# Comparison of Laboratory Wastewater Treatment Systems with Settlers

## Affect of cycloheximide and recirculation ratio

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*The influence of cycloheximide and recirculation ratio upon system performance and microbial viability was studied in two continuous lab-scale wastewater treatment systems. Each system consists of an aerobic bioreactor, a settler, a sludge recycling line, and a sludge wastage line (purge). The addition of cycloheximide, which is a eukaryotic inhibitor that primarily impacts microbial predators, significantly ($p < 0.05$) reduced chemical oxygen demand (COD) removal efficiencies in the systems, but had no affect on ammonia removal (i.e., biological nitrification). However, complete nitrification was more apparent in the reactor system with the lower sludge recirculation ratio, especially when cycloheximide was being added. Although the temporal dynamics of living versus dead bacterial cell densities in the reactors did not differ as a function of cycloheximide addition or recirculation ratio, the data show that a stable and diverse predator guild creates a more stable treatment community and more consistent treatment performance.*

*Keywords: recycle ratio, purge ratio, time scale, wastewater treatment process, bioreactor, live/dead ratio*

Biological treatment of wastewater involves organic matter removal, as well as elimination of the nitrogen-based compounds and other pollutants (sulphur, phosphorus, heavy metals). These processes depend upon complex bacterial communities, which include heterotrophic, nitrifying, denitrifying and other bacteria, but also on protozoa; all species acting together or separately in wastewater treatment. In fact, there are many physical, chemical or biological factors that might influence the microbial community structure in treatment bioreactors (e.g., variety of species and number of individuals) and their activity. Examples include wastewater composition, dissolved oxygen (DO), *p*H, temperature, the recirculation ratio and cell wastage rates, system configuration, and the presence of toxic compounds among other factors. The actual bacterial component of the community is also affected by ecological factors, such as grazing of predators [1]. Therefore, the performance of the whole treatment system depends on various aspects, but ultimately its success depends on the active biomass present [2], which can be modified among others by predatory activity.

In general terms, heterotrophic microorganisms are responsible for the majority of organic matter degradation under aerobic conditions. In the absence of oxygen (i.e., anoxic conditions), some facultative heterotrophs alternately use nitrate and nitrite (from nitrification) as final electron acceptors (i.e., denitrification) [3,4]. The oxidation of ammonia to nitrate is carried out by nitrifiers in two steps: ammonia-oxidizing bacteria (AOB) transform ammonia to nitrite, while nitrite-oxidizing bacteria (NOB) transform the latter to nitrate. Both categories of nitrifiers tend to have low growth and nutrient processing rates, making nitrification a slow process that can be operationally unstable. At the same time, bacteria are the primary food source for protozoa. As predators, protozoa are a major cause of mortality for both heterotrophic and autotrophic bacteria in wastewater systems; therefore, they play a major role in controlling the bacterial biomass [5,6]. However, it is generally considered that protozoa, especially ciliated protozoa, present a beneficial role to the process performance by grazing on free-swimming bacteria (suspended or dispersed) and enhancing floc formation, which both improves solids clarification in reactor effluents [6-8].

The protozoa display a certain degree of selective feeding [9], which depends upon the abundance, size, shape and viability of the prey [10,11]. Moussa et al. [3] showed that a dramatic increase in active biomass was observed when no predators were present. Furthermore, changes in the protozoan guild may shape the whole food web in the wastewater community [12], thus affecting the biological performance of wastewater treatment plants. Finally, predators can be bio-indicators of the activated sludge process state [13]. For example, Carchesium sp. and Opercularia microdiscus indicate a lack of dissolved oxygen in the aeration tank, whereas crawling ciliates, such as Chilodonella sp. and Aspidisca cicada, signify at low F/M ratio. Aspidisca cicada, Chilodonella sp. and V. triata tend to be prevalent in aged sludge with elevated solid retention times (SRT) [14]; Vorticella campanula correlates with low effluent biological oxygen demand (BOD) levels [14]; and the abundance of nematodes suggest low organic loadings [15].

