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Research article

The effect of malathion on the activity, performance, and microbial ecology of activated sludge

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ABSTRACT

This study evaluated the effect of a VX (*O*-ethyl *S*-[2-(diisopropylamino)ethyl] methylphosphonothioate) surrogate (malathion) on the activity, performance, and ecology of activated sludge bioreactors. In the presence of malathion, the maximum observed respiration rates varied between 43 and 53 μg/O_2 min, generally similar to the 49 μg O_2/min rates observed in controls. Malathion did not alter the respiration ratio of O_2 consumed-to-CO_2 produced nor did it impact the shape of the oxygen consumption curves during respirometry. Shorter term (12 h) batch tests showed that both chemical oxygen demand (COD) and ammonia removal were not negatively impacted by the presence of 0.1–3 mg/L malathion. Longer term continuous addition (i.e. 40 days) of 0.1 mg/L of malathion also had no effect on COD and ammonia removal. In contrast to shorter term exposures, longer term continuous addition of 3 mg/L of malathion negatively impacted both COD and nitrogen removal and was associated with shifts in the abundance of species that are common to activated sludge. These results illustrate the impact that chemicals like malathion may have on COD removal, and nitrification, as well as the robustness of activated sludge microbial communities.

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1. Introduction

Decontamination activities following an accidental or intentional chemical release may generate enormous quantities of contaminated water/washwater. While some of this contaminated water will be contained on-site, a sizable quantity may enter the wastewater collection system where it could impact biological wastewater treatment plants. The biological process at such facilities normally functions to remove conventional pollutants but may also serve to prevent the spread of chemical weapons agents (CWAs) into the aquatic environment. For instance, some CWAs appear to be partially biodegradable (Bell, 1986; Imran et al., 2006; Shan et al., 2009; Walters and Thesis, 2013); however, there are concerns about microbial inhibition that may be caused by CWAs or their metabolites (Walters and Thesis, 2013). Further understanding is needed to determine the potential for wastewater treatment plants to effectively remove CWAs from water.

This study focused on the effect of malathion, a surrogate for the CWA VX (Bartelt-Hunt et al., 2008), on an activated sludge system. Janeczko et al. (2014) demonstrated that activated sludge can degrade malathion in laboratory-scale bioreactors. There is also circumstantial evidence showing that isolates common to activated sludge can biodegrade malathion (Singh et al., 2013). These findings stand in contrast to the results of Tsezo and Bell (1991), who used radiolabeling to demonstrate that sorption and abiotic degradation, not microbial activity, were primarily responsible for malathion removal in activated sludge. Thus, biodegradation of malathion may not be universally observed in activated sludge, perhaps due to the different community structures present at various facilities.

The effect of malathion on sludge activity is also a concern. For

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example, Guo et al. (2009) found that malathion can inhibit the hydrolase enzyme responsible for pyrethroid (insecticide) degradation. Janeczko et al. (2014) found that 3 mg/L of malathion inhibited COD removal in batch tests with activated sludge, and Pai et al. (2009) found that the heterotrophic growth rate constant decreased by 66% in the presence of 0.5 mg/L of malathion. (Tazdait et al., 2013) also reported inhibition of heterotrophic growth by malathion, but at a considerably higher concentration (i.e.140 mg/L) than that of (Pai et al. (2009).

This study utilized respirometry, a test which measured microbial activity. Respirometry has been used to reveal metal-induced inhibition of COD removal and nitrification in activated sludge (Madoni et al., 1999) and to describe the effects caused by organic chemicals which can serve as both substrate and inhibitor (Ricco et al., 2004). Respirometry has not been performed with malathion in activated sludge and may help resolve uncertainty about the malathion concentrations responsible for inhibition. This data, used in combination with water quality data and ecological profiles, may shed more light on the short and longer-term effects of malathion on biological wastewater treatment systems.

2. Experimental

2.1. Sequencing batch reactors

Three 2.0 L sequencing batch reactors (SBRs) were constructed and operated as described previously Janeczko et al. (2014). The SBRs were seeded with activated sludge taken from the Fairborn Water Reclamation Facility, Fairborn, OH. All three reactors operated continuously on identical 12 h cycles consisting of fill, aeration (11 h), and a 1 h settling phase. Mixing was achieved with a stir plate. The dissolved oxygen (DO) concentration was between 3 and 4 mg/L. Wasting took place during the aeration period to maintain a solids retention time of 27 days. The hydraulic retention time was 36 h. The synthetic feed consisted of dissolved chemical constituents designed to provide carbon sources and nutrients. Sodium bicarbonate was provided to maintain a pH between 6.5 and 7.5, while allowing for a relatively consistent alkalinity and an inorganic carbon source during nitrification. Peptone (87% of influent COD) and sodium acetate (13% of influent COD) provided the influent COD for the reactors. The feed stock was as described by previously Janeczko et al. (2014). During the first 4 months of SBR operation the reactors were monitored for COD, ammonia (NH_3), nitrite (NO_2), and nitrate (NO_3) concentrations in the effluent. This period allowed for the development of long term baseline data and for periodic removal of activated sludge for both the respirometry and bench scale batch test experiments.

Following the respirometry and batch tests experiments, the feed for two of the reactors was augmented with malathion at concentrations of 0.3 mg/L and 9.6 mg/L respectively. The malathion concentrations supplied to each reactor, along with feed A and feed B volumes mentioned earlier, brought the initial concentration of malathion of reactors I and II to 0.1 mg/L and 3.0 mg/L (respectively). The effluent was monitored for COD, NH_3, NO_2, and NO_3 concentrations during longer term malathion exposure testing. Reactor III was not fed with malathion.

