Fate of effluent organic matter during soil aquifer treatment: biodegradability, chlorine reactivity and genotoxicity

David M. Quanrud, Robert G. Arnold, Kevin E. Lansey, Carmen Begay, Wendell Ela and A. Jay Gandolfi

ABSTRACT

Hydrophobic acid (HPO-A) and transphilic acid (TPI-A) fractions of dissolved organic matter (DOM) were isolated from a domestic secondary wastewater effluent that was polished via soil aquifer treatment (SAT). Fractions were isolated using XAD resin adsorption chromatography from samples obtained along the vadose zone flowpath at a full-scale basin recharge facility in Tucson, Arizona. Changes in isolate character during SAT were established via biodegradability (batch test), specific ultraviolet light absorbance (SUVA), trihalomethane formation potential (THMFP), and Ames mutagenicity assays. The dissolved organic carbon (DOC) concentration decreased by >90% during SAT. A significant fraction (up to 20%) of isolated post-SAT HPO-A was biodegradable. The (apparent) refractory nature of DOM that survives SAT may be a consequence of low DOC concentration in groundwater as well as the nature of the compounds themselves. Specific THMFP (μg THM per mg DOC) of HPO-A and TPI-A varied little as a consequence of SAT, averaging 52 and 49 μg THM per mg DOC, respectively. The nonbiodegradable fractions of HPO-A and TPI-A exhibited higher reactivities: 89 and 95 μg THM per mg DOC, respectively. Genotoxicity of HPO-A (on a per mass basis) increased after SAT, suggesting that responsible compounds are removed less efficiently than bulk organics during vadose zone transport.

Key words | effluent organic matter, genotoxicity, secondary effluent, soil aquifer treatment, trihalomethanes

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INTRODUCTION

Among the notable distinctions between water and wastewater treatments is that wastewater treatment focuses on bulk organics removal while water treatment primarily targets pathogens and particles. Since water reclamation and reuse have become more common, however, wastewater treatment and potable water quality issues are linked. In his 2001 Stockholm Water Prize Laureate lecture, Takashi Asano called water reuse the greatest challenge of the 21st century (Asano 2002). A key task in meeting this challenge is specification of cost-effective wastewater treatment and effluent polishing techniques (including soil aquifer treatment, SAT) for control of trace organic levels. Preliminary to process selection, however, is the determination of process-dependent changes in critical reclaimed water parameters and the estimation of dose-dependent risk from exposure to trace levels of organic residuals.

Public ambivalence concerning indirect potable reuse is reflected in the difficulties encountered in formulating regulations governing aquifer recharge with reclaimed water (National Academy Press 1998). Trace organics are a primary regulatory concern. The genotoxicity and carcinogenicity of trace organics present in effluent organic matter (EfOM) and the fates of those organics during

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SAT have not been adequately established. As an example, interim guidelines for SAT leading to potable water reuse in the State of California rely on bulk organic measurements, limiting organic carbon of wastewater origin to 1.0 mg l^{-1} at points of groundwater withdrawal for potable use (Asano 1993). Concern arises due to uncertainty of organic species survival during SAT processes and production of disinfection byproducts when such waters are chlorinated prior to reuse.

Epidemiological studies have associated the consumption of chlorinated drinking water and cancers of the digestive and urinary tracts (Cantor *et al.* 1985; Hildesheim *et al.* 1991; Vena *et al.* 1993; King & Marrett 1996; Freedman *et al.* 1997; Doyle *et al.* 1997). Increased risk of early term miscarriage has been associated with consumption of tap water containing >75 mg l^{-1} trihalomethanes (THMs) (Swan *et al.* 1998; Waller *et al.* 1998). The maximum contaminant limit (MCL) for total THMs was lowered from 100 to 80 μg l^{-1} in the United States under Stage I of the Disinfectants/Disinfection Byproduct Rule and will probably be further lowered in the next few years (USEPA 1994; Pontius 1998).

Hydrophobic acids (HPO-A) tend to exhibit higher reactivity with free chlorine in forming disinfection byproducts than other dissolved organic fractions in natural waters (Singer 1999). Thus, chlorine reactivity measurements (e.g. trihalomethane formation potential, THMFP) have been used in several studies on HPO-A isolates from natural organic matter (NOM) (e.g. Collins 1985; Teng & Veenstra 1996; Klevens *et al.* 1996; Singer 1999). In a few cases, other fractions of isolated NOM (e.g. transphilic acids, TPI-A) have also been subject to chlorine reactivity measurements (e.g. Debroux 1998).

