria at a high chlorine dose. Hwang and Tanaka (6) found in batch tests that M. parvicella remained intact at very high chlorine doses, while the microbial flocs were completely destroyed. Fontaine (3) could not overcome filamentous bulking in a pilot plant using chlorination. More recently, Madoni et al. (12) reported in a survey in Italy that filamentous bulking results in less dense and less settleable sludge flocs, and in severe cases the operation of the plant can be totally compromised. Addition of disinfectants (mostly chlorine based) to the sludge to selectively kill off the causative filamentous bacteria of the sludge in the final clarifier. The proliferation of filamentous bacteria in the microbial community of the sludge, referred to as “filamentous bulking,” has often been reported to hamper this solid-liquid separation (7, 18). Filamentous bulking results in less dense and less settleable sludge flocs, and in severe cases the operation of the plant can be totally compromised. Addition of disinfectants (mostly chlorine based) to the sludge to selectively kill off the causative filamentous bacteria is often used in practice as a short-term and cost-effective solution (7). Jenkins et al. (7) presented sludge chlorination as a method of choice in the United States to combat filamentous bulking. They reported several case histories of successful control of bulking at full-scale treatment plants using chlorine and described the method as a “universal success” with any filamentous bacteria. Yet cases of partial success and cases of failure can be found in the literature. Lakay et al. (11) obtained only a partial elimination of Microthrix parvicella bacteria at a high chlorine dose. Hwang and Tanaka (6) found in batch tests that M. parvicella remained intact at very high chlorine doses, while the microbial flocs were completely destroyed. Fontaine (3) could not overcome filamentous bulking in a pilot plant using chlorination. More recently, Madoni et al. (12) reported in a survey in Italy that use of chlorination was successful in only 63% of the cases. Most often, no relevant explanation was offered for these unsuccessful cases of sludge chlorination. Thoroughly examined unsuccessful cases have been ascribed to the presence of well-known resistant filamentous bacteria with hydrophobic cell walls such as M. parvicella and Nostocoida limicola (6, 18). This paper presents and discusses the effects of chlorine on two sludges, one dominated by a chlorine-vulnerable type 021N bacterium and the other dominated by a chlorine-resistant type 021N bacterium, as a relevant explanation of the discrepancies noted in the effectiveness of chlorine in the cure of bulking sludge.

Experimental procedures. The two types of sludges (sludges A and B) involved in this study originated from two industrial wastewater treatment plants (plants A and B) located in the region of Ghent, Belgium. Plant A employed a two-stage activated sludge system fed continuously with the wastewater resulting from the manufacture of mayonnaise and soups. The sludge loading rate (SLR) of sludge A was 0.4 g of COD (g of VSS · day)−1 (where COD is the chemical oxygen demand and VSS is the volatile suspended solids). The 5-day biochemical oxygen demand (BOD)5/N/P ratio for this plant was variable, and addition of extra nutrients (N and P) was often necessary. Plant B, a small and poorly supervised plant, operated at an average SLR of 0.3 g of COD (g of VSS · day)−1. On the day of sampling, the value measured gave an SLR of 0.6 g of COD (g of VSS · day)−1, probably due to a recent wastage of overestimated excess sludge. This plant employed a sequencing batch reactor (SBR) system using approximately 20 h of aeration followed by 2 to 3 h of sedimentation and 1 h of effluent decantation. Thus, unlike plant A, plant B was fed discontinuously with the wastewater generated from the processing of potatoes to make chips. For both systems, sludge samples were collected and transported to the lab within less than 2 h in vessels no more than half filled, thus providing for a headspace with oxygen. Both sludges reached the laboratory on the same day and were tested immediately for the appropriateness of chlorine to cure the filamentous bulking. The rest of the samples were kept at 4°C in vessels no more than half filled. The pHs were 7.3 for sludge A and 7.1 for sludge B. The concentrations of mixed-liquor suspended solids (MLSS) and VSS were 3.00 and 2.43 g liter−1, respectively, for sludge A and 2.00 and 1.69 g liter−1, respectively, for sludge B. Filamentous bacteria were similarly abundant in both sludges and reached level 6 (excessive) on the scale of 0 (none) to 6 based on the filament scoring system proposed by Jenkins et al. (7). Sludge A was dominated by larger flocs (up to 700–μm diameters), while sludge B contained mostly smaller flocs (60–μm diameters). The sludge volume indexes (SVIs) were 667 ml g−1 for sludge A and 960 ml g−1 for sludge B, compared to 150 ml g−1 for a well-settling sludge.