Microbial activity and the performance of the wastewater treatment plant can also be influenced by the presence of inhibitory and toxic substances in the feed [16], the protozoa often being generally the most affected [12,17]. In fact, chemical inhibition of protozoa has been used to quantify the grazing mortality of bacteria [18]. One of the substances useful for assessing the affect of inhibitors on the protozoan guild is cycloheximide, an antibiotic that blocks protein synthesis in eukaryotic cells [19], but does not affect bacteria in most aerobic systems. Cycloheximide inhibits most protozoa except ciliates, which are more tolerant of the chemical [20,21], and has been used to study community demographics in wastewater treatment

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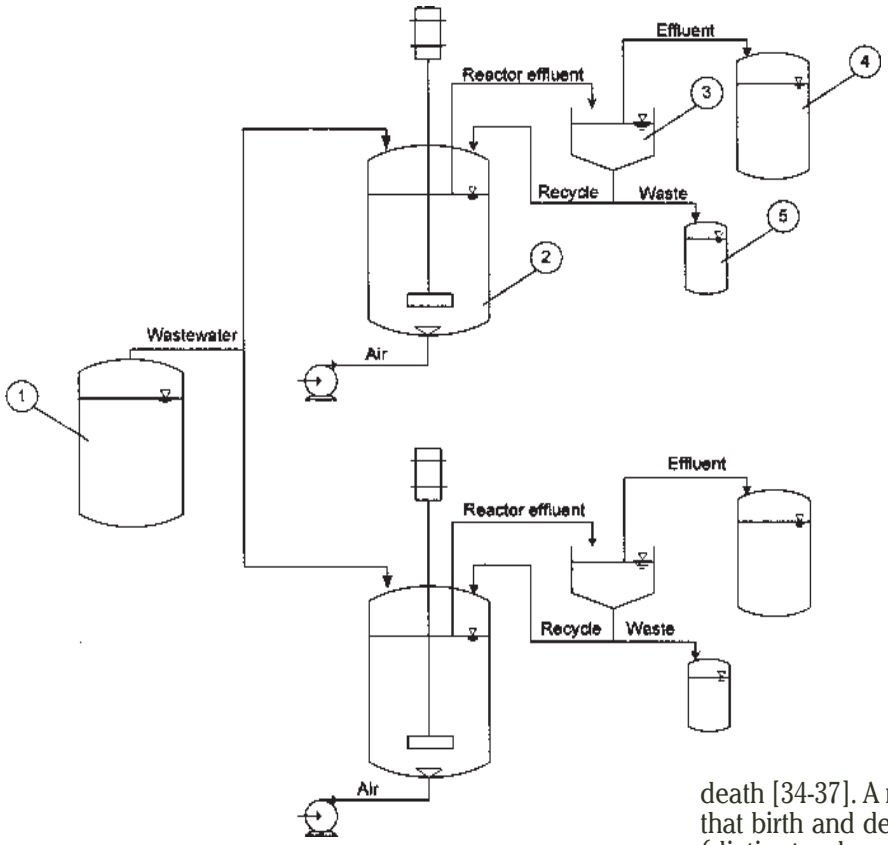



Fig. 1. Experimental set-up for bother reactor systems: 1. wastewater feed tank; 2. aerobic reactor; 3. settler; 4. effluent recovery tank; 5. wasted sludge tank

processes. For example, cycloheximide levels greater than 0.05 mg/L completely inhibited growth, and at least 50% of grazing capacity of Tetrahymena pyriformis in a wastewater environment [22]. At higher concentrations (over 1 mg/L), grazing was 85% inhibited, although a concentration of 20 mg/L mildly stimulated the growth of free-swimming forms [23].

Another important component of a continuous biological wastewater treatment process is sludge recirculation, which keeps the effluent free of biomass and increases the biomass concentration in the system. The accumulation of the inert in the system is avoided by biomass wastage, thus controlling the solid retention time (SRT) [24-27]. In fact, the recycle ratio is a very important control parameter that affects overall performance of the biological system, including the microbial ecology, system hydraulics, and the effluent quality [28,29]. Recirculation also reduces tank sizes be returning active biomass to the main reactor after clarification.

