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Investigating the *in situ* degradation of atrazine in groundwater†

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Abstract: This study focused on whether or not atrazine could be degraded by indigenous groundwater bacteria as part of an *in situ* remediation approach. Groundwater was taken from an unconfined middle upper chalk site where concentrations of atrazine and nitrate were typically in the ranges 0.02–0.2 μg litre^{-1} and 11.6–25.1 mg NO_3-N litre^{-1} respectively. Sacrificial batch studies were performed using this groundwater spiked with atrazine at a concentration of 10 μg litre^{-1} in conjunction with a minimal mineral salts liquid (Glu-MMSL) medium which contained glucose as the sole carbon source. Treatments comprised either the Glu-MMSL groundwater cultured bacteria or *Pseudomonas* sp. strain ADP. Results from sacrificial batches indicated the occurrence of bacterial growth and denitrification as monitored by optical density (absorbance at 600 nm) and NO_3-N content. Analysis of atrazine content by solid phase extraction coupled with high-performance liquid chromatography showed no degradation of atrazine over a period of 103 days in either treatment. These results indicated that no acclimatised bacterial community featuring positive degraders to the herbicide atrazine had become established within this chalk aquifer in response to the trace levels encountered.
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Keywords: atrazine; *Pseudomonas* sp. strain ADP; *in situ* bioremediation and natural attenuation

1 INTRODUCTION

Current legislation in the form of the European Community (EC) drinking water directive EC 98/83EEC provides parametric values for the acceptable concentrations of organic and inorganic contaminants allowed within potable water.[1] Of those contaminants cited within this legislation, a significant number are of agricultural origin. The use of pesticides and fertilisers has subsequently been linked in the literature[2–6] to the contamination of groundwaters. With a drinking water pesticide content of 0.1 μg litre^{-1} as the maximum admissible concentration within the EC's legislation, the number of potable groundwater sites potentially exceeding this may increase.

It has, however, been found that natural attenuation of pesticides can occur in some of these contaminated groundwaters. A variety of studies have focused on recording this process either *in situ*[7–10] or through laboratory observations using indigenous bacteria from contaminated sites.[11–15] In these studies, biological degradation was found to occur with a varying level of efficacy where aquifer pesticide concentrations exceeded 40 μg litre^{-1}. Where this occurred, it was associated with acclimatised bacterial communities featuring positive degraders of the compound.[8–10] From these studies we find that pesticide concentrations >40 μg litre^{-1} can select for an indigenous bacterial community with specific positive degraders which are capable of undertaking pesticide degradation.

Unfortunately, few directed studies have been undertaken[16–19] to monitor natural attenuation of diffuse concentrations of pesticide in groundwater (<10 μg litre^{-1}). Results by Purcarević *et al.*[16] indicated the presence of both atrazine (average 0.198 μg litre^{-1}) and common metabolites in a large-scale survey of near-surface aquifers in Vojvodina province, Serbia. Although degradation products of atrazine were found, these results can be questioned as to whether they were derived by the actions of soil or groundwater bacteria. Larsen and Aamand[17] indicated that no degradation of a 25 μg litre^{-1} spike of atrazine occurred independently of the oxidative condition used in relation to the two sandy aquifers that were tested. This was in agreement with an earlier paper by Larsen *et al.*[18] where atrazine degradation

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had been found, albeit very slowly (the doubling time for $^{14}CO_2$ evolution from ^{14}C-atrazine was 5 years), within the ploughed layer (>0.7 m) and could not be attributed to the groundwater bacterial community at increased depths. These examples suggest that the atrazine degradation products monitored in the groundwaters of Vojvodina province[16] were simply from the leaching of the soil profile. These examples do not demonstrate any evidence that the diffuse groundwater pesticide concentration has resulted in an acclimatised bacterial community capable of undertaking degradation.

The work reported in this paper was designed to investigate whether any prevalent microbial adaptation had occurred to degrade atrazine (2-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,6-diamine) within a diffusely contaminated ($\sim$0.2 μg litre^{-1}) shallow chalk aquifer. We used a simple nutrient regime featuring glucose as the sole carbon source and *Pseudomonas* sp. strain ADP[20] as a positive control. These treatments were designed on the basis of providing efficacy information in response to the application of future *in situ* bioaugmentation methods.

