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# The impact of gamma radiation on sediment microbial processes

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Running Head: Microbial communities and gamma radiation

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## Abstract

Microbial communities have the potential to control the biogeochemical fate of some radionuclides in contaminated land scenarios or in the vicinity of a geological repository for radioactive waste. However, there have been few studies of ionizing radiation effects on microbial communities in sediment systems. Here, acetate and lactate amended sediment microcosms irradiated with 0.5 or 30 Gy $h^{-1}$ gamma radiation for 8 weeks all displayed $NO_3^-$ and Fe(III) reduction, although the rate of Fe(III) reduction was decreased in 30 Gy $h^{-1}$ treatments. These systems were dominated by fermentation processes. Pyrosequencing indicated that the 30 Gy $h^{-1}$ treatment resulted in a community dominated by two Clostridial species. In systems containing no added electron donor, irradiation at either dose rate did not restrict $NO_3^-$, Fe(III) or $SO_4^{2-}$ reduction. Rather, Fe(III)-reduction was stimulated in the 0.5 Gy $h^{-1}$ treated systems. In irradiated systems, there was a relative increase in the proportion of bacteria capable of Fe(III)-reduction, with *Geothrix fermentans* and *Geobacter* sp. identified in the 0.5 Gy $h^{-1}$ and 30 Gy $h^{-1}$ treatments respectively. These results indicate that biogeochemical processes will likely not be restricted by dose rates in such environments and electron accepting processes may even be stimulated by radiation.


## Introduction

In many countries, including the UK, the current policy for the long term disposal of intermediate-level radioactive waste (ILW) is to a deep geological disposal facility (GDF). In UK disposal concepts for higher–strength rocks and lower-strength sedimentary rocks, much of the ILW will be immobilised with a cementitious grout in stainless steel containers that are then surrounded with a cementitious backfill prior to closure of the facility (1). The vicinity of a GDF

39 will not be a sterile environment and microbial activity in the surrounding geosphere could have
40 important implications for the evolution of biogeochemical processes, including microbial gas
41 generation and utilization, microbially-induced corrosion of waste containers and contents, and
42 the mobility of radionuclides (2). In addition, there will be elevated concentrations of potential
43 electron donors in and around the repository, including organics from the degradation of
44 cellulose in the waste (3) and also molecular hydrogen from the radiolysis of water and the
45 anaerobic corrosion of steel drums (4). Indeed, the availability of alternative electron acceptors
46 will likely not be limited, as nitrate can be present in nuclear waste materials (5), and Fe(III) will
47 be present due to aerobic corrosion of waste components and engineered infrastructure during
48 the operational phase of the GDF.

49 The stimulation of an Fe(III)-reducing community due to an increase in electron donors and
50 acceptors is of particular interest as this may promote the reduction and precipitation of redox-
51 active radionuclides via the production of biogenic Fe(II)-bearing phases (2, 6). Indeed, many
52 key Fe(III)-reducing species may also possess cytochromes and hydrogenases capable of directly
53 reducing multi-valent elements, such as Tc(VII), Np(V) and U(VI), with radionuclides of interest
54 in safety assessments (7, 8). As these processes could lower the mobility of these elements, the
55 microbial ecology and potential for Fe(III)-reduction in geodisposal environments has been the
56 focus of recent research.

57 These environments, and the microbially driven processes that occur within them, may be
58 subject to significant radiation doses. Firm values for total absorbed doses and dose rates are
59 difficult to predict as they are likely to be highly heterogeneous and dependent on the activity of
60 the waste, the radiation type, decay dynamics and the absorbing materials in the waste. For
61 example, Canadian researchers predict the maximum dose rate to be 52 Gy $h^{-1}$ at the surface of a

waste container (9). Similarly, Allard and Calas (2009) suggest that dose rates in silicate clays used for backfill material may be in the order of 72 Gy $h^{-1}$ over the first 1000 years of the repository lifetime. For Swedish spent fuel disposal, on the other hand, the maximum estimate of dose rate outside the canister is 0.5 Gy $h^{-1}$ over the first 1000 years, followed by significant decay after this (11).

Significant radiation fluxes may also be associated with near surface sites contaminated by radionuclides, for example, activities up to 0.37 GBq $kg^{-1}$ have been measured at contaminated DOE sites (12, 13). Again, it is difficult to predict how activities such as this relate to dose rates and total absorbed doses, however, as a reference, it has been calculated by particle track calculation and Monte Carlo simulation that activities of 8.1 MBq $kg^{-1}$ $^{90}Sr$ and 9.6 MBq $kg^{-1}$ $^{137}Cs$ in Chernobyl soils equate to dose rates of 51.7 Gy $y^{-1}$ and 14.8 Gy $y^{-1}$ respectively (14).

Ionizing radiation is potentially lethal to organisms as the energies involved are sufficient to cause strand breaks in DNA. Despite this, most bacteria encode conventional enzymatic DNA repair mechanisms, rendering much of the damage repairable. However, cytoplasmic water radiolysis generates quantities of reactive oxygen species (e.g. $HO^{\bullet}$, $H_2O_2$, $O_2^{\bullet-}$) which may react indiscriminately with essential biomolecules, such as nucleic acids, proteins and lipids, causing damage (15–17). Indeed, radiation induced protein oxidation has been quantifiably related to bacterial viability (17).