In summary, biological treatment processes are sustained by maximizing the active microbial community, which makes the cell viability of importance. The degree of viability of microorganisms is determined by the reproductive and metabolic activities together with the integrity of the cytoplasmic membrane [31-33], the latter separating the cell from its environment. To reproduce, a cell requires both metabolic activity and cellular membrane integrity. Cells with damaged membrane have the internal structure exposed to the environment and therefore the metabolic activity stops and, consequently, they can be considered dead cells.

The objectives of this study was to investigate the effects of sludge recycling and chemical inhibition on the viability of the activated sludge and its correlation with system performance, and also the influence of recirculation across two time scales (i.e., retention and generation times). Recent papers showed that during the apparent steady-state behaviour, complex non-linear population dynamics exists due to elementary events like birth, growth, and cell death [34-37]. A new paradigm appeared which assumes that birth and death shape the population in age-classes (distinct sub-groups of individuals born at the same physical time and dead after the same period of time), producing oscillatory behaviour at population and process levels. Despite these interesting reports, limited experimental data exist linking community dynamics and cell birth and death cycles. Therefore, here were quantified live-to-dead bacterial cell ratios in activated sludge communities to determine if this ratio altered the dynamic behaviour. Given microbial dynamics in bioreactors are known to be influenced by prey-predator activity; thus, cycloheximide was used as inhibitor to reduce the abundance of predators in the system. In theory, if dynamic behaviour continues in the presence of cycloheximide, this might be explained also by factors other than predation, including live-dead cell effects [36,37].

*Materials and methods*

Microbial community and synthetic wastewater

The sludge used for inoculation was collected from the recirculation pipe of a municipal wastewater treatment plant located in Spennymoor (County Durham, UK, 54°41'0" N, 1°36'0" W) and kept at 4°C until the inoculation of the two reactors. The synthetic wastewater (100x) used for reactors feeding was prepared as follows: 64 g peptone, 38 g meat extract (Lab "LEMCO" powder), 6 g yeast extract, 6 g urea, 13.2 g, $(NH_4)_2SO_4$, 5.6 g $K_2HPO_4$, 0.4 g $CaCl_2 . 2H_2O$ and 0.4 $MgSO_4 . 7H_2O$, added to 2000 mL MQ water. The reactors were provided 0.5 mL/L sterile trace-metal solution and 0.7 g/L $NaHCO_3$. The trace metals solution content in 1000 mL MQ water was: 2.0 g $FeCl_2 . 4H_2O$, 0.05 g $H_3BO_3$, 0.05 g $ZnCl_2$, 0.03 g $CuCl_2 . 2H_2O$, 0.05 g $MnCl_2 . 4H_2O$, 0.05 g, $NH_4MoO_4 . 4H_2O$, 0.05 g $AlCl_3$, 0.05 g $CoCl_2 . 6H_2O$, 0.05 g $NiCl_2 . 6H_2O$, 0.50 g EDTA and 1 mL HCl. The solution was autoclaved and then stored in the refrigerator.

System operation

The study was carried out in a lab-scale system which was built in a laboratory at Newcastle University, UK. A schematic diagram of the experimental set-up is provided