2.2. Respirometry

Respirometry was conducted with 250 ml Pyrex bottles. The sludge was settled and then re-suspended with synthetic feed (including malathion) at the start of the oxygen uptake test. The respirometer (Columbus Instruments, Inc., Columbus, OH) consisted of a micro-oxymax-BGM system sample pump, a sample drier, an oxygen sensor, a carbon dioxide sensor, and two 10 port expansion interface units. The samples were continually mixed utilizing an IKA 15 position stirring plate at approximately 330 rpm. The system was controlled utilizing the micro-oxymax software V2.3.5 running on Windows 98. Oxygen consumption and carbon dioxide production were determined over a 12 h period.

2.3. Batch tests

Batch tests were conducted in three 1000 ml beakers. Sludge taken from the SBRs was exposed to malathion concentrations up to 3 or (in some cases) 5 mg/L. Concentrations of malathion, soluble COD, NH_3, and NO_3 were measured hourly for 12 h. Additionally, NO_2 was measured every 3 h and gravimetric measurements were taken every 6 h. Mixing was achieved with a stir plate and the DO concentration was between 3 and 4 mg/L.

2.4. Detection of malathion

Water samples were extracted using BD Luev-lok syringes (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) and then passed through syringe filters (Cole Parmer, Vernon Hills, IL, USA) with surfactant-free cellulose acetate membranes with glass fiber pre-filters and opening sizes of 0.2 μm) to remove any suspended solids. The filtered sample was placed in a 2 ml crimp top amber auto sampler vial and stored at 5 °C. All samples were taken in duplicate with the exception of the batch test samples, which were taken in triplicate. Malathion concentrations were determined with an Agilent 7000C GC/MS Triple Quad (Agilent Technologies, Santa Clara, CA, USA) with a 7890B GC system utilizing a 7693 Auto Sampler, a 7693A Auto injector module with a 10 μl syringe, an Agilent 5190–2293 inlet liner, and an Agilent HP-5MS Ultra inert column (0.25 μm film thickness, 0.250 mm internal diameter x 30 m length).

2.5. Other analytical methods

Concentrations of COD, ammonia, nitrate, and nitrite were measured using HACH methods 8000 (Low range), 10,031, 10,020, and 8153. Total suspended solid (TSS) and volatile suspend solid (VSS) were measured according to the standard methods for the examination of water and wastewater methods 2540D and 2540E, respectively (APHA et al., 2016). All HACH measurements were conducted in triplicate; all TSS and VSS measurements were conducted in duplicate.

2.6. Microbial community analysis

DNA was extracted using the Mo Bio Laboratories, Inc. PowerSoil DNA extraction kit (Mo Bio Laboratories, Inc., Carlsbad, CA) and protocols (Mobio, 2014) and procedures adopted from Janeczko et al. (2014). A detailed description of the methods is included in the Appendix. Briefly, the reactor samples were evaluated for initial quantity using the Qubitr 2.0 u-fluorometer (Thermo Fisher Scientific, Waltham, MA) and a dsDNA HS (High Sensitivity) Assay Kit (Thermo Fisher Scientific, Waltham, MA). After sequencing, a Binary Alignment/Map (BAM) file was generated with raw sequencing data. Sequence adapter trimming was accomplished using Cutadapt 1.2.1. Quality control and assembly of the sequence reads was conducted using Mira 3.9.1.7. A standalone version of NCBI's BLAST 2.2.30 + was downloaded along with the 16S Microbial database. Using the command line interface, the FASTA files for each barcode set of contiguous (contig) sequences were parsed using a nucleotide query (blastn) and NCBI's 16S microbial database. The BLAST query used default standalone values. The BLAST output file was imported into MEGAN. A Least Common Ancestor

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(LCA) parameter filter was applied. The minimum bit score for results was 50.0. The maximum expected value (e-value) was 0.01. All returned values below 10.0% of the maximum percentage hit were discarded. Finally, the 16S Percent Identity Filter was enabled enforcing rank-based match percentage requirements of Species 99%, Genus 97%, Order 90%, Class 85%, and Phylum 80%. The normalized number of sequence reads was used as a metric for determining the abundance of microbial species.

3. Results and discussion

3.1. The effect of malathion on microbial respiration

Maximum respiration rates were approximately 49 μg O_2/min (±4%) when the activated sludge was not exposed to malathion (Fig. 1, shown for duplicate experiments for each concentration). When malathion was added, the maximum respiration rates varied between 43 and 53 μg O_2/min (also ± 4%). The maximum respiration rate measured without malathion was statistically similar to most of the maximum respiration rates measured after adding malathion but it was statistically different from the lowest maximum respiration rate measured after adding 1 mg/L malathion ($\alpha = 0.1$). The shape of the oxygen consumption curves were similar in all cases, beginning with a rapid oxygen consumption rate during first 1.5–2 h, followed by a gradual, nonlinear decline in the respiration rates until the experimental time reached approximately 6 h. The respiration rates after hour 6 were between 5 and 15 μg O_2/min. Cumulative O_2 consumption varied between 10 and 13.5 g of oxygen over the 12 h experiment across all 6 trials, but it was typically smaller when malathion was present (see Fig. A1). However, neither the respiration rates nor the cumulative oxygen consumption exhibited a clear trend with increasing malathion concentration. When the oxygen uptake data were normalized with the VSS concentration, the findings showed no systemic relationship between specific oxygen uptake rates (SOUR) and malathion concentration (data not shown). For example, the highest 1 h SOUR (i.e. 16 mg O_2/g VSS-hr) was observed at 0 and 5 mg/L of malathion, the lowest 1 h SOUR (i.e. 14.3 mg O_2/g VSS-hr) was observed at 0.1 mg/L.