2 MATERIALS AND METHODS

2.1 Field site

Groundwater was used from an unconfined middle upper chalk aquifer with a shallow water table <5 m in depth. Groundwater monitoring had previously indicated that this site contained atrazine and nitrate concentrations periodically in excess of the 0.1 μg litre^{-1} and 50 mg litre^{-1} parametric values (PVs) of the EC drinking water directive EC 98/83EEC. Monitored concentrations of atrazine and nitrate had typically been shown to occur within the ranges 0.02–0.2 μg litre^{-1} and 11.6–25.1 mg NO_3-N litre^{-1} respectively. This water is currently being treated using ion exchange and granular activated carbon to ensure compliance with the UK Water Supply (Water Quality) 2000 regulations.

2.2 Chemicals

Atrazine (97.4% pure) was a gift from Oxon Italia SpA. Methanol and the far-UV-absorbent acetonitrile used in experiments and analysis were of high-performance liquid chromatography (HPLC) grade (Fisher Scientific, Loughborough, UK). All water used (MilliQ water) in experiments and analysis was supplied from an ELGA Purelab Option-S 7/15 module used in conjunction with an ELGA Ultrapure genetic module (ELGA, High Wycombe, UK). All other chemicals were supplied by Fisher Scientific and were of reagent grade or better.

2.3 Microbial growth media and atrazine spiking solution

The minimal mineral salts liquid (Glu-MMSL) medium was derived from a concentrated stock solution and designed for periodic amendment at low volume from this.[21] Two separate concentrated stock solutions were used (buffer and salts), giving the following concentrations per litre of batch fluid when added at volumes of 4 and 0.5 ml litre^{-1} respectively. The buffer constituent of the Glu-MMSL medium created batch fluid concentrations of glucose 159, KH_2PO_4 25, $NaH_2PO_4{\cdot}2H_2O$ 100, $MgSO_4{\cdot}7H_2O$ 10 and NH_4Cl 300 mg litre^{-1}. The salts constituent of the Glu-MMSL medium created batch fluid concentrations of H_3BO_4 3, $MnSO_4{\cdot}H_2O$ 2, $CuSO_4$ 0.4, $ZnCl_2$ 0.2, $CoCl_2{\cdot}6H_2O$ 0.4, $Na_2MoO_4{\cdot}2H_2O$ 0.3 and $FeCl_3{\cdot}6H_2O$ 1 mg litre^{-1}, with concentrated H_2SO_4 5 ml litre^{-1} to retard precipitation of the salts. Both Glu-MMSL media constituents were prepared in MilliQ water and filter sterilised before use. The glucose in the buffer solution was the sole amended carbon source at a ratio of Glu-C/NO_3-N = 4:1 based on an analysed groundwater NO_3-N concentration of 15.90 mg litre^{-1} (Merck Nitrate Spectroquant cells in a Nova 60 spectrophotometer, Merck Ltd, West Drayton, UK). The actual addition of glucose was calculated in relation to this groundwater NO_3-N load but in reference to batches being composed 80% by volume of groundwater. The remaining 20% by volume was composed of inoculum and the relevant media. Liquid and solid atrazine media containing sucrose and sodium citrate carbon sources at 1 g litre^{-1} were used as described by Mandelbaum *et al*.[20] This methodology was altered for the atrazine spikes, which were not dissolved in methanol, and cycloheximide 50 mg litre^{-1} was omitted.

The atrazine spiking solution was prepared in MilliQ water, as no additional carbon sources could be added to the sacrificial batch trials. Owing to the relative insolubility of atrazine in water ($\sim$70 mg litre^{-1}), a 10 mg mass was added to 1 litre of autoclaved MilliQ water, shaken for 24 h at 140 rpm (Gallenkamp Orbital incubator, Sanyo, Crawley, UK) and filtered (Millipore 0.22 μm × 47 mm GSWP filters, Fisher Scientific) before being analysed by direct HPLC analysis. All apparatus used throughout was autoclaved before use (121 °C for 15 min in a Priorclave autoclave, Priorclave Ltd, Woolwich, UK). All media solutions were filter sterilised before use (Millipore 0.22 μm × 47 mm GSWP filters, Fisher Scientific).

2.4 Indigenous groundwater bacteria and *Pseudomonas* sp. strain ADP inocula

Inocula used were at 10% of batch volume. The indigenous groundwater bacterial inoculum was obtained from groundwater amended with the Glu-MMSL medium. This was grown under shaken (140 rpm; Gallenkamp Orbital incubator, Sanyo) aerobic conditions, amended with Glu-MMSL medium at 0, 2, 4 and 6 weeks, and used after 7 weeks. The purpose of this inoculum was to remove the lag phase in the experiment, allowing the indigenous groundwater batch system to be comparable to the inoculation of a liquid culture of *Pseudomonas* sp.

strain ADP in an equivalent system, which was used as the positive control.