When the generation of reactive oxygen species exceeds the scavenging capacity of the cell, oxidative stress is incurred. This, when combined with the inability of a cell's metabolism to replenish damaged molecules as a result of radiation stress, likely results in fatality. The dose at which this occurs in a specific species is very variable and as such, there has long been a focus on determining radiation sensitivity in environmentally important species. For example, the

extreme radiation resistance of *Deinococcus radiodurans* and the sensitivity of subsurface bacterial species such as Fe(III)-reducing *Shewanella* sp. have been assessed (15, 18). However, many of these studies were conducted with pure cultures at high acute doses, and whilst acute dose laboratory studies may predict canister vicinities to be sterile (9, 19), survival may actually be possible under dose rates more relevant to nuclear environments. For example, under a chronic dose rate of ~2 Gy $h^{-1}$, microorganisms isolated from a spent nuclear fuel pond were capable of surviving total absorbed doses five times greater than tolerated in acute dose experiments (>426 Gy $h^{-1}$) (20, 21). Furthermore, microbes from the indigenous endolithic community of a proposed repository were capable of surviving low gamma doses in a viable but non-culturable state (22), such that resuscitation may be possible when environmental conditions become more favourable (23). This highlights the importance of gathering low dose rate data, particularly as lower dose rates may allow species to respond via up-regulating repair mechanisms (24) or even adapting over geological timescales, relevant to radwaste disposal scenarios. Similarly, the survival data from pure culture studies may not be applicable to relatively nutrient limited sediments, where there is competition from different species of the community, and where radiation is perhaps not the only selective stress. Indeed, Bruhn et al. (2009) showed that the survival of the usually radioresistant *D. radiodurans* in a mixed culture was somewhat limited, probably as a result of competition with *Pseudomonas* spp. However, this study was conducted in a rich Tryptic Soy Broth medium that is far from representative of *in situ* GDF conditions.

Whilst it is important to examine the radiation tolerance of microbial community members, radiation may also impact upon the extracellular environment, which may consequently influence the capacity for microbial processes. For instance, the radiolysis of water generates



Applied and Environmental Microbiology

molecular hydrogen which may be used as an electron donor for a range of microbial electron accepting processes (2, 25–27). Furthermore, radiation has been shown to break down natural organic matter in soils resulting in increases in dissolved organic carbon (DOC) (28, 29). This radiolytic degradation of organic matter may enhance the bioavailability of organic carbon for microbial metabolism.

The oxidation state of potential electron acceptors may also be altered by ionizing radiation. For instance, irradiation led to Fe(III)-reduction in a range of materials, including clays and goethite (28, 30–32). On the other hand, irradiation induced oxidation of Fe in steel and aqueous Fe(II) solutions led to the generation of the Fe(II)/(III) oxides lepidocrocite, maghemite and magnetite (33, 34). Such changes to the oxidation state of Fe may have important implications for the bioavailability of Fe(III) for microbial respiration.

Even when no radiation induced oxidation/reduction is observed, Fe(III) in both ferrihydrite and hematite may be made more available for microbial reduction via alteration to the crystalline structure (35). As Fe is likely to be a significant component of waste packaging and repository infrastructure, such radiation effects could have important implications to deep subsurface microbial communities. With regard to other electron acceptors, many studies have shown a decrease in the concentration of nitrate in irradiated soils (36). On the other hand, sulfate concentrations increased in a soil by 17% after 30 kGy gamma-irradiation, albeit this was attributed to releases from lysed cells (37).

It is, therefore, evident that radiation may impact upon both cellular physiology and the bioavailability of growth substrates; i.e. electron donors, acceptors and presumably nutrients (36). However, despite the potential consequences to the evolution of biogeochemical processes in nuclear environments, there is a lack of information on the combined effect of all these

131 radiological processes on microbial metabolism at low dose rates. Here, we address the impact of
132 low-dose chronic gamma-irradiation upon a sediment microbial community and the
133 biogeochemical processes controlled by this community both during irradiation and throughout a
134 subsequent recovery stage. In addition, Fe(II) concentrations were probed to assess the ability of
135 an irradiated community to carry out Fe(III) reduction. To the authors' knowledge, this was
136 conducted using the lowest dose rate over the longest irradiation period of any comparable study
137 to date. Two dose rates were employed: 30 Gy $h^{-1}$, representative of dose rates at radwaste
138 canister surfaces, and 0.5 Gy $h^{-1}$, simulating dose rates further afield, or after decay of radiation
139 levels and microbial repopulation of the repository vicinity. This is in sharp contrast to the acute
140 radiation levels used in other pure culture studies.

141

142 **Materials and Methods**

143 **Sediment collection.** Sediment samples were taken from a location representative of the
144 Quaternary, unconsolidated alluvial flood-plain deposits in the vicinity of the UK Sellafield
145 reprocessing site. This site was selected as our group has extensive experience studying the
146 biogeochemistry of sediment from this area. Samples were collected from the shallow sub-
147 surface at a locality ~2 km from the Sellafield site, in the Calder Valley, Cumbria (38–40).
148 Samples were transferred to sterile containers, sealed, and stored in the dark at 4 °C prior to use.
149 **Sediment microcosms.** To assess the impact of gamma radiation on the indigenous
150 microorganisms of the sediment, microcosms were prepared in sterile 100 mL serum bottles by
151 the addition of a sterile synthetic groundwater representative of the region (41) to samples of
152 sediment (10 ± 0.1 g sediment; 100 ± 1 mL groundwater buffered at pH 7 using 0.24 g $L^{-1}$
153 $NaHCO_3$). After addition of the buffered groundwater, the pH of the microcosms was

154 approximately 6.4. Sodium lactate and sodium acetate were added as electron donors, where
155 necessary, to give final added concentrations of 7 mM for each. Thus, a range of microcosm
156 conditions were produced, as shown in Table 1. Triplicates of each of the different microcosms
157 were then sealed with butyl rubber stoppers prior to irradiation.

158 Microcosms containing no added electron donor were also irradiated prior to the addition of an
159 active *Geobacter sulfurreducens* culture to investigate the effect of radiation on Fe(III)-reduction
160 in the sediments, whilst evaluating the impact of radiation toxicity on the indigenous
161 microorganisms. After irradiation, microcosms were purged with an $N_2$-$CO_2$ (80:20) gas mixture
162 to render the sediments anoxic to support microbial Fe(III) reduction. Suspensions of *G.*
163 *sulfurreducens* (100 µL) were added, where necessary, to give a final cell density of
164 approximately $1 \times 10^7$ cells $mL^{-1}$. Cultures were initially prepared by growing *G. sulfurreducens*
165 at 30 °C in a fully defined anaerobic medium, as described previously (42). Sodium acetate (20
166 mM) and fumarate (40 mM) were added as electron donor and acceptor, respectively. After 24 h,
167 late log/early stationary phase cultures were harvested anaerobically by centrifugation at 4920*g*
168 for 20 min under $N_2$-$CO_2$ (80:20) and washed twice with sterile nitrogen purged 30 mM sodium
169 bicarbonate (pH 7.2).

