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Full Paper

Impact of pre-treatments on nitrifying bacterial community analysis from wastewater using fluorescent in situ hybridization and confocal scanning laser microscopy

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Fluorescent in situ hybridization (FISH) and confocal scanning laser microscopy (CSLM) are the key techniques used to investigate bacterial community structure at wastewater treatment plants. An optimum nitrifying bacterial population is necessary for nitrification, which plays a significant ecological role in regulating the overall quality of water. Nitrifying bacteria mainly appear as dense aggregates within activated sludge flocs. The impacts of five different pre-treatment methods (physical, chemical, enzymatic and combinations) on floc dispersion from two different wastewater treatment plants were determined. The effect of pre-treatment on the enumeration of the nitrifying bacterial population was also investigated. This study on floc dispersion using CSLM images showed sonication was the superior method for all the samples tested, irrespective of the sludge type. For samples from industrial wastewater plants, an optimized sonication level of 8 W for 8 min could reduce the floc size to 10 μm, whereas for domestic wastewater samples, the floc size was reduced to 10 μm at 8 W for 5 min. The maximum number of nitrifying bacterial cells was observed at this optimized level for different samples. A decrease in the number of cells was observed beyond this optimized level for both the plants. The results presented here highlight the importance of optimizing pre-treatment methods for different types of wastewater for accurate bacterial community analysis using FISH-CSLM.

Key Words——CSLM; FISH; nitrifying bacteria; pre-treatment; wastewater

Introduction

The activated sludge process is one of the most extensive biological processes used for the treatment of wastewater, and is dependent on the formation and arrangement of microbial flocs to which many bacteria are attached (Snidaro et al., 1997). This bacterial aggregation and flocculation has been attributed primarily to an exopolymeric matrix. The structural arrangement of activated sludge flocs is too complicated to assess with conventional techniques alone (Bourrain et al., 1999).

CSLM in combination with FISH has been effectively used for the direct analysis of whole cells within microbial populations in activated sludge systems. FISH represents a highly efficient technique that enables the culture-independent identification of bacteria (Carr et al., 2005). Combining FISH with CSLM and digital image analysis makes it feasible to analyze the spatial distribution of nitrifying bacteria in activated sludge flocs and removes disturbing auto-fluorescent signals.

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The optical sectioning properties of CSLM also significantly improved the in situ detectability of nitrifying bacteria in activated sludge (Delatolla et al., 2009).

There are reports of considerable variation in the nitrifying bacterial population among activated sludge samples from industrial and municipal wastewater treatment plants (Layton et al., 2000). Nitrifying bacteria mainly appear as dense aggregates within activated sludge flocs (Manser et al., 2005). For accurate quantification, it is essential to disrupt the flocs for the release of the bacterial cells from within the sludge sample (Biggs and Lant, 2000). Sonication is one of the commonly used techniques to disperse activated sludge flocs and enumerate bacteria (Daims et al., 2006). This technique does not contaminate the sample and is efficient for dispersing bacterial aggregates, provided excessive energy which could lyse the cells and contaminate the sample with intracellular polymers, is not used (Sears et al., 2005). Other methods tested include enzymatic pre-treatment with lysozyme. Lysozyme opens up the peptidoglycan layer and allows for increased cell permeability which might otherwise result in insufficient penetration of high molecular weight reagents into bacterial cells (Moter and Göbel, 2000). Chemical pre-treatment with a non-ionic surfactant (nonidet) was also tested because it alleviates cell clumping without any obvious damage to the cells and when used at appropriate concentrations, enhances cell dispersion (Stahl and Amann, 1991).

Although there are few reports on floc dispersion, the effect of pre-treatments on the microbial community has not been well analyzed. Many of these procedures have been employed for years; however, little work has been done to compare the efficiency of these pre-treatments on different types of wastewater. Thus, the aim of this study was to investigate the impact of pre-treatments on bacterial floc dispersion and to expose single cells for accurate enumeration of the nitrifying bacterial community, using FISH-CSLM.

