

2,4-D removal via denitrification using volatile fatty acids

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ABSTRACT

Many countries have waters contaminated with both herbicides and nitrates; however, information is limited with respect to removal rates for combined nitrate and herbicide elimination. This research investigates the removal of 2,4-D via denitrification, with a particular emphasis on the effect of adding naturally generated volatile fatty acids (VFAs). The acids were produced from an acid-phase anaerobic digester with a mean VFA concentration of 3153 ± 801 mg/L (as acetic acid). Initially, 2,4-D degrading bacteria were developed in an SBR fed with both sewage and 2,4-D (30–100 mg/L). Subsequent denitrification batch tests demonstrated that the specific denitrification rate increased from 0.0119 ± 0.0039 using 2,4-D alone to 0.0192 ± 0.0079 g NO₃-N/g VSS per day, when 2,4-D was combined with natural VFAs from the digester. Similarly, the specific 2,4-D consumption rate increased from 0.0016 ± 0.0009 using 2,4-D alone to 0.0055 ± 0.0021 g 2,4-D/g VSS per day, when using 2,4-D plus natural VFAs. Finally, a parallel increase in the percent 2,4-D removal was observed, rising from 28.33 ± 11.88 using 2,4-D alone to 54.17 ± 21.89 using 2,4-D plus natural VFAs.

Key words | 2,4-D degradation, acid-phase anaerobic digester, denitrification, SBR, volatile fatty acids

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INTRODUCTION

The main use of the chlorinated organic herbicide, 2, 4-dichlorophenoxy acetic acid (2,4-D) is on cereal crops. In addition, it is used for the control of broadleaf aquatic weeds in lakes, ponds and reservoirs. Agriculture is a major revenue sector for New Zealand (Li *et al.* 2005), thus 2,4-D has been extensively utilised, comprising 68% of all herbicides used in New Zealand (Holland & Anis 1999). New Zealand's topography makes ground-based application of 2,4-D difficult, thus aerial application is the most common method of dispersing herbicides (Elefsiniotis & Li 2008). A recent study estimated that only 5% of sprayed chemicals actually reached the targeted area, with the remaining 95% aerial-drifting into surrounding regions (deBlanc-Knowles & Motavali 2003). It is not surprising therefore that many countries have reported waters contaminated by 2, 4-D (Main 2004; Mitchell *et al.* 2005).

In regions where herbicides control weed growth, nitrogen-based fertilizers are often used to increase agricultural productivity. Nitrogen is a cause for environmental concern because of deterioration of water quality in aquifers, eutrophication of receiving waters, and an increase in nitrate-

related health problems. Nitrates can however be removed by denitrification, a reaction requiring a carbon source. If commercially-available carbon sources are used (eg. methanol), significant costs can be incurred (Thalasso *et al.* 1997); thus, alternative carbon sources are of great interest. These include municipal and agricultural effluents that are often rich in volatile fatty acids (VFAs).

Although denitrifying micro-organisms can degrade many toxic carbon compounds (Sherwood *et al.* 1998; Rockne & Strand 2001); there seems to be a limited number of studies that focus on the ability of denitrifying organisms to degrade 2,4-D (Sanford & Tiedje 1992). Indeed, most research into 2,4-D degradation has involved pure batch cultures under aerobic conditions, usually with a focus on the biochemical or genetic aspects (Smith *et al.* 1994). Furthermore, although easily-degradable co-substrates enhance degradation of toxic compounds (Oleszkiewicz *et al.* 1991), very few denitrification studies have focused on pesticide co-metabolism. Notable exceptions include denitrification research by Aslan & Türkman (2005, 2006) investigating the ability of wheat straw and ethanol to enhance degradation of tifluralin-contaminated

waters. These latter studies provide impetus for investigating other co-substrates for herbicide degradation under denitrification conditions.

The aim of this research was therefore to investigate the biodegradation capability of the herbicide 2, 4-D using naturally-generated VFAs as the co-substrate for the simultaneous removal of nitrates. The study sought to provide information on denitrification and VFA consumption rates, as well as 2, 4-D biodegradation rates and removal efficiencies.

MATERIALS AND METHODS

Acid-phase anaerobic digester

Three separate physical systems were used in this research. The first was an acid-phase anaerobic digester to generate VFAs. The digester consisted of a 30 L stainless steel cylinder with a sealed base and removable lid. A landfill gas analyser measured gas while an external pump continuously mixed the digester contents. The 2 L daily feeding and wasting regime of the 20 L liquid volume yielded an SRT/HRT of 10 days. The digester was inoculated with digested sludge from the Bromley wastewater treatment plant (WTP) in Christchurch, New Zealand, while a full-fat, enzyme-active soy flour (40 g/L) was chosen as synthetic feed (approximately 46% protein, 38% carbohydrate and 7% lipid).

Sequencing batch reactor (SBR)

A sequencing batch reactor (SBR) provided the source of 2, 4-D acclimated biomass. The SBR was constructed from stainless steel with an operating liquid volume was 22 L. The target mixed liquor suspended solids (MLSS) value was 4000 mg/L at an SRT of 20 days. The dissolved oxygen (DO) in the mixed liquor was held at 2 mg/L during aerobic periods. Filling, mixing, reaction times, settling and decanting functions were controlled *via* a programmable controller set for a 340 minutes cycle. At the end of the settle phase, 11 L of effluent was decanted while the same volume of influent was added resulting in an HRT of 11.3 hours. The SBR was inoculated with activated sludge from the Lyttleton WTP, New Zealand while the feed was municipal wastewater from the Bromley WTP. No herbicide was added during start-up, however, after 24 days, the SBR was fed with wastewater contaminated with 30 mg/L of 2, 4-D. After 80–90% 2, 4-D degradation, the 2, 4-D feed concentration was increased to 50 mg/L, then finally to 100 mg/L.

Denitrification batch tests

Denitrification batch tests were conducted in duplicate, in 5 L glass batch reactors at $21 \pm 2^\circ\text{C}$. The reactors were filled with a mixture of (i) 2, 4-D acclimated biomass from the SBR, (ii) settled, acid-phase anaerobic digester effluent providing VFAs, and (iii) NaNO_3 to provide nitrogen. Continuous stirring ensured solids were suspended, and a mean biomass concentration of 1727 ± 177 mg/L was achieved. To quantify abiotic losses, three reactors were operated as controls (no biomass) and fed 2, 4-D only, digester effluent (VFAs) only and $\text{NO}_3\text{-N}$ only. To start, 10 “base-line” tests had NaNO_3 added to make C:N ratios of 0.1–5.0 (i.e. carbon-limited to carbon-excess conditions). Following that, 18 tests were conducted using 2, 4-D as the sole carbon source. To make C:N ratios of 0.1–3.5, 2, 4-D was added in concentrations of 30–100 mg/L with 20–100 mg/L of $\text{NO}_3\text{-N}$. Finally, 18 tests used VFAs and 2,4-D as the carbon source were tested. Anaerobic digester effluent and 30–100 mg/L of 2, 4-D were added, resulting in C:N ratios from 0.5–5.0.

Chemical analysis

The digester was sampled daily for the first few months, and then every 2–3 days thereafter. Samples were analysed for pH, temperature, TS, VS, TSS, VSS, soluble COD, TOC and VFAs according to Standard Methods (A.P.H.A. *et al.* 1998). The SBR system was regularly sampled for pH and MLSS, while during cycle track studies, filtered samples were analysed for TOC, SCOD, $\text{NO}_3\text{-N}$, and 2, 4-D. The concentration of 2, 4-D was determined by HPLC. Samples from the denitrification batch tests were taken at 0, 4, 8 and 12 h and every 4 h between 24–32 h and 48–56 h. Finally, after 72 h, samples were drawn twice a day for one week. Samples were analysed for VFAs (acetic (HAc), propionic (HPr), n-butyric (n-HBu) and iso-valeric (i-HVa) acids), 2, 4-D, $\text{NO}_x\text{-N}$, pH, alkalinity, and VSS as per the methods described earlier.

RESULTS AND DISCUSSION

Acid-phase anaerobic digestion

Table 1 characterizes the soy flour feed solution and the digester effluent. The feed VSS/VS ratio was 73.5%, thus a large fraction of the VS had potential to be solubilised. The VSS value however dropped very little from influent to effluent (14%), implying that the particulate solubilization of the feed was poor. Since the VSS/VS ratio increased to

86.5%, the 8,600 mg/L difference between the influent and effluent VS resulted from a reduction in the dissolved fraction of the VS, attributed to the conversion of volatiles to gas. The composition of gas averaged 10% CH₄, 90% CO₂ and less than 1% N₂ and H₂, suggesting that the low pH value (4.98 ± 0.18) and the short SRT (10 days) largely suppressed methanogenesis. The specific TOC solubilization rate was 0.007 mg TOC/mg VSS/day while the specific COD solubilization rate was 0.022 mg soluble COD/mg VSS/day. The relatively low solubilization rates suggest that the particulate matter in the soy flour was not entirely amenable to solubilization.

Table 1 indicates that the digester was generating VFAs, either from the conversion of particulate matter and/or the fermentation of complex soluble organics. Given the low VSS destruction (14%) and the low particulate solubilization rates, VFA generation was inferred to arise from the conversion of complex soluble organics. The specific VFA production rate was 0.014 mg VFA as HAc/mg VSS/day, which is comparable to other studies (Min *et al.* 2002). The VFA concentrations were converted to COD equivalents and though net VFA production (as HAc) appeared to be substantial (2805 mg/L), effluent VFAs (5156 mg COD/L) accounted for only 34% of the soluble COD in the digester. Thus, other compounds were contributing to the SCOD, presumably unused soluble substrate, cell lysis products and extracellular intermediate metabolites.

The digester effluent (total VFAs equalling 3153 mg/L as HAc), contained mean concentrations of 1621 mg/L HAc (51.4%), 1071 mg/L HPr (27.5%), 909 mg/L n-HBu (19.6%) and 75 mg/L i-HVa (1.4%). In general, the distribution of HAc and HPr was relatively consistent with other researchers operating at similar SRTs, where HAc ranged from 33% to 73% and HPr from 22% to 44% (Mavinic *et al.* 2000; Bouzas *et al.* 2002).

Acclimation and biodegradation of 2, 4-D in the SBR

After running for 24 days, a concentration of 30 mg/L of 2, 4-D was added to the feed (Figure 1). On day 33, the SBR

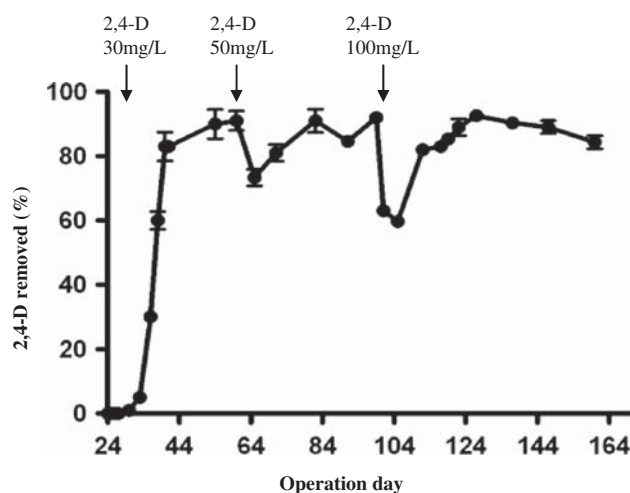


Figure 1 | Removal of 2, 4-D during acclimation phase in the SBR.

exhibited the first sign of biodegradation with an increasing trend until 90% removal was achieved on day 54. When the 2, 4-D concentration was raised from 30 to 50 mg/L (day 60), the 2, 4-D degradation efficiency dropped from 91% to 75%; however, the biomass quickly adapted and the removal efficiency increased to 80% on day 70 (reaching approximately 90% on day 82). The second 2, 4-D increase on day 99 (from 50 to 100 mg/L) led to a large drop in MLSS (from nearly 4000 to 2600 mg/L) and a drop in 2, 4-D degradation efficiency to 63%. Less sludge was wasted until the MLSS increased and on day 127, 93% removal was achieved.

Track studies were carried out after each point of 2, 4-D increase and Figure 2 shows a representative plot during one SBR cycle. The removal of 2, 4-D during the non-aerated period was minimal, while most of the 2, 4-D was removed within the 240 minutes of aeration. For each track study, the specific 2, 4-D degradation rate was calculated and a mean specific 2, 4-D degradation value of 0.026 ± 0.005 mg/mg VSS/day was calculated when the initial 2, 4-D concentration was 30 mg/L. The mean specific 2, 4-D degradation rate increased to 0.043 ± 0.007 mg/mg VSS/day when the initial 2, 4-D concentration increased to 50 mg/L, finally reaching a mean rate of 0.062 ± 0.015 mg/mg VSS/day when the initial 2,

Table 1 | Characteristics of anaerobic digester feed solution and acid-phase digester effluent

Parameters		pH	TS (mg/L)	VS (mg/L)	TSS (mg/L)	VSS (mg/L)	Soluble COD (mg/L)	TOC (mg/L)	VFAs (mg/L) (as HAc)
Influent	Mean ± σ	6.7	34245	32267	24200	23722	10473	3815	348
		0.1	1317	1095	421	410	1573	174	65
Effluent	Mean ± σ	4.98	25679	23625	21057	20441	14990	5246	3153
		0.18	1016	935	833	825	238	605	801

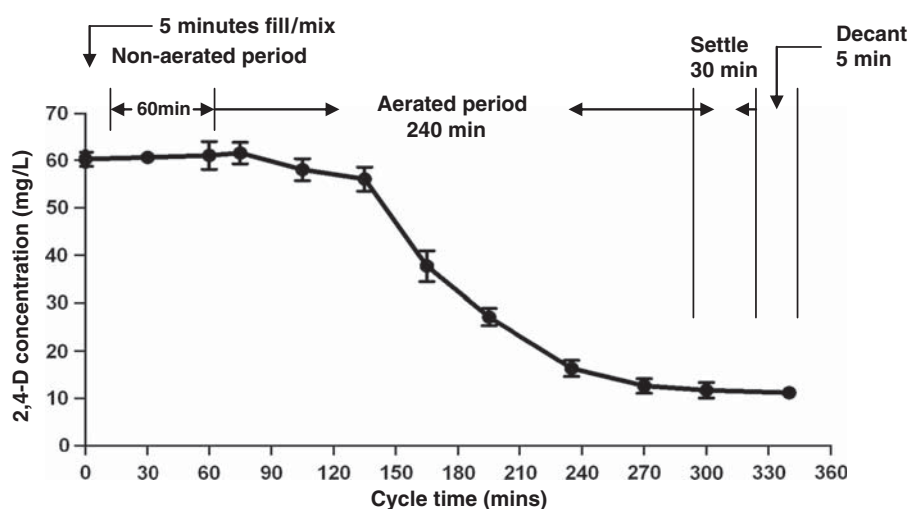


Figure 2 | Track study of 2, 4-D removal in the SBR.

4-D concentration increased to 100 mg/L. The increase in specific 2, 4-D degradation rates suggests that the biomass had the ability to degrade the 2, 4-D but the system had not reached its full biodegradation potential. The overall mean specific 2, 4-D degradation rate for all track studies was 0.046 ± 0.018 mg/mg MLSS per day which is comparable to the rates of 0.035 to 0.216 mg/mg MLVSS/day given by Mangat & Elefsiniotis (1999).

Denitrification batch tests

Observations of the controls indicated that 2, 4-D, VFA-C and $\text{NO}_3\text{-N}$ concentrations had less than 5% variation, indicating that abiotic losses were negligible. Denitrification proceeded successfully in the 10 “base-line” tests using naturally-generated VFAs as evidenced by alkalinity recovery, $\text{NO}_3\text{-N}$ disappearance and VFA-C consumption. A preferential order for utilization of VFA species was observed with HAc being the most preferred VFA species for denitrification, followed by HPr, followed by n-HBu and i-HVa which were consumed at nearly the same time.

A typical plot of $\text{NO}_3\text{-N}$ consumption and 2, 4-D degradation (Figure 3) for the 18 tests using 2, 4-D as the sole carbon source indicates that the $\text{NO}_3\text{-N}$ concentration decreased significantly in the first 70 hours, completely disappearing by the end of the test. The 2, 4-D concentration however did not drop significantly in the first 30 hours, indicating $\text{NO}_3\text{-N}$ reduction utilising endogenous carbon. The lack of 2, 4-D removal complements the observation from the anoxic portion of the SBR, in that the bacteria were acclimated to the electron donor (i.e. 2, 4-D) (since they showed no adverse affects); however they were not accli-

mated to the $\text{NO}_3\text{-N}$ electron acceptor, since all degradation occurred during the aerobic phase. It was conjectured that during a batch test however, sufficient enrichment time would allow expression of enzymes to remove 2, 4-D under anoxic conditions. This was substantiated in that Figure 3 indicates that 42 % of the 2, 4-D was removed using $\text{NO}_3\text{-N}$ as the terminal electron acceptor, from 30 hours onwards to the end of the test.

As mentioned, 18 batch tests were conducted using mixtures of digester effluent and 2, 4-D as carbon sources. A representative plot (Figure 4) of VFA-C consumption, $\text{NO}_3\text{-N}$ consumption and 2, 4-D degradation indicates approximately 30 hours needed for the biomass to commence significant degradation of the 2, 4-D, but only after approximately 50% of the VFAs had been consumed. Figure 4 thus confirms a sequential substrate utilization pattern with simpler (non-chlorinated) organic compounds degraded first, followed by chlorinated organic compounds. The degradation pattern agrees with research using non-chlorinated phenol to

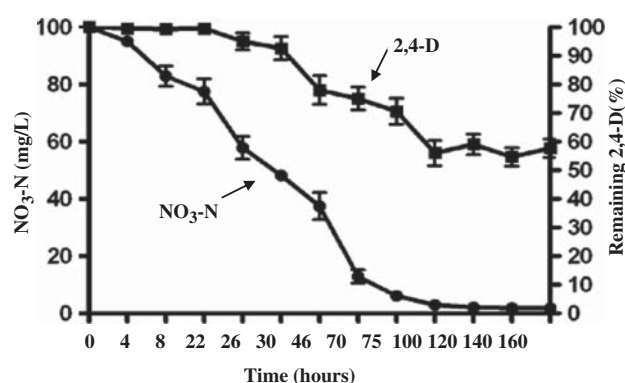


Figure 3 | Plot of $\text{NO}_3\text{-N}$ and 2, 4-D degradation using 2, 4-D as a sole carbon source.

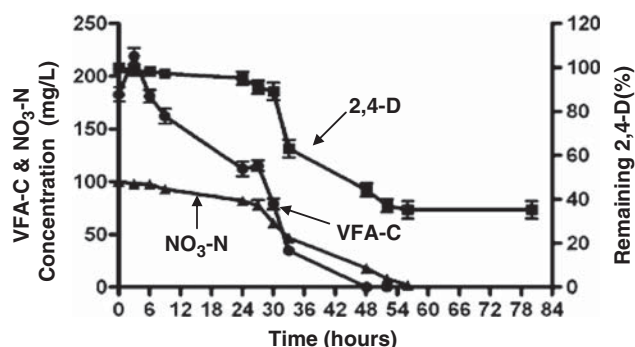


Figure 4 | VFA-C consumption, $\text{NO}_3\text{-N}$ degradation and 2,4-D removal using VFAs and 2,4-D.

biodegrade a chlorinated organic compound (3-chlorophenol) through cometabolism (Chiavola *et al.* 2004).

Calculation of specific rates

Table 2 lists the specific nitrate and carbon consumption rates, as well as the percent removals for the carbon compounds during the denitrification batch tests. Denitrification rate results from this study for VFAs only ($0.024 \pm 0.003 \text{ g NO}_3\text{-N/g VSS per day}$) appear to be low however most published rates are associated with continuous, flow-through systems, which generally have higher rates than batch systems; primarily due to acclimation of bacteria to carbon sources at steady-state conditions.

The average specific VFA carbon consumption rate was $0.046 \pm 0.017 \text{ g VFA-C/g VSS per day}$; however, since the initial C:N ratio influences the rate, the data was divided into two distinct sets based on C:N ratios. The results (not shown) indicate that the specific carbon consumption rate was affected by the limitation of external carbon, dropping from an average of $0.058 \pm 0.011 \text{ g VFA-C/g VSS per day}$ at C:N ratios above 2.0 to $0.033 \pm 0.013 \text{ g VFA-C/g VSS per day}$ at C:N ratios below 2.0. The mean rates from these two sets proved statistically different at the 95% confidence level.

From Table 2, the average specific denitrification rate using dual-substrates ($0.0192 \pm 0.0079 \text{ g NO}_3\text{-N/g VSS per}$

day) was slightly lower than the rate using natural VFAs as a sole carbon source; however, the specific VFA-C consumption rate using dual-substrates ($0.1216 \pm 0.0335 \text{ g VFA-C/g VSS per day}$) was much higher than that using natural VFAs as a sole carbon source. These rates, statistically different at the 95% confidence level, suggest that in this research, the addition of 2, 4-D below 100 mg/L inhibited the nitrate reduction process; while, at the same time not inhibiting the VFA-C consumption pattern. This implies non-denitrifying activity. As anticipated, the nitrate reduction process was further inhibited when 2,4-D was used as a sole carbon source ($0.0119 \pm 0.0039 \text{ g NO}_3\text{-N/g VSS per day}$) as compared to when VFAs and 2,4-D were carbon sources. Compared with the aerobic degradation of 2, 4-D in the SBR, the mean specific 2, 4-D degradation rate from the denitrification tests was approximately 30 times lower.

Table 2 also demonstrates that the addition of a digester effluent rich in naturally-produced VFAs increases the specific 2, 4-D consumption rate as well as the 2, 4-D biodegradation efficiency. In particular, the average specific 2, 4-D consumption rate increased from 0.0016 ± 0.0009 to $0.0055 \pm 0.0021 \text{ g 2, 4-D/g VSS per day}$, when using 2, 4-D alone versus using 2, 4-D plus natural VFAs as carbon sources. These again proved statistically different at the 95% confidence level. The 2, 4-D removal showed a parallel increase from $28.33\% \pm 11.88\%$ using 2, 4-D alone to $54.17\% \pm 21.89\%$ using 2, 4-D plus natural VFAs as carbon sources.

The results imply that the degradation rate of 2, 4-D using nitrates can be enhanced by VFAs. Obviously though, if drinking water standards were the objective, then biomass settling and disinfection would have to occur, in addition to perhaps further treatment such as activated carbon.

CONCLUSIONS

This research focused on 2,4-D removal under anoxic conditions with an emphasis on providing information on removal

Table 2 | Summary of specific rates in denitrification batch tests

Carbon Source	Natural VFAs Mean $\pm \sigma$	Natural VFAs plus 2,4-D Mean $\pm \sigma$	2, 4-D Mean $\pm \sigma$
Specific Denitrification Rate (g $\text{NO}_3\text{-N/g VSS per day}$)	0.024 ± 0.003	0.0192 ± 0.0079	0.0119 ± 0.0039
Specific Carbon Consumption Rate (g VFA-C/g VSS per day)	0.046 ± 0.017	0.1216 ± 0.0335	-----
Specific 2, 4-D Consumption Rate (g 2, 4-D/g VSS per day)	-----	0.0055 ± 0.0021	0.0016 ± 0.0009
2, 4-D Removal Percentage (%)	-----	54.17 ± 21.89	28.33 ± 11.88

rates. Naturally generated VFAs from an acid-phase anaerobic digester were used as a co-substrate during denitrification batch tests. The results indicated that the specific denitrification rate, the specific 2,4-D consumption rate and the percent 2,4-D removal all increased when 2,4-D was combined with natural VFAs as compared to when 2,4-D was used alone.

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