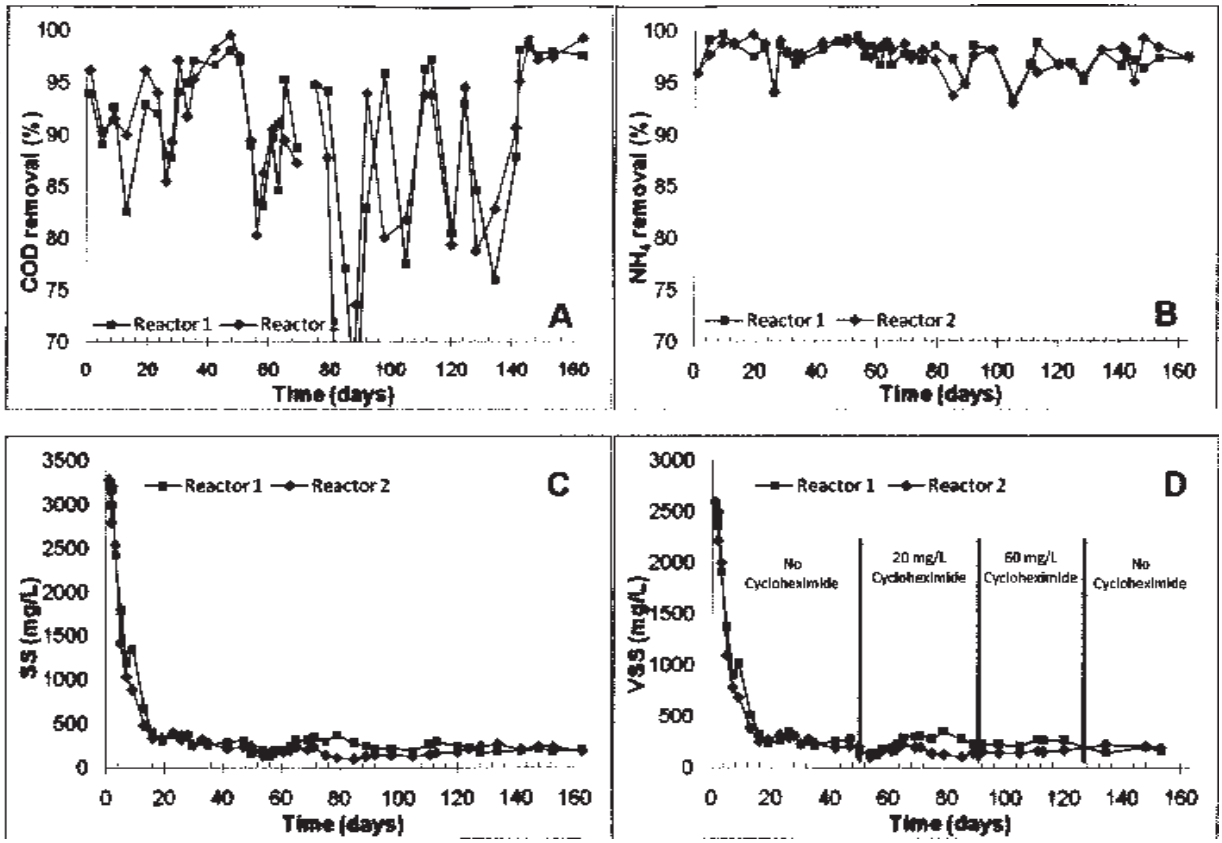



Fig. 2. The performance of the two systems in terms of (A) COD and (B) ammonia removal, and ambient (C) suspended and (D) volatile solids over the complete duration of the study. The different phases of cycloheximide addition in the experiment are noted on panel (D). With one exception, COD removal was always > 75%, whereas ammonia removal was always > 95%.

as figure 1. The experiment was performed using two continuous-flow set-ups operated in parallel (named R1 and R2, respectively), each having a "completely" mixed continuously aerated bioreactor, a settler and a sludge recirculation line. Both bioreactors were fed with the same synthetic wastewater, continuously mixed, and maintained in the refrigerator prior to input. Although rates were changed lately in the experimental program, R1 initially had a lower recirculation flow rate (0.15 mL/min) than R2 (1 mL/min). For inoculation 1.75 L of sludge and 1.75 L culture medium were used. At the beginning of the experiment, the bioreactors were operated in the batch mode for 48 h and then switched to the continuous operating mode with an influent flow rate of 0.48 mL/min. The cycloheximide was used at two different concentrations: 20 mg/L and 60 mg/L, respectively.

Reactor monitoring and analyses

Samples from the two reactors were collected every 3 days and routinely analyzed for suspended solids (SS), volatile suspended solids (VSS), COD and ammonium–nitrogen content ($NH_4^+$). The COD and ammonium–nitrogen were measured with Merck Cell Tests (St Louis, MO). Nitrite and nitrate concentrations were measured using ion chromatography (Dionex ICS-1000). The *p*H was kept in the optimal range by providing supplemental bicarbonate, as needed (i.e., *p*H 7.8 - 8.1).