Materials and Methods

Sampling and cell fixation. Samples of mixed liquor were collected from the aeration basins of two different activated sludge treatment plants, treating domestic and industrial wastewater. Sample bottles were half-filled with the mixed liquor to maintain aerobic conditions during sample transit, and stored at 4°C until further use. Samples were washed twice with distilled water and re-suspended in phosphate buffered saline solution (PBS: 130 mM sodium chloride, 10 mM sodium phosphate buffer, pH 7.2). For in situ hybridization, all activated sludge samples were fixed immediately in 4% paraformaldehyde (Amann, 1995), to render the nitrifying bacterial cells permeable to probes. Fixed samples were stored in a 1 : 1 mixture of PBS and absolute ethanol at −20°C until further hybridization.

Pre-treatment of activated sludge samples using physical, chemical and enzymatic methods. For physical treatment, floc dispersion was achieved by sonication of the fixed sample, placed in an ice bath, using a probe sonicator (Virtis, Virsonic 100; USA). Sonication was optimized by comparing the effects of five power levels of 5, 6, 7, 8 and 9 W. The duration of sonication varied from 3 to 9 min at a time interval of 1 min for each power level. For chemical treatment, all fixed samples were treated with Nonidet P-40 (Sigma, Germany) 0.1% (v/v). A combination of pre-treatments involving sonication together with nonidet was also tested.

For enzymatic treatment, all fixed samples were treated with lysozyme (Sigma). Lysozyme permeabilization buffer (1 mg/ml) was applied to samples for 30 min at 37°C (Wallner et al., 1993). Thereafter samples were exposed to a combination pre-treatment method of sonication together with lysozyme.

Whole cell hybridization and DAPI staining. Teflon-coated microscope slides were pre-treated with 1 : 10 (v/v) Poly-L-Lysine solution (Sigma), which is an effective tissue adhesive. Poly-L-Lysine solution was brought to room temperature (18–26°C) and slides were immersed in this solution for 5 min. Slides were subsequently dried overnight at room temperature before use. The samples for all five pre-treatment methods were mixed thoroughly and hybridized with 16S rRNA-targeted oligonucleotide nitrifying bacterial probes (Table 1). Hybridized, pre-treated samples (10 µl) were applied to each well. Spots were air dried prior to dehydrating through an ethanol series (60, 80 and 100%, v/v) for 3 min. Filter paper was soaked in the appropriate hybridization solution and placed in a polypropylene tube to allow the chamber to equilibrate for 15 min at 46°C. The spotted slides were placed in the pre-warmed chamber and incubated to allow for hybridization at 46°C for 2 h. Wash buffer was allowed to pre-heat in a water bath at 48°C. Thereafter, hybridized slides were placed into the wash buffer and into a water bath for 20 min at 48°C. Slides were thereafter

Table 1. List of 16S rRNA-targeted oligonucleotide nitrifying bacterial probes for FISH.

Probe	Sequence	Specificity	Reference
NIT3	CCTGTGCTCCATGCTCCG	*Nitrobacter* sp.	Wagner et al. (1996)
NEU	CCCCTCTGCTGCACTCTA	*Nitrosomonas* sp.	Juretschko et al. (1998)
Nsv443	CCGTGACCGTTTCGTTCCG	*Nitrospira* sp.	Mobarry et al. (1996)

rinsed twice with 1× PBS and air-dried. Nitrifying bacterial cells were thereafter stained with 10 µl of 0.25 µg/ml DAPI (4,6-diamidino-2-phenylindole dihydrochloride hydrate — Sigma) for 10 min in the dark for image analysis. The slides were thereafter rinsed with PBS solution (pH 7.2), air dried and mounted in Vectashield® anti-fading agent (Vector Laboratories, Burlingame, USA).

Image analysis and quantification using FISH and CSLM. Following pre-treatment and whole cell hybridization, samples were visualized using epifluorescence microscopy (1,000× oil immersion) and enumerated accordingly. The slides were viewed using an Axiolab HBO50/AC microscope (Carl Zeiss, Germany). Images were captured using a Zeiss AxioCam MRc camera and image analysis was carried out using the Zeiss AxioVision Release 4.6 (12-2006) imaging software. A total of three fields per sample were enumerated. For CSLM, the slides were viewed using a Zeiss confocal scanning laser microscope (LSM 710 and Axio Observor Z1) and images were captured to ascertain floc structure and its dispersion across the various pre-treatment methods.