Previous research has indicated that malathion may inhibit protein synthesis (Kim et al., 2005) or serve as a substrate for nonspecific cometabolic enzymes such as monooxygenases (Janeczko et al., 2014). The current findings suggest that inhibition and degradation could be occurring simultaneously. Known models for substrate utilization and chemical inhibition did not describe the relationship between the respiration kinetics and the malathion concentration (data not shown). However, an empirical dual use model (Blanch and Clark, 1997) was used to estimate the value of model parameters K_i and K_s (data not shown), which are, in principle, related to the concentration range at which malathion acts as an inhibitor (in the case of K_i) or a substrate (in the case of K_s). The best fit values were $K_i = 1$ mg/L and a $Ks = 100$ mg/L, which suggests that malathion acts primarily as an inhibitor at a malathion concentration near 1 mg/L. Future research should continue

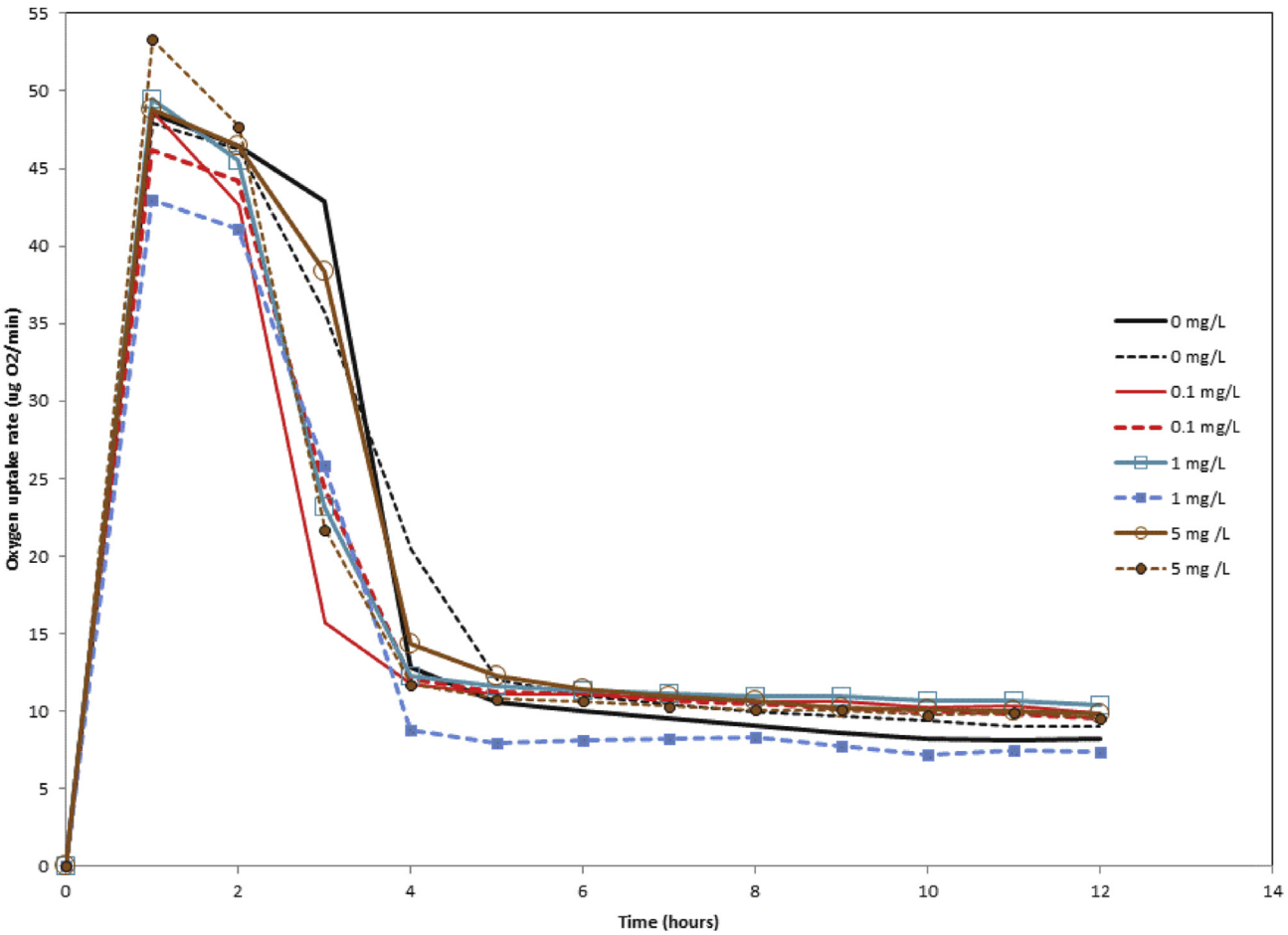

Fig. 1. The effect of malathion concentration on oxygen uptake rates during respirometry. Error for these measurements is ±2%. Data from duplicate experiments is shown for each malathion concentration.

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to investigate the competing mechanisms impacting cellular respiration.

The stoichiometric O_2 consumption-to-CO_2 production ratio reflects the underlying respiratory pathways used to generate cellular energy and it depends on the identity of the principle substrate. For example, when acetate was used for aerobic growth, the bioenergetically expected O_2 consumption-to-CO_2 production ratio was computed as described previously (Blanch and Clark, 1997) and it was 2.48 mol O_2/mole CO_2 and 0.98 mol O_2/mole CO_2 when peptone was the growth substrate (Fig. 2). The peptone-driven ratio was lower than the acetate-driven ratio in large part because each electron-equivalent (eeq) of peptone produces 0.24 mol of CO_2, while each eeq of acetate produces just 0.13 mol of CO_2 (Blanch and Clark, 1997). When the feed COD was 87% peptone and 13% acetate and when nitrification was accounted for, the theoretically expected O_2/CO_2 ratio was 1.76. This stoichiometric value was in good agreement with the measured O_2 consumption-to-CO_2 production ratios, which were generally between 1.7 and 1.85 mol O_2/mole CO_2. These data suggest that malathion did not impact the underlying respiratory pathways used to generate cellular energy.