The *Pseudomonas* sp. strain ADP liquid inoculum was produced by inoculating single actively degrading colonies monitored on solid atrazine medium[20] into 20 × 20 ml glass universals (Fisher Scientific) containing liquid atrazine medium.[20] Universals that indicated degradation of atrazine after 7 days (data not shown) were combined to form a 10% inoculum (100 ml) in 2 × 1 litre glass Duran bottles (Fisher Scientific) containing fresh liquid atrazine medium.[20] Growth and atrazine degradation by *Pseudomonas* sp. strain ADP were monitored by optical density (OD) readings at 600 nm using 1.5 ml plastic cuvettes (Fisher Scientific) on a Jenway 6505 UV–visible spectrophotometer (Jenway, Felsted, UK) and HPLC analysis respectively. These batches were subsequently used as the 10% inoculum in sacrificial batch trials.

2.5 Sacrificial batch studies

These studies were performed unshaken within a temperature-controlled room at 16 °C under both open and sealed conditions. Duplicate batches were created for testing the groundwater bacteria (GW-MMSL) and the *Pseudomonas* sp. strain ADP batches, where MilliQ water was used as controls.

Each individual batch contained a start volume of 1 litre. This facilitated a zero extraction (400 ml volume) to determine the start concentration, and then a later second extraction (again 400 ml by volume) to ascertain the time-dependent value. Open (500 ml glass Erlenmeyers with 50 mm polyurethane foam stoppers, both supplied by Fisher Scientific) and closed (500 ml screwtop bottles, Fisher Scientific) bottles were used to expose bacteria to varying oxidative states. Treatments (Table 1) contained, by volume, 80% unsterilised groundwater and 10% bacterial inoculum (cultured indigenous groundwater bacteria in response to the Glu-MMSL media or *Pseudomonas* sp. strain ADP). The remaining 10% by volume contained the atrazine MilliQ water spiking solution (1.5 ml at 10 mg litre^{-1}) and either the liquid atrazine medium for *Pseudomonas* sp. strain ADP treatments[20] or autoclaved MilliQ water for reference controls. Both treatments subsequently received additions of Glu-MMSL medium at 0 and 23 day intervals; this was substituted by MilliQ water in the negative controls.

Atrazine concentration, pH (Hanna H18424 pH meter, Leighton Buzzard, UK), NO_3-N and bacterial growth were monitored in triplicate at 0, 23, 63, 83 and 103 day intervals in both of the two sacrificial batch samples per monitoring point. Bacterial growth was monitored on a Jenway 6505 UV–visible spectrophotometer in 1.5 ml plastic cuvettes from Fisher Scientific. NO_3-N analysis was completed using the Hach nitrate, high-range test (N tube, 0–30.0 mg litre^{-1} NO_3-N, chromotrophic acid method 10020 for water and wastewater; Hach Lange Ltd, Salford, UK).

2.6 Sample preparation for atrazine analysis

Direct HPLC analysis of atrazine batch concentrations >1 mg litre^{-1} was achieved after centrifugation (15 min at 13 400 × *g*; MSE Micro Centaur, Sanyo) on 1.5 ml samples. For atrazine batch concentrations <1 mg litre^{-1}, solid phase extraction (SPE) was used to pre-concentrate samples prior to HPLC analysis. Sample clean-up was by filtration (70 mm GF 52 glass fibre filters, Schleicher & Schuell, Dassel, Germany), with the exception of the 83 and 103 day sample intervals where centrifugation was used (10 310 × *g* for 15 min using a Hettich Rotanta 96 R centrifuge; Global Medical Instrumentation Inc., Albertsville, MN, USA) to achieve a greater degree of clean-up in response to elevated bacterial growth.

SPE concentration was conducted using Phenomenex Strata-X 60 mg/3 ml cartridges in a Phenomenex 12-position vacuum manifold (Phenomenex, Macclesfield, UK). The Strata-X cartridges were conditioned using 3 ml of methanol, equilibrated using 3 ml of water and loaded at 4 ml min^{-1} under a vacuum pressure of 15 mmHg using a 400 ml sample. After sample loading, cartridges were washed with 1 ml of MilliQ water, dried for 5 min and subsequently eluted with 2 × 1 ml of acetonitrile into a 2 ml volumetric flask to check elution volume, before completing HPLC analysis.