A stock solution of 1.90 g of Cl2 liter−3, prepared by the dilution of a commercially available solution of sodium hypochlorite (12% Cl2 = 38.04 g of Cl2 liter−3) with distilled water, was used. For each type of sludge, the following doses were
added to 1-liter volumes of sludge contained in 2-liter Erlenmeyer flasks and used as activated sludge units: 0 (control), 2, 5, 10, 15, and 40 g of Cl₂ (kg of MLSS)⁻¹ (designated treatments T0, T1, T2, T3, T4, and T5, respectively). After 20 min on a rotary shaker (90 rpm), 5 ml of mixed liquor was taken from each Erlenmeyer flask for viability staining and 200 ml was taken for oxygen uptake rate (OUR) measurements. The remaining sludge (about 800 ml) for each treatment was further fed with 200 ml of a skim milk solution at an SLR of 0.4 g of COD (g of VSS · day)⁻¹ for 24 h using materials and equipment similar to those described by Seka et al. (16). After this period of incubation, the SVI and the residual soluble COD were measured for each treatment. Sludge samples were examined microscopically with light microscopy (Polyvar; Reichert-Jung, Vienna, Austria), and digital images were captured with a charge-coupled device camera (Hamamatsu Photonics GmbH, Herschching, Germany). Filament scoring (7) was further determined for each treatment.

Floc diameter and filament length and width were determined with digital image analysis software (Micro Image 4.0; Olympus Optical Co., Hamburg, Germany). COD, MLSS, and VSS testing were performed as described by the American Public Health Association (5). The SVI was measured as the diluted SVI (5). OUR measurements were conducted using equipment and protocol similar to those described by Gernaey et al. (4). Viability staining was performed using the commercial Live/Dead stain (L-13152) (Live/Dead BacLight bacterial viability kit technical information sheet, Molecular Probes Europe BV, Leiden, The Netherlands) and equipment and procedures similar to those described by Seka et al. (16). For each sludge, three preparations per treatment were examined by fluorescent microscopy. Bacteria with intact membranes were
stained green and scored as “alive,” and those with damaged membranes were stained red and scored as “dead.” Digital images were also captured.

The main filamentous bacteria in both sludges were first identified using the criteria of Jenkins et al. (7) and Eikelboom (2). These criteria include morphological features and results of Gram and Neisser staining. The sulfur oxidation test using sodium thiosulfate and the sodium hypochlorite test for the visualization of sheaths described by Jenkins et al. (7) were also conducted. The presence of a sheath was further investigated by means of scanning electron microscopy using a JEOL JSM 840 instrument. Samples of 10 ml of each sludge type were first centrifuged and subjected to double fixation with glutaraldehyde and OsO₄ (20) before being subjected to scanning electron microscopy. The identification of the filamentous bacteria dominating both sludges was further double-checked by means of fluorescent in situ hybridization (FISH). On the basis of the morphological features, probe 21N (17), complementary to the 16S rRNA sequences of the filamentous bacterium type 021N, was used. This probe, obtained from Genset (Paris, France), was labeled at its 5’ end with Cy3. Probe EUB338 (Eurogentec, Liège, Belgium), labeled with fluorescein and hybridizing with all bacteria, was used simultaneously with probe 21N to check the vitality of the filamentous bacteria that would not react to probe 21N. The hybridization was carried out as described by Wagner et al. (17) and Manz et al. (13). FISH preparations were viewed by means of an Eclipse E600 microscope (Nikon Europe BV, Badhoevedorp, The Netherlands).

Characteristics and identification of the main filamentous bacteria in both sludges. The filaments dominating both sludges were very long (0.3 to 1.2 mm in sludge A and 0.5 to 1.1 mm in sludge B), unbranched, coiled or smoothly curved, Gram and Neisser stain negative, sheathless, and multicellular. Individual cells within the filaments were not uniform in shape (discoid or barrel) or size (1.0 to 1.8 μm in width and 0.9 to 1.3 μm in length in sludge A and 1.5 to 1.8 μm in width and 0.8 to 1.5 μm in length in sludge B). Septa between the cells were clearly visible. Before the sulfur oxidation test, no sulfur granules were present in sludge B while very few could be seen in sludge A. After the sulfur oxidation test, few granules were present in sludge A and very few were present in sludge B. Overall, the filaments dominating both sludges showed quite similar morphological traits and were identified as type 021N. When FISH was used, probe 21N hybridized with the filamentous type dominating both sludges, confirming the identification according to morphology. The fluorescence obtained with both probes was in general of low intensity, and it could be seen that not all cells in a given filament produced fluorescence. Probe EUB338 also hybridized with filamentous bacteria, which did not hybridize with probe 21N (data not shown). The weak fluorescence generally observed on the filaments could be explained by the fact that the bacteria were less active. Indeed, prior to hybridization, the sludge samples were kept at 4°C for 2 weeks, corresponding to the delay in receiving the molecular probes. The viability staining performed at the end of the 2 weeks revealed a high number of dead cells on most of the filaments.