3.2. The effect of malathion on COD and nitrogen removal in shorter term (batch) experiments

Sets of batch experiments were performed in the presence of 0, 0.1, and 3 mg/L of malathion. Fig. 3 and Figs. A2–A3 (replicate trials) show influent soluble COD removal during batch experiments. COD removal followed a nearly first-order trend during the first 2 h, with an effective first order rate constant of 0.6 per hour. Very little COD removal was observed after 2 h and COD removal was not stopped by the addition of 0.1 or 3 mg/L of malathion. In each trial, the COD removal profiles showed an increase in the COD concentration after 8 h when the initial malathion concentration was 3 mg/L; this may be due in part to the production of poorly-degradable metabolites or the excretion of other microbial byproducts. Ammonia-N removal proceeded after 2 h at rates of approximately 1.4 mg N/L-hr (control), 2 mg N/L-hr (0.1 mg/L initial malathion concentration) and 0.94 mg N/L-hr (3 mg/L initial malathion concentration) (Fig. 4). The 2 h lag in ammonia removal can be understood by considering both the NH_3-N and COD data together. This data suggests that heterotrophic activity dominated the 2 h of the batch tests, successfully out-competing the slower-growing nitrifiers for oxygen and permitting nitrification only after the COD levels had been substantially reduced. Malathion removal was observed between hours 2 and 9 (data not shown), which suggests cometabolic autotrophic activity observed previously (Janeczko et al., 2014). The ammonia-N results from the second batch test (Fig. A4) also showed the same 2-h lag, while the results of the third batch test (Fig. A5) showed no discernable lag in ammonia-N removal, but also showed a more immediate reduction of COD. Thus in each case, ammonia-N removal proceeded when COD was substantially reduced. Although each batch test showed that the lowest ammonia-N removal rate was observed at the highest malathion concentration of 3 mg/L, all of these results showed that both COD and NH_3 removal proceeded during batch exposure to malathion at 0.1 and 3 mg/L. Short term spikes of malathion (at 3 mg/L or less) did not stop COD or NH_3 removal.

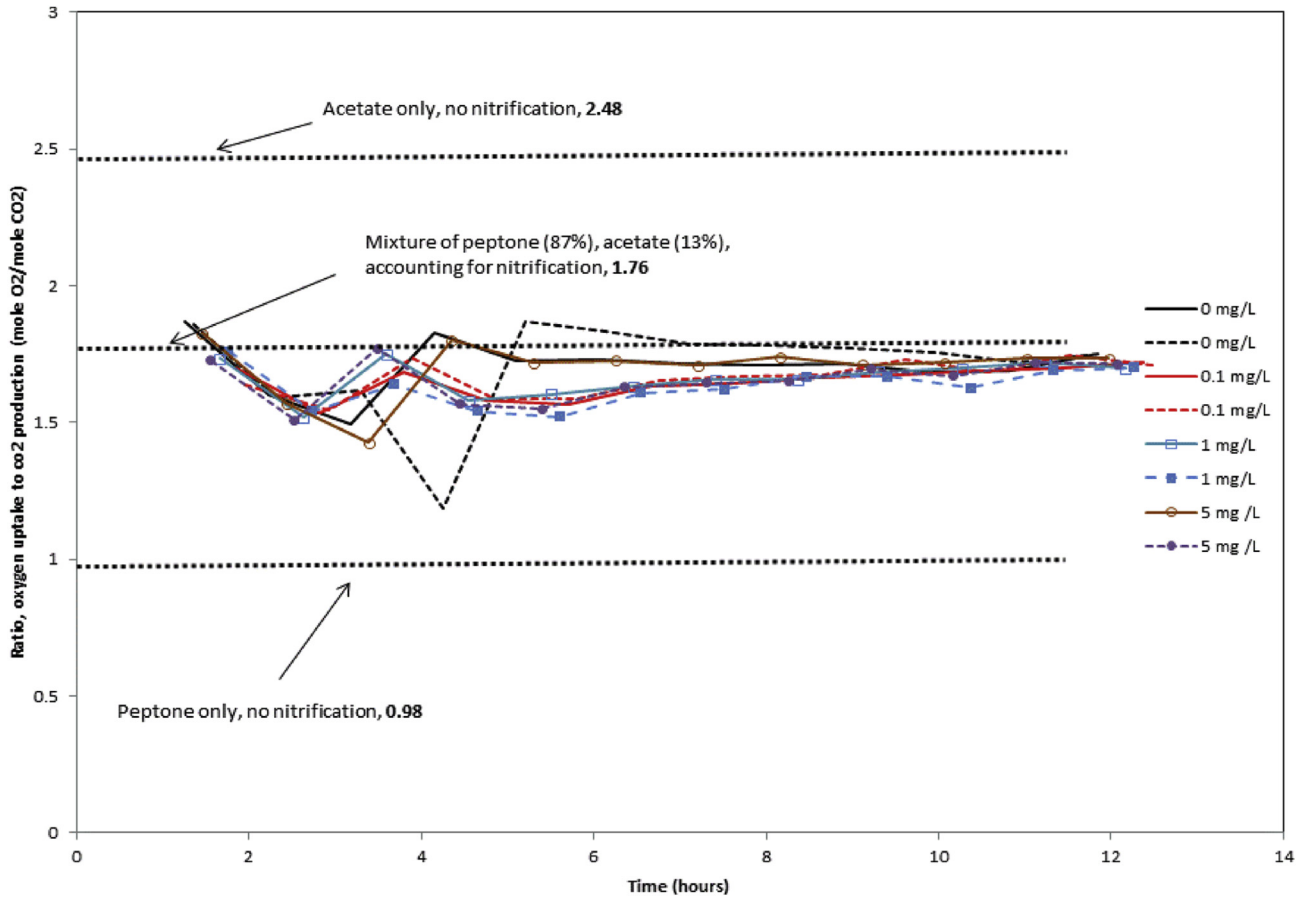

Fig. 2. The effect of malathion concentration on O2-to-CO2 molar ratio. Theoretically-derived ratios were determined for growth on acetate, peptone, or their mixture (COD weight basis). Data from duplicate experiments is shown for each malathion concentration.