2.7 SPE extraction efficiency

Direct analysis of six individual samples of the filtered 10 mg litre^{-1} atrazine MilliQ water solution indicated that it had a concentration of 7.66 ± 0.1 mg litre^{-1}. This solution when diluted at 1.5 ml litre^{-1} in batch fluid gave a calculated concentration of 11.50 μg litre^{-1}. The average zero concentration for all batches created (totalling 50 for both treatments and controls), after allowing for the SPE concentration step

Table 1. Sacrificial batch flask parameters: treatment designation, oxidative state, replicate number, inoculum and media additions used

Treatment designation	Oxidative state	Replicate	Inoculum	Medium
Control A	Open bottle	1	MilliQ water	MilliQ water
GW Glu-MMSL (A)	Open bottle	2	Glu-MMSL culture	Glu-MMSL only
GW ADP (A)	Open bottle	2	*Pseudomonas* sp. strain ADP	Glu-MMSL + liquid atrazine
Control B	Sealed bottle	1	MilliQ water	MilliQ water
GW Glu-MMSL (B)	Sealed bottle	2	Glu-MMSL culture	Glu-MMSL only
GW ADP (B)	Sealed bottle	2	*Pseudomonas* sp. strain ADP	Glu-MMSL + liquid atrazine

(×200), was $10.80 \pm 1.18\,\mu g\,litre^{-1}$. The extraction efficiency of the SPE system was calculated to be 93.98% and results have been corrected for this efficiency.

Ideally, atrazine spiking solutions would have been prepared and spiked in a solvent such as methanol. However, owing to the relative insolubility of atrazine in pure water ($70\,mg\,litre^{-1}$), this could not be done, as it would have introduced more methanol-based carbon into the batch systems than the trace concentrations of glucose-based carbon being tested. The resulting bacterial community would have been induced more in response to methanol, rather than glucose, metabolism.

2.8 Analysis for atrazine content

Detection and calibration of atrazine between 1 and $50\,mg\,litre^{-1}$ were carried out on a Shimadzu class VP HPLC with a UV detection system (Shimadzu Deutschland GmbH, Duisburg, Germany). The limit of detection of this system was $0.1\,mg\,litre^{-1}$. A Phenomenex security guard cartridge holder was used in conjunction with a C8 (octyl, MOS) 4 mm × 3.0 mm i.d. guard cartridge placed in line before a Phenomenex Luna 5μ C8(2) 150 mm × 4.6 mm column. The solvent system was acetonitrile + MilliQ water (40 + 60 by volume) pumped at a flow rate of $1\,ml\,min^{-1}$. This corresponded to an atrazine retention time of ~7.8 min on 15 min sample runs determined at a wavelength of 210 nm.

For atrazine concentrations $>1\,mg\,litre^{-1}$ as used in monitoring *Pseudomonas* sp. strain ADP inocula, duplicate samples were taken per monitoring point, with triplicate HPLC analysis per sample. Error deviations presented in the results are based on six analyses of two samples per monitoring point, and data are presented for the two 1 litre batches separately.

For spiked atrazine concentrations $<1\,mg\,litre^{-1}$, SPE pre-concentrated these samples to concentrations $>1\,mg\,litre^{-1}$, and analysis was performed in triplicate but on one extracted sample per batch. Results from these three analyses were averaged. Error deviations are not given for control flasks, which were not replicated, but they are given for the treatment batches.

3 RESULTS

The correlation of growth and atrazine degradation by *Pseudomonas* sp. strain ADP on liquid atrazine medium under sterile conditions, before inoculation into the batch trials, is presented in Fig. 1. Initially, OD readings decreased during a 140 h lag period as underlying bacterial growth used media constituents. After this period, OD measurements were dominated by the exponential growth of bacterial biomass, occurring concomitantly with the degradation of atrazine. Growth of *Pseudomonas* sp. strain ADP in batch culture reached a stationary phase after approximately 233 h, giving OD measurements of approximately 0.300 and 0.260 for batches 1 and 2 respectively.

Atrazine degradation rates averaged $13.4\,mg\,litre^{-1}\,day^{-1}$, compared with $100\,mg\,litre^{-1}$ of [^{14}C]atrazine in 25 h reported previously.[20] Clearly, the *Pseudomonas* sp. strain ADP used within these experiments was not degrading atrazine optimally in comparison with previous findings. In our experiments this culture was grown at 16 °C, compared with 30 °C used by Mandelbaum *et al.*,[20] and this probably caused the difference.

Glu-MMSL and *Pseudomonas* sp. strain ADP groundwater inocula were subsequently used to start the sacrificial batch trials (Figs 2(a) and 2(b)). As expected, these results depended on the different media used. *Pseudomonas* sp. strain ADP batches

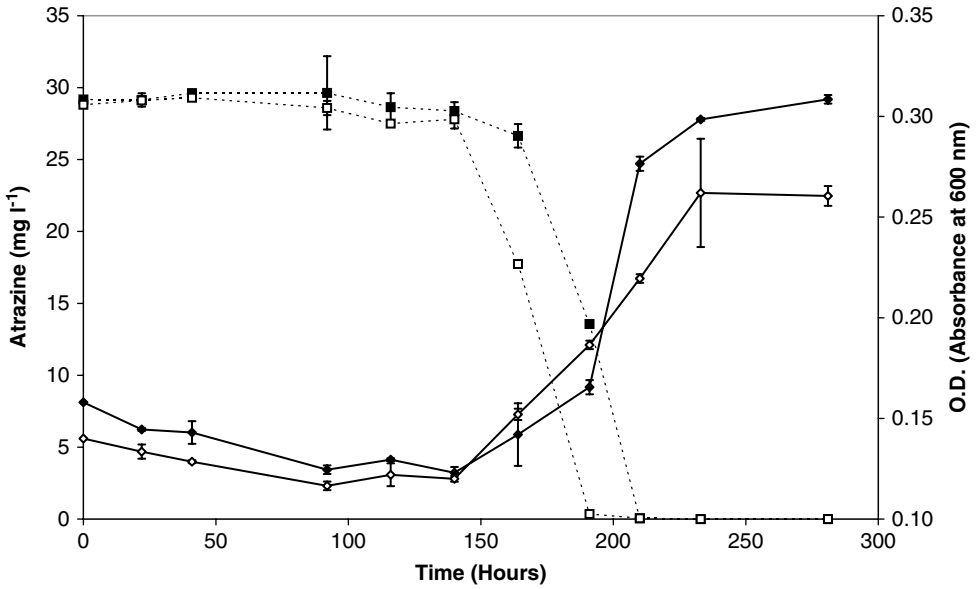


Figure 1. Growth of *Pseudomonas* sp. strain ADP inoculum in two 1 litre sterile batches: full symbols, batch 1; open symbols, batch 2; (■, □) atrazine degradation; (♦, ◊) growth.

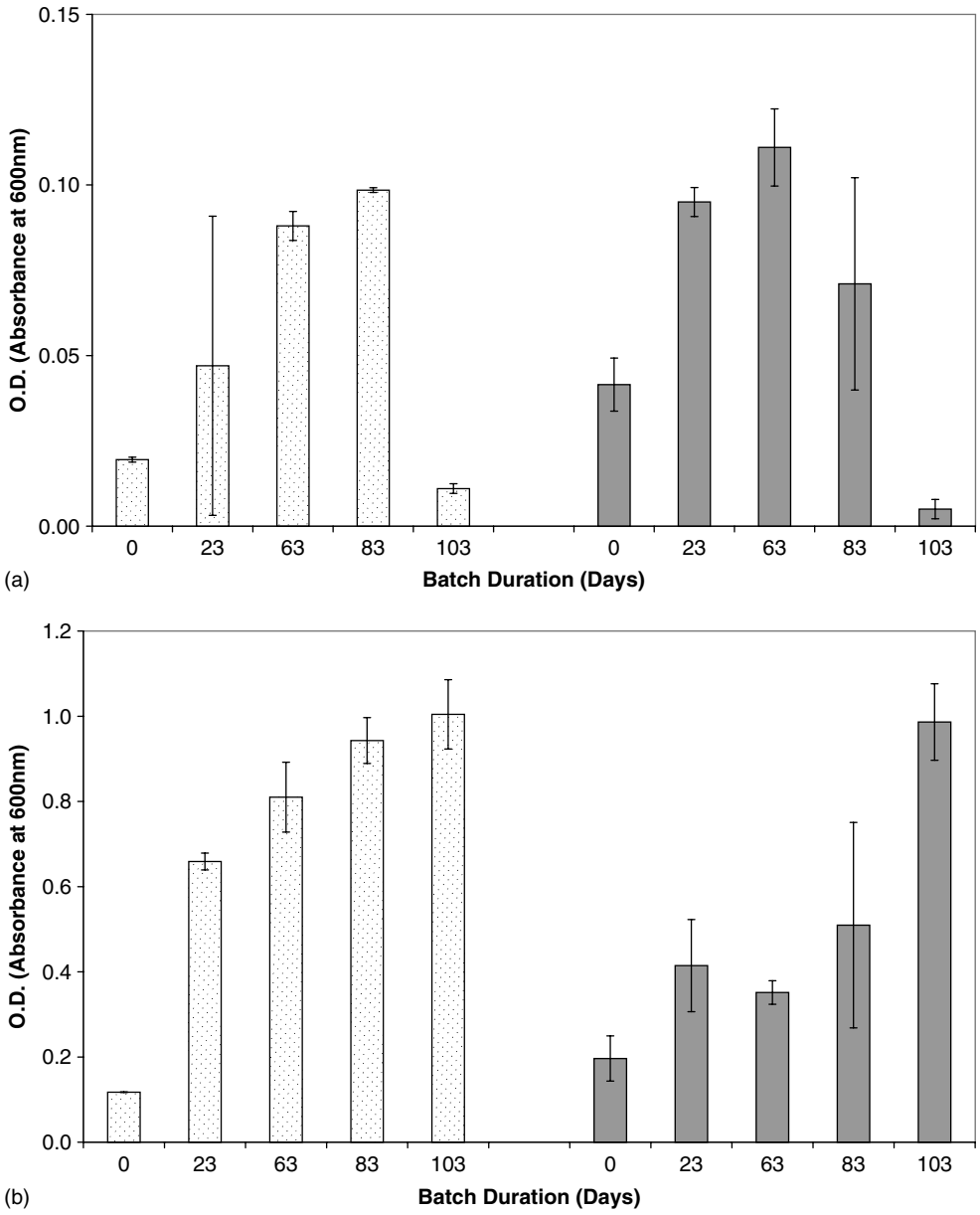


Figure 2. Growth of (a) Glu-MMSL cultured groundwater and (b) *Pseudomonas* sp. strain ADP within sacrificial batches monitored by OD measurements at 600 nm. Open bottle conditions are denoted by speckled bars, closed bottle conditions by grey bars.

which received both liquid atrazine medium[20] as well as Glu-MMSL amendments grew much better in both open and closed bottle conditions (peak OD values of 1.00 and 0.98 respectively; Fig. 2(b)) than cultures inoculated with Glu-MML cultured groundwater bacteria (peak OD values of 0.10 and 0.11 respectively; Fig. 2(a)). OD-monitored growth occurred throughout the *Pseudomonas* sp. strain ADP inoculated groundwater over the 100 day period (Figs 2(a) and 2(b)) owing to the use of liquid atrazine medium.[20] Comparatively, in the groundwater Glu-MMSL test case, OD-monitored growth declined after 83 days, most probably owing to complete metabolism of glucose having occurred.

Different oxidative conditions were generated using both open and closed batches, but these showed no marked difference in growth throughout, irrespective of treatment. These data can be understood by looking at the available NO_3-N concentration data (Fig. 3). Zero nitrate concentrations in all batches were as expected when compared with the original analysis of the groundwater NO_3-N content (15.90 mg litre^{-1} NO_3-N). As groundwater only comprised 80% of total batch volume, inevitably there is less in the groundwater Glu-MMSL cultured batches, especially compared with the elevated starting value in the *Pseudomonas* sp. strain ADP batch flasks. This is perhaps due to the liquid atrazine medium[20] containing $Co(NO_3)_2{\cdot}6H_2O$ as a trace salt.

In all treatments and oxidative conditions a notable reduction in NO_3-N content was observed to occur between the 0 and 23 day monitoring points. Within the Glu-MMSL cultured treatments, removal of NO_3-N was >90% in all cases, although there were occasional residual NO_3-N concentrations present in some batches. In *Pseudomonas* sp. strain ADP

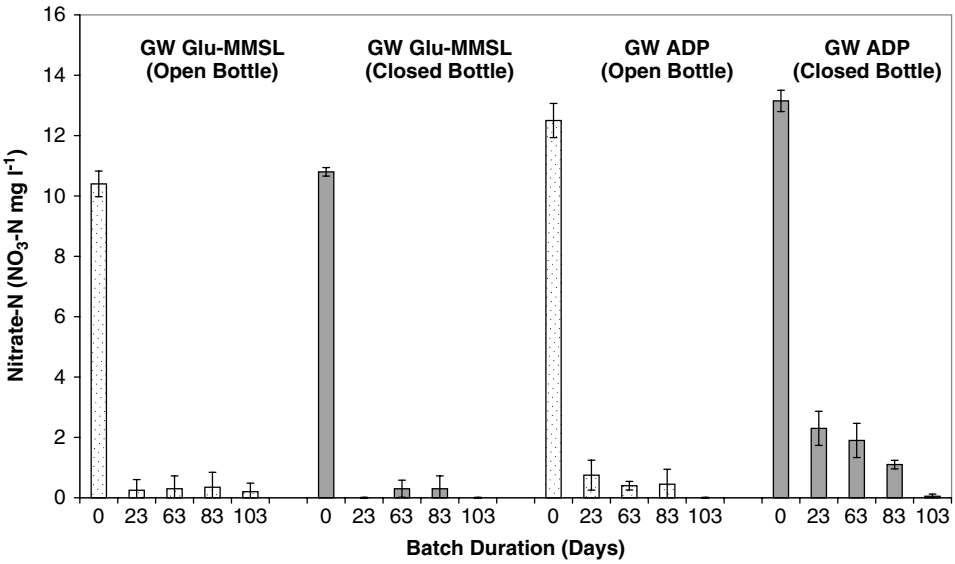


Figure 3. Changes in nitrate-N (mg litre^{-1}) concentrations in sacrificial batches. Open bottle conditions are denoted by speckled bars, closed bottle conditions by grey bars.