Beyond the reactivity of bulk organics with free chlorine, the possibility of adverse health effects due to long-term exposure to low levels of largely uncharacterized organics that escape wastewater treatment has now come under scrutiny (Ding *et al.* 1996; Fujita *et al.* 1996). Potential health effects associated with exposure to trace organics in reclaimed water cannot be adequately evaluated with a chemical monitoring approach, however, since even the most in-depth characterization can cover only a fraction of the multitude of compounds potentially present in complex environmental mixtures (Schoen 1998). Moreover, toxicological data are not available for all compounds and the sum of interactions between compounds in a complex mixture cannot be predicted at the present time.

Bioassays are a useful screening tool for evaluating genotoxicity effects in environmental samples. The *Salmonella*/mammalian-microsome reverse mutation screening test (Maron & Ames 1983) is the most commonly used genotoxicity bioassay (e.g. Ringhand *et al.* 1987; Helma *et al.* 1998; Monarca *et al.* 1998; Schoen 1998; Liu *et al.* 1999; Carraro *et al.* 2000; Monarca *et al.* 2000; Haider *et al.* 2002) due to its ease of use and low cost.

Previous SAT field studies at the Sweetwater Recharge Facilities (SRF) in Tucson, Arizona, established that dissolved organic carbon (DOC) and adsorbable organic halides (AOX) are attenuated by 90% and 80%, respectively, during percolation of secondary effluent to the local unconfined aquifer at 37 m (120 feet) below land surface (BLS) (Wilson *et al.* 1995; Quanrud *et al.* 2003*a*). In this study, we extended these results by evaluating changes in genotoxicity and biodegradability of HPO-A and TPI-A fractions of EfOM during percolation at the SRF. The biodegradability of effluent organics, before and after SAT, was examined by concentrating organics prior to testing.

METHODS

Samples were collected at three locations at the SRF during January/February 1999 and June/July 1999. Waters were obtained from the pond in recharge basin no. 1 (RB-1), a 5.1 cm (2.0 inch) I.D. PVC well (MW#5) intercepting a shallow perching zone at 5.2 m (17 feet) BLS, and local monitoring wells (WR-199A, WR-068A) that take water from near the groundwater table at approximately 37 m (120 feet) BLS. A submersible groundwater pump (Keck) was used to obtain samples from MW#5. Source water for recharge at the SRF is chlorinated secondary effluent from the Roger Road Wastewater Treatment Plant. Secondary treatment is provided in biotowers, followed by clarification and chlorination. For a more detailed description of the SRF, see Wilson *et al.* (1995) and Quanrud *et al.* (2003*a*).

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Samples were collected in 20-l low-density polyethylene (LDPE) cubitainers (Cole Parmer) and filtered through pre-rinsed Filtersoft® Maxima glass microfiber pleated filter cartridges (12.0 and 0.2 μm, nominal) connected in series. Filtered samples were transferred to rinsed 20-l cubitainers, acidified to pH 1.95 ± 0.05 using concentrated HCl, and stored at 4°C until processed.

XAD resin chromatography

HPO-A and TPI-A fractions were isolated by solid-phase extraction onto XAD8/XAD4 Amberlite resins (Rohm and Haas) following established methods (Malcolm 1991; Aiken *et al.* 1992). Prior to use, resin was cleaned by Soxhlet extraction for 48 h using methanol, followed by a second 48-h cleaning cycle using acetonitrile. This sequence was repeated once. Clean resin was stored in methanol pending use. Glass chromatography columns (Spectrum) were filled with resin/methanol slurry and rinsed with at least 50 pore volumes of reagent grade (Milli-Q) water to remove methanol. The packed columns were then prepared for use by rinsing (15 pore volumes per h) with three pore volumes of 0.1 N NaOH followed by three pore volumes of 0.1 N HCl. This cleaning sequence was repeated twice.

Samples were acidified (pH 2) using concentrated HCl and applied at a flow rate of 15 pore volumes per h to the columns containing XAD8 and XAD4 resins in series. The volume of sample was determined using:

$$V_e = 2V_o \times (1 + k') \quad (1)$$

where: V_e is the volume of water sample,
V_o is the void volume of the column, and
k' is the column capacity factor (selected by the user).

According to Equation 1, only those organics having a k' value larger than the chosen capacity factor will be completely retained during the isolation procedure. A value of 50 was selected for the k' cutoff (Aiken *et al.* 1992).

After the specified volume of sample had passed through the column array, the columns were re-plumbed to allow for separate back elution with 0.1 N NaOH. The basic solution was applied in upflow mode (opposite the direction of water application) at one-third the flowrate used for sample application. The HPO-A and TPI-A eluates were collected, acidified to a pH of 1.95 ± 0.05 using concentrated HCl, and stored in muffled amber glass bottles at 4°C.