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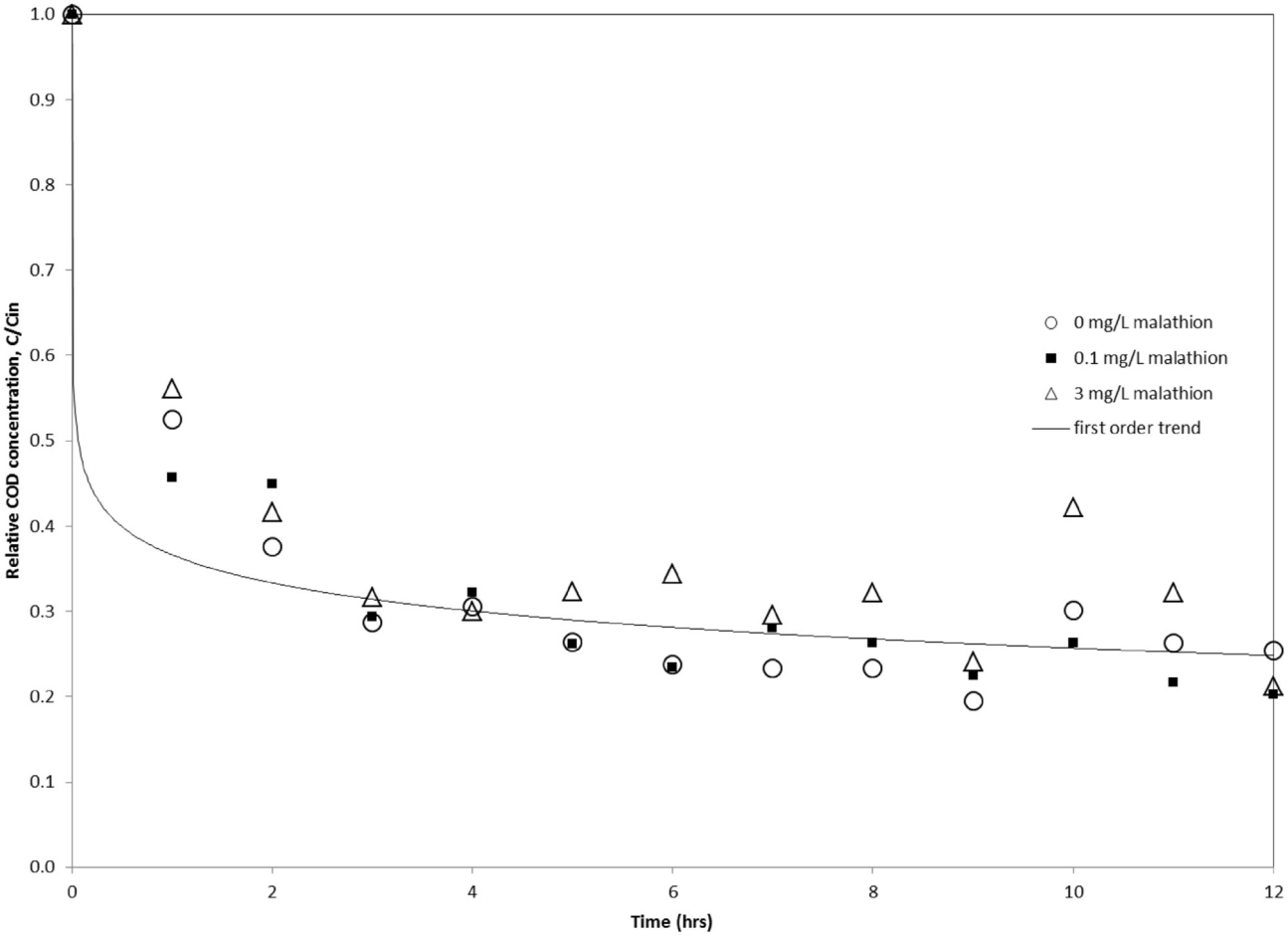

Fig. 3. Trial 1, The effect of malathion concentration on COD removal in batch experiments.

3.3. The effect of malathion on long-term SBR performance

The effect of the influent malathion concentration on long-term SBR performance, measured via COD and NO_3, is shown in Fig. 5. The activated sludge in reactor I, with an initial malathion concentration of 0.10 mg/L, was not significantly inhibited by the presence of malathion. The mean effluent COD concentration for reactor I prior to malathion addition was 12 ± 26 mg/L compared with 12 ± 7.8 mg/L after the addition of malathion. A two tailed *t*-test, assuming unequal variance, indicated that there was no significant ($\alpha = 0.1$) difference in effluent COD concentrations when comparing the 11 samples taken before and the 11 samples taken after the addition of malathion. The mean NO_3 concentration for reactor I prior to malathion addition was 28 ± 5.8 mg/L, compared with 28 ± 2.6 mg/L following exposure. Utilizing the same statistical test, mean NO_3 concentrations were not significantly different before and after malathion addition. These results show that 0.10 mg/L of malathion did not interrupt the heterotrophic and nitrifying bacterial communities.