The relative viability of the microbial community was quantified using LIVE/DEAD® BacLight Bacterial Viability Kit, L13152 (Molecular Probes, Invitrogen, Paisley, UK). The kit includes the green fluorescent DNA-binding stain SYTO 9 and the red fluorescent DNA-binding stain propidium iodide (PI). The differences between these two stains are related to their spectral characteristics and ability to penetrate the membrane of bacterial cells: the SYTO 9 stain labels bacteria with both intact and damaged membranes, while the larger molecules of propidium iodide penetrate only the bacteria with compromised membranes. When mixed in recommended proportions, SYTO 9 stain produces green fluorescence signal for bacteria with intact cell membranes and propidium iodide produces red fluorescence signal for bacteria with damaged membranes, the background remaining virtually non-fluorescent.

Typically, 60-µL of raw sample was mixed with 40 µL 0.85% NaCl in the wells of a 96-well flat-bottom microplate. When all the wells were filled with liquid, 100 µL staining mixture were added in each well. The plate was incubated at room temperature in the dark for 15 minutes, so that the stains should permeate the cell membrane. Then, the signals for the two dyes were measured using a Fluoroskan Ascent FL combined microplate fluorometer and luminometer (Thermo Scientific, USA) and the ratio of live and dead cells was computed as the ratio of the green and red signals.

The results obtained using the kit are expressed here as the normalized ratio of live versus dead cells in the sludge, computed as follows:

$$\bar{y} = \frac{y - y_{min}}{y_{max} - y_{min}}, \quad (1)$$

where:

y is the value of the live/dead ratio as it was read at the fluorometer,;

$y_{min}$ and $y_{max}$ are the minimum and the maximum values of the live/dead ratio for a certain staining experiment;

$\bar{y}$ is the normalized value of the ratio y. This system was used to be compatible with similar previous applications [39-41].

## Results and discussions

Operating plan

The reactors were operated for 163 days, during which both systems were monitored for carbon and nitrogen treatment performance and live-dead cell dynamics over time. The reactors were run under four main regimes: 0 – 49 days with synthetic wastewater feed; 49 – 90 days with 20 mg/L cycloheximide amended to the wastewater; 90 – 127 days with 60 mg/L cycloheximide added; and 127 – 163 days with cycloheximide removed to assess recovery (fig. 2D). This pattern was chosen to first quantify baseline operations prior to modification, and then progressively assess the effect of increasing cycloheximide levels (i.e., altered predation) on system performance and dynamics. The units were then studied during recovery to the original operating conditions. Therefore, the experiment had four phases: a first phase where the microbial community was allowed to develop without constraint; a second phase with low cycloheximide addition inhibits more susceptible predators in the reactors; a third phase with higher cycloheximide additions to suppress "all" possible predators; and a fourth phase without cycloheximide to determine whether the bioreactors return to these original states after prolonged stress.

Initial reactors operations without cycloheximide addition

The bioreactors are initially operated in batch mode and then in continuous-flow recirculation. Therefore, figure 2C and D show that biosolids decreased significantly over the first week as evidenced dropping SS and VSS levels. After ten days, recirculation was commenced with different recirculation rates for the two systems: 0.15 mL/min for R1 and 1.0 mL/min for R2, respectively. After about 16 days, SS and VSS levelled off and did not substantially change for the duration of the study, attaining confined dynamic equilibrium common to such bioreactor systems.

As the bioreactor operations proceeded, communities in the two bioreactors progressively changed, probably resulting from the different recirculation ratios. In general, the bioreactor with the higher recirculation rate (R2) had larger and more flocs that settled better, while the lower recirculation rate unit (R1) had smaller flocs that settled poorly. These patterns may reflect different species selection in the lower (R2) versus higher (R1) SRT systems; i.e., shorter SRTs often favour higher growth rate heterotrophs, and smaller flagellate and ciliated protozoa, whereas longer SRTs tend to favour slower growing heterotrophs, autotrophs, and crawling and stalked ciliated protozoa [42].