170 **Irradiations.** Microcosm irradiations were carried out in the dark at Cell 5, AMEC, Harwell,
171 UK. Co-60 gamma (1.25 MeV) was supplied to two separate sets of microcosms at dose rates of
172 0.5 Gy $h^{-1}$ ± 10% and 30 Gy $h^{-1}$ ± 10% over a 56 day period. Total absorbed doses after the 56
173 day period are shown in Table 1. The total absorbed dose in microcosms irradiated at 30 Gy $h^{-1}$
174 for 28 days, when aliquots were removed for chemical analysis, was 19.2 kGy ± 10%. Dose
175 measurements were made with instrumentation traceable to national standards. The temperature
176 inside Cell 5 was 18 ± 1 °C. External control experiments were maintained at the same

177 temperature. After irradiation, all microcosms were returned to the University of Manchester
178 Geomicrobiology laboratory and incubated in the dark at 19 °C.

179 **Geochemical analyses.** Experiments were sampled periodically for geochemical analyses and
180 microbial community analysis using aseptic techniques under anoxic conditions. Microbial
181 Fe(III)-reduction was monitored in all microcosms by spectrophotometric determination of Fe(II)
182 using the ferrozine assay (43). Biogenic Fe(II) was determined by digestion of 100 µL of
183 sediment slurry in 5 mL 0.5 N HCl for 1 h. Total bioavailable Fe was determined by digestion of
184 100 µL of sediment slurry in 5 mL 0.25 N HCl and 0.25 N hydroxylamine-HCl, followed by the
185 ferrozine assay (44).

186 A sediment slurry (2 mL) from each replicate microcosm was centrifuged at 3000*g* for 3 min.
187 The supernatant was used for analysis by ion chromatography and the sediment was used for
188 microbiological characterisation. Samples were stored at -20 °C prior to analysis.

189 **Ion chromatography.** Chloride, nitrate, nitrite, sulfate, phosphate and organic acids were
190 measured using a Dionex IC5000 system with a Dionex Capillary AS11-HC 4µ column.
191 Aliquots of 0.4 µL were injected into a potassium hydroxide mobile phase with a flow rate of
192 0.015 mL $min^{-1}$ and a gradient of 1 mM – 36 mM KOH over 40 minutes.

193 **16S amplicon pyrosequencing and data analysis.** Samples for 16S rRNA gene pyrosequencing
194 were taken from the microcosm that was the most representative of the mean of chemical
195 analyses of the three replicates. DNA was isolated from microcosm samples (200 µL slurry)
196 using the MoBio PowerSoil™ DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA,
197 USA), following the manufacturer's instructions. PCR of the V1-V2 hypervariable region of the
198 bacterial 16S rRNA gene was performed using universal bacterial primers 27F (45) and 338R
199 (46), synthesised by IDTdna (Integrated DNA Technologies, BVBA, Leuven, Belgium).

The fusion forward primer (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG**XXXXXXXX**AGAGTTTGATGMTGGC TCAG-3') contained the 454 Life Sciences "Lib-L Primer A", a 4 base "key" sequence (TCAG), a unique eight-base barcode "MID" sequence for each sample (**XXXXXXXX**), and bacterial primer 27F. The reverse fusion primer (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGTGCTGCCTCCCGTAGGAGT-3') contained the 454 Life Sciences "Lib-L Primer B", a 4 base "key" sequence (TCAG), and bacterial primer 338R. The PCR amplification was performed in 50 µL volume reactions using 0.5 µL (2.5 units) Fast Start High Fidelity DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany), 1.8 mM $MgCl_2$, 200 µM of each dNTP, 0.4 µM of each forward and reverse fusion primers, and 2 µL of DNA template. The PCR conditions included an initial denaturing step at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 45 sec, and a final elongation step at 72 °C for 5 min.

PCR products were loaded in an agarose gel, and following gel electrophoresis, bands of the correct fragment size (approximately 410 bp) were excised, cleaned up using a QIAquick gel extraction kit (QIAGEN, GmBH, Hilden, Germany), and eluted in 30 µL of DNAse free $H_2O$. The cleaned up PCR products from this study (22 samples in total) were quantified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA), and pooled so that the mixture contained equal amounts of DNA from each sample. The emulsion emPCR and the pyrosequencing run were performed at the University of Manchester sequencing facility, using a 454 Life Sciences GS Junior system (Roche).

The 454 pyrosequencing reads were analysed using Qiime 1.6.0 release (47). Low quality reads (mean quality score less than 25) and short sequences (less than 300 bp) were discarded, and



Applied and Environmental Microbiology

both forward and reverse primers were removed from further analysis. De-noising and chimera removal was performed during operational taxonomic unit (OTU) picking (at 97% sequence similarity) with 'usearch' (48) in Qiime, and a representative sequence for each OTU was identified. Taxonomic classification of all reads was performed in Qiime using the Ribosomal Database Project (RDP) at 80% confidence threshold (48), while the closest GenBank match for the OTUs that contained the highest number of reads (the representative sequence for each OTU was used) was identified by Blastn nucleotide search.

## Results and Discussion

**Biogeochemistry of irradiated microcosms containing added electron donor.** To assess the impact of chronic gamma irradiation on the biogeochemical processes in the sediment, a series of microcosms were prepared with and without added electron donor and irradiated for 56 days at 0.5 and 30 Gy $h^{-1}$. In control and irradiated microcosms spiked with lactate and acetate (final added concentrations of 7 mM each), electron acceptor usage progressed in the order nitrate > Fe(III) > sulfate during the irradiation period (Figure 1). Treatment with 30 Gy $h^{-1}$ gamma radiation did not appear to affect the reduction of nitrate, which was removed completely from porewaters after 28 days in both treated and control microcosms. However, 0.5 N HCl extractable Fe(II) concentrations in microcosms after treatment with 30 Gy $h^{-1}$ for 56 days were ~0.5 mM compared to ~2mM in non-irradiated controls. This limited Fe(III)-reduction was likely due to decreased viability of Fe(III)-reducing microorganisms arising from a total absorbed dose of 38.6 kGy. After completion of the irradiation, levels of 0.5 N HCl extractable Fe(II) increased gradually over 176 days to levels comparable to those in non-irradiated microcosms, suggesting that significant Fe(III)-reduction was still possible, albeit at a slower