However, higher concentrations of malathion affected the ability of activated sludge to degrade COD and produce NO_3. In the bioreactor exposed to 3 mg/L malathion, the mean effluent COD concentration prior to malathion exposure was 12 ± 21 mg/L, compared with 20 ± 150 mg/L following malathion exposure. The difference between the pre- and post-exposure COD concentrations was statistically significant ($\alpha = 0.1$). The large standard deviation for the COD values can be attributed to the observed spike in effluent COD concentration on day 6. This spike was consistent with previous findings (Janeczko et al., 2014). NO_3 production was also impacted by the addition of 3 mg/L malathion. The mean effluent concentration of NO_3-N prior to malathion exposure was 31 ± 1.3 mg/L compared with 25 ± 4 mg/L following exposure. These statistically significant changes in effluent quality showed that the heterotrophic and nitrifying bacteria were negatively affected by exposure to 3 mg/L of malathion.

The effluent concentrations of malathion from both reactors I and II were below the detection limit of 1 μg/L. Malathion was likely removed via both sorption (Tsezo and Bell, 1991) and biodegradation (Janeczko et al., 2014; Leoni et al., 1992) to produce byproducts. Previous research indicated that malathion may be partially incorporated into cell tissue (Subramanian et al., 1994) and may be partially transformed into byproducts including malaoxon, malathion diethylthiomalate, malathion monocarboxylic acid, and dimethyl malathion, each with potential to act as inhibitors (Kralj et al., 2007; Lai et al., 1995). Thus, the inhibition that may be observed after malathion addition may be due in part to the presence of the metabolites.

3.4. The effect of malathion on the microbial community in activated sludge

Longer term addition of malathion was also associated with changes in the relative abundance of a wide variety of bacteria commonly found in activated sludge. Changes in the normalized

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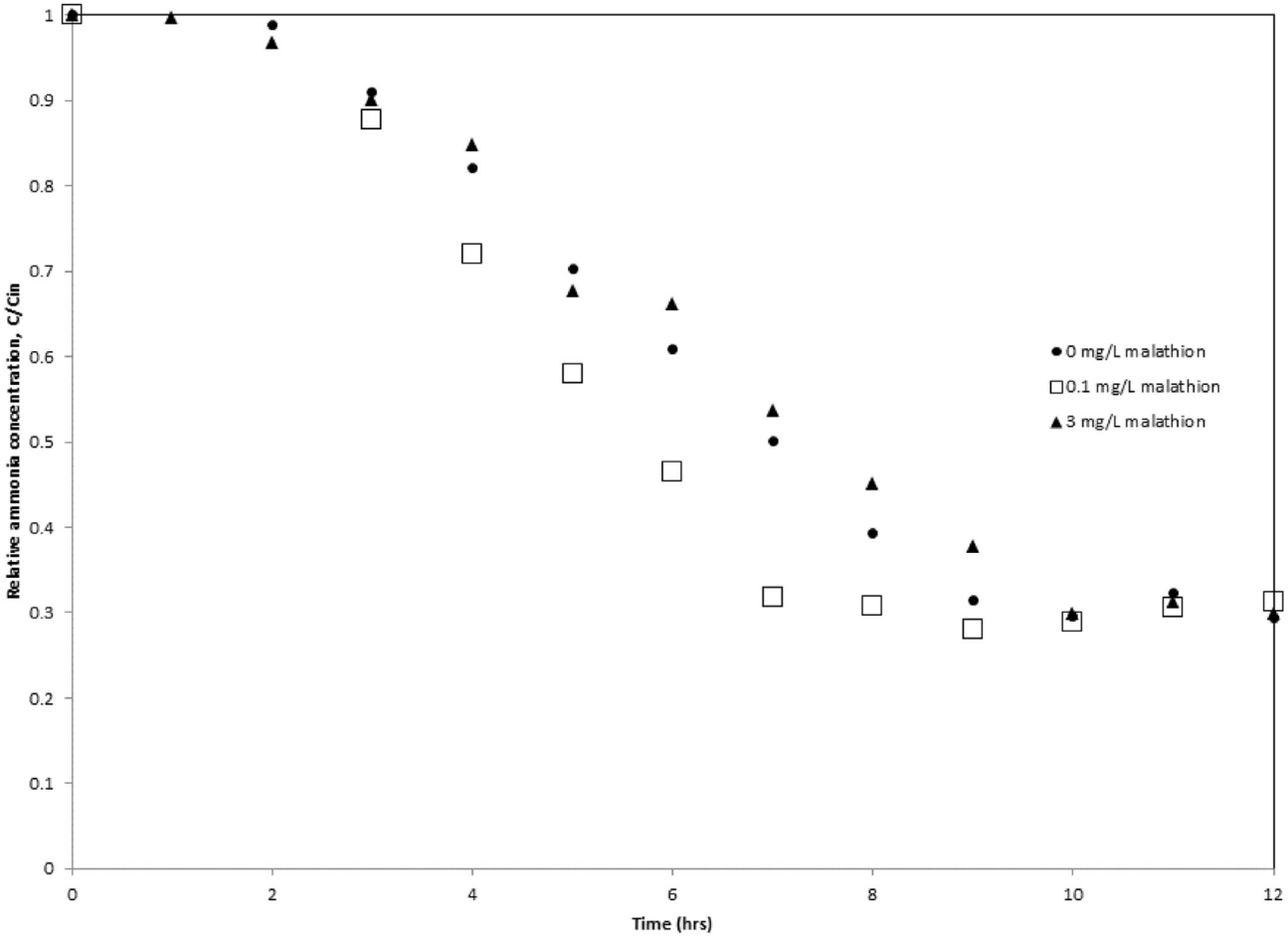

Fig. 4. Trial 1, The effect of malathion concentration on NH3-H removal in batch experiments.