Statistical analysis. Statistical analyses were performed using a Microsoft Excel spreadsheet including the 'Analysis Toolpak add-in'. Differences between means were evaluated with the independent-samples *t*-test.

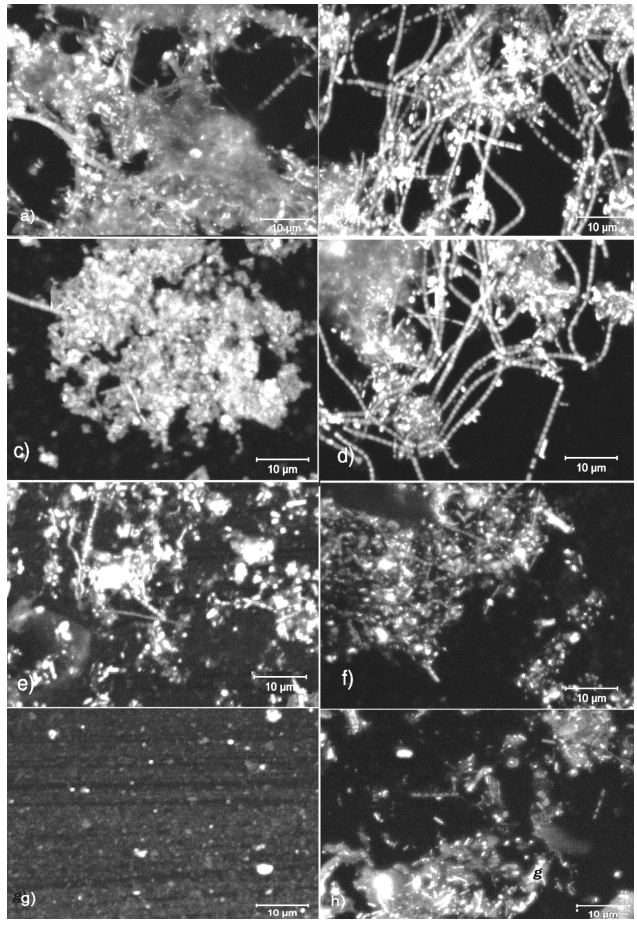

Fig. 1. FISH-CSLM images showing floc dispersion using different pre-treatment methods.

a) Control; b) Lysozyme only; c) Lysozyme+Sonication; d) Nonidet only; e) Nonidet+Sonication; f) Sonication only; g) Sonication after 5 min for domestic sludge samples; h) Sonication after 8 min for industrial sludge samples.

Results

Effect of pre-treatment on floc dispersion of different wastewater samples

Five pre-treatment methods were evaluated for floc dispersion using CSLM images (Fig. 1). An untreated sample (Fig. 1a) was used as a control. The images clearly indicate the variation in floc size and dispersion for each treatment. Sonication was found to be the best method for floc dispersion for both the plants tested (Fig. 1f). All the other pre-treatment methods were ineffective for the complete dispersion of the floc (Fig. 1b–e). To evaluate the different degrees of dispersion with sonication, further tests were carried out at a power range of 5–9 W across a time range of 3 to 9 min. For samples from industrial wastewater plants, an optimized sonication level of 8 W for 8 min reduced the floc size to 10 µm. For domestic wastewater samples, the floc size was reduced to 10 µm, at 8 W for 5 min. Beyond this sonication level there was no decrease in floc size for either of the tested samples. The cell integrity was lost beyond 8 min for industrial sludge sam-

ples (Fig. 1g) and beyond 5 min for domestic sludge samples (Fig. 1h).

Effect of pre-treatment on enumeration of nitrifying bacterial populations from different wastewater samples

The results of the preliminary experiments on the effects of pre-treatment on the enumeration of nitrifying bacterial populations are presented in Fig. 2. All cell enumerations were conducted in triplicate. Error bars represent the standard deviations of the median measurements. There was a significant difference in the number of individual cells observed for the same sample under different pre-treatments. Sonication was the best pre-treatment method for individual bacterial counts. Pre-treatment with lysozyme and nonidet were ineffective since they resulted in low individual cell counts (Fig. 2) for the same sample.