rate, even after the maximum radiation dose was applied. This was likely a result of the gradual regrowth of surviving Fe(III)-reducers after completion of the irradiation. Sulfate concentrations in the porewaters of the non-irradiated microcosms decreased after 29 days. This is consistent with the onset of microbial sulfate reduction, with complete sulfate removal from solution observed after 280 days. Conversely, in systems irradiated with 30 Gy $h^{-1}$, sulfate concentrations doubled during the 56 day irradiation period from ~0.35 mM to ~0.7 mM, with most of this increase occurring during the first 28 days of irradiation. Marschner (1993) also reported sulfate increases of 17% after 30 kGy gamma irradiations due to release from dead microbial biomass. On the other hand, Ishii et al. (2011) suggested that for a rice paddy sediment irradiated with 1 Gy $day^{-1}$, increases in sulfate may be a result of radiation induced activation of mineralization processes. Sulfate may also be generated via the oxidation of sulphide bearing minerals by radiolytically produced oxidants, such as hydrogen peroxide (50, 51).

Sulfate began to be removed from solution after day 57, once irradiation had ceased, followed by complete removal after 280 days. These results indicate that microbial sulfate reduction occurred despite treatment with a total dose of 38.6 kGy. As for Fe(III)-reduction, this suggests that limited numbers of sulfate reducing bacteria survived irradiation, followed by their gradual regrowth after the irradiation was terminated.

Treatment with 0.5 Gy $h^{-1}$ gamma radiation did not have a significant impact on the amount of nitrate, Fe(III) or sulfate reduction noted (Figure 1). Indeed, the extent of nitrate and Fe(III) reduction after irradiation of the microcosms for 58 days was the same as in the non-irradiated controls. These data suggest that irradiation at this lower dose rate did not have a significant effect on the microbial communities which control electron acceptor turnover.



Applied and Environmental Microbiology

268 Lactate concentrations in all spiked systems decreased throughout the irradiation period,
269 resulting in the complete removal from solution after 56 days in both the 0.5 and 30 Gy $h^{-1}$
270 treated systems and in non-irradiated control microcosms (Figure 2). This suggests that lactate
271 was likely used as a carbon source or as an electron donor for the electron accepting processes
272 described earlier. Lactate removal was not as rapid in 30 Gy $h^{-1}$ treated systems and this may be
273 related to a reduction in microbial activity/viability associated with radiation toxicity at this
274 higher dose rate.

275 Acetate concentrations did not change significantly in any of the microcosms during the
276 irradiation period, however, acetate was completely removed from solution in control systems
277 and in the 0.5 Gy $h^{-1}$ treated microcosms after a 48 day recovery period. This is consistent with
278 the use of acetate as an electron donor, as observed in previous studies with this sediment type
279 (39), albeit after more thermodynamically favourable processes had consumed other electron
280 donors, such as lactate. However, in microcosms treated with 30 Gy $h^{-1}$, acetate concentrations
281 increased significantly to ~11 mM after 147 days, followed by its complete removal from
282 solution after 280 days. The increase in acetate levels is likely a result of fermentation reactions
283 catalysed by more radiation resistant members of the community (see below). Indeed, the
284 delayed removal from solution may suggest an initial decrease in viability of members of the
285 microbial community capable of respiring acetate as a result of irradiation, although these
286 processes appear able to recover after a period of removal from the radiation source.

287 Propionate appeared in both control systems and systems irradiated with 0.5 Gy $h^{-1}$ and increased
288 throughout the irradiation period to a concentration of ~3.5 mM. After 56 days, when the
289 experimental microcosms were removed from the radiation source, propionate concentrations
290 decreased throughout the recovery period at approximately the same rate as in the non-irradiated



Applied and Environmental Microbiology

291 microcosm controls, resulting in complete removal from solution after 205 days. This generation
292 and removal of propionate is consistent with its production via fermentation of lactate (52) and
293 subsequent use as an electron donor. Propionate was not detected in the 30 Gy $h^{-1}$ treated
294 systems at any sampling point, suggesting that other metabolic pathways were more dominant
295 for this treatment.

296 A slight increase in formate concentrations to ~50 µM was observed in the non-irradiated
297 microcosms and 0.5 Gy $h^{-1}$ treatments during the recovery period after 147 days. In the 30 Gy
298 $h^{-1}$ treatments, on the other hand, formate appeared during the latter half of irradiation to a
299 concentration of ~120 µM. In addition to formate, a large increase in malate was also observed
300 in the 30 Gy $h^{-1}$ treated microcosms only, with a significant increase during the recovery period
301 to ~12 mM after 105 days. Whilst DOC has previously been observed to increase as a result of
302 sediment gamma irradiation (29), the significant production of malate during the recovery period
303 and formate during the latter half of the irradiation period, suggests they are likely fermentation
304 products. Furthermore, their subsequent removal from solution is consistent with their use as
305 electron donors.

306 **Microbial community changes in irradiated microcosms containing added electron donor.**
307 Analysis of the bacterial community in the oxic starting sediment revealed a relatively diverse
308 community with 16 phyla detected through pyrosequencing of 16S rRNA gene amplicons.
309 Communities were dominated by species representing the Acidobacteria (47%) and
310 Proteobacteria (32%), consistent with previous studies conducted on Sellafield-type sediments
311 (38, 39). Of the most dominant individual species, an uncharacterized Acidobacterium and a
312 bacterium of the Bradyrhizobiaceae family (Proteobacteria) represented 5% and 4% of the
313 complex microbial community, respectively.