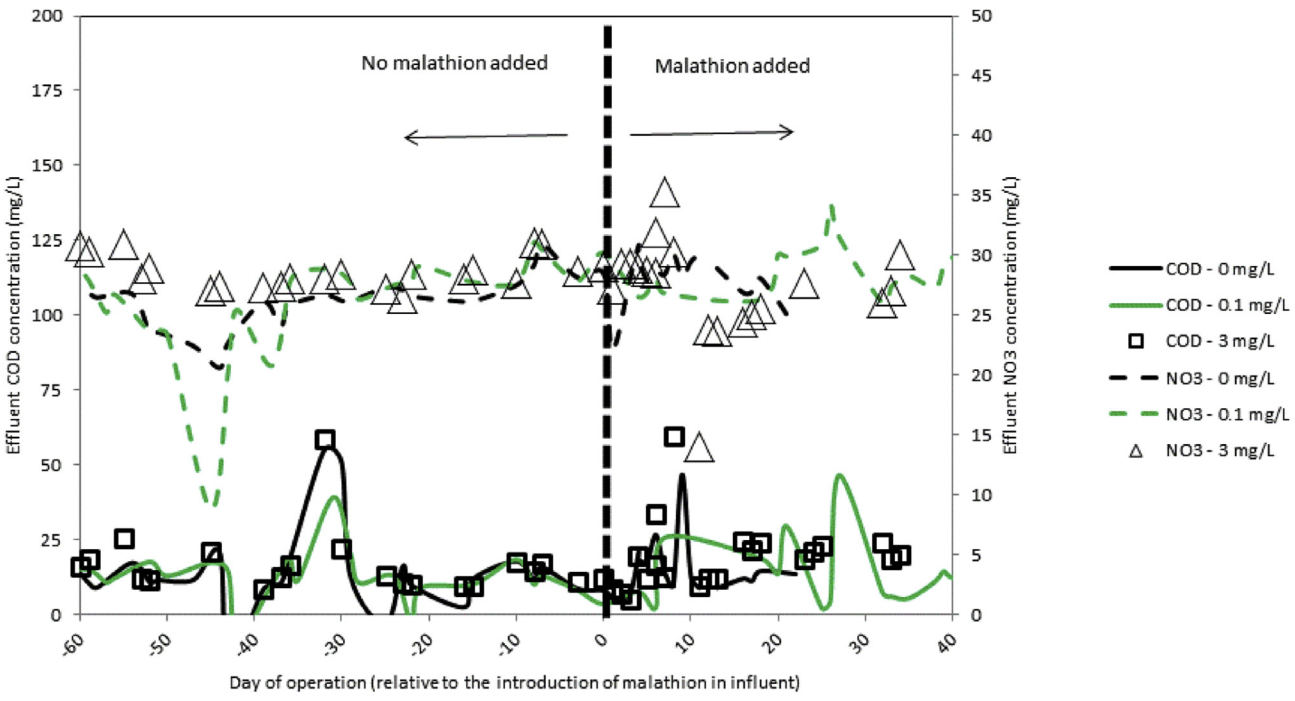

Fig. 5. The effect of influent malathion concentration on effluent quality. The legend shows malathion concentrations associated with data.

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number of sequence reads was used as a metric for the relative abundance of microbial species (Fig. 6). Several bacterial groups exhibited a decline in relative abundance after exposure to 3 mg/L of malathion including: *Meganema*, a well-known filamentous organism (Thomsen et al., 2006); lactic acid bacteria, namely: *Leucobacter*, *Lactococcus*, *Leuconostoc* (ASM Press, 2007), *Crocinitomix*; and *Bdellovibrio*, a gram-negative, rod-shaped aerobic strain suspected of preying on other gram-negative bacteria (Davidov and Jurkevitch, 2004). *Meganema* and *Leucobacter* also declined in the control reactor (data not shown), indicating that malathion may not have affected these two groups in reactor II. Little information is available about the growth characteristics of *Crocinitomix*, which was previously isolated from a polar habitat (Bowman et al., 2003); the current study appears to be the first to report its presence in activated sludge.

Upon exposure to 0.1 mg/L of malathion, the abundance of *Meganema*, *Bdellovibrio*, *Opitutus*, *Lewinella*, *Algoriphagus*, and *Nitrospira* decreased. Both *Lewinella*, anaerobic chemoorganotroph, and *Opitutus*, a fermentative bacterium, have been detected in activated sludge previously (Whitman et al., 2012). *Algoriphagus* is known to subsist on peptone and organic nitrogen compounds with some groups capable of degrading ammonia (Dworkin et al., 2006). *Nitrospira* is a common nitrifying species found in activated sludge, and it is known to become inhibited in the presence of a wide range of industrial chemicals including pesticides (Whitman et al., 2012).

There are several groups that increased in relative abundance after exposure to malathion. Upon exposure to 3 mg/L of malathion, *Dysgonomonas*, a facultative anaerobe, and *Nitrospira* increased in relative abundance. The increase in the relative abundance of *Nitrospira* in reactor II was unexpected and unlikely to be generally observed as it is in conflict with previous findings Janeczko et al. (2014) as well as results from reactor I. Upon exposure to 0.1 mg malathion/L, several common heterotrophs increased in relative abundance, including: *Bryobacter*, *Pontibacter*, *Cryomorpha*, *Dyadobacter*, *Amaricoccus*. These groups all contain genes that permit the expression of nonspecific oxidizing enzymes (oxygenases), thus, in principle, it is possible that these species were able to biochemically oxidize malathion (Wackett and Hershberger, 2001). However, further research is warranted to prove the presence of these transformations.

There are two final points, regarding the impact of malathion on the ecology of activated sludge. First, previous research has suggested that malathion may select for novel, unknown microbial groups. For example, Janeczko et al. (2014) discovered that malathion increased the abundance of unclassified *Actinobacteria* species by 31%–81% (in various samples) and of unclassified *Bacteriodetes* species between 83% and 95%. Thus, it is possible that unknown, novel species increased in abundance after malathion was added into the SBRs. The second point is related to functional redundancy. The application of molecular methods has permitted scientists to show that numerous microbial species can carry out the same biochemical functions during wastewater treatment (He et al., 2010; Hesselsoe et al., 2009; Siripong and Rittmann, 2007; Valentin-Vargas et al., 2012). This redundancy permits COD and nutrient removal to continue in the presence of inhibitors; more resistant subgroups substitute for those that are impaired. This explains why 0.1 mg/L malathion inhibited several heterotrophs and a nitrifier without interrupting COD and nitrogen removal. One

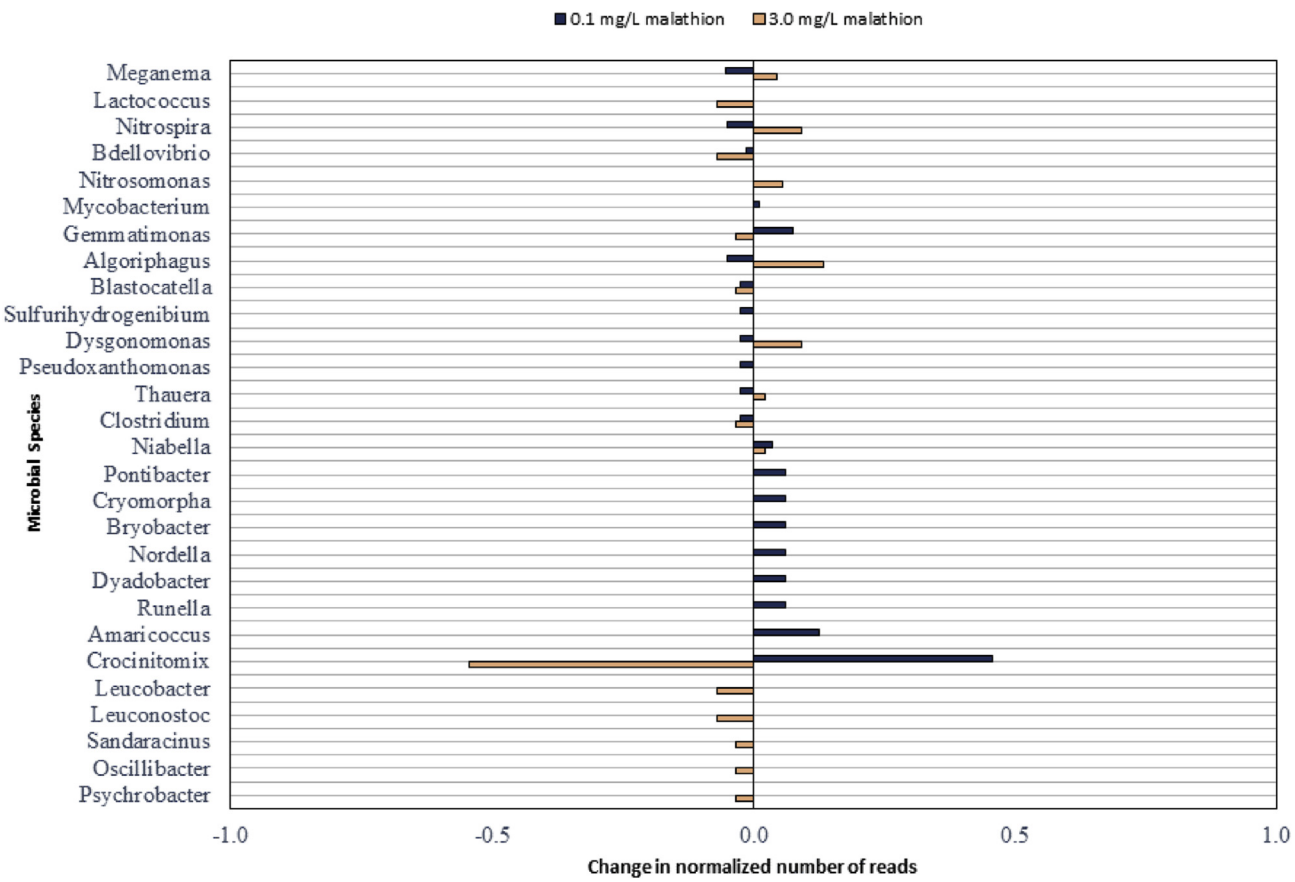

Fig. 6. The change in the normalized number of reads for SBRs exposed to malathion.

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should expect such observations at properly-managed activated sludge systems where low levels of malathion (i.e. $\leq$ 0.1 mg/L) may inhibit a few of the microbial species without stopping proper treatment because other groups rise to fill the void. On the other hand, treatment plants that are not properly controlled (such as those that suffer from poor pH control) are likely to have less functional redundancy (Seviour and Nielsen, 2010), making them more vulnerable to failure in the presence of chemicals like malathion.

4. Conclusions

This study demonstrated that short term exposure to malathion did not stop respiration, COD removal, or nitrification in 12-h batch tests. Stoichiometric O_2 consumed-to-CO_2 produced ratios indicate that malathion did not alter the underlying metabolic pathways responsible for respiration during these short term exposures. Longer term continuous exposures (i.e. 40 days) to both 0.1 mg/L and 3 mg/L of malathion were associated with shifts in the abundance of species that are common to activated sludge. Further, longer term exposures to 3 mg/L of malathion inhibited both COD and nitrogen removal. Although, no detectible malathion was present in the effluent during longer term continuous exposure tests, the results indicate that exposures, especially to 3 mg/L malathion, may adversely affect activated sludge performance. Washdown waters contaminated with chemicals like malathion may potentially be treated at activated sludge treatment plants without interrupting normal biological functions, especially when the process is normally well-controlled and when such treatment is only required for short periods of time with relatively low contaminant concentrations (i.e. ppb levels).

5. Disclaimer

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jenvman.2016.08.076

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