Original Article

Chlorine Dose Determines Bacterial Community Structure of Subsequent Regrowth in Reclaimed Water

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ABSTRACT

Bacterial regrowth in reclaimed water, specifically observed when residual chlorine concentration declines along the distribution system, causes undesirable changes in water quality and hampers its acceptability. To study the impact of chlorination on regrowth and bacterial community structure, unchlorinated tertiary treated reclaimed water was collected and dosed with chlorine such that the initial doses were 1, 3, 5 mg-Cl₂/L before being stored at ambient temperature under dark condition. Chlorine measurement, cell counts and bacterial community profiling were carried out at regular intervals for 21 days. Addition of chlorine caused rapid decline in intact cell concentration and no regrowth was observed until free chlorine decayed below detection limit (0.03 mg-Cl₂/L). Upon regrowth, intact cell concentrations reached the initial level except in the case of 5 mg-Cl₂/L where the intact cell concentration was lower by 1-log₁₀. The dominant species that regrew under each condition were distinct, based on their capacity to withstand chlorine. The most chlorine-tolerant groups belonged to the order Sphingomonadales and Rhizobiales, which have been previously reported to initiate biofilms. This study demonstrates that chlorination selects specific bacterial groups which have the potential to regrow in the distribution network.

Keywords: bacterial regrowth, chlorination, community profile, reclaimed water

INTRODUCTION

Reclaimed water is gaining importance as an alternate source of fresh water, due to its stable volume and reduction in pollutant load which gets discharged into the environment [1,2]. Tertiary treated reclaimed water is popular for non-potable urban uses like toilet flushing, landscaping and industrial uses, however, deterioration in microbial water quality during storage and distribution is still a concern for reclaimed water industry [3]. Owing to its higher nutrient content than drinking water [4,5], intermittent consumption pattern and being sourced from wastewater, reclaimed water provides a conducive environment for microbes to grow. Hence, it is disinfected before being supplied to the customer to prevent accidental contact with harmful pathogens. Among disinfectants, chlorination is widely used to disinfect reclaimed water as it is economical and offers residual disinfectant properties. Despite chlorination, bacterial regrowth

is observed when residual chlorine declines along the distribution network, providing the opportunity for regrowth of opportunistic pathogens [6,7], causing biofouling of cooling towers [8] and corrosion of the infrastructure [9]. This situation calls for a greater understanding of the microbial communities in reclaimed water, which is currently unavailable.

Most studies which have investigated bacterial regrowth in reclaimed water were based on cultivation dependent techniques like Heterotrophic Plate Count (HPC), selective media to detect pathogens (frank and opportunistic) [6,10,11] or indicator organisms. The basic problem with these culture based methods are: (a) not all live bacteria can be cultured on a single medium in the case of HPC [12]; (b) on contact with disinfectants, bacterial cells can convert to viable but not culturable state (VBNC) and hence cultivation-dependent techniques may underestimate viable bacteria; (c) most indicators are based on enteric bacteria, which are sensitive to chlorine and are hence not likely to regrow after chlorina-

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Fig. 1 Diagrammatic representation of the Reclaimed Water Facility.

tion [7,11]. Thus it is essential to adopt culture-independent techniques like enumeration by flow-cytometer (FCM) and 16S rRNA gene based community profiling to capture the regrowth phenomenon. A systematic understanding of chlorine-tolerant bacteria, their growth dynamics and relationship with chlorine may help us in developing efficient disinfection regime. This paper aims to understand (i) relationship between chlorine dose, bacterial inactivation and regrowth, and (ii) accompanying changes in bacterial community profile.