Effect of chlorine on the sludges. The effect of chlorine on the sludges was studied using a variety of methods and parameters: viability staining, OUR inhibition, inhibition of COD removal, decrease of filament abundance, and decrease of SVI. Overall, the viability staining evidenced increasing numbers of damaged type 021N bacteria and largely intact microbial flocs in sludge A with chlorine doses increasing from treatment T0 to T4. Microbial flocs were significantly affected only with treatment T5, at which dose all filaments were killed. In contrast, type 021N bacteria were largely intact and microbial flocs were increasingly damaged in sludge B with chlorine doses increasing from treatment T0 to T4. Type 021N filaments were significantly damaged at treatment T5, at which dose no microbial floc survived. This contrasting effect of chlorine on the sludges was best illustrated by the results from treatments with 15 g of Cl₂ (kg of MLSS)⁻¹ (Fig. 1). It suggests the existence of a chlorine-susceptible type 021N in sludge A and a chlorine-resistant type 021N in sludge B. The results obtained for the OUR, the residual soluble COD, the SVI, and the filament abundance are shown in Fig. 2. Standard deviations were derived from the coefficients of variation determined according to OUR (sludge A, 10%; sludge B, 12%), COD (A, 12%; B, 15%), and SVI (A, 8%; B, 10%).
13%), SVI (A, 6%; B, 4%), and filament scoring (0% for A and B) from four replicates of treatment T0. According to the OUR and COD measurements, the microbial activity decreased with increasing chlorine doses for both sludges. For the same chlorine dose, this decrease appeared more rapidly in the case of sludge B than of sludge A. SVI and filament abundance decreased with increases in the chlorine dose in the case of sludge A, while in the case of sludge B these parameters hardly varied. The micrographs corresponding to the control and to treatment T4 (Fig. 3) illustrate the different evolutions of filament content between sludge A and sludge B after the 24-h incubations on a shaker. These observations are in agreement with the preliminary effects of chlorine on the filamentous bacteria as revealed by the viability staining. Indeed, the rationale for curing filamentous bulking by means of chlorination is that the causative filamentous bacteria protruding from flocs are more exposed to toxic compounds than the bacteria inside the flocs (18). Therefore, a reduction in the filamentous bacteria in the microbial population of a bulking sludge, accompanied by an improvement of the sedimentation of the sludge, is a sign of the effectiveness of chlorine. In the present study, this was observed with sludge A but not with sludge B. Thus, chlorination was effective against type 021N in sludge A but not in sludge B, although the filaments were protruding out of flocs in both sludges. The decrease in microbial activity with increasing chlorine dose shown by OUR and residual COD measurements confirms the adverse effects of chlorine on the microbial populations of the sludges. This excludes the hypothesis of an important influence of the chlorine demand from the water content of the sludges. Nevertheless, OUR and residual COD measurements did not reflect the effects of chlorine on the filamentous bacteria alone. The decrease in microbial activity was even more pronounced with sludge B than sludge A. This result confirms the inappropriateness of the activity parameters for monitoring the effect of chlorine on filamentous bulking sludges mentioned by Bitton and Koopman (1). It should be mentioned that all the observations and trends reported above were confirmed by repeating the experiments on the effects of chlorine on the sludges (data not shown).

Thus, viability staining, filament abundance, and SVI, used to examine the effects of chlorine on the filaments, led to the identification of a chlorine-vulnerable type 021N bacterium in sludge A and a chlorine-resistant type 021N bacterium in sludge B. Bacteria within the same strain or species that exhibit variations in resistance to chlorine have already been mentioned by other authors (10, 14). Type 021N is traditionally known as a chlorine-susceptible filamentous bacterium (7). The exact causes for the development of the chlorine-resistant
variant that was observed in this study have not been investigated. However, the growth resulting from the starvation conditions imposed by the endogenous periods of the sequencing batch reactor is suspected to be the most probable cause. Indeed, according to Roszak and Colwell (15), factors such as starvation may alter cell membrane composition and affect its permeability. The fact that the disinfecting efficiency of chlorine depends on penetration rate through the cell wall (19) suggests that type 021N in sludge B developed a less permeable cell wall. Moreover, the slow or difficult staining of certain bacteria by viability stains mentioned in other studies (9, 16) and attributed to poor cell membrane permeability was also noticed with type 021N in sludge B (data not shown).

The two different reactions of type 021N to chlorine may also imply the existence of different species of type 021N bacteria, as suggested by Kanagawa et al. (8). This could not be confirmed by our relatively limited FISH study. Unfortunately, additional FISH assays of freshly collected sludge samples were unsuccessful because, due to the dynamic feature of the activated sludge, the microbial populations had changed.

So far, unsuccessful bulking correction using chlorination is poorly understood and is often attributed to inappropriate application. The results of this study, particularly concerning sludge B, showed that chlorination will not always be efficient in curing bulking, even if the rules established by Jenkins et al. (7) are followed. These results offer striking evidence that may in curing bulking, even if the rules established by Jenkins et al. (7) are followed. These results offer striking evidence that may

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