After 147 days, the microbial community of non-irradiated sediment microcosms containing added lactate and acetate showed a decrease in the relative contributions of Acidobacteria (21%) and Proteobacteria (17%) (Figure 3). However, the most marked shift was an increase in organisms affiliated with the Bacteroidetes (29% of the community) and Firmicutes (22%; of which, 97% were affiliated with Clostridia). The Bacteroidetes included uncultured *Prolixibacter* spp. (7% of the total microbial community), two uncultured Bacteroidetes bacteria (4% and 3%) and an organism affiliated with *Paludibacter propionicigenes* (2%). The *Prolixibacter* genus comprises facultative anaerobes capable of sugar fermentation (53), with *P. propionicigenes* an anaerobic propionate producing strain which can utilize a range of sugars to produce acetate and propionate as major fermentation products (54). In addition, organisms affiliated with the Clostridial group (Firmicutes) catalyse a mixed acid fermentation under anoxic conditions (55). Thus, the relative increase in Clostridia and *Paludibacter* species is likely related to the significant production of propionate observed during the first 56 days (Figure 2) (52). Furthermore, Clostridia, such as *Pelotomaculum* spp. (10% of the community), includes species capable of oxidizing propionate (56). The increase in such species may be related to the decrease in propionate observed after 56 days (Figure 2).

Species of the known Fe(III)-reducing genus *Geobacter* showed a slight increase to represent 1% of the community in control systems. This correlates with the increase in Fe(III)-reduction observed during the first 56 days (Figure 1). Although Fe(III)-reduction is clearly a significant electron-accepting process in these sediments, *Geobacter* spp. or other known Fe(III)-reducing bacteria were not dominant components of this community, probably due to the dominance of fermentative processes as a result of the addition of significant organic carbon concentrations.



Applied and Environmental Microbiology

Similar community shifts were also observed in 0.5 Gy $h^{-1}$ treatments. An organism affiliated with the Bradyrhizobiaceae was also present in the irradiated microcosm community at a proportion similar to that in the non-irradiated microcosm community (4%). In contrast to control systems, bacteria of the Firmicutes phylum were not as well represented in the microcosm community irradiated with 0.5 Gy $h^{-1}$ (13%). However, an organism closely related to a member of the genus *Pelotomaculum* (97% match) was again the main representative of this class (3%), and this may be related to the similar levels of propionate observed in these two treatments. The Proteobacteria appeared slightly enriched in this treatment (24%) compared to the control sample (17%), with *Geobacter* spp. comprising 8% of this group. Thus, significant Fe(III)-reduction by this genus was likely more important in these systems. A Betaproteobacterium closely related to species of the genus *Janthinobacterium* also represented a significant proportion of the community at 4%. Species of this genus were well represented in a previous study using similar sediments containing added nitrate, and it was suggested that Betaproteobacteria such as this may be involved in the reduction of nitrate (57). Thus, the appearance of this genus in sediments treated with 0.5 Gy $h^{-1}$ may be related to nitrate reduction observed early on in the irradiation period.

In the microcosm irradiated at 30 Gy $h^{-1}$, a marked loss in diversity occurred after 147 days, with a strong shift toward species of the Firmicutes phylum (91%) (Figure 3). Two close relatives to known Clostridial species were the main components of this phylum. The first, an uncultured Clostridiacea bacterium, represented 83% of the total community. This species is most closely related to an organism isolated from a sulfate-reducing enrichment of sediments from an acid mine lake (95% match) (58). The second, an organism most closely related to a novel *Clostridium bowmanii* species (98% match) originally isolated from a microbial mat in the



Applied and Environmental Microbiology

359 McMurdo Dry Valley region of Antarctica (59), represented 8% of the total community.
360 Members of the Clostridial family catalyse a mixed acid fermentation, with *C. bowmanii* capable
361 of generating butyrate; acetate; formate; ethanol and lactate (59). As such, it is possible that
362 these species may have been involved in fermentation processes, including acetate and formate
363 production, observed throughout the incubation period (Figure 2). In addition, most of the
364 species within this family are able to form endospores, which may allow cells to survive a range
365 of environmental stresses (60). As such, it is likely that both these species represent
366 radioresistant members of the sediment community. Thus, in environments with significant
367 radiation fluxes, in conjunction with available fermentable substrates such as lactate, species
368 from the Clostridiaceae family could predominate.

369 Despite Clostridiaceae species dominating the bacterial community after 147 days in the 30 Gy
370 $h^{-1}$ treatment, subsequent incubation to 280 days in the absence of radiation led to a significant
371 reduction in the contribution of these organisms to the total microbial community. Firmicutes
372 comprised ~13% of the community, with *Bacillus* spp. the main component (~10% of the total
373 community), whereas relatives of Clostridiaceae only comprised ~1% of the total community. Of
374 the remaining Clostridia, species of the *Desulfosporosinus* genus comprised 1.5% of the total
375 microbial community. This genus contains known spore formers capable of reducing Fe(III) and
376 sulfate (61) and thus, the increase in relative abundance of species of this genus may indicate
377 radiation tolerance (via endospore formation), and subsequent contribution to significant Fe(III)
378 and sulfate reduction observed throughout the incubation period to 280 days.

379 In addition, there was a significant increase in members of the Bacteroidetes phylum (~71% of
380 the total community), of which members of the order Bacteroidales comprised ~57% of the total
381 community. Deeper phylogenetic classification of these organisms was not possible in this

382 analysis; however, the emergence of members of this class after the 30 Gy $h^{-1}$ treatment is
383 somewhat surprising as this taxon comprises non-spore-forming, Gram-negative anaerobes. This
384 result, therefore, suggests that even after exposure to high dose rates of gamma radiation, some
385 non-spore-forming microbial species may be able to recover to become dominant members of a
386 sediment microbial community.

387 Although Fe(II) concentrations in the 30 Gy $h^{-1}$ treatment returned to the same level as noted in
388 non-irradiated controls during the period prior to phylogenetic analysis after 280 days, relatives
389 of known Fe(III)-reducing species were not well represented in the microbial community. A
390 close relative of *Geothrix fermentans* (Acidobacteria; 97% match) comprised 1.9% of the total
391 microbial community, whilst *Geobacter* spp. contributed 0.8% to the community. Despite this,
392 these results highlight the potential for electron accepting processes to recover in sediments
393 subject to significant radiation fluxes with available organic carbon substrates present.