MATERIALS AND METHODS

Sampling and site description

Reclaimed water was collected from a Water Reclamation plant located in Tokyo metropolitan area, Japan. This reclamation facility receives secondary treated effluent, which is then subjected to biofiltration, ozonation and chlorination before being stored in the storage tank of 2000 m³ capacity (**Fig. 1**). Chlorine is added to the recirculation line in order to maintain total chlorine concentration in the tank to about 1 mg-Cl₂/L. Hydraulic retention time in the tank was about 2 – 3 days and chlorine dose per unit volume of treated water was 6 mg-Cl₂/L. In the present study, reclaimed water was sampled after ozonation in order to perform chlorination under laboratory conditions. The samples were transferred to the laboratory and were stored at 4°C. The experiments were conducted within 6 h after sampling.

Preparation of carbon free glassware

In this study, carbon free glassware were used to avoid organic matter contamination as well as to minimize chlorine demand. They were prepared by thoroughly washing with detergent, rinsing with Milli-Q, and then by 0.1 N HCl acid, followed by several rounds of Milli-Q wash, after which they were wrapped in foil and were baked at 550°C for 6 h. The plastic caps were subjected to a similar procedure except that

they were air-dried instead of being baked.

Experimental setup

Five carbon-free 2000-mL screw cap glass bottles were filled with 1800 mL of reclaimed water samples. Sodium hypochlorite stock solution was added such that the initial doses were 1, 3 or 5 mg-Cl₂/L, which were lower than the actual dosage (6 mg-Cl₂/L) at the plant, to observe the effects of different chlorine doses on selection of bacterial groups with differing chlorine-resistance. One set was kept unchlorinated and served as control. The bottles were covered with aluminium foil and were kept stationary under ambient temperature (~23°C) in order to simulate the microbial regrowth that occurs during storage and distribution. Samples were collected using aseptic plastic pipettes after briefly shaking the bottles. Residual free chlorine was measured until it fell below detection limit. Samples for bacterial counts and microbial community profiling were collected at regular intervals for 21 days. Other physico-chemical analyses were performed on the samples collected at the start and end of the experimental duration.

Physico-chemical analyses

Temperature and pH were noted down at the sampling site. Other physicochemical parameters were analysed after filtering through 0.22 µm filters (Isopore GTTP, Millipore, USA) in the laboratory and stored in carbon-free glass vials. Dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were analysed by TOC-L (Shimadzu, Japan). Ammonia nitrogen (NH₄-N) was measured by Ammonia TNT830 (Hach, USA). Anion concentration was analysed by ion chromatography (Metrohm 861 compact IC, Switzerland). Residual free chlorine concentration was analysed during the experiments by the DPD colorimetric method using DPD Test and Tube kit (Hach, USA) and HACH DR/890 colorimeter (Hach, USA).

Table 1 Water quality of the sample collected.

Parameter	Values
pH	7.3
Temperature (°C)	20
DOC (mg-C/L)	3.0
TDN (mg-N/L)	9.3
NH ₃ -N (mg-N/L)	0.043
NO ₃ ⁻ -N (mg-N/L)	9.25
PO ₄ ³⁻ -P (mg-P/L)	0.6
TCC (cells/mL)	$(3.0 \pm 0.2) \times 10^6$
ICC (cells/mL)	$(8.8 \pm 2.4) \times 10^5$
HPC (CFU/mL)	$(1.5 \pm 0.1) \times 10^5$

Enumeration of bacteria

Samples for bacterial count were stored at 4°C until analysis after the residual chlorine was quenched with 3% sodium thiosulphate. The total cell concentration (TCC) and intact cell concentration (ICC) of bacteria were obtained by staining samples with SYBR Green I (Molecular probes, USA) and SYBR Green I along with Propidium iodide (PI) respectively, followed by incubation in dark and counting by BD Accuri™ C6 flow cytometer (BDBiosciences, USA) [13]. SYBR Green I stains all cells whereas PI specifically stains membrane damaged cells. HPC was carried out on R2A medium using spread plate technique and incubating the petriplates at 20°C for 7days. HPC gives a count of cultivable bacteria in a given sample.