Varied recirculation rate between bioreactors only slightly influenced COD and ammonia removal efficiencies. Figure 2A shows that COD removal ranged from 82% to 98% when cycloheximide was not added and ammonia removal efficiency was usually > 95%. Mean COD and ammonia removal efficiencies were about 1 to 2% higher in the high recirculation rate bioreactor (R2), which can be the influence of a longer SRT, but the difference between bioreactor performance was not statistically significant (Wilcoxon Rank-Sum Test; $p > 0.05$).

Reactor operations with 20 mg/L cycloheximide added to feed

After reactor operations had stabilised (~49 days), cycloheximide addition was commenced by providing the chemical to both the influent feed and the reactors to a final concentration of 20 mg/L. The goal was to selectively inhibit predatory activity in the systems to assess how process might change and also whether changes related to altered demographics or live and dead bacterial cells in the systems. At this concentration, the most susceptible predators should be impacted, which in turn, should influence both bacterial abundances and dynamics. Protozoan and metazoan predators can be quite selective in feeding due to their different physical sizes and also the characteristics of the bacterial prey, such as different cell morphologies (like filament formation and attaching in the micro-colonies), cell size, swimming speed, and toxin production as a defence mechanism [43-46].

Microscopic and other investigation of the sludge before and after 20 mg/L cycloheximide addition indicated the inhibitor did altered predators demographics, but SS and VSS data in figures 2C and 2D suggested that overall biomasses did not significantly change. In addition, cycloheximide appeared to impact floc formation in the systems. Specifically, smaller and more dispersed aggregates become more common, especially in the low recirculation rate unit (R1), which influenced settler performance [25, 49].

Bioreactor performance significantly declined after cycloheximide addition, both in terms of efficiency and stability; however, the significant reduction in performance was only seen in COD removal and not ammonia removal. This is consistent with previous observations that nitrification is less affected by cycloheximide that other wastewater treatment reactions [47]. Figure 3 shows that COD removal efficiency declined significantly ($p < 0.01$) from ~93% to 83% before and after cycloheximide addition, and figure 3A suggests much less consistent COD removals over time. In contrast, ammonia removal did not change, staying roughly at 97% during the period of COD instability. We suspect this greater process instability mostly resulted from disappearance of susceptible small ciliated protozoa and reduced predator diversity because of the selective inhibition [20], although we only have

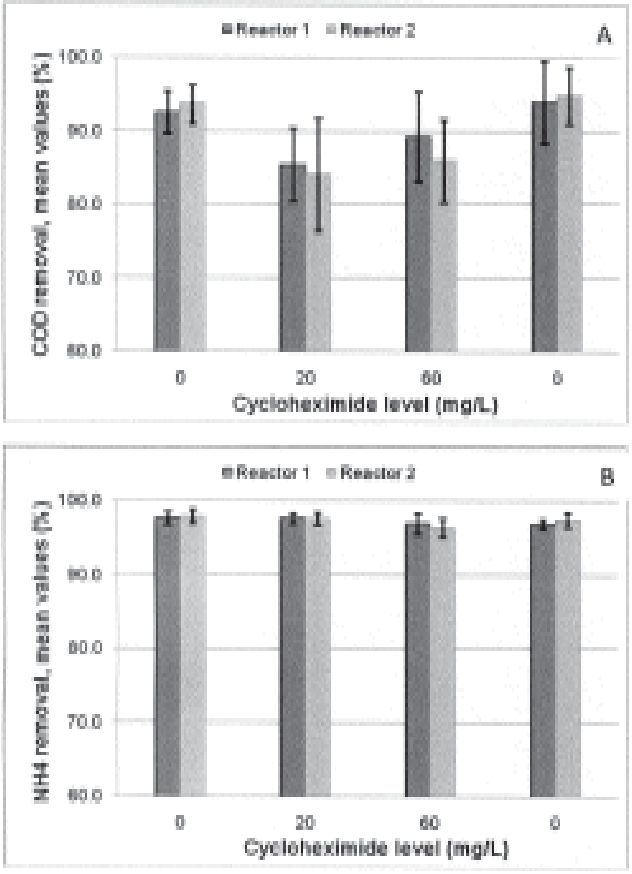
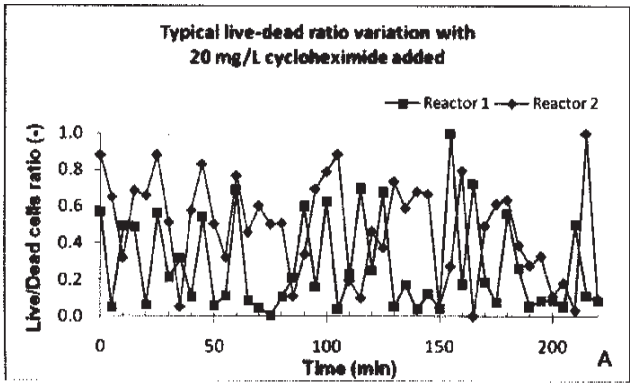