394 **Biogeochemistry in microcosms containing no added electron donor.** In addition to systems
395 containing added carbon, the impact of gamma radiation on sediment biogeochemistry and
396 microbial communities was also assessed with microcosms containing no added electron donor
397 (lactate or acetate). Radiation had no significant effect on the generation or reduction of sulfate
398 throughout the irradiation period. However, after irradiation, significant Fe(III)-reduction was
399 observed in microcosms treated with 0.5 Gy $h^{-1}$ gamma radiation, whereas Fe(III)-reduction in
400 control and 30 Gy $h^{-1}$ treated microcosms was not observed until day 105. Indeed, Fe(III)-
401 reduction in the 0.5 Gy $h^{-1}$ treated systems continued throughout the incubation period at an
402 enhanced rate. No increase in 0.5 N HCl extractable Fe(II) was observed in control or irradiated
403 microcosms during the 56 day irradiation period as the absence of added electron donor likely
404 precluded microbial Fe(III)-reduction during this initial period.

In contrast to systems containing added lactate and acetate, nitrate removal in the unamended microcosm controls and in those irradiated with 30 Gy $h^{-1}$ was slower. However, nitrate concentrations in the 30 Gy $h^{-1}$ treatment were slightly lower (~70 µM) than in non-irradiated microcosms (~120 µM) (Figure 4). General removal of nitrate in both systems is likely related to the activity of denitrifying bacteria. However, the increased removal in the 30 Gy $h^{-1}$ treated microcosms is consistent with the abiotic removal of nitrate in previous studies of gamma sterilisation of sediments (36). The reasons for this are unclear; however, radiolysis studies have shown that abiotic decomposition of nitrate to nitrite is possible (62). It is not possible to say whether this process also occurred in the 0.5 Gy $h^{-1}$ treatments because nitrate concentrations were not determined during the irradiation of these microcosms. However, these results suggest that radiolysis of nitrate may promote the removal of nitrate in irradiated sediments. In turn, this may have resulted in the early onset of Fe(III)-reduction followed by enhanced sulfate reduction (after 150 days) observed in the 0.5 Gy $h^{-1}$ treated microcosms, due to a decreased competition for the alternative electron acceptor. It is not clear why this enhanced Fe(III)-reduction was not observed in the 30 Gy $h^{-1}$ treated microcosms; however, this may be precluded by increased radiation toxicity associated with a higher dose.

Formate was generated in all treated and untreated microcosms (up to ~50 µM) throughout the recovery period and was likely a product of fermentation (Figure 5). Acetate, on the other hand, was not observed in control microcosms or in the microcosms irradiated at 0.5 Gy $h^{-1}$; however, ~0.2 mM acetate was produced in the 30 Gy $h^{-1}$ treated microcosms during the latter half of the irradiation period. Acetate generation in this irradiated system continued throughout the recovery period until 105 days, but by the end of the incubation period, acetate had largely been removed from solution. The production of acetate (during the latter part of irradiation only) and its



Applied and Environmental Microbiology

428 subsequent removal suggests its production by microbial fermentation, followed by its oxidation
429 as an electron donor. It is possible that these processes occurred in the non-irradiated systems
430 and in the 0.5 Gy $h^{-1}$ treated systems, however, acetate may have been metabolised as quickly as
431 it was formed. Thus, the detection of acetate in the 30 Gy $h^{-1}$ treated systems may be a result of
432 radiation toxicity in acetate-oxidizing species.

433 It is unclear from these data whether the enhanced reduction of Fe(III) in the 0.5 Gy $h^{-1}$ treated
434 microcosms was related to an increase in the availability of organic electron donors, increases in
435 the bioavailability of Fe(III), or a decrease in electron acceptor competition arising from
436 enhanced nitrate removal.

437 **Microbial community changes in the absence of added electron donor.** To assess potential
438 changes to the microbial community which may have led to the enhanced Fe(III)-reduction
439 observed in 0.5 Gy $h^{-1}$ treated microcosms, bacterial phylogenetic diversity was assessed in
440 samples taken immediately after irradiation (T = 57 days; Figure 6). Both the non-irradiated and
441 0.5 Gy $h^{-1}$ treated microcosms showed slight enrichment of Proteobacterial species, including a
442 representative of the Bradyrhizobiaceae (Alphaproteobacteria) (6% in controls and 8% in 0.5 Gy
443 $h^{-1}$ treatments). Controls were also enriched in a relative of a known *Janthinobacterium* sp.
444 (Betaproteobacterium) (5%), but this was not observed in the treated systems. However, the 0.5
445 Gy $h^{-1}$ treated microcosms did show a slight increase in an organism affiliated with *Rhodoferax*
446 spp. (99% sequence similarity) (2% in treated versus <1% in controls). The closest known
447 relative was originally identified in Arctic glacier melt water and has 98% sequence similarity to
448 a *Rhodoferax ferrireducens* strain which exhibits dissimilatory Fe(III)-reduction (63, 64). Whilst
449 the increase in abundance of relatives of this organism may be consistent with the enhanced
450 levels of Fe(III)-reduction which was observed in the 0.5 Gy $h^{-1}$ treated system after the removal

451 of experiments from the radiation source, this organism was not detected in the microbial
452 community analysed at 147 days, after significant Fe(III) reduction had been observed in these
453 microcosms.

454 As with electron donor spiked systems, community analysis after 147 days of incubation of the
455 non-irradiated system containing no added carbon revealed a relative increase in the
456 Bacteroidetes (9%) and Firmicutes (3%) phyla (Figure 6). As with electron donor spiked
457 systems, the Bacteroidetes phylum was strongly represented by *Prolixibacter* related species
458 (6%). A significant increase in abundance of a relative of *Geothrix fermentans* (Acidobacteria)
459 was also observed (from <1% in the T = 57 sample to 5% in the T = 147 sample) and, as this is a
460 known Fe(III)-reducing species (65), this increase is likely related to the Fe(III)-reduction
461 observed in this system after 105 days.

462 Community analysis of the 0.5 Gy $h^{-1}$ treatment after 147 days displayed a further relative
463 increase in representatives of the Bacteroidetes (19%) and Firmicutes (7%) phyla. Unclassified
464 species of the Bacteroidales order showed a significant increase, representing 17% of the total
465 microbial community, with respect to the control sample (6%). Uncultured *Prolixibacter* spp.
466 also showed an increase with respect to control samples, and were well represented in this
467 treatment at 10% of the total community. The increase in representatives of the Firmicutes
468 phylum mainly arose from a general increase in Clostridial species, which may indicate an
469 increase in fermentation activity or is perhaps related to spore formation and enhanced survival.