Bacterial 16S rRNA gene profiling

Samples (100 mL volume) collected at various time points were filtered through 0.22 µm membrane (Isopore GTTP, Millipore, USA) and these filter membranes were stored at -20°C till DNA extraction was carried out. DNA extraction was performed using RapidWater® DNA extraction kit (MoBio, USA) as per the manufacturer's instructions and the obtained DNA was suspended in 100 µL of 10 mM Tris buffer at the final step.

For construction of 16S rRNA gene sequence library, 2 µL of extracted DNA was amplified using primers 515F and 806R targeting constant regions flanking V4 hypervariable region of 16S rRNA gene. The PCR amplification conditions were: initial denaturation at 94°C for 2 min; followed by 25 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, elongation at 72°C for 30 sec; and a final elongation at 72°C for 5 min. The PCR products were purified using MinElute® PCR purification kit (Qiagen, Germany). Three microlitre of the purified PCR product was used as

template for 2nd PCR. Primers (519F and 802R) linked with barcodes were used to amplify the products of 1st PCR. The PCR amplification conditions were: Initial denaturation at 94°C for 2 min; followed by 12 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 30 sec, elongation at 72°C for 30 sec; and a final elongation at 72°C for 5 min. The 50 µL of 2nd PCR products were loaded onto 1% agarose gel, stained with 1× SYBR® Safe stain and viewed on UV trans illuminator. Bands corresponding to 450 bp were excised from the gel and were eluted with MinElute® Gel Extraction Kit (Qiagen, Germany). The obtained products were quantified using Picogreen® (Molecular Probes, Invitrogen, USA) and Nanodrop 3300 fluorospectrometer (Thermo Scientific, USA). Equal amounts of PCR products were mixed and sent to FASMAC, Japan, for sequencing by Illumina MiSeq gene sequencer.

The raw reads obtained from sequencer were subjected to quality filtering by Sickel (ver. 1.33) [14], sequence trimming by FASTX-toolkit (ver. 0.0.13.2) [15], forward and backward sequences merged with FLASH (ver. 1.2.10) [16], chimera checking by usearch (ver. 7.0.1090_i86linux64) [17] and classification at Operational Taxonomic Unit (OTU) level using QIIME (ver. 1.8.0) [18].

RESULTS AND DISCUSSION

Quality of the reclaimed water sample before chlorination

Water quality data of the reclaimed water sample collected are listed in **Table 1**. Absence of nitrite (< 0.05 mg-N/L), presence of high amounts of nitrate and low concentrations of ammonia-N indicate that complete nitrification had occurred before the sample was chlorinated. This could be attributed to the presence of biofiltration unit in the treatment chain. As a result, formation of inorganic chloramines is negligible and hence free chlorine is the major disinfectant. Typical nutrient ratio for optimal microbial activity is reported to be in the ratio C:N:P = 100:10:1 [19], hence carbon is the limiting substrate in this sample as nitrogen and phosphorus are abundant. The bacterial counts enumerated by three methods showed that out of the TCC of around 3×10^6 cells/mL, only about 30% were intact cells and 5% were cultivable (HPC) on R2A agar.

Chlorine decay

Chlorine decay observed during the course of experiment is depicted in the **Fig. 2(a)**. The decay of free chlorine was rapid up to 180 min after which it slowed down, which was

Fig. 2 (a) Chlorine decay observed in bulk water during the experiment with different chlorine doses. The points where chlorine concentration falls below the detection limit (i.e., 0.03 mg-Cl₂/L) is depicted with circle and arrow pointing downward. (b) A semi-log plot of residual chlorine concentration vs. time shows that chlorine decay doesnot follow first order decay kinetics.

Fig. 3 ICC under different chlorine doses. Points marked with circle and arrow pointing downwards represent points below Limit of Quantification (LoQ) of FCM (200 cells/mL).

clearly observed in 3 mg-Cl₂/L and 5 mg-Cl₂/L samples. This varying rate of chlorine decay cannot be explained by simple first order decay (**Fig. 2 (b)**). Besides decay rate being proportional to initial chlorine dose, rate could also be dependent upon the presence of 2 or more groups of chlorine-demanding components differing in their reactivity with chlorine. Such models have been developed to explain chlorine decay in drinking water and reclaimed water [20,21]. Residual chlorine reached below the detection limit (i.e., 0.03 mg-Cl₂/L) within 1, 2 and 3 days for samples chlorinated with 1, 3 and 5 mg-Cl₂/L of initial chlorine, respectively.