inoculated batches, after an initial rapid decline in NO_3-N content, denitrification slowed during the rest of the trial period. Such high denitrification efficiencies have been reported previously in the context of the use of *Pseudomonas* sp. strain ADP.[22,23]

These results for NO_3-N content indicate that denitrification occurred within all batches, irrespective of open or closed bottle conditions. This would not normally be expected from an open batch system, but was probably due to a gradient in oxidative conditions ensuring anaerobic conditions at the base of the batches.

Having established by OD monitoring and denitrification that bacterial growth occurred in these flasks, the effects on atrazine concentration are shown in Figs 4(a) and 4(b) respectively for open and closed sacrificial batches. Data are presented both for the time duration batches together with the relevant zero values.

These data for atrazine concentrations are treated qualitatively, as in all cases there was no degradation. A step change in atrazine concentration would provide evidence of positive degradation, but no obvious changes were encountered in either the controls, groundwater or *Pseudomonas* sp. strain ADP inoculated flasks under either open or closed bottle conditions. In the case of the Glu-MMSL cultured bacteria this agrees with similar findings cited in the literature.[17,18]

4 DISCUSSION

Use of SPE coupled with HPLC is perhaps less than ideal for these analyses, and either GC/MS or LC/MS would be more sensitive. At best, the methods used here should detect changes in concentration of 10 μg litre^{-1} or more, and this should be sufficient to detect a positive change in atrazine concentration associated with degradation caused by bacteria.

In inoculated batches, *Pseudomonas* sp. strain ADP degraded atrazine slowly at a tenth of the cited rate.[20] Whether a 16 °C incubation temperature accounts for this reduction in degradation is unclear, but, as the inoculum was used in an impure culture, the unsterilised groundwater matrix in conjunction with a high nutrient pressure (liquid atrazine medium[20]) may have produced conditions under which *Pseudomonas* sp. strain ADP could not compete successfully.

A similar observation has been reported within fluidised bed reactors,[22] where the loss of atrazine degradation activity by *Pseudomonas* sp. strain ADP was due to contamination by other denitrifying bacteria that were unable to degrade atrazine. It is therefore unclear how effective bacteria are in degrading trace levels of their associated pollutant. In the environment, positive degrading bacteria are notably found at sites that feature high concentrations of their associated pollutant, and have been isolated and grown under laboratory conditions in the presence of high pollutant concentrations.[20,21] Given the use of a pure culture of a positive degrading bacterium in sterile laboratory conditions, degradation of the pollutant would be expected to reach a zero value if monitored using equipment sensitive enough for the purpose. However, whether bacteria can sustain these adaptations in the environment under low pollutant concentrations and microbial competition is not self-evident, and has been suggested by these data and in other papers.[13]

Under environmental conditions, positive degraders may not necessarily degrade their target pollutant when exposed to trace concentrations (<10 μg litre^{-1}), favouring instead use of alternative and more readily available carbon or nitrogen sources. For instance, *Pseudomonas* sp. strain ADP does not use atrazine as either a carbon or energy source, but as a nitrogen source.[12]

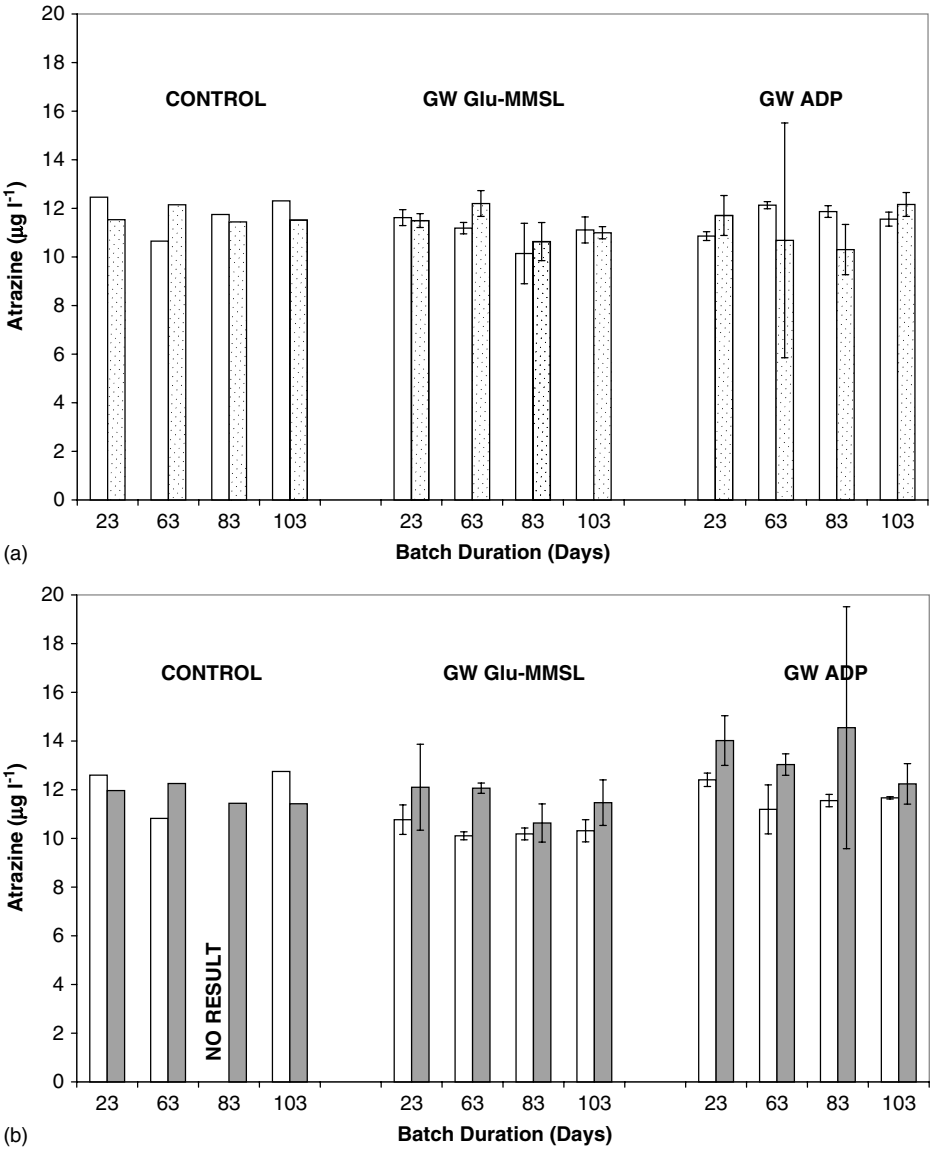


Figure 4. Averaged replicate data for atrazine concentration within (a) open and (b) closed bottle sacrificial batches. White bars denote zero atrazine concentrations per batch, with grey bars denoting time-dependent atrazine concentrations per batch.

Where degradation is incomplete, only residual concentrations of pollutants may be encountered. Invariably, these levels may be low, as exemplified by the case of pesticides in drinking water, yet may still be greater than required for legislative purposes ($<0.1\,\mu g\,litre^{-1}$). Consequently, positive degrading bacterial strains cannot be presumed to completely degrade pollutants, whether they are introduced through use in bioaugmentation programmes or as a result of natural selection occurring *in situ*. Our results for bacteria from groundwater, where atrazine concentrations were typically $0.02-0.2\,\mu g\,litre^{-1}$, indicated no degradation of the herbicide atrazine when spiked to concentrations of $10\,\mu g\,litre^{-1}$. This suggests that the aquifer atrazine concentration may have been insufficient to generate an adaptation to positive degrader status within the indigenous bacteria.

The presence of nitrate within this groundwater, coupled with the use of ammonium chloride in the Glu-MMSL media as an additional nitrogen source conducive with denitrification, may have provided non-limited nitrogen conditions. It is known that *Pseudomonas* sp. strain ADP will degrade atrazine as a sole nitrogen source and does not require atrazine as either a carbon or energy source.[12,20,22] The prevailing NO_3-N content at this site could therefore have been responsible for no adaptation occurring within the indigenous bacterial community to degrade atrazine as nitrogen source, and under laboratory conditions a readily available nitrogen source may have stopped these bacteria from undertaking atrazine degradation.

Overall, our studies indicate that atrazine degradation does not readily occur at trace levels in groundwater where diffuse atrazine contamination is encountered. Consequently, it cannot be assumed that pesticides that are readily degradable at high concentrations in soils will automatically be degraded at trace levels in groundwaters.

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