Typically, a series of isolation runs was performed on successive days until the entire sample was processed. After each day in the series, columns were re-acidified using 0.1 N HCl. At the beginning of the next day in the series, the columns were washed three times with 0.1 N NaOH and 0.1 N HCl, ending with the acid solution. Columns were packed with fresh (Soxhlet cleaned) resin before isolations were initiated for a new water sample.

Eluates derived from the XAD8/XAD4 procedure were desalted primarily to remove sodium and chloride ions that were added during pH adjustments. Eluates were reapplied onto the respective columns at about four pore volumes per h (approximately one-fourth the flowrate used during the initial isolation step). The columns were then flushed with Milli-Q water to remove chloride ions, until the electrical conductivity of the column effluent was <750 mS/cm. Retained organics were re-eluted using 0.1 N NaOH. Sodium was removed from the (chloride-free) eluates by passage through a column containing hydrogen-saturated cation exchange resin (AG-MP 50, Biorad).

Cation exchange resins (CER) convert organic acids from their sodium salts to free acid forms. A potential drawback of CER is retention of organics during the desalting procedure (MacCarthy & O'Cinneide 1974; Thurman & Malcolm 1983). McKnight *et al.* (1997) noted that CER can selectively remove fulvic acid molecules with N-containing basic moieties. Since the amine and amino acid content of EfOM is higher than that of NOM, it is possible that losses during cation exchange are more significant for EfOM than NOM. Malcolm (1991) indicated that non-macroporous cation exchange resins (e.g. AG 50W-X8) are preferred for desalting operations. Macroporous resins allow penetration of organic solutes into the beads, resulting in less efficient elution. The CER used in this study was analytical grade AG-MP 50, the same resin used by Aiken *et al.* (1992). This resin employs a styrene divinylbenzene matrix with SO_3^- functional

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groups, has a specific surface area of 35 m^2/g, and is specified as macroporous for compounds with molecular weight less than 100,000 Daltons (BioRad 1997). It is possible that incomplete elution from the AG-MP 50 CER contributed to organic losses during desalting operations.

The salt-free eluates were further concentrated using a Büchi 011 Rotavapor rotary evaporator to reduce liquid volume prior to lyophilization. The water bath temperature was maintained at 30°C, and vacuum was supplied by a Welch duofold two-stage vacuum pump operating at approximately 686 mm (27 inches) Hg. Under these conditions, eluates would 'boil' for the first few minutes under vacuum. A Brinkmann RM6 Lauda chiller containing a 70/30% mixture of ethylene glycol and water was used to chill the condenser coils of the rotary evaporator. Eluates were reduced to a final volume of 150 ml in this manner.

The concentrated eluates were lyophilized using a homemade freeze-drying system in the Department of Pharmacology at the University of Arizona. Samples were frozen as 'shells' onto the interior of 1000-ml Labconco freeze-dryer flasks using a dry ice/methanol bath. Typically, samples were lyophilized for 48 h under a vacuum of 25–50 microTorr, resulting in complete primary and secondary water removal. The finished freeze-dried product was transferred to airtight 112 ml (4 oz) wide-mouthed glass jars for long-term storage (in the dark at ambient temperature).

Biodegradability, specific absorbance and chorine reactivity

Possible relationships between specific ultraviolet light absorbance (SUVA), biodegradability and chlorine reactivity were examined using reconstituted isolate solutions. Each lyophilized organic isolate was redissolved to a target concentration of 9.0 mg DOC per litre. For each isolate, the mass required to achieve a 9.0 mg l^{-1} solution was calculated using the non-ash-corrected percent carbon obtained from elemental analysis (Huffman & Stuber 1985). Prior to dissolution, isolates were placed in an oven (65°C) for 24 h to remove residual moisture. To promote fast, complete dissolution, dried material was suspended in a few ml of 0.1 N NaOH for several minutes. When dissolution was visually evident, the mixture was immediately brought to 500 ml using Milli-Q water containing 1.0 mM KH_2PO_4, 1.0 mM NH_4Cl, and 0.5 mM $CaCO_3$. Solution pH was adjusted to 7.0 ± 0.05 using 0.1 M HCl. Solutions were stored in muffled amber glass bottles at 4°C pending use.

The suite of parameters measured in reconstituted isolates included DOC concentration, ultraviolet light absorbance at 254 nm (UV-254), specific UV absorbance (defined as UV-254 divided by DOC concentration, l m^{-1} mg^{-1}), biodegradable (BDOC) and nonbiodegradable (NBDOC) DOC fractions, and trihalomethane formation potential (THMFP). The nonbiodegradable organic fractions remaining after the 5-day BDOC test were subjected to the same suite of analyses. Methods for these analyses are described in Quanrud *et al.* (2003*a*).

Evaluation of mutagenicity

Selected HPO-A and TPI-A isolates were evaluated for mutagenic activity using the *Salmonella*/mammalian-microsome reverse mutation screening assay (Maron & Ames 1983) by either Covance Laboratories, Inc. (Vienna, Virginia) or the Department of Pharmacology and Toxicology, The University of Arizona. The assay measured the ability of organic isolates and/or metabolites derived from isolates to induce reverse mutations at the histidine locus in the genome of *Salmonella typhimurium* tester strains. Tests were conducted in the presence and absence of an exogenous metabolic activation system consisting of mammalian microsomal enzymes derived from Aroclor™-induced rat liver (S9). The tester strains used in the mutagenicity assay were TA98 and TA100. TA98 registers frameshift mutagens whereas TA100 responds to base pair substitutions. When >100 mg of an organic isolate was available, the assay was conducted using both TA98 and TA100 at seven dose levels in the presence and absence of S9 mix, along with appropriate negative and positive controls. Organic isolates were solubilized and adjusted to pH 7.0 using 0.1 N NaOH. Dilutions were in water containing 0.9% NaCl. The dosage levels were 10, 33, 100, 333, 1,000, 3,333 and 5,000 μg (dry weight) of isolate per plate, and tests were conducted in duplicate.

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An NOM isolate was also tested up to 10,000 μg (dry weight) of isolate per plate. Negative and positive controls were plated in triplicate and duplicate, respectively. Compatible negative controls included deionized water, dimethylsulfoxide, ethanol and dimethylformamide. Positive controls included 2-nitrofluorene, 2-aminoanthracene and sodium azide for TA98 (w/o S9), TA100 (with S9) and TA100 (w/o S9), respectively. Results were scored as positive when two conditions were met: (1) revertants in the highest-dosage test plate were at least 2× (3× for TA98) the number of spontaneous revertants in the negative control plate, and (2) there was a statistically clear dose-response relationship.

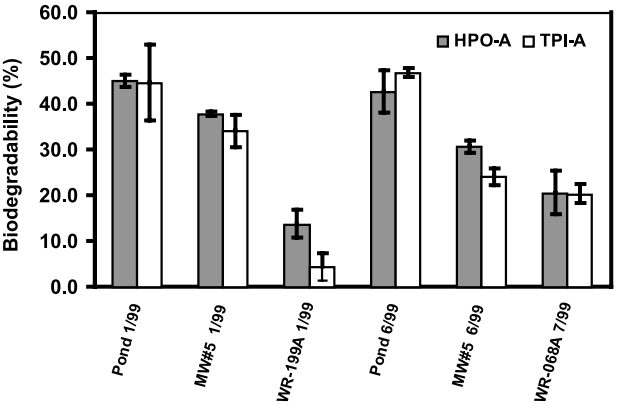

Figure 1 | Biodegradability (5-day BDOC) of reconstituted HPO-A and TPI-A isolates obtained from the Sweetwater Recharge Facilities. Biodegradability is defined here as the fraction of DOC lost during a 5-day incubation period.

RESULTS AND DISCUSSION

Fate of DOC during SAT

Dissolved organic carbon is typically reduced by >90% during percolation of secondary effluent through the vadose zone at the Sweetwater recharge site (Wilson *et al.* 1995; Quanrud *et al.* 2003*a*). Changes in the molecular characteristics of HPO-A and TPI-A isolates during SAT at this site are described in Quanrud *et al.* (2003*b*).

Analyses on reconstituted isolates

Biodegradabilities (based on 5-day BDOC tests) of reconstituted HPO-A and TPI-A isolates ($C_o = 9$ mg DOC l^{-1} for all) are summarized in Figure 1. Measurements were conducted in triplicate; error bars represent plus/minus one standard deviation from the mean value. As expected, the biodegradability of isolates decreased from the pond to the aquifer. Nevertheless, the biodegradability of the HPO-A aquifer isolates was surprisingly high (4–20%) since these organics were expected to be biorefractory. Consequently, additional experiments were performed to determine whether levels of biodegradation in groundwater isolates were sensitive to the initial DOC concentration in the NBDOC test. The working hypothesis was that the organics in these isolates are potentially biodegradable at higher dissolved concentrations. Minimum sustainable substrate concentrations required to maintain biofilm activity in saturated sediments are thought to be in the order of a fraction of a mg per litre to several mg per litre, depending on the substrate itself and electron acceptor condition (McCarty *et al.* 1985). The DOC concentrations of the two groundwater samples (WR-199A, WR-068A) collected in this study were 1.4 and 2.2 mg l^{-1}, respectively. It is likely that local microbial activity was substantially impaired by lack of available substrate at these DOC concentrations.

To test this hypothesis, BDOC tests were conducted (in triplicate) at initial DOC concentrations ranging from 3 to 20 mg l^{-1} using reconstituted solutions made from the HPO-A isolate from WR-068A (collected in July 1999). Results suggest that there is a weak relationship between starting DOC concentration (C_o) and DOC loss during the first 5 days of the experiment (Figure 2). At a starting concentration of 3 mg l^{-1}, there was a 10% reduction of DOC after 5 days. At initial concentrations of 6 and 20 mg l^{-1}, DOC was attenuated by 20% after 5 days. Reductions in DOC were due to aerobic biological activity; there was no significant attenuation in negative controls spiked with 2 mM sodium azide ($C_o = 6$ mg l^{-1}). Previous work (Quanrud *et al.* 1996) showed that aerobic respiration is essentially eliminated in sediment suspensions containing 2 mM sodium azide. Increases in DOC that occurred after 5 days were probably due to breakdown and lysis of biofilm microorganisms. The greatest evidence of bacterial decay at $t = 10$ days occurred in reactors with the lowest starting DOC concentration

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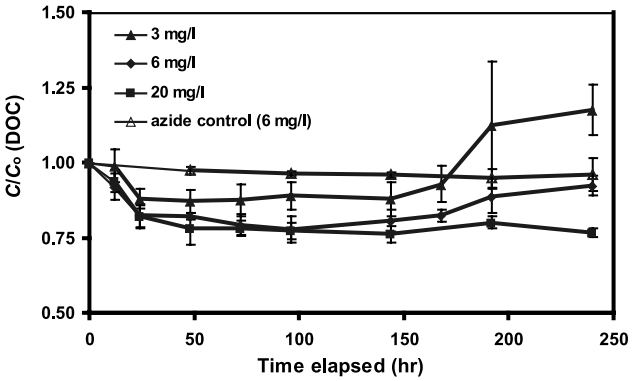

Figure 2 | Kinetics of BDOC exertion for reconstituted groundwater HPO-A obtained from well WR-068A. Reactors differed primarily in terms of initial DOC (in the range of 3 to 20 mg l^{-1}). The DOC in the azide control was 6 mg l^{-1}.

Table 1 | Specific absorbance values (1 m^{-1} mg^{-1}) of reconstituted isolates before and after the BDOC test procedure

Isolate description	SUVA (pre-BDOC test)	SUVA (NBDOC)	Increase (%)
HPO-A			
Pond 1/99	2.23	3.12	39.8
MW#5 1/99	2.90	3.78	30.0
WR-199A 2/99	2.63	2.70	2.7
Pond 6/99	2.36	3.20	35.4
MW#5 6/99	3.36	4.00	19.3
WR-068A 7/99	2.37	2.62	10.7
TPI-A			
Pond 1/99	1.75	2.60	48.5
MW#5 1/99	2.13	2.92	37.2
WR-199A 2/99	2.37	2.53	6.8
Pond 6/99	1.68	2.37	40.4
MW#5 6/99	2.78	3.20	15.2
WR-068A 7/99	2.39	2.57	7.6

(3 mg l^{-1}). This experiment suggests that 5 days (120 h) is an appropriate time period for determination of biodegradable DOC in the batch test used here. That is, DOC levels were relatively stable after 5 days, suggesting that most biodegradable organics had been eliminated, and decay of the test bacterial community was not yet important.

SUVA values were higher for HPO-A than for corresponding TPI-A isolates (Table 1). For HPO-A, uniform trends in SUVA were apparent; an initial increase in SUVA was followed by a decrease during percolation through the vadose zone. This trend is consistent with changes in isolate aromaticities established using carbon-13 nuclear magnetic resonance and Fourier transform infrared spectroscopies for molecular characterization of these isolates (Quanrud *et al.* 2003*b*).

Relationships among biodegradability, SUVA and chlorine reactivity of Sweetwater isolates were also examined. Using all the data, no relationship between biodegradability and SUVA is apparent (Figure 3). If the aquifer isolates are neglected, however, there is a strong inverse linear relationship between biodegradability and SUVA for both HPO-A and TPI-A (Figure 3), suggesting that aromaticity is an important factor controlling biodegradability in waters taken from near-surface sediments. After the more easily biodegradable components of EfOM are removed in near-surface sediments, slower processes act to decrease aromaticity during transport through the remainder of the vadose zone (i.e. from 6 to 37 m BLS at the Sweetwater recharge site).

The specific THMFP of HPO-A and TPI-A did not change significantly as a consequence of SAT, averaging 52 and 49 μg THM per mg DOC, respectively (Figure 4). These values are similar to specific THMFP yields reported in Leenheer *et al.* (2001) for reclaimed water organic isolates after recharge into groundwater at the Montebello Forebay in Los Angeles County, California. SUVA measurements suggest that there are significant changes in the characters of HPO-A and TPI-A during percolation through the vadose zone (Table 1). Nevertheless, differences in specific THMFP of isolated HPO-A and TPI-A during SAT were minor (Figure 4).

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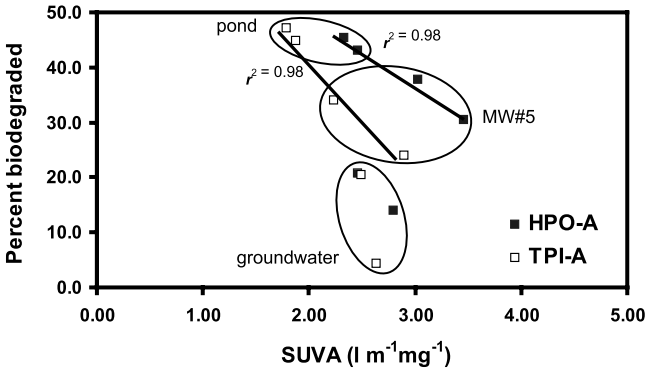

Figure 3 | Relationship between biodegradability and SUVA for reconstituted HPO-A and TPI-A isolates from the Sweetwater Recharge Facilities. For purposes of biodegradability measurements, all samples were redissolved to produce an initial DOC concentration of 9 mg l^{-1}.

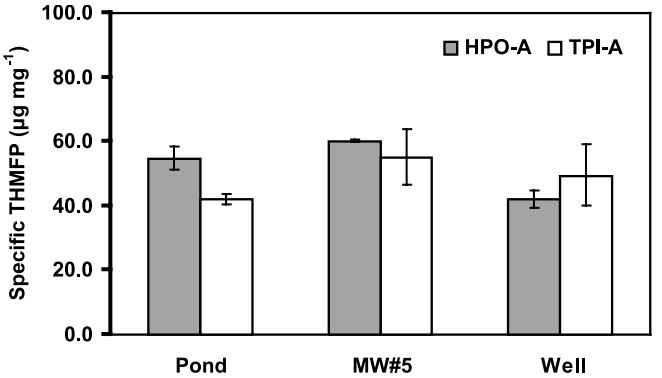

Figure 4 | Specific THMFP (µg mg^{-1}) for reconstituted HPO-A and TPI-A isolates from the Sweetwater Recharge Facilities. The 'well' refers to groundwater from WR-199A and WR-068A.

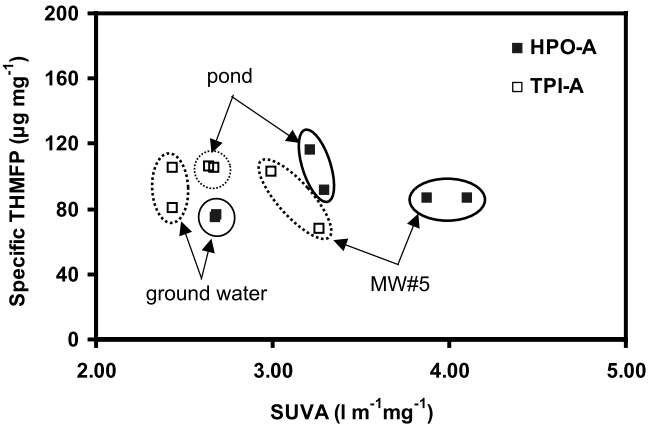

Figure 5 | Relationship between $SUVA_{NBDOC}$ and specific THMFPNBDOC for reconstituted HPO-A and TPI-A isolates from the Sweetwater Recharge Facilities. That is, figures represent the fractional increase in SUVA measurements during the 5-day BDOC procedure for the samples and isolates indicated.

The nonbiodegradable component remaining after BDOC testing was subjected to the same suite of analyses. Aromaticity of all isolates increased after the BDOC test procedure, as evidenced by increased SUVA values (Table 1). The magnitude of SUVA percentage increase (relative to secondary effluent) decreased after percolation through the vadose zone (Table 1), indicating that nonaromatic carbon removal during SAT occurs primarily during passage through near-surface sediments.

To evaluate the importance of sorption as a removal mechanism for THM precursors during SAT, THMFP tests were conducted on the NBDOC component of all isolates. Here, it was assumed that organics remaining after the BDOC procedure represented the EfOM component available for removal via sorption during SAT. Regression analyses indicated that the modest decrease in specific $THMFP_{NBDOC}$ from pond to groundwater was statistically significant ($p \leq 0.05$) for HPO-A but insignificant for TPI-A ($p = 0.22$). That is, these results indicate that sorption plays a role in removal of HPO-A THM precursors during SAT but is not significant for removal of TPI-A THM precursors. The mean $THMFP_{NBDOC}$ values for HPO-A and TPI-A (Figure 5) were 89 and 95 µg THM per mg DOC, respectively, significantly higher than mean THMFP values (52 and 49 µg THM per mg DOC) of the same organic fractions prior to the BDOC procedure (Figure 4).