Inactivation of bacteria by chlorine and subsequent regrowth

ICC represent the bacteria with intact cell membranes and is a conservative measure of cell viability. Addition of chlorine resulted in decline of intact bacterial numbers, however, the regrowth resumed once the residual chlorine levels declined below limit of detection i.e., 0.03 mg-Cl₂/L (**Figs. 2 and 3**). At higher doses-3 and 5 mg-Cl₂/L, the regrowth had a time lag. Despite different doses of chlorine administered, the maximum cell counts were similar in all cases except 5 mg-Cl₂/L, where cell counts were 1-log₁₀ order lower.

Fig. 4 (a) Cell counts by various methods and (b) change in major OTUs when reclaimed water is not chlorinated. Major OTUs are the OTUs that occupy more than 5% in at least one of the samples sequenced. Those OTUs which never exist more than 5% were combined as “others”. Red arrows at the top represent points when samples were collected for sequencing.

Bacterial community in the unchlorinated reclaimed water sample

Bacterial community structure in the unchlorinated reclaimed water sample collected from the reclamation plant was analysed. Proteobacteria was found to be the most abundant phylum (68%) followed by Bacteroidetes (11%) and Spirochetes (7%). This is similar to previous studies where Proteobacteria was the most abundant phylum in activated sludge [22,23], surface water and sewage [24] and drinking water distribution system [25]. At OTU level, the bacterial community had 4 major species i.e., species with relative abundance greater than 5% – *Acidovorax* sp., *Flavobacterium* sp., *Leptonema* sp. and unidentified Oxalobacteraceae. *Acidovorax* sp. and *Flavobacterium* sp. are commonly found microbes in sewage and activated sludge [23,24] and are likely to be detected in reclaimed water.

No chlorine addition

The TCC and ICC remained constant since the start of monitoring period, however, HPC count showed an increase by 1-log₁₀ order within 6 h and reached the same count as that of the ICC. All types of cell counts dipped at Day 3, followed by a growth. Bacterial community profiling at Day

5 reveals a drastic change in the community structure where *Sediminibacterium* sp., *Novosphingobium* sp., unclassified Alcaligenaceae are major OTUs and the groups at Day 0 have become less significant. Hence, the dip at Day 3 could be attributed to the death of major groups due to substrate limitations, followed by emergence of a new set of bacteria which probably used cell components released by dying bacteria as substrate. Thus, it was found that the bacterial community was dynamic when chlorine was not added (**Fig. 4**).

Initial chlorine dose of 1 mg-Cl₂/L

When spiked with chlorine dose of 1 mg-Cl₂/L, inactivation of bacteria was observed across the three measurements, however, the decline was more pronounced in HPC and intact cell counts. HPC fell below 300 CFU/mL within 30 min of contact time, achieving more than 3-log₁₀ reduction. Relative abundance of dominant species – *Acidovorax* sp., unclassified Oxalobacteraceae, *Flavobacterium* sp. – decline within 6 h, however *Leptonema* sp. still constitutes 20% of the relative abundance. Significant regrowth was observed at the end of Day 1, correlating with the chlorine decay. OTU belonging to *Rheinheimera* sp. attained relative

Fig. 5 (a) Cell counts by various methods and (b) change in major OTUs when 1 mg-Cl₂/L of free chlorine is added to reclaimed water. Red arrows at the top represent points when samples were collected for sequencing. Open symbols represent LoQ of HPC: 300 CFU/mL.

abundance > 5% and grew with time. *Acidovorax* sp., unclassified *Oxalobacteraceae* and *Sediminibacterium* sp. were found to increase in abundance after chlorine decay. By the end of Day 3, all types of counts reached around maximum value, and continued to remain at that value for up to 10 days (Fig. 5). Upon chlorination, slight shift in dominant species, miniscule changes in total counts and a quick repopulation suggest that the population probably consists of resuscitated/ repaired as well as regrown cells.

Initial chlorine dose of 3 mg-Cl₂/L

The addition of 3 mg-Cl₂/L of chlorine caused a decline in all types of bacterial counts. HPC fell below 300 CFU/mL within 30 min of contact time whereas counts by flow cytometer – both TCC and ICC – declined slowly to about 3-log₁₀ orders and showed no signs of regrowth until the end of Day 3. The concomitant increase in total counts, ICC and HPC to 10⁶ cells/mL at Day 7 indicate regrowth. Sample collected on the Day 5 indicates emergence of new groups of bacteria – unclassified Betaproteobacteria, which constituted 70% of the relative abundance, *Methylobacterium mobilis* and *Pseudomonas* sp._2. Over the experimental duration, other OTUs like unclassified Comamonadaceae, *Pseudomonas* sp._1 and *Acidovorax* sp. were detected at greater than 5%

of total abundance in addition to the ones dominant at Day 5. The communities that regrew were strikingly different from the community at Day 0 demonstrating that regrown bacteria belonged to groups that survived disinfection and multiplied (Fig. 6).

Initial chlorine dose of 5 mg-Cl₂/L

The addition of 5 mg-Cl₂/L of chlorine caused a decline in all types of bacterial counts. HPC fell below 10 CFU/mL, ICC fell below detection limit of 200 cells/mL and TCC decayed to 5 × 10³ cells/mL within a day. Beyond 5 days, TCC and ICC increased slowly reaching maximum value at day 13 (Fig. 7). Samples for sequencing collected at Day 13, the first sample after TCC recovered above 10⁵ cells/mL, reveal that unidentified Sphingomonadaceae (belonging to Order Sphingomonadales, Class Alphaproteobacteria) – which constituted about 86% of the total abundance – and *Hyphomicrobium sulfonivorans* (belonging to Order Rhizobiales, Class Alphaproteobacteria) were present. At day 16 and onwards, bacteria belonging to unidentified Sphingomonadales and *Methylobacterium* sp. (Order Rhizobiales) were detected. Members belonging to Order Sphingomonadales have been reported to possess high chlorine tolerance, are important biofilm-forming species and grow when other bac-

Fig. 6 (a) Cell counts by various methods and (b) change in major OTUs when 3 mg-Cl₂/L of free chlorine is added to reclaimed water. Red arrows at the top represent points when samples were collected for sequencing. Open symbols represent LoQ of HPC: 300 CFU/mL.

Fig. 7 (a) Cell counts by various methods and (b) change in major OTUs when 5 mg-Cl₂/L of free chlorine is added to reclaimed water. Red arrows at the top represent points when samples were collected for sequencing. Open symbols represent LoQ of FCM: 200 cells/mL and HPC: 300 CFU/mL; open symbol with arrow represent HPC below 10 CFU/mL.

terial species are low in numbers [26–28]. Members belonging to Sphingomonadaceae and *Methylobacterium* sp. were detected throughout drinking water distribution network (from raw water to tap water) employing chlorination [25]. Thus chlorination selects closely related groups that possess chlorine tolerance as previously described by Chiao *et al.* [29] and can regrow upon decline in residual chlorine in the system. The repopulated bacterial levels are 1-log_{10} lower than the previous cases either due to low cell number yield of these groups or difference in substrate requirements.

Impact of initial chlorine dose on bacterial community structure in reclaimed water

The microbial community before addition of chlorine had *Flavobacterium* sp., *Acidovorax* sp., unclassified Oxalobacteraceae and *Leptonema* sp. as major groups and ICC in the range of 10^6 cells/mL. When no chlorine was added, bacterial ICC remained roughly in the same range, however, the community profile changed. Major groups that grew were *Sediminibacterium* sp., *Novosphingobium* sp. and unclassified Alcaligenaceae. When reclaimed water samples were chlorinated with initial concentration of $1\text{ mg-Cl}_2/\text{L}$, bacterial counts declined and regrew to 10^6 cells/mL within 3 days with *Rheinheimera* sp., *Acidovorax* sp., unclassified Oxalobacteraceae and *Sediminibacterium* sp. as major groups. Similarly samples treated with $3\text{ mg-Cl}_2/\text{L}$, bacterial counts declined and regrew to 10^6 cells/mL within 7 days with major groups being unclassified Betaproteobacteria, *Methylobacterium* sp., *Pseudomonas* sp._2, *Pseudomonas* sp._1, unclassified Comamonadaceae, and *Acidovorax* sp. However, when subjected to $5\text{ mg-Cl}_2/\text{L}$, bacteria could regrow upto 10^5 cells/mL by 13 days where major regrowing groups were chlorine-tolerant species of unidentified Sphingomonadaceae, unidentified Sphingomonadales, *Methylobacterium* sp. and *Hyphomicrobium sulfonivorans*. Differing doses of chlorine selected bacteria based on their chlorine tolerance, thus altering the OTUs that regrew.

Comparison of the total cell counts, intact cell counts and HPC

The three modes of bacterial enumeration provide insight into different facets of bacterial viability. ICC represents intact cells while TCC incorporates cells with both intact and injured membranes. HPC, on the other hand, is a culture-based method which represents the cultivable fraction of live bacteria.

Although TCC in each case attains a constant value upon regrowth, the corresponding bacterial community structure

does not stabilise but changes with time. Thus, the total count is more likely to represent an “apparent” value while underlying bacterial communities multiply, die and turnover.

After chlorination, the HPC values dropped quicker than the ICC and TCC, which is a phenomenon seen in various drinking water and reclaimed water disinfection studies. The difference could be attributed to the loss of cultivability on contact with chlorine before cell membrane damage can be detected by FCM-live/dead stain [30,31]. It is interesting to note that unlike drinking water where HPC is 1-log_{10} order or more lower than FCM counts [25], we found that the HPC and ICC upon regrowth were within similar range. This indicates that a major portion of bacteria that regrow in reclaimed water are cultivable on R2A media, which in turn provides a good opportunity to isolate chlorine tolerant bacteria, characterise them and devise methods to control their growth.

CONCLUSIONS

To understand the relationship between bacterial regrowth in reclaimed water and chlorine dose applied, reclaimed water was chlorinated such that initial free chlorine concentration was 1, 3 or $5\text{ mg-Cl}_2/\text{L}$. The presence of higher chlorine demand in reclaimed water caused the chlorine levels to drop quickly, paving way for regrowth. Once residual free chlorine declined below detection limit, bacterial regrowth was observed, however, addition of higher dose delayed regrowth.

With the increase in chlorine dose, the bacterial communities that regrew differed; chlorine-tolerant species increasingly survived and multiplied. On addition of initial chlorine doses up to $3\text{ mg-Cl}_2/\text{L}$, groups belonging to Proteobacteria (mainly Beta- and Gammaproteobacteria) namely *Acidovorax* sp., unidentified Comamonadaceae, unidentified Betaproteobacteria and *Pseudomonas* sp. were detected on regrowth whereas at an even higher dose of $5\text{ mg-Cl}_2/\text{L}$, only groups belonging to Sphingomonadales and Rhizobiales (both under Class Alphaproteobacteria) survived and regrew. Many of these chlorine tolerant species are known to form biofilms and this explains why despite high levels of chlorine being administered, regrowth and biofilm formation are often reported. To the best of our knowledge, this is a new study that captures bulk water bacterial regrowth after chlorination in reclaimed water alongwith the microbial communities involved.

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