470 In addition, an organism most closely related to *Geothrix fermentans* (Acidobacteria) (~98%
471 sequence similarity) showed a significant increase with respect to the control sample,
472 representing 22% of the total microbial community. *Geobacter* spp. were increasingly
473 represented with respect to controls, with an organism most closely related to *G. chapellei*

474 comprising 3% of the community, compared to the most populous in control samples: a *G.*
475 *bremensis* relative (0.2% of the total community). This relative increase in *Geothrix* and
476 *Geobacter* spp. after 147 days, rather than the emergence of the *Rhodoferax* relative described
477 earlier (after 57 days), is more consistent with the enhanced level of Fe(III)-reduction in the 0.5
478 Gy $h^{-1}$ treated microcosms.

479 In response to the 30 Gy $h^{-1}$ treatment, further relative increases were observed in the
480 Bacteroidetes and Firmicutes phyla (Figure 6). Unclassified species from the order Bacteroidales
481 represented 37% of the total microbial community. This comprised two dominant species, the
482 first (12% of the community) was most closely related to an uncultured bacterium isolated from
483 moss pillars at an Antarctic lake (66) and the second, an uncultured *Prolixibacter* sp. (11%).
484 *Paludibacter* spp., also of the order Bacteroidales, represented 5% of the total community and
485 species of the family Chitinophagaceae (Bacteroidetes phylum) comprised 7% of the total
486 community. Two uncultured Sphingobacteria (Bacteroidetes phylum) represented 14% of the
487 community. Interestingly, these observations are similar to those from microcosms amended
488 with acetate and lactate and irradiated at 30 Gy $h^{-1}$, in which members of the Bacteroidales
489 represented ~57% of the total microbial community after 280 days. These results suggest that
490 members of the Bacteroidetes phylum may exhibit high levels of radiation resistance, and
491 potentially represent a group of respiratory generalists capable of dominating a community after
492 significant radiation stress.

493 Of the key Fe(III)-reducing species in the microcosms irradiated at 30 Gy $h^{-1}$, 18% of the total
494 community was affiliated with known *Geobacter* species. However, unlike in the microcosms
495 irradiated at 0.5 Gy $h^{-1}$, *Geothrix* species were not well represented, comprising <0.1% of the
496 total microbial community. In addition, a close relative of the *Herbaspirillum frisingense*

(Betaproteobacteria) comprised 5% of the total community (98% sequence similarity). This species is capable of nitrate reduction and nitrogen fixation and can oxidise a broad range of sugars and alcohols (67). As in the microcosms irradiated at 0.5 Gy $h^{-1}$, the increase in Firmicutes mainly arose from a general increase in Clostridial species.

These results indicate that despite sediments receiving a total absorbed dose of nearly 40 kGy, Fe(III)-reduction was still possible in sediments without added electron donor. Furthermore, irradiation of these sediments resulted in significant increases in abundance of Fe(III)-reducing species compared to non-irradiated systems. This suggests that, although Fe(III)-reduction was not enhanced in the 30 Gy $h^{-1}$ treated systems, these sediments may be poised for Fe(III)-reduction.

**Fe(III)-reduction in irradiated microcosms inoculated with *Geobacter sulfurreducens.*** To assess the potential for enhanced Fe(III)-reduction in the microcosms irradiated at 30 Gy $h^{-1}$, irradiated and control microcosms were inoculated with cultures of *G. sulfurreducens* and 0.5 N HCl extractable Fe(II) was monitored (Figure 7). Both 0.5 and 30 Gy $h^{-1}$ treated microcosms showed enhanced Fe(III)-reduction with respect to the control systems, 21 days after inoculation. Fe(III)-reduction was observed in inoculated non-irradiated microcosms after 35 days. Fe(II) concentrations approached those in the irradiated microcosms after 92 days, albeit at a slower rate. These results suggest that, as in the microcosms irradiated at 0.5 Gy $h^{-1}$, a potential for enhanced Fe(III)-reduction existed in the microcosms irradiated at 30 Gy $h^{-1}$ (Figure 4), but reduced viability of Fe(III)-reducing species at this radiation dose precluded it.

Radiation has been previously shown to release significant quantities of DOC into solution in a range of soils exposed to 25 kGy to 60 kGy (29, 68, 69). This could potentially increase the availability of carbon for use as a carbon source or electron donor. Irradiation at 30 Gy $h^{-1}$ did



Applied and Environmental Microbiology

520 lead to increased concentrations of organic acids representative of the bioavailable organic
521 fraction in sediments (Table 2). However, such µmolar increases were probably not sufficient to
522 account for the observed Fe(III)-reduction. Moreover, no significant increases in organic acids
523 were observed in microcosms irradiated at 0.5 Gy $h^{-1}$, nor were there significant radiation
524 induced increases during the irradiation period of the non-inoculated microcosms.

525 Previous experiments indicated that gamma radiation may lead to an increase in the availability
526 of Fe(III)-oxides for microbial Fe(III)-reduction (35). It is possible that the enhanced Fe(III)-
527 reduction observed here may be related to this phenomenon. Whilst the previous study observed
528 this effect after acute irradiation to 1 MGy, our results may suggest that a similar, more subtle
529 process may also occur at lower doses.

530 On the other hand, the enhanced Fe(III)-reduction may also be related to the removal of nitrate
531 by radiolysis, as in the irradiation of sediments containing no added *G. sulfurreducens* cells or
532 electron donors. Nitrate concentrations in irradiated microcosms (0.13 mM in 30 Gy $h^{-1}$
533 treatments and ~0.3 mM in 0.5 Gy $h^{-1}$ treatments) were significantly lower immediately after
534 irradiation than in non-irradiated systems (~0.5 mM). Again, these results are consistent with
535 radiation enhanced removal of nitrate and the early onset of Fe(III)-reduction, as discussed
536 previously.

537 **Implications to the geodisposal of radioactive waste.** This study highlights microbial activities
538 under dose rates representative of gamma radiation emitted from radioactive waste canister
539 surfaces in the near field of a geological disposal facility. We then assessed microbial activities
540 under a simulated recovery period that would exist after significant radioactive decay had
541 occurred.