Although $SUVA_{NBDOC}$ decreased substantially during SAT (Table 1), $THMFP_{NBDOC}$ did not follow this trend (Figure 5). That is, the utility of the SUVA measurement as a predictor of THMFP decreased for nonbiodegradable fractions of HPO-A and TPI-A during SAT.

Evaluation of mutagenicity

Results from *Salmonella* microsomal mutagenicity (Ames) tests performed on HPO-A and TPI-A isolates (pond water, shallow well MW#5, and groundwater wells

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Table 2 | Summary of Ames Test results on HPO-A and TPI-A isolates

Sample	HPO-A Assay result	HPO-A Mean increase[a]	TPI-A Assay result	TPI-A Mean increase
Pond (DOC = 2.5 mg l^{-1})				
TA98 w/o S9	Neg[b]	2.25	—[c]	—
TA98 w/S9	Neg	0.90	—	—
TA100 w/o S9	Neg	1.09	—	—
TA100 w/S9	Neg	1.64	—	—
MW#5 (DOC = 7.4 mg l^{-1})				
TA98 w/o S9	Neg	1.44	Neg	1.26
TA98 w/S9	Neg	1.36	Neg	1.14
TA100 w/o S9	Neg	1.12	Neg	0.94
TA100 w/S9	Pos	2.13	Neg	1.31
WR-199A (DOC = 1.4 mg l^{-1})				
TA98 w/o S9	Pos	8.25/2.18[d]	—	—
TA98 w/S9	Pos	3.77/2.12	—	—
TA100 w/o S9	Neg	1.57/1.38	—	—
TA100 w/S9	Neg	1.34/2.09	—	—
WR-068A (DOC = 2.4 mg l^{-1})				
TA98 w/o S9	Pos	8.27	Neg	0.74
TA98 w/S9	—	—	Neg	1.93
TA100 w/o S9	Neg	1.67	Neg	0.87
TA100 w/S9	Neg	1.48	Neg	1.08
Wetland NOM (DOC = 2.5 mg l^{-1})				
TA98 w/o S9	Neg	1.18	Neg	0.95
TA98 w/S9	Neg	0.84	Neg	0.96
TA100 w/o S9	Neg	0.66	Neg	0.95
TA100 w/S9	Neg	1.41	Neg	1.18

[a]Defined as highest dose response divided by negative control response.
[b]This test was not scored a positive because it was not clearly dose responsive.
[c]Not assayed.
[d]Performed by Covance Laboratories, Inc./performed by University of Arizona Department of Pharmacology and Toxicology.

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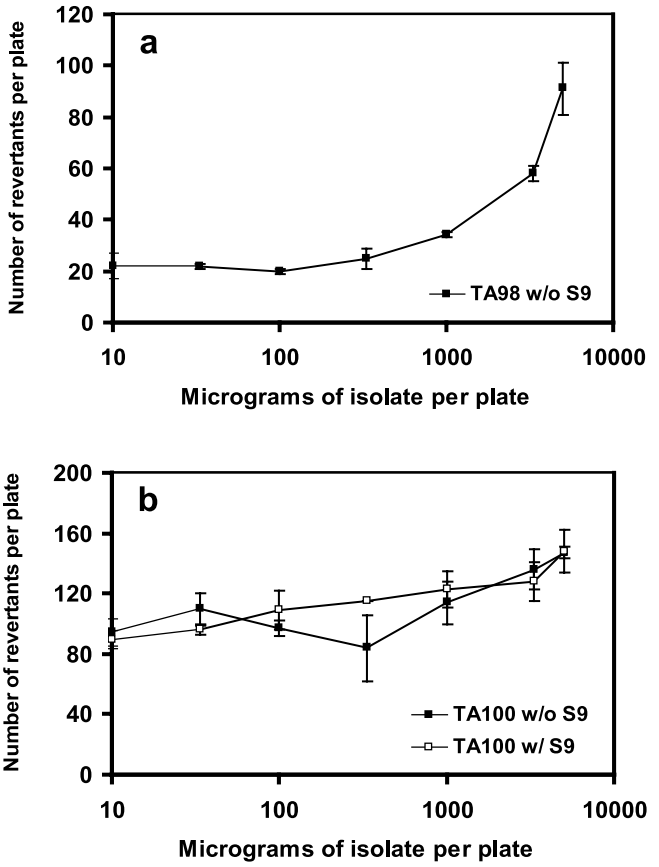

Figure 6 | Dose-dependent number of revertants produced during Ames testing of groundwater HPO-A isolate from well WR-068A: (a) strain TA98, (b) strain TA100) (TA98 with S9 was not performed due to sample mass limitation).

WR-199A, WR-068A) are summarized in Table 2. The pond HPO-A isolate did not yield a positive test response (based on criteria provided above) under any of the four test conditions. A 2.3-fold increase in mean number of TA98 revertants per plate occurred in the absence of S9 mix (Table 2). However, that increase was not clearly dose responsive, so the response was considered negative. Linear regression analysis of the TA100 data without S9 indicated that the slope of the regression line representing the relationship between chemical dose and reversion frequency was significantly different from zero ($p = 0.01$). This suggested a dose-response relationship, even though the TA100 assay did not meet the two-fold increase criteria for scoring as a 'positive' result. The HPO-A MW#5 isolate yielded a positive response under one test condition (TA100 w/S9) (Table 2).

In contrast, both groundwater HPO-A isolates (WR-199A, WR-068A) stimulated dose-related increases in mean number of revertants per plate for strain TA98 (Table 2). Dose-response data for the WR-068A HPO-A isolate are shown in Figure 6a (TA98) and Figure 6b (TA100). In both groundwater HPO-A samples, there was an 8.3-fold increase (relative to the negative control) in the mean number of TA-98 (without S9) revertants per plate at the highest dose tested (5,000 μg per plate). Both groundwater HPO-A isolates produced <2-fold increase in TA100 revertants (with and without S9). However, dose-response relationships were again evident in results of regression analyses of the dosage-dependent data ($p < 10^{-4}$). The corresponding TPI-A isolates from the groundwater isolates were nonmutagenic, suggesting that responsible compounds are relatively hydrophobic (captured on XAD8 resin).

Results indicate that there was a significant increase in specific mutagenicity (number of revertants per mg carbon) of the HPO-A isolates between the pond/MW#5 and groundwater samples for the TA98 strain in either the absence or presence of S9 mix. A smaller, yet significant increase occurred for strain TA100 in the absence of S9. Previous work with aquatic organic extracts also showed greater mutagenic response with TA98 than with TA100 (Monarca *et al.* 1998; Guzzella & Sora 1998). Results suggest that compounds responsible for mutagenicity comprise a larger fraction of the DOC remaining after SAT. That is, mutagenic compounds may, on average, be less biodegradable or less sorbable than bulk organics in domestic wastewater effluents. Alternatively, the compounds responsible for measured mutagenicity may contain metabolic by-products that are not as prevalent in the pre-SAT water.

Procedural artifacts contributing to the mutagenic response of the groundwater isolates are unlikely. Results from parallel Ames tests on wetland-derived natural organic matter HPO-A and TPI-A isolates obtained using the same XAD isolation procedures (Quanrud *et al.* 2003*c*) were nonmutagenic (Table 2). Since the DOC concentration (2.5 mg l^{-1}) of the wetland effluent was not substantially greater than that of the groundwaters tested, it

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follows that the isolation/fractionation procedure did not produce false positive results. Ringhand *et al.* (1987) also concluded that mutagenic artifact formation during XAD concentration procedures was negligible.

Two of the HPO-A isolates tested (pond, WR-199A) were also analyzed for AOX. These tests were conducted at the Technical University of Berlin using methods described in Drewes & Jekel (1998). Specific AOX values (AOX normalized to DOC concentration) for pond and groundwater HPO-A isolates were 10.2 $\mu g\ mg^{-1}$ and 30.0 $\mu g\ mg^{-1}$, respectively, paralleling the relative mutagenic responses in these samples. This is not to suggest that AOX is responsible for the mutagenicity of the waters tested – only that potential mutagens may be less susceptible to SAT-dependent transformations than are most residual organics in treated wastewater.

SUMMARY AND CONCLUSIONS

The following conclusions are supported by experimental results.

1. The biodegradable fraction of both HPO-A and TPI-A in EfOM decreased with depth through the vadose zone. Residual EfOM that survived vadose zone percolation was potentially amenable to further biochemical oxidation; however actual biotransformation depended on substrate concentration.
2. The aromatic component of EfOM, as indicated by SUVA, biodegraded more slowly than bulk EfOM in the upper vadose zone, but its level of removal was not significantly different from that of bulk organics after percolation through the 37-m vadose zone.
3. The reactivity of HPI-A and TPI-A with chlorine (Specific THMFP) did not change appreciably as a consequence of SAT.
4. The nonbiodegradable component of all HPO-A and TPI-A isolates exhibited greater THMFP.
5. Trace organics in HPO-A isolates of EfOM that were responsible for mutagenicity in Ames tests were removed less efficiently during SAT than were bulk organics in the HPO-A isolate fraction.

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DISCLAIMER

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