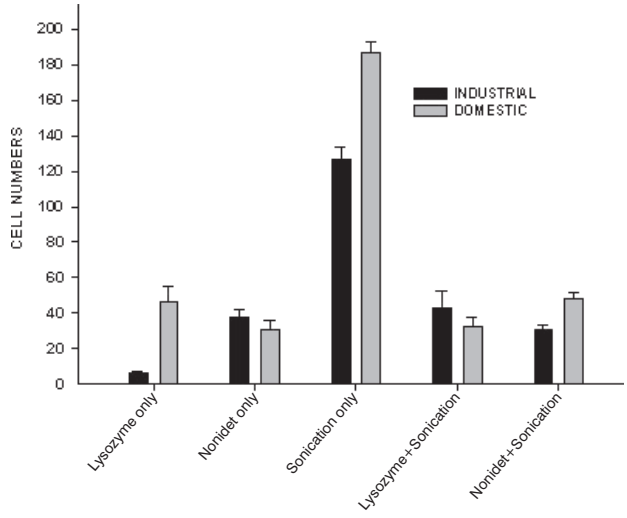

Fig. 2. Comparison of pre-treatment methods based on cell quantification.

Results are given as medians and standard deviations of the three replicates. Error bars represent the standard deviations of three replicate measurements of the median.

Further optimization of sonication was carried out to determine the optimum time and power level for bacterial cell dispersion from both plants (Fig. 3a and b). A power range of 5 to 9 W was tested across a time range of 5 to 9 min to evaluate the different degrees of dispersion. There were significant differences with regard to the number of cells observed. High individual cell counts were observed with sonication at 8 W for 8 min for samples from industrial wastewater. Beyond 8 min, there was a drastic decline in the number of single whole cells enumerated (Fig. 3a), resulting in cell lysis. On the other hand, there was a decrease in number of cells enumerated after 5 min for the domestic sludge samples (Fig. 3b).

Discussion

The generally low abundance of nitrifying bacteria in activated sludge and their appearance in densely packed aggregates create the need for effective pre-treatment methods for the successful enumeration of this population. A quantification method using FISH was successfully applied in this study to reproducibly determine the representative abundance of nitrifying bacteria in activated sludge. The analysis revealed significant differences among the five pre-treatment methods tested for the two different sludge samples.

Digital CSLM images (Fig. 1a–f) depict the comparative analysis of all five pre-treatment methods tested. All five methods showed varying degrees of floc dispersion. Sonication alone proved to be the consistent-

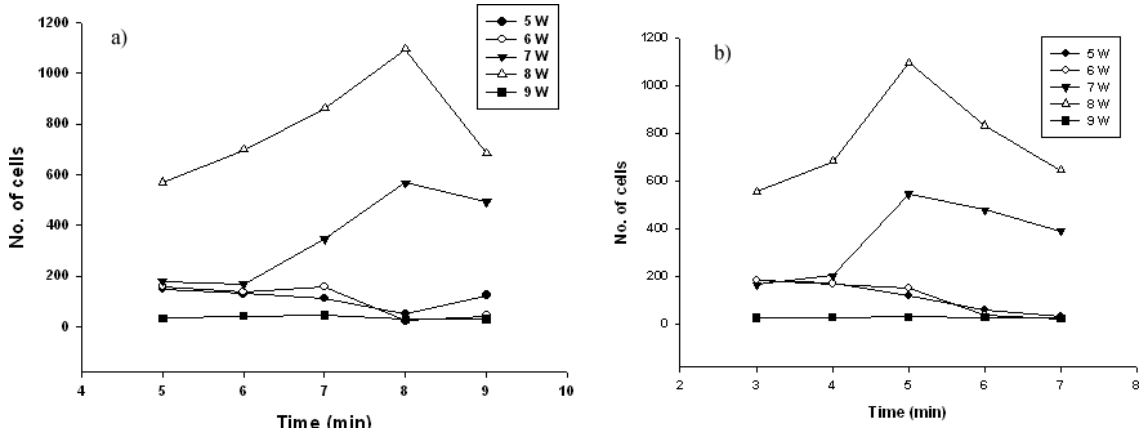

Fig. 3. Sonication at a power range of 5–9 W across a time-frame of 3–9 min.
a) Industrial sludge samples and b) Domestic sludge samples.

ly superior method of dispersion, since there is a clear correlation between sonication and the highest number of single whole cells dispersed out of the floc (Fig. 1f). The floc size diminished from the control (Fig. 1a) across the different pre-treatment methods (Fig. 1b–f). Snidaro et al. (1997) reported a reduction in floc size to 10 μm by sonication at 3 W for 3 min. Beyond this, there was no decrease in floc size. For the present study, the maximum dispersion of the floc was observed at 8 W and 8 min for the industrial and 8 W and 5 min for domestic wastewater samples. At this stage the size of the floc was 10 μm. Beyond this time interval, cell integrity was lost (Fig. 1g and h), where very few individual cells could be enumerated since cell lysis had occurred. These findings could be attributed to the nature of the sludge. Industrial wastewater used in this study comprised primarily of textile and poultry abattoir wastes as the major constituents. The sludge samples from the industrial wastewater treatment plant consisted mainly of non-degradable substances such as textiles, plastics and animal wastes from an animal waste processing facility, none of which are easily disrupted. Additionally, textile dyes were also present in this wastewater. Most industrial processes generate toxic wastes which rely on chemical and physical treatment to remove these toxic compounds.

Comparisons of the five different pre-treatment methods, based on cell quantification are depicted in Fig. 2. Individual whole cells dispersed out of the floc were only enumerated. Sonication resulted in the highest number of cells that could be dispersed from the flocs and quantified for both plants. Sonication helps in dissociation of activated sludge bacteria from organic and inorganic materials and flocs (Biggs and Lant, 2000; Daims et al., 2006). The most inefficient method was with lysozyme alone, where the least number of cells were quantified. This was probably due to the fact that enzymatic pre-treatment with lysozyme allows for cell permeability and is mainly used for cells surrounded by a thick peptidoglycan layer, i.e. Gram-positive cells (Furukawa et al., 2006). Nitrifying bacteria are Gram-negative; thus the lysozyme might have had little effect on floc dispersion.

Subsequently, the most significant sonication parameters (sonication duration and power input) were further optimized. Previous studies by Snidaro et al. (1997) showed that total disruption of the micro-colonies by sonication without causing significant cell lysis was highly unlikely. Therefore, for this study, sonication was applied to disrupt the flocs into micro-colonies only, which prevented complete sample destruction and any change in the floc characteristics. To determine the minimum time required to produce micro-colonies which would not cause significant cell lysis, the sludge samples were sonicated at different time intervals, ranging from 3 to 9 min across power levels of 5 to 9 W (Fig. 3a and b). Preliminary experiments showed that sonication time varies according to the type of sludge samples. The floc size decreased drastically (150 μm to 10 μm) and further there was no reduction in floc size. This result, however, is in contrast to the findings of Biggs and Lant (2000) where the optimum sonication time for the production of micro-colonies was 3 min. This could be possibly influenced by the difference in sludge type. The industrial sludge was very compact and dense and thus more time was required to break up the cell matrix before cell lysis occurred. In this study, pre-treatment methods were tested on samples from an industrial and a domestic wastewater treatment plant. The domestic sludge used for the present study was also receiving 10% industrial waste. Previous findings show that short sonication time intervals destroy sludge floc agglomerates, but do not affect the cells. Long sonication time intervals, however, break cell walls as well as the sludge solids which will release dissolved organic compounds, which leads to the loss of cell integrity (Guangming et al., 2006). This work experimentally elucidated the effects of 5 different pre-treatment methods on the integrity of individual cells and dispersion of activated sludge flocs and showed sonication was the superior method for cell dispersion. It also indicated the need for a longer sonication time and power level to disperse the highly compact flocs which emphasized the importance of optimization of pre-treatments for different types of sludge.

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