542 Previous studies suggested that microbial activity will be suppressed in these environments. For
543 instance, studies of survival of microorganisms from clay buffer material have suggested that
544 typically only 10% of the population survives after doses of ~1.6 kGy (9) and that dose rate may
545 not have a significant impact on the viability of microbial populations (19). On the other hand,
546 indigenous members of an endolithic microbial community from a proposed high-level
547 radioactive waste repository may have been able to survive in a non-culturable state after
548 irradiation (9.34 kGy at 1.63 Gy $min^{-1}$), to be rejuvenated when conditions become favourable
549 (22, 23).

550 In contrast, the results presented here indicate that a sediment community can survive long-term
551 gamma irradiation and components of these communities can remain active and catalyse
552 biogeochemical processes, including Fe(III)-reduction. We have shown this to be the case for
553 doses of up to ~38 kGy using a lower, environmentally relevant dose rate of 30 Gy $h^{-1}$. Indeed,
554 dose rate had a strong influence on the community structure in systems with and without added
555 carbon. This demonstrates the importance of acquiring low dose rate data, particularly as lower
556 dose rates may allow species to respond via up-regulating repair mechanisms (24) or adapting
557 over the geological timescales involved.

558 Radiation led to significant changes in the microbial communities, with fermentative bacteria,
559 such as Clostridia, dominant in systems with added carbon. Such changes may be important in
560 environments where there is an excess of carbon substrates, such as in cellulosic wastes (3).
561 Despite this loss of diversity, these results suggest that Fe(III) reduction can still be an important
562 electron accepting process in such sediments. Furthermore, in environments with lower electron
563 donor concentrations, an Fe(III)-reducing community may be selected by radiation. This may
564 occur both directly, by making Fe(III) more bioavailable through radiation-induced changes to

the mineralogy; or indirectly, by radiation induced removal of other electron acceptors, such as nitrate, which may lead to the early onset of microbial Fe(III) reduction. Regardless, a relative increase in Fe(III)-reducing species was also observed in irradiated systems which did not display enhanced Fe(III)-reduction. These results have positive implications for the geodisposal of radioactive waste, whereby the stimulation of an Fe(III)-reducing community by radiation may enhance the reduction and subsequent precipitation of radionuclides by direct enzymatic or indirect (e.g. biogenic Fe(II)-mediated) mechanisms. Furthermore, the oxidation of molecular hydrogen by the radiolysis of water coupled to the enhanced reduction of alternative electron acceptors by low dose gamma radiation could provide the basis of a novel ecosystem in the deep biosphere. Future studies will focus on the radiolysis of recalcitrant organic matter and the potential for enhanced carbon mineralization by subsurface microbial communities. Further work would be required to assess how these altered communities may affect the mobility of key radionuclides.

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**Figure Legends and Tables**

**Figure 1.** Concentrations of nitrate, 0.5 N HCl extractable Fe(II) and sulfate in microcosms containing added lactate and acetate (final added concentrations of 7 mM each). The grey shaded area indicates the duration of the irradiation. Error bars represent the standard error of the mean of triplicate experiments and where not visible, error bars are within the symbol size.

**Figure 2.** Concentrations of lactate, acetate, propionate, formate and malate in microcosms containing added lactate and acetate (final added concentrations of 7 mM each). The grey shaded area indicates the duration of the irradiation. Error bars represent the standard error of the mean of triplicate experiments and where not visible, error bars are within the symbol size.

**Figure 3.** Bacterial phylogenetic diversity in microcosms containing added lactate and acetate (final added concentrations of 7 mM each). T = time (days).

**Figure 4.** Concentrations of nitrate, 0.5 N HCl extractable Fe(II) and sulfate in microcosms containing no added electron donor. The grey shaded area indicates the duration of the irradiation. Error bars represent the standard error of the mean of triplicate experiments and where not visible, error bars are within the symbol size.

814 **Figure 5.** Concentrations of lactate, acetate and formate in microcosms containing no added
815 electron donor. The grey shaded area indicates the duration of the irradiation. Error bars
816 represent the standard error of the mean of triplicate experiments and where not visible, error
817 bars are within the symbol size.
818
819 **Figure 6.** Bacterial phylogenetic diversity in microcosms with no added electron donor.
820 Microcosms were removed from the irradiation cell at T = 56. T = time (days).
821
822 **Figure 7.** 0.5 N HCl extractable Fe(II) concentrations in control and irradiated microcosms
823 inoculated with *G. sulfurreducens*. Microcosms were removed from the irradiation cell and
824 inoculated at T = 0. Error bars represent the standard error of the mean of triplicate experiments
825 and where not visible, error bars are within the symbol size.
826
827

828 **Table 1.** Initial microcosm compositions and treatments. Lactate and acetate were added, where
829 required, to give the final concentrations shown below.

| Experimental system | Dose rate (Gy $h^{-1}$) | Total absorbed dose (kGy) | Added lactate (mM) | Added acetate (mM) |
|---|---|---|---|---|
| Sediment + electron donor | Non-irradiated | 0 | 7 | 7 |
| | 0.5 ± 10% | 0.6 ± 10% | 7 | 7 |
| | 30 ± 10% | 38.6 ± 10% | 7 | 7 |
| Sediment | Non-irradiated | 0 | 0 | 0 |
| | 0.5 ± 10% | 0.6 ± 10% | 0 | 0 |
| | 30 ± 10% | 38.6 ± 10% | 0 | 0 |
| Sediment + *G. sulfurreducens* | Non-irradiated | 0 | 0 | 0 |
| | 0.5 ± 10% | 0.6 ± 10% | 0 | 0 |
| | 30 ± 10% | 38.6 ± 10% | 0 | 0 |

830

831 **Table 2.** Concentrations of bioavailable Fe, inorganic anions and fatty acids in sediment microcosms immediately after irradiation and

832 after addition of a fresh *G. sulfurreducens* inoculum. Errors indicate the standard error of the mean of triplicate measurements.

| Treatment | Bioavailable Fe mM | $NO_3^-$ mM | $NO_2^-$ mM | $SO_4^{2-}$ mM | Lactate µM | Acