JACOBS PUBLISHERS

OPEN ACCESS

Jacobs Journal of Biotechnology and Bioengineering

Research Article

Assessment of the Role of Extracellular DNA and Protozoan Grazing in Aerobic Granular Sludge Development

Sodhi N[1], Scott A Rice[1, 2], Staffan Kjelleberg[1, 2], Diane McDougald[*1,2]

[1]*Centre for Marine Bio-Innovation, School of Biotechnology and Biomolecular Science, University of New South Wales, Sydney, Australia*

[2]*Singapore Centre on Environmental Life Sciences Engineering, Nanyang Technological University, Singapore*

**Corresponding author: Dr. Diane McDougald, Nanyang Technological University, Singapore, Tel: +65 63162818; E-mail: MDIANE@ntu.edu.sg*

Received: 08-14-2014

Accepted: 11-10-2014

Published: 12-29-2014

Abstract

A scaled-down batch reactor system, modelled on traditional laboratory scale batch reactors, was developed for the preliminary assessment of the role of a number of factors on microbial aggregation. Long-term batch reactors were used to investigate the role of extracellular DNA in the re-granulation of crushed granular sludge, while short-term reactors were used to assess the impact of protozoan grazing on aggregation and microbial community structure. Extracellular DNA was observed as halos and honeycomb structures within granules and was found to be integral to granular architecture. Grazing by heterotrophic protists was shown to increase and maintain aggregate size as well as significantly affect the microbial community structure.

Keywords: Aerobic Granular Sludge; Batch Reactor; Extracellular DNA; Protozoan Grazing; Aggregation; Wastewater Treatment

Introduction

The treatment of wastewater, imperative for both human and environmental health, has traditionally involved the use of activated sludge, a native microbial consortium used to removal nutrients from wastewaters. Anaerobic granular technology has been widely studied and implemented for wastewater treatment in the form of upflow anaerobic sludge blanket reactors (UASB). The disadvantages of anaerobic granular systems include long start up times, the inability to treat low nutrient loads due to incomplete nutrient removal and the requirement for further treatment of effluent [1]. Aerobic granule technology offers more efficient treatment, as these self-aggregating microbial communities encased in an extracellular matrix, have faster settling times [2], the capacity to withstand low and high nutrient loads [3-6] and the ability to degrade more toxic wastewaters [7-9]. Sequencing batch reactors (SBRs) used for aerobic granulation combine both aerobic and anaerobic conditions, and thus they can be successfully manipulated to achieve simultaneous nitrate and phosphorous removal [10-13].

In the laboratory, scaled down SBRs are used to assess the capacity of sludge to treat a range of wastewaters in real time. These reactors mimic full scale SBRs in all aspects, including the 4 major steps of operation; influent fill, reaction, settlement and effluent removal, while a range of parameters are used to assess reactor performance. The installation, running and maintenance of laboratory-scale SBRs can be costly as they require specialised laboratories and equipment, and are typically controlled and monitored by online systems. Moreover, the onset of granulation can be extremely variable and unpredictable, which makes the investigation of operational variables difficult. This time and labour intensive approach to assessing granulation of sludge also means there is limited scope to study other factors, such as biological and molecular influences. Manipulation of the system and the running of replicates are often not feasible, and thus there is little capacity to assess the multitude of ecological variables which may affect granulation. Here we report the implementation of a simplified, manually operated batch reactor system, which we propose can be used as a relatively quick and simple approach for screening of a range of environmental factors involved in granulation. Here this system was used to study the impact of extracellular DNA and predation by heterotrophic protists on granule formation.

Many aspects of granule formation, such as the production of extracellular polymeric substances (EPS) [14-17] and resistance to antimicrobials and other toxic compounds [9,18], mirror that of surface attached microbial communities (known as biofilms) [19-21]. Thus, granules can be viewed as self-immobilised biofilms. Granulation has been largely studied in the context of engineering principals and reactor design, however, as with other surface-associated communities, a range of biological factors may also play significant roles in community structure and function.

Extracellular DNA (eDNA) is observed to be an important component of biofilm EPS, with emerging evidence suggesting a significant role for eDNA in biofilm development in both Gram-negative and Gram-positive bacterial species. Extracellular DNA had been shown to be important for initial biofilm formation of *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* species [22,23] and for dispersal of *Caulobacter crescentus* and *Acidovorax temperans* biofilms [24,25]. Nucleic acids, DNA in particular, have also been consistently observed as part of the extracellular matrix of both granules and activated sludge [14,26,27]. Emerging evidence suggests that eDNA is important for floc structure in activated sludge biofilms, with Dominiak et al [28] demonstrating a localisation of eDNA around microcolony clusters and deflocculation with DNase I addition [28]. The abundance of eDNA has also been shown to vary among bacterial clusters within both aerobic activated and granular sludge, suggesting that active secretion of eDNA may also occur within these wastewater communities [28,29]. Despite the growing evidence for the importance of eDNA in the development and maintenance of complex biofilm structures, the role of eDNA in granulation remains largely unexplored.

Heterotrophic protists are single-celled eukaryotic organisms common to aquatic environments, and indigenous to activated sludge communities. Known to graze on bacteria, protozoa use a variety of feeding strategies to capture and ingest their prey [30,31]. Due to their sensitivity to changes in environmental conditions, protozoa have long been used as indicator organisms for sludge health in wastewater treatment systems [32]. The presence and activity of indigenous protozoa can affect the bacterial community composition. For example, grazing by protozoa has been shown to decrease *Escherichia coli* numbers in both estuarine and wastewaters [33,34], while protozoan predation in activated sludge systems can reduce the number of suspended solids in the effluent [35].

Protozoan grazing may also impact processes such as nitrification, which are vital for nutrient removal in wastewater treatment systems. Decreased grazing of activated sludge by protozoa has been correlated to a decrease in the rate of nitrification [36], while overgrazing of nitrifying populations by protozoa has been reported to decrease the overall nitrifying capacity of sludge [35]. Despite the evidence suggesting that protozoa play an important and complex role in activated sludge processes, very little work has been done to explore the role of protozoa in granulation. Here we assess the role of predation by heterotrophic protists, both a native sludge isolate and a previously isolated cilate, and eDNA in the aggregation of sludge using a simplified, scaled-down reactor system.

Materials and Methods

Experimental Overview

Reactors were run initially over a 43 day period to assess feasibility of re-granulating crushed granular sludge. Once this was established, experiments adding DNA and protozoa were performed in reactors of working volume 250 and 50 ml, respectively. Two separate experiments involving protozoa were performed. In the first, protozoa native to the granular sludge that was used throughout all experiments were isolated, grown and added to reactors with crushed sludge in controlled numbers. In a second set of experiments, a previously isolated freshwater ciliate was added to the reactors with crushed sludge in controlled numbers and made inactive using the eukaryotic antimicrobial cycloheximide.

Batch reactor setup and operation

Carbon-removing granular sludge was collected from bioreactors established for use in batch reactor experiments at the

Institute of Environmental Science and Engineering Pte Ltd (IESE), Nanyang Technological University (NTU), Singapore. Two hundred millilitres of granular sludge were crushed to smaller flocs using a mortar and pestle or homogenised manually. One hundred millilitres of crushed sludge were added to two 500 ml measuring cylinders containing 400 ml synthetic wastewater (mg l^{-1}: NaOAc, 1000; NH_4Cl, 100; K_2HPO_4, 22.5; $CaCl_2 \bullet 2H_2O$, 15; $MgSO_4 \bullet 7H_2O$, 12.5; $FeSO_4 \bullet 7H_2O$, 10) and 1 ml trace elements (mg l^{-1}: H_3BO_3, 0.05; $ZnCl_2$, 0.05; $CuCl_2$, 0.03; $MnSO_4 \bullet H_2O$, 0.50; $(NH_4)_6 \bullet Mo_7O_{24} \bullet 4H_2O$, 0.05; $AlCl_3$, 0.05; $CoCl_2 \bullet 6H_2O$ 0.05; $NiCl_2$, 0.05). Continuous mixing was achieved by bubbling air through reactors with air stones. At 10 and 14 h intervals, biomass was allowed to settle and 250 ml of the spent media was removed using a syringe and replaced initially with 250 ml of synthetic wastewater. The reactors were run for several days using these cycle conditions in order to acclimatise the sludge. For the experiments where the sludge was removed and tested, the air supply was stopped and the sludge was allowed to settle for a fixed period before the supernatant was removed using a syringe.

Sample collection and determination of aggregate size

Sampling of three 1 ml aliquots of the settled biomass in batch reactors was performed every 2-3 days. Two aliquots were centrifuged and the cell pellets were frozen and stored for DNA extraction, and the third was used immediately for microscopy. Suspended biomass samples were also collected (up to 3 x 10 ml), centrifuged and stored at -20°C for DNA extraction.

Aggregate size was determined by analysis of micrographs using the image analysis program Image J [37](Microscope images were taken using a Nikon Eclipse 80i for initial floc to granule experiments and a Leica DM LB phase microscope for eDNA addition experiments.

Extracellular genomic DNA addition to batch reactors

Extracellular DNA was obtained by extraction of genomic DNA from *E. coli* 4295 and added to batch reactors (250 ml working volume) at final concentrations of 1 and 10 μg ml^{-1}. At 10 and 14 h cycles, 100 ml of supernatant were removed from the reactors and replaced with 100 ml of fresh media containing eDNA. Control reactors with no eDNA were also included and all treatments were run in triplicate and repeated two times.

Genomic DNA was extracted from *E. coli* 4295 using a modified XSP buffer extraction method [38]. Briefly, 500 ml of an overnight culture of *E. coli* 4295 was centrifuged (Beckman Coulter, Avanti ™ J-25I) and the cell pellet was resuspended in 10 ml XS buffer (1% w/v potassium ethyl xanthogenate, 20% v/v 4 M ammonium acetate, 1% v/v 1 M Tris-HCl, pH 7.2, 4% 0.45 M EDTA, pH 8.0, 4% v/v SDS, water to 50 ml) and 10 ml of phenol (Research Organics) and incubated at 65°C for 15 min. After incubation, the tubes were vortexed briefly and cooled on ice for 1 - 2 min, followed by centrifugation (5min at 10,000 x g). The upper layer was extracted twice with equal volumes of a 1:1 phenol/chloroform solution; DNA was precipitated by the addition of 2 volumes of ethanol and 1/10 volume of 3 M sodium acetate and the tubes were incubated on ice for 45 min. The DNA was pelleted by centrifugation (15 min at 13,000 x g), the supernatant was removed and the pellet was washed with 1 ml of 70% ethanol and air-dried. The DNA was resuspended in 1 ml of molecular grade water and the concentration was determined using a spectrophotometer (Thermo Scientific NanoDrop™1000).

Visualisation of eDNA in sludge

One-millilitre aliquots of sludge samples were centrifuged for 5 min at 16,060 x g and the cell pellet was resuspended in 1 ml of PBS. One microliter of a 1:10 dilution of the nucleic acid stain, BOBO-3 (Molecular Probes®), and the EPS stain, FITC-conA (Molecular Probes®) were added and the sample was incubated for 20 min in the dark and observed by microscopy (Olympus FV1000).

Assessment of DNase activity in sludge

E. coli 4295 genomic DNA was added to 1 ml aliquots of granular sludge, cell-free supernatants and molecular water at final concentrations of 50 or 100 μg ml^{-1}. The aliquots were left to incubate at room temperature for 24 h, with 50 μl being removed every 2 h for the first 8 h and at 24 h. Ten microliters from each time point were electrophoresed on a 1% (w/v) agarose gel run at 80 V, 400 A for 30 min. DNA was visualised under UV (BioRad, GelD°C XR) after staining with 1 μg ml^{-1} ethidium bromide.

Maintenance of protozoa

Native sludge protozoa isolated from an Enhanced Biological Phosphorous Removal (EBPR) reactor and the freshwater ciliate *Tetrahymena pyriformis* were routinely maintained in synthetic wastewater and fed heat-killed *Pseudomonas aeruginosa* PAO1. Protozoa were enumerated by fixing with Lugol's Solution and counted by light microscopy (Leica DM LB phase microscope).

Native sludge protozoa addition

Replicate 50 ml falcon tubes were used for mini reactors containing 40 ml crushed sludge in synthetic wastewater. Before use, native protozoa isolated from the sludge were incubated at room temperature for several days in order for cell numbers to reach a minimum of 10^3 cells ml^{-1}. One millilitre of cells was added to each triplicate reactor and the protozoa were enumerated as described above.

Reactors were aerated for a total of 72 h, with media being replenished every 24 h. At the end of each 24 h cycle, samples were taken for aggregate size determination and community analysis. Reactors were allowed to settle for 5 min, after which 20 ml of supernatant was removed. Protozoa in this spent supernatant were enumerated and the same number of them was added back to the reactor along with fresh media. Suspended biomass was collected from the supernatant by centrifugation at 10,000 × g for 10 min and used for community analysis.

T. pyriformis addition

Experiments using *T. pyriformis* were performed as above (section 2.7.1), but were run for a total of 168 h. Initially, a single reactor was inoculated with crushed sludge and 1 ml of a 105 cells ml^{-1} culture of *T. pyriformis*, and run for 96 h to acclimatise the sludge. A control reactor with *T. pyriformis* addition was also included. After this acclimation period, half of the sludge in test reactors with *T. pyriformis* was transferred to new reactors and both sets of reactors were made up to 40 ml with synthetic wastewater. To one reactor, a final concentration of 10 μg ml^{-1} of cycloheximide was added to inactivate the protozoa. Reactors with *T. pyriformis* addition, cycloheximide addition and control reactors were aerated for a further 48 h, with fresh media being replaced every 24 h. Cycloheximide was also replenished at a final concentration of 10 μg ml^{-1} at the end of each cycle. Enumeration of protozoa and collection of suspended biomass were also performed as above (section 2.7.1). All conditions were run in triplicate.

Analysis of genomic DNA from sludge samples

Genomic DNA from sludge samples was extracted as described by Griffiths et al [39]. DNA concentration was determined by spectrophotometry (Thermo Scientific NanoDrop™1000), while DNA quality was determined by electrophoresis (1% agarose gel in 1 x TBE).

Variable regions of the 16S ribosomal DNA were amplified using the forward primer 341F with a 33 base pair GC-clamp at the 5' end of the primer and reverse primer 534R [40]. The PCR reaction contained 40 ng of DNA template, 5 μl of 10 x buffer, 100 nmol $MgCl_2$, 12.5 nmol dNTPs, 10 pmol each of forward and reverse primer, 0.2 μl of Taq polymerase and molecular grade water for a final volume of 50 μl. The PCR conditions were as follows: 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 90 s, with a final extension of 72°C for 10 min.

Regions of the 18S ribosomal RNA gene were amplified using the forward primer 300F with a 39 base pair GC-clamp at the 5' end of the primer (5'-CGCCCGCCGCGCCCCCGCCCCGGCCCGCCGCCCCCGCCCGAGGGTTCGATTCC-3') and reverse primer EK555sR (5'-GCTGCTGGCACCAGACT-3') [41]. The PCR reaction contained 80 ng DNA template, 5 μl of 10 x buffer, 75 nmol $MgCl_2$, 2.5 μl of DMSO, 10 nmol dNTPs, 20 pmol each of forward, and reverse primer, 0.5 μl of Taq polymerase and molecular grade water to a final volume of 50 μl. The PCR conditions were as follows: 95°C for 2 min, followed by 25 cycles of 95°C for 30 s, 61°C for 30 s, 72°C for 30 s, with a final extension of 72°C for 10 min. PCR products were electrophoresed on a 1% agarose gel and stained with 1 μg ml^{-1} ethidium bromide.

Amplified DNA was submitted for pyrosequencing (Research and Testing Laboratories LLC, Texas, USA) [42]. Primers 28F (5'-AGAGTTTGATCMTGGCTCAG-3') and 519R (5'-GWATTACCGCGGCKGCTG-3') were used for sequencing the bacterial community, while 300F (5'-AGGGTTCGATTCCGGAG-3') and EK555sR (5'-GCTGCTGGCACCAGACT-3') were used for sequencing of the eukaryotic community. Analysis of the pyrosequencing data was performed using Mothur v.1.16.1 [43]. Briefly, reads obtained from pyrosequencing were trimmed to remove primer and barcode sequences as well as to remove poor quality sequences. Unique sequences were then extracted and aligned to the SILVA reference database, after which chimeric sequences were removed. The remaining sequences were then pre-clustered to reduce sequencing noise from the pyrosequencing data. Operational taxonomic units (OTUs) were then assigned to sequences that were 3% divergent for bacterial communities and unique for eukaryotic communities.

Statistical analyses

Statistical analyses were performed using Prism software (GraphPad Software Inc.). Pair-wise comparisons were performed using unpaired two-tailed t-tests with confidence intervals of 95% or more. Where the variance was unequal, Welch's correction was applied. One-way ANOVAs were performed in Prism using the nonparametric Kruskal-Wallis test and the Dunns post-test. Again, P values with confidence intervals of 95% or more were used.

Results

Long-term batch reactors

Initial batch reactors with a working volume of 500 ml, were set up in duplicate and run in parallel to assess whether crushed granules would re-granulate under simplified conditions. After 6 days, the mean aggregate size for reactors 1 and 2 was 9.62×10^4 $\mu m^2 \pm 7.64 \times 10^4$ and 8.45×10^4 $\mu m^2 \pm 9.15 \times 10^4$, respectively (Figure 1). The aggregate size increased significantly ($p < 0.001$) over time with the mean aggregate size for reactors 1 and 2 at day 43 reaching 6.30×10^5 $\mu m^2 \pm 3.80 \times 10^5$ and 9.85×10^5 $\mu m^2 \pm 4.81 \times 10^5$, respectively. The transition from small, irregular aggregates to large, smooth granules was also tracked visually and is summarised in Figure 2.

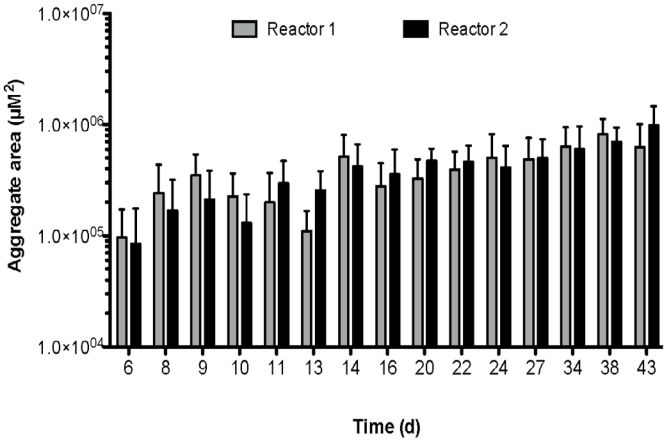

Figure 1. Mean aggregate area of sludge from batch reactors operated over 43 days.

The mean aggregate area of duplicate reactors was calculated by microscopy (Nikon Eclipse 80i) over a period of 43 days as sludge transitioned from flocs to granules. By day 43, the aggregate size had increased significantly ($p < 0.001$). Error bars represent standard deviations. Experiments were run in duplicate and repeated two times.

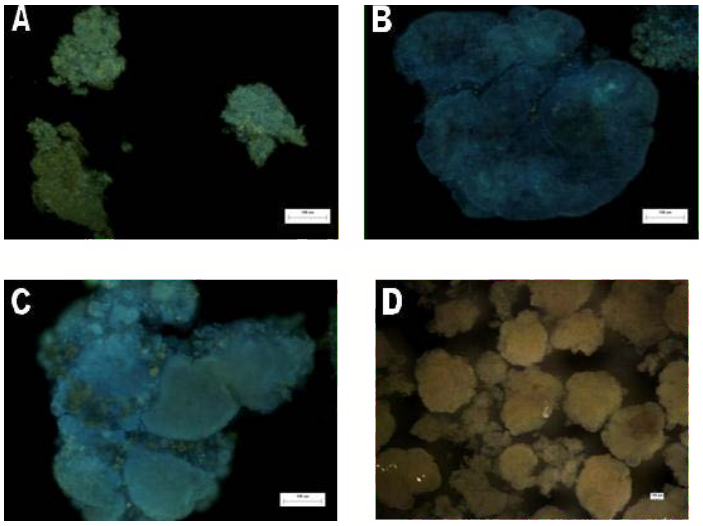

Figure 2. Microscope images of sludge samples over a 43-day period.

Samples from days 4 (A), 12 (B) and 20 (C) were stained with the fluorescent dye DAPI and visualised by fluorescent microscopy (Nikon Eclipse 80i) while visualisation of day 43 (D) samples was done using a stereomicroscope. Scale bars for all images represent 100 μm.

Role of extracellular DNA in granules

Batch reactors of 250ml working volumes were run over 43 days, with genomic DNA from *E. coli* 4295 added at final concentrations of 1 or 10 μg ml^{-1}, every 10 and 14h. Control reactors with no DNA addition were also included. The addition of eDNA to sludge in this manner had no significant effect on granule size or the rate of granulation (data not shown). Treatment of granules with varying concentrations of DNase I resulted in no significant difference in mean aggregate size. However, the presence of the enzyme did result in differences in the aggregate size range, the smallest aggregate size in DNase I treated samples was at least 10-fold smaller than that in untreated controls (Figure 3).

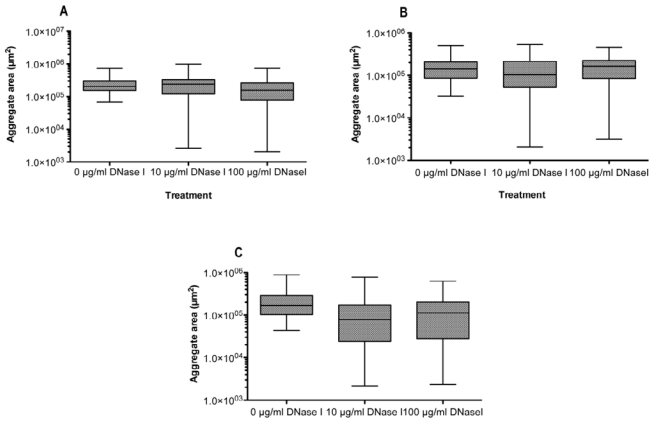

Figure 3. Box and whisker plots of aggregate sizes in reactors treated with DNase I.

Samples from control reactors (A), reactors with 1 μg ml^{-1} eDNA addition (B) and reactors with 10 μg ml^{-1} (C) taken after 40 days of treatment with 10 and 100 μg ml^{-1} DNase I. The bars represent the maximum and minimum values, while the box represents the 25th to the 75th percentile range, the line represents the median value. Experiments were run in triplicate and repeated twice.

To confirm the penetration of DNase I into the granules, samples were stained with BOBO-3 and FITC-conA in order to visualise eDNA and EPS, respectively, via confocal microscopy. There was less eDNA visible in DNAse I treated samples, as indicated by the decrease in red fluorescence in treated samples, particularly those treated with the higher (100 μg ml^{-1}) concentration of DNase I (Figure 4C). The red fluorescing filaments seen in all images, particularly in Figure 4C, were auto-fluorescing filamentous microbes and not eDNA.

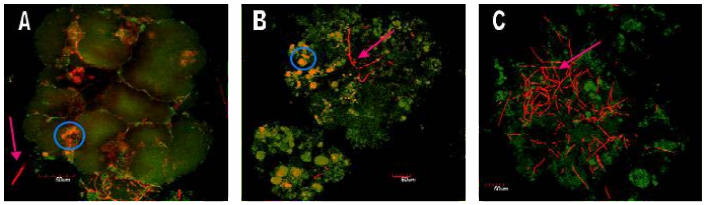

Figure 4. Detection of eDNA in DNase I treated sludge.

Control sludge (A), and sludge treated with 10 (B) and 100 μg ml^{-1} (C) of DNase I were stained with the DNA stain BOBO-3 (red) and the EPS stain FITC-conA (green) and visualised by fluorescent confocal microscopy (Olympus FV1000). The circles indicate areas of eDNA, while arrows indicate fluorescent filaments. The scale bars represent

50 µm.

Extracellular DNA within granules is protected from native sludge DNase activity

E. coli 4295 g DNA was added to samples of sludge and cell free supernatants from reactors at 50 and 100 µg ml^{-1}, and incubated at room temperature (Figure 5). Aliquots were sampled over 24 h and the DNA visualised on agarose gels. The eDNA started to visibly degrade after 6 h in sludge samples and was completely digested after 24 h (Figure 5).

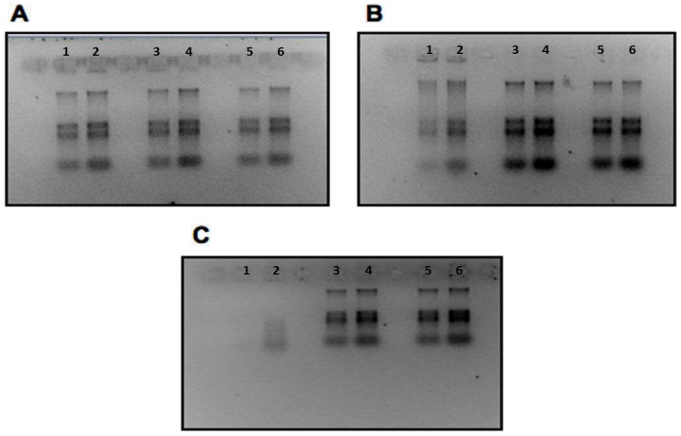

Figure 5. Detection of *E. coli* 4294 genomic DNA.

Agarose gel electrophoresis of *E. coli* 4295 gDNA incubated in sludge and cell free supernatant for 1 (A), 6 (B) and 24 h (C). Fifty (lanes 1, 3, 5) and 100 µg ml^{-1} (lanes 2, 4, 6) of DNA was incubated in sludge (lanes 1, 2), cell free supernatant (lanes 3, 4) or water (lanes 5, 6). Controls with no DNA were also included. Agarose gels were 1 % (w/v) and stained with 1 µg ml^{-1} ethidium bromide.

eDNA within granules were observed as halos and honeycomb structures (Figure 6). Halos were consistently observed around dead or membrane compromised cell clusters (i.e., red fluorescing cells) within granules, while honeycomb structures were consistently free of both dead (red fluorescing) cells and EPS (green fluorescence), indicating an association with live cells.

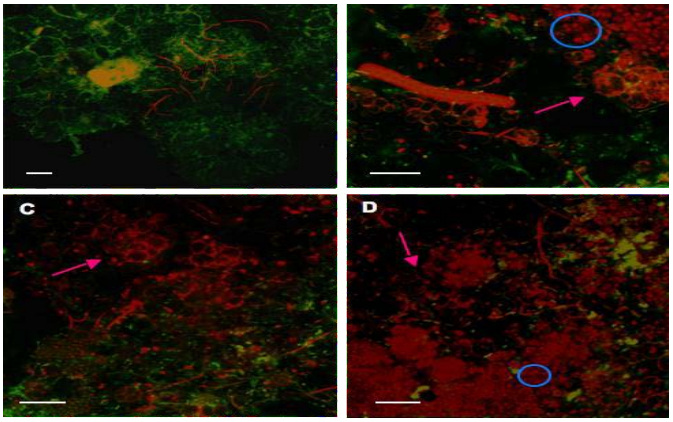

Figure 6. Visualisation of eDNA in granules.
Granules were stained with BOBO-3 (red) and FITC-conA (green) and visualised by confocal microscopy (Olympus, $FV^{-1}000$) at 20 x magnification (A) at 200 x magnification (B) and at 100 x magnification (C and D) and show halos (circles) and honeycomb structures (arrows) of eDNA. Scale bars are 50 µm for A and 5 µm for B – D.

Addition of protozoa to small-scale, short-term batch reactors

Protozoa isolated from sludge and the freshwater ciliate, *T. pyriformis*, were added to small scale (50 ml), short-term batch reactors to assess their effect on aggregate formation. The addition of native sludge protozoa to crushed sludge resulted in a shift in aggregate size distributions, with a trend towards larger aggregate size compared to the untreated control (Figure 7). The addition of *T. pyriformis* to crushed sludge did not affect aggregate formation over time (Figure 8A). The removal/inactivation of protozoa by cyclohexamide significantly reduced aggregate size in reactors with protozoa addition, but not controls reactors (Figure 8B).

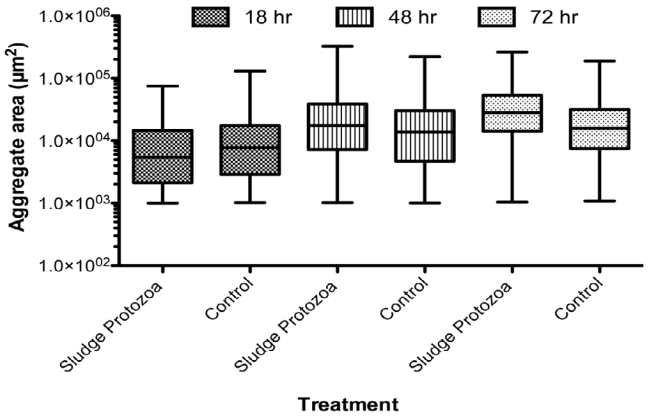

Figure 7. Effects of native sludge protozoa on sludge aggregate size.

Aggregate size distributions were calculated for crushed sludge with the addition of excess ciliates isolated from an EBPR reactor. The bars represent the maximum and minimum values, while the box represents the 25th to the 75th percentile range, with the line representing the median value. Experiments were performed in triplicate and repeated 3 times.

DNA extracted from samples was submitted for pyrosequencing. Analysis of pyrosequencing data showed that microbial community structure (both bacterial and eukaryotic communities), was affected more by time than by protozoa addition (data not shown). The addition of *T. pyriformis* to sludge did however significantly alter the contribution of a number of individual members (OTUs) to the overall community. These OTUs were referred to as being differentially represented or differentially abundant.

While no significant changes in the overall community structure were observed between treated and control samples, there were a number of OTUs in both bacterial and eukaryotic communities that were differentially represented with *T. pyriformis* addition (Table 1). In eukaryotic communities, 13 OTUs were represented differentially, all of which increased significantly ($p < 0.05$) with the addition of *T. pyriformis*. In bacterial communities, there were a total of 29 OTUs that were differentially abundant ($p < 0.05$), with 9 OTUs increasing and 20 decreasing with *T. pyriformis* addition. In eukaryotic communities, all the OTUs differentially abundant with treatment were also differentially abundant with time; however, of the 29 OTUs in the bacterial community, there were 4 OTUs that were differentially represented with treatment only, and not over time. This suggests that the abundance of these 4 taxa was affected by the presence of *T. pyriformis*. These OTUs were identified and are summarised in Table 2.

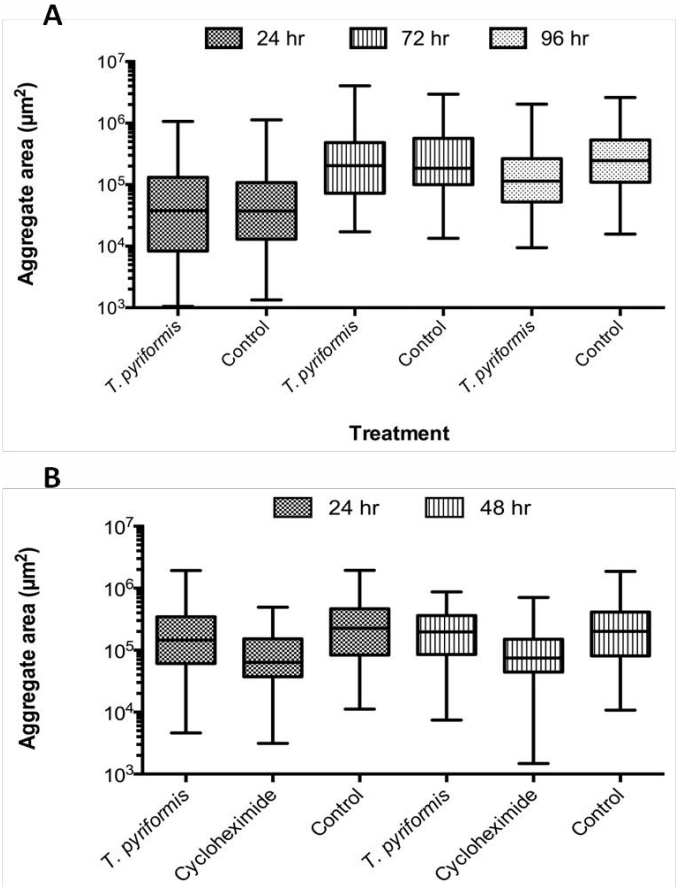

Figure 8. Effects of *T. pyriformis* grazing on aggregate size.

Aggregate size distributions were calculated for crushed sludge with excess *T. pyriformis* addition (A) and after removal of protozoa by cycloheximide addition (10 µg ml^{-1}) (B). The bars represent the maximum and minimum values, while the box represents the 25th to the 75th percentile range, with the line representing the median value. Experiments were performed in triplicate and repeated 2 times.

Table 1. Summary of differentially represented OTUs in batch reactors with *T. pyriformis* addition ($p < 0.05$).

Community	Bacterial	Eukaryotic
Total OTUs	29	13
Increased OTUs	9	13
Decreased OTUs	20	0

Table 2. Summary of differentially represented OTUs in the bacterial community with *T. pyriformis* addition ($p < 0.05$).

OTU	Taxa	Identity	Increase/Decrease
44	Family	Planctomycetaceae	Increase
80	Genus	*Steroidobacter*	Increase
90	Genus	*Stella*	Decrease
101	Order	Rickettsiales	Decrease

Discussion

Development of Mini Batch Reactors

Scaled down bioreactor systems have traditionally been used to screen large numbers of experimental conditions [44], with small-scale microbial bioreactors often used to characterise a range of bioprocesses. Bioreactors scaled from 7 L down to 10 ml have been used to characterise microbial fermentation processes of *E. coli* cells [45]. Similarly, scaled down bioreactors were used to characterise transcriptional responses of *E. coli* to dissolved oxygen gradients in order to better understand scale-up parameters for ethanol and organic acid production [46]. Based on these suspension bioreactors, small-scale bioreactors are also used as reproducible systems to culture stem cells, both for research and clinical use [47,48].

In the work presented here, an experimental design that uses scaled down batch reactors based on laboratory scale SBRs, with smaller working volumes, was explored. This system allows manipulation of various biological parameters to be tested for short-term effects on granulation. Batch reactors of 500 ml working volume were run in duplicate and granule development of crushed sludge was monitored daily (Figure 1). Granulation occurred by day 43, where mean granule size had plateaued over time but was significantly larger ($p < 0.001$) than the starting sludge. Thus, using this small-scale batch reactor system, crushed granules were able to re-granulate.

Crushed granules have also been shown to be effective in decreasing reactor start-up times, maintaining reactor biomass and retaining nutrient removal capacity throughout granula-

tion in laboratory scale systems. In a recent study by Pijuan et al. [49], aerobic bioreactors containing a mixture of floccular sludge and crushed granules not only decreased granulation time, but also resulted in biomass retention, when compared to control reactors without crushed granules. The use of fluorescent beads and confocal microscopy revealed that the crushed granules acted as an anchor for the floccular sludge, thereby reducing biomass washout and retaining both biomass and nutrient removal [50].

Effect of eDNA on granule development

It is becoming increasingly evident that extracellular DNA plays an important role in intercellular adherence and biofilm formation in a range of bacterial species. Extracellular DNA has been shown to contribute to the architecture of *P. aeruginosa* biofilms [51], promote biofilm formation of *Staphylococcus epidermidis* [52] and provides structural support in *Staphylococcus aureus* biofilms [53]. Moreover, eDNA has been observed in planktonic aggregates of *P. aeruginosa* [54] and addition of DNA to *E. coli* cultures has been shown to promote cellular aggregation [55].

The batch reactor system described in this paper was therefore used to assess the role of eDNA in granule formation and stability, by using externally added genomic DNA to mimic eDNA. Batch reactors of 250 ml working volume were set up in triplicate with the addition of 1 or 10 μg ml^{-1} of *E. coli* genomic DNA and a triplicate control set in which no DNA was added. The aggregate size of sludge in the reactors was followed over a 44-day period, until it was established that granulation had occurred. A significant increase in aggregate size was observed from day 0 to day 44 across all treatments, however no significant differences in aggregate size was observed between reactors with 1 μg ml^{-1} DNA addition, 10 μg ml^{-1} DNA addition and control reactors (data not shown). These results indicated that the external addition of DNA to crushed sludge did not influence the granule size and its development.

In order to determine if native eDNA plays a role in the development and stability of granules, batch reactors were treated with DNase I. Results indicated that the enzyme was able to penetrate and was active within the granules (Figure 4). Treatment with DNase I resulted in an increase in the number of smaller aggregates compared to untreated controls (Figure 3), indicating that the enzyme had an effect on aggregate size. These results suggested that eDNA was important for granule stability. However, there was no significant change in the overall mean aggregate size for DNase I treated samples compared to controls, indicating that eDNA alone did not maintain granule architecture, but rather did so in combination with other EPS components. EPS has been previously shown to be important for granule stability with granule disintegration on exposure to enzymatic treatments reported [56].

The crushed granular biomass used in this study conferred native DNase activity, degrading externally added genomic DNA within 6 h, with complete degradation after 24 h, while cell-free sludge supernatants showed no enzymatic activity (Figure 5). The results indicated that DNase activity was associated with cellular biomass and was not extracellular. This observation may explain why the addition of DNA to batch reactors had no effect on granule development; the externally added DNA was digested by native enzymes rather than incorporated into the granules. In contrast, a previous study by Vallom and McLoughlin [57] demonstrated that the addition of DNA extracted from sludge was able to induce flocculation in sludge isolates, while Lui et al. [55] showed the addition of various DNA types (herring sperm, pUC18 and lambda DNA) promoted aggregation of *E. coli* cells. The biomass used in this study comprised a complex microbial community, compared to the individual isolates and single species used by both the above studies, and is the probable explanation accounting for these contrasting results.

Despite the ability of sludge biomass to degrade gDNA (Figure 5), the presence of DNA observed in the extracellular matrix within granules using fluorescent dyes and confocal microscopy (Figure 6), indicated that this eDNA was protected from native sludge DNase activity. These results suggested that this eDNA was excreted from within the granules themselves, and not incorporated from the externally added gDNA. Two differing structures of eDNA were observed within the granules, halos and honeycomb structures, which were reported here for the first time in granules (Figure 6). To date, highly organised honeycomb structures within biofilms have been reported for a number of bacteria including S. *epidermidis* [58,59] and *P. fluorescens* [60], however there is little evidence that eDNA is a component of these structures.

The appearance of distinct eDNA structures suggests that different subpopulations or species within the granules are responsible for eDNA production. These distinct structures may also reflect varying mechanisms of eDNA release, namely through cell lysis or active excretion. The release of eDNA via the lysis of a subpopulation of cells and has been demonstrated for a number of bacterial species including *P. aeruginosa*, S. *aureus* and S. *epidermidis* [52,61-63], while active secretion of DNA without the occurrence of cell lysis has been reported for *Neisseria gonorrhoeae*, which is able to excrete eDNA via a specific type IV secretion system [64].

Effect of heterotrophic protists on granulation

Protozoa are a common constituent of microbial sludge communities and are consistently observed in wastewater granules [65,66]. They are known to affect biological processes and sludge communities in wastewater treatment, their presence can be both advantageous and detrimental to the treatment process. Dempsey et al. [67] reported that the overgrowth of a

stalked ciliate reduced the existing nitrifying biofilm community resulting in the breakdown of the treatment process of an expanded bed reactor. The batch reactor concept developed in this work was utilised to investigate the role of protozoan grazing under short-term conditions, using both a native sludge protozoan isolate and the ciliate *T. pyriformis*.

The addition of a native sludge protozoa resulted in a shift towards larger aggregates, suggesting that native sludge protozoa contribute, in part, to the formation of aggregates. The observed increase in aggregation could be a consequence of the ingestion of planktonic (ie: non-aggregating) bacteria by the protozoa, leaving behind aggregating populations. Alternatively, aggregation may be a strategy used by bacterial sludge community for grazing resistance. Bacterial aggregate formation in response to predation has been reported for freshwater *Sphingobium*sp. [68], *Pseudomonas* sp. MWH1 [69] and *P. aeruginosa* [70]. The presence of protozoan grazers has been reported to increase the rate of microcolony formation in *P. aeruginosa* and *Vibrio cholerae* species, while cells within differentiated biofilms remain resistant to grazing [70,71]. By forming cell aggregates and biofilms, bacteria are able to avoid predation by making themselves inaccessible to grazers.

While grazing by *T. pyriformis* has been shown to induce biofilm formation in *V. cholerae* [71], no difference in aggregate size was observed with the addition of *T. pyriformis* to batch reactors. However, inhibition of grazing via cycloheximide addition resulted in a decrease in aggregate size (Figure 8B). While the majority of the literature on the role of protozoa in activated sludge communities credit protozoa for maintaining effluent clarity [32,35] and acting as indicator organisms for healthy sludge [32], the results presented here suggest that protozoa also play a role in driving aggregate formation and maintaining aggregates.

The effect of grazing on the microbial community structure was also investigated. While analysis of pyrosequencing data showed that microbial community structure was affected more by time than by protozoa addition (data not shown), the addition of *T. pyriformis* to sludge did significantly alter the contribution of some members to the overall community (Table 1). In eukaryotic communities, there was an increase across all 13 differentially represented OTUs, suggesting that the presence *T. pyriformis* may stimulate the growth of certain eukaryotes. Of the 29 bacterial OTUs that were significantly different with *T. pyriformis* addition, 4 of these were unique to the treatment and not to time (Table 2). Of these, 2 increased, while 2 decreased. One explanation for these results is that OTUs with decreased reads, namely Stella and Rickettsiales were consumed by *T. pyriformis*, thus allowing the growth of Planctomycetaceae and Steroidobacter, the reads of which increased. Protozoan grazing has also been demonstrated to alter the community structure of SBRs bioaugmented with nitrifying communities, with predation affecting nitrifiers but not reactor performance [72].

Conclusion

This research focused on establishing a simplified, manually operated batch reactor set-up that allows for the manipulation of a range of biological parameters in replicate. We have demonstrated the viability of this system by re-granulating crushed granular sludge. Using this system, we have demonstrated the importance of eDNA in maintaining granule architecture. However, it is only eDNA released from the community itself, and not externally added DNA, which plays a role. We also reported for the first time distinct halo and honeycomb eDNA structures within granules. Based on the data presented, both grazing by indigenous sludge protozoa and inhibition of grazing by the ciliate *T. pyriformis* played a significant role in the formation and maintenance of granular sludge aggregates. Altered microbial community structure in the presence of *T. pyriformis*, suggests that the effect of protozoa on this specific sludge community is complex, and the impact of protozoa on the granulation process, microbial sludge community composition and reactor performance warrant further investigation. We propose this simplified system as a tool in the preliminary assessment of a range of ecological factors and conditions that may be involved in granulation.

Acknowledgements

This research was supported by the Environmental Biotechnology Cooperative Research Centre and the University of New South Wales. Part of this research was conducted at the Advanced Environmental Biotechnology Centre (AEBC), Nanyang Technological University, Singapore. The authors would like to acknowledge the input and advice of Dr Zhou Yan of the AEBC, and thank the Institute of Environmental Science and Engineering Pte Ltd (IESE), Nanyang Technological University (NTU), Singapore for providing the initial aerobic granular sludge.

References

1. Seghezzo L, Zeeman G, van Lier JB, Hamelers HVM, Lettinga G. A review: The anaerobic treatment of sewage in UASB and EGSB reactors. Bioresource Technology. 1998, 65(3), 175-190.

2. Liu Y, Tay JH. State of the art of biogranulation technology for wastewater treatment. Biotechnol Adv. 2004, 22(7), 533-563.

3. Uyanik S, Sallis PJ, Anderson GK. The effect of polymer addition on granulation in an anaerobic baffled reactor (ABR). Part II: compartmentalization of bacterial populations. Water Res. 2002, 36(4), 944-955.

4. Forster CF. Anaerobic upflow sludge blanket reactors: aspects of their microbiology and their chemistry. J Bacteriol. 1991, 17(3): 221-231.

5. Batstone, DJ, Keller J, Blackall L. The influence of substrate kinetics on the microbial community structure in granular anaerobic biomass. Water Res. 2004, 38(6): 1390-1404.

6. Ye FX, Shen DS, Feng XS. Anaerobic granule development for removal of pentachlorophenol in an upflow anaerobic sludge blanket (UASB) reactor. Process Biochem. 2004, 39(10): 1249-1256.

7. Tay ST, Moy BY, Maszenan AM, Tay JH. Comparing activated sludge and aerobic granules as microbial inocula for phenol biodegradation. Appl Microbial Biotechnol. 2005, 67(5): 708-713.

8. Qin L, Liu Y. Aerobic granulation for organic carbon and nitrogen removal in alternating aerobic-anaerobic sequencing batch reactor. Chemosphere. 2006, 63(6): 926-933.

9. Jiang HL, Tay JH, Tay ST. Aggregation of immobilized activated sludge cells into aerobically grown microbial granules for the aerobic biodegradation of phenol. Lett Appl Microbiol. 2002, 35(5): 439-445.

10. Gieseke A, Arnz P, Amann R, Schramm A. Simultaneous P and N removal in a sequencing batch biofilm reactor: insights from reactor- and microscale investigations. Water Res. 2002: 36(2): 501-509.

11. Lee DS, Jeon CO, Park JM. Biological nitrogen removal with enhanced phosphate uptake in a sequencing batch reactor using single sludge system. Water Res. 35(16): 3968-3976.

12. Patureau D, Helloin E, Rustrian E, Bouchez T, Delgenes JP. Combined phosphate and nitrogen removal in a sequencing batch reactor using the aerobic denitrifier, Microvirgula aerodenitrificans. Water Res. 2001, 35(1): 189-197.

13. Tsuneda S, Ohno T, Soejima K, Hirata A. Simultaneous nitrogen and phosphorus removal using denitrifying phosphate-accumulating organisms in a sequencing batch reactor. Biochem Eng J. 2006, 27(3): 191-196.

14. Wang Z, Liu L, Yao J, Cai W. Effects of extracellular polymeric substances on aerobic granulation in sequencing batch reactors. Chemosphere. 2006, 63(10): 1728-1735.

15. Zhang L, Feng X, Zhu N, Chen J. Role of extracellular protein in the formation and stability of aerobic granules. Enzyme Microb Technol. 2007, 41(5): 551-557.

16. Xiu-Fen L, Yan-Jun L, He L, Zhao-Zhe H, Guo-Cheng D et al. Correlation between extracellular polymeric substances and aerobic biogranulation in membrane bioreactor. Sep Purif Technol. 2008, 59(1): 26-33.

17. Di Iaconi C, Ramadori R, Lopez A, Passino R. Influence of hydrodynamic shear forces on properties of granular biomass in a sequencing batch biofilter reactor. Biochem Eng J. 2006, 30(2): 152-157.

18. He-Long Jiang, Joo-Hwa Tay, Abdul Majid Maszenan, Tay STL. Bacterial diversity and function of aerobic granules engineered in a sequencing batch reactor for phenol degradation. Appl Environ Microbiol. 2004, 70(11): 6767-6775.

19. Danese PN, Pratt LA, Kolter R. Exopolysaccharide production is required for development of *Escherichia coli* K-12 biofilm architecture. J Bacteriol. 2000, 182(12): 3593-3596.

20. Watnick PI, Lauriano CM, Klose KE, Croal L, Kolter R. The absence of a flagellum leads to altered colony morphology, biofilm development and virulence in *Vibrio cholerae* O139. Mol Microbiol. 2001, 39(2): 223-235.

21. Mukherjee PK, Chandra J. Candida biofilm resistance. Drug Resist Update. 2004, 7(4-5): 301-309.

22. Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS. Extracellular DNA required for bacterial biofilm formation. Science. 2002, 295(5559): 1487.

23. Moscoso M, García E, López R. Biofilm formation by *Streptococcus pneumoniae*: Role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion. J Bacteriol. 2006, 188(22): 7785-7795.

24. Berne C, Kysela DT, Brun YV. A bacterial extracellular DNA inhibits settling of motile progeny cells within a biofilm. Mol Microbiol. 2010, 77(4): 815-829.

25. Heijstra B, Pichler F, Liang Q, Blaza R, Turner S. Extracellular DNA and Type IV pili mediate surface attachment by *Acidovorax temperans*. Antonie van Leeuwenhoek. 2009, 95(4): 343-349.

26. Rikke Palmgren, Per Halkjær Nielsen. Accumulation of DNA in the exopolymeric matrix of activated sludge and bacterial cultures. Water Sci Technol. 1996, 34(5-6), 233-240.

27. Bura R, Cheung M, Liao B, Finlayson J, Lee BC et al. Composition of extracellular polymeric substances in the activated sludge fl°C matrix. Water Sci Technol. 1998, 37(4-5), 325-333.

28. Dominiak DM, Nielsen JL, Nielsen PH. Extracellular DNA

is abundant and important for microcolony strength in mixed microbial biofilms. Environ Microbiol. 2011, 13(3): 710-721.

29. Cheng M, Cook AE, Fukushima T, Bond PL. Evidence of compositional differences between the extracellular and intracellular DNA of a granular sludge biofilm. Lett Appl Microbiol. 2011, 53(1): 1-7.

30. Fenchel T. Ecology of protozoa: The biology of free-living phagotrophic protists, Science Tech Publishers, 1987.

31. Boenigk J, Arndt H. Comparative studies on the feeding behavior of two heterotrophic nanoflagellates: the filter-feeding choanoflagellate Monosiga ovata and the raptorial-feeding kinetoplastid Rhynchomonas nasuta. Aquat Microb Ecol. 2000, 22(3): 243-249.

32. Curds CR, Cockburn A. Protozoa in biological sewage-treatment processes--I. A survey of the protozoan fauna of British percolating filters and activated-sludge plants. Water Res. 1970, 4(3): 225-228, IN221-IN222, 229-236.

33. Curds CR, Fey GJ. The effect of ciliated protozoa on the fate of *Escherichia coli* in the activated-sludge process. Water Res. 1969, 3(11): 853-867.

34. J McCambridge and T A McMeekin. Protozoan predation of *Escherichia coli* in estuarine waters. Water Res. 1979, 13(7): 659-663.

35. Ghyoot W, Verstraete W. Reduced sludge production in a two-stage membrane-assisted bioreactor. Water Res. 2000, 34(1): 205-215.

36. Petropoulos P, Gilbride KA. Nitrification in activated sludge batch reactors is linked to protozoan grazing of the bacterial population. Can J Microbiol. 2005, 51(9): 791-799.

37. Image J. Image Processing and Analysis in Java.

38. Daniel Tillett, Brett A. Neilan. Xanthogenate nucleic acid isolation from cultured and environmental cyanobacteria. J Phycol. 2000, 36(1): 251-258.

39. Robert I. Griffiths, Andrew S. Whiteley, Anthony G. O'Donnell, and Mark J. Bailey. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. Appl Environ Microbiol. 2000, 66(12): 5488-5491.

40. Tang X, Gao G, Zhu L, Chao J, Qin B. DNA extraction procedure affects organic-aggregate-attached bacterial community profiles from a shallow eutrophic lake. Can J Microbiol. 2009, 55(6): 776-782.

41. Moreno AM. Understanding bacteria-protozoa interactions: from grazing resistance mechanisms to carbon flow in bacteria-protozoa food webs, The University of New South Wales, Sydney, 2008.

42. Data Analysis Methodology

43. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol. 2009, 75(23): 7537-7541.

44. Van Winkle A, Gates ID, Kallos MS. Embryonic Stem Cells - Basic Biology to Bioengineering. Kallos MS (ed), InTech, 2011.

45. Betts J, Baganz F. Miniature bioreactors: current practices and future opportunities. Microb Cell Fact. 2006, 5(1): 21.

46. Lara AR, Leal L, Flores N, Gosset G, Bolívar F et al. Transcriptional and metabolic response of recombinant *Escherichia coli* to spatial dissolved oxygen tension gradients simulated in a scale-down system. Biotechnol Bioeng. 2006, 93(2): 372-385.

47. Cormier JT, zur Nieden NI, Rancourt DE, Kallos MS. Expansion of undifferentiated murine embryonic stem cells as aggregates in suspension culture bioreactors. Tissue Eng. 2006, 12(11): 3233-3245.

48. Schroeder M, Niebruegge S, Werner A, Willbold E, Burg M et al. Differentiation and lineage selection of mouse embryonic stem cells in a stirred bench scale bioreactor with automated process control. Biotechnol Bioeng. 2005, 92(7): 920-933.

49. Pijuan M, Werner U, Yuan Z. Reducing the startup time of aerobic granular sludge reactors through seeding floccular sludge with crushed aerobic granules. Water Res. 2011, 45(16): 5075-5083.

50. Verawaty M., Pijuan M, Yuan Z, Bond P. Determining the mechanisms for aerobic granulation from mixed seed of floccular and crushed granules in activated sludge wastewater treatment. Water Res. 2012, 46(3): 761-771.

51. Allesen-Holm M, Barken KB, Yang L, Klausen M, Webb JS. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. Mol Microbiol. 2006, 59(4): 1114-1128.

52. Qin Z, Ou Y, Yang L, Zhu Y, Tolker-Nielsen T et al. Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. Microbiology. 2007, 153(7): 2083-2092.

53. Rice KC, Mann EE, Endres JL, Weiss EC, Cassat JE et al. The cidA murein hydrolase regulator contributes to DNA release

and biofilm development in *Staphylococcus aureus*. Proc Natl Acad Sci U S A. 2007, 104(19): 8113-8118.

54. David Schleheck, Nicolas Barraud, Janosch Klebensberger, Jeremy S Webb, Diane McDougald et al. *Pseudomonas aeruginosa* PAO1 preferentially grows as aggregates in liquid batch cultures and disperses upon starvation. PLoS ONE. 2009, 4(5): e5513.

55. Liu HH, Yang YR, Shen C, Zhang ZL, Shen P et al. Role of DNA in bacterial aggregation. Curr Microbiol. 2008, 57(2): 139-144.

56. Adav SS, Lee DJ, Tay JH. Extracellular polymeric substances and structural stability of aerobic granule. Water Res. 2008, 42(6-7): 1644-1650.

57. Vallom JK, McLoughlin AJ. Lysis as a factor in sludge flocculation. Water Res. 1984, 18(12): 1523-1528.

58. Robinson DH. Pleomorphic mammalian tumor-derived bacteria self-organize as multicellular mammalian eukaryoticlike organisms: morphogenetic properties in vitro, possible origins, and possible roles in mammalian 'tumor ecologies.'. Med Hypotheses. 2005, 64: 177-183.

59. Christoph Schaudinn, Paul Stoodley, Aleksandra Kainović, Teresa O'Keeffe, Bill Costerton et al. Bacterial biofilms, other structures seen as mainstream concepts. Microbes. 2007, 2(5): 231-237.

60. Baum MM, Kainović A, O'Keeffe T, Pandita R, McDonald K et al. Characterization of structures in biofilms formed by a *Pseudomonas* fluorescens isolated from soil. BMC Microbiol. 2009, 9: 103-115.

61. Mann EE, Rice KC, Boles BR, Endres JL, Ranjit D et al. Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. PLoS ONE. 2009, 4(6): e5822.

62. Webb JS, Thompson LS, James S, Charlton T, Tolker-Nielsen T et al. Cell death in *Pseudomonas aeruginosa* biofilm development. J Bacteriol. 2003, 185(15): 4585-4592.

63. Das T, Manefield M. Pyocyanin promotes extracellular DNA release in *Pseudomonas aeruginosa*. PLoS ONE . 2012, 7(10): e46718.

64. Dillard JP, Seifert HS. A variable genetic island specific for *Neisseria gonorrhoeae* is involved in providing DNA for natural transformation and is found more often in disseminated infection isolates. Mol Microbiol. 2001, 41(1): 263-277.

65. Weber SD, LudwigW, Schleifer KH, Fried J. Microbial composition and structure of aerobic granular sewage biofilms. Appl Environ Microbiol. 2007, 73(19): 6233-6240.

66. Elbanna K, Lütke-Eversloh T, Van Trappen S, Mergaert J, Swings J et al. Schlegelella thermodepolymerans gen. nov., sp. nov., a novel thermophilic bacterium that degrades poly(3-hydroxybutyrate-co-3-mercaptopropionate). Int J Syst Evol Microbiol. 2003, 53(4): 1165-1168.

67. Dempsey MJ, Lannigan KC, Minall RJ. Particulate-biofilm, expanded-bed technology for high-rate, low-cost wastewater treatment: Nitrification. Water Res. 2005, 39(6): 965-974.

68. Blom JF, Zimmermann YS, Ammann T, Pernthaler J. Scent of danger: Floc formation by a reshwater bacterium Is induced by supernatants from a predator-prey co-culture. Appl Environ Microbiol. 2005, 76(18): 6156-6163.

69. Hahn MW, Moore ER, Höfle MG. Role of microcolony formation in the protistan grazing defence of the aquatic bacterium *Pseudomonas* sp. MWH1. Microb Ecol. 2000, 39(3): 175-185.

70. Matz C, Bergfeld T, Rice SA, Kjelleberg S. MicroColonies, quorum sensing and cytotoxicity determine the survival of *Pseudomonas aeruginosa* biofilms exposed to protozoan grazing. Environ Microbiol. 2004, 6(3): 218-226.

71. Matz C, McDougald D, Moreno AM, Yung PY, Yildiz FH et al. Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio cholerae*. Proc Natl Acad Sci USA. 2005, 102(46): 16819-16824.

72. Yu L, Peng D, Ren Y. Protozoan predation on nitrification performance and microbial community during bioaugmentation. Bioresour Technol. 2011, 102(23): 10855-10860.