Fig. 3. Treatment performance for COD and ammonia removal associated with different cycloheximide level supply levels. Errors refer to 95% confidence intervals based on between 8 and 12 samples, depending on reactor and phase



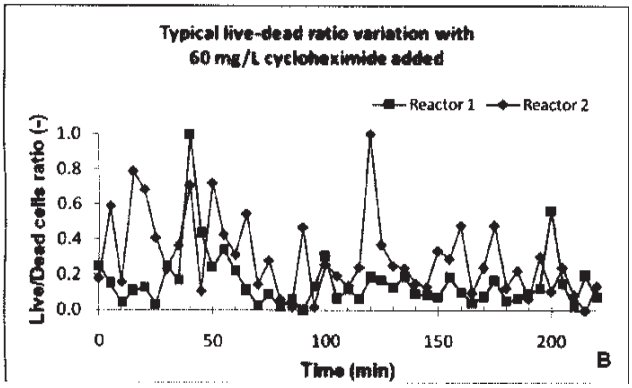




Fig. 4. Typical normalized ratio of live and dead cells dynamics when reactors were amended with (A) 20 and (B) 60 mg/L cycloheximide.

Operations with 60 mg/L cycloheximide added to feed

The 20 mg/L cycloheximide addition has been expected to significantly reduce predation so that live and dead cell dynamics could be examined in the absence of major predators. However, predation still existed at 20 mg/L, therefore cycloheximide levels were increased further to 60 mg/L in bother reactor systems. In addition, cell wastage from the recirculation line was also commenced to allow greater regrowth of new species within the main aerobic reactors; i.e., one-third of the recycle flow from the settlers were directed to waste and two-thirds were recirculated to aerobic bioreactor.

Overall, these changes in operation did not alter process efficiencies between 20 mg/L and 60 mg/L cycloheximide addition. Figure 4 shows COD removal efficiencies (i.e., ~86%) were still significantly lower that operations without cycloheximide ($p < 0.05$), although figure 3A indicates that day-to-day effluent COD levels were more variable when higher cycloheximide was provided. As before, cycloheximide addition, even at 60 mg/L, did not significantly alter nitrification efficiencies. Further, qualitative observation of the sludge at the higher cycloheximide levels suggested that predatory demographics will not be substantially altered by increasing the level of the inhibitor.

Figure 4 provides an example of associated live and dead bacterial cell dynamics associated with cycloheximide amended reactors. It had been speculated that dynamic oscillations often seen in live-dead cell levels might disappear if predation was altered unless another factor like time-delayed birth and death was also important to dynamics. With both 20 mg/L (fig. 4A) and 60 mg/L (fig. 4B) cycloheximide additions, live-dead cell ratios oscillated, although with limited symmetry. Greater amplitudes of live-dead oscillations were apparent at 20 mg/L cycloheximide without cell wastage compared with 60 mg/L with cell wastage, but the oscillations were not

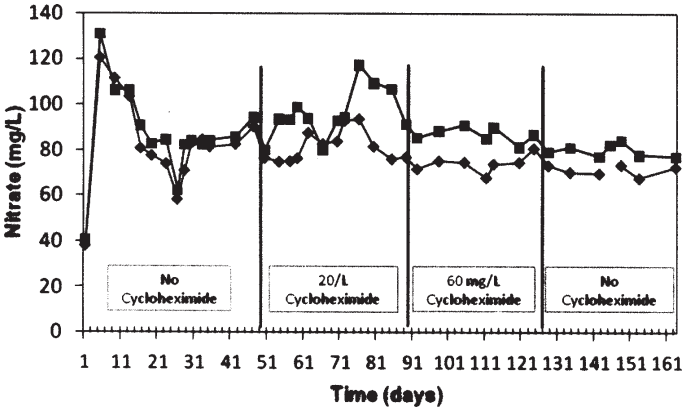



Fig. Effluent nitrate levels over time throughout the whole study.

patterned, which was clearly seen with E. coli populations in pure culture [37]. However, it is unclear if this lack of patterned oscillations was due to the complexity of the wastewater treatment community versus a pure culture, or due to disturbing the ecosystem by cycloheximide addition. In general, oscillations were more slightly patterned when cycloheximide was not provided, but did not changed significantly with cycloheximide. Therefore, no major conclusions can be made about live and dead cell dynamics, apart that the presence or absence of predators seems not to affect it strongly.

Recovery operations after cycloheximide was removed from feed

To verify that the wastewater systems could recover after a period of toxicant addition, cycloheximide addition was ceased after 129 days operation. Overall, when cycloheximide was removed, process performance returned to its original state before the inhibitor was added. COD removal returned to about 94%, which was not significantly than prior to cycloheximide addition ($p > 0.05$). Ammonia removal stayed between 97 to 98% throughout all phases of the experiment. In summary, cycloheximide clearly impacted COD removal in the reactor systems, possibly associated with altered feeding patterns by system predators, although the relative abundances of living versus dead cells did not seem to alter or being consistently affected by differing grazing patterns.

Affect of recirculation rate of complete nitrification

Although nitrification rates as reflected by ammonia removal were always high, the extent of complete nitrification did differ between the low (R1) versus high (R2) recirculation rate reactors. Figure 5 shows that effluent nitrate levels, which suggest complete nitrification, were consistently higher in the lower recirculation rate unit. Interestingly, the difference between the two systems was greater after cycloheximide addition, which suggests that changes in predator demographics affected complete nitrification. This result could either be due to different grazing patterns among surviving predators on ammonia-oxidizing bacteria (AOB) versus nitrite-oxidizing bacteria (NOB) within the flocs, or alternate increased susceptibility of NOB to cycloheximide.

## Conclusions

Parallel laboratory scale treatment systems were used to examine the affect of recirculation ratio and a chemical inhibitor (cycloheximide) on COD and ammonia removal from wastewaters. In general, the reactors performed well, with removal efficiencies almost always being greater than 80% for COD and 95% for ammonia. However, the inclusion

of the cycloheximide in the feed, which selectively targets eukaryotes (i.e., predators) in the system, significantly reduced COD removal efficiencies, caused the flocs to be more disaggregated, and appeared to change predator demographics. Further, day-to-day treatment performance was more variable when cycloheximide was present. In contrast, inclusion of cycloheximide had no effect on net ammonia removal efficiencies, although incomplete nitrification was more apparent in the high recirculation ratio units. Finally, the inclusion of the chemical inhibitor did not appear to significantly alter live versus dead cell dynamics.

In summary, although the inclusion of cycloheximide did not grossly alter average waste treatment performance in our systems, predator demographics were clearly affected and relative day-to-day stability in performance were influenced. This suggests that net COD and ammonia utilization is hugely influenced by the presence or absence of predators, which is not surprising given that the bacteria are responsible for soluble carbon and nitrogen processing. However, the data suggest that a stable and diverse predator guild does create a more stable treatment community. We suggest that more work focused specifically on that role is needed, especially regarding predator diversity and its affect on complete nitrification.

## Abbreviations

AOB – ammonia oxidizing bacteria
BOD – biological oxygen demand, mg/L
CSTR – continuous-flow stirred tank reactor
COD – chemical oxygen demand, mg/L
DO – dissolved oxygen, mg/L
NOB – nitrite oxidizing bacteria
SRT – solids residence time, d
VSS – volatile suspended solids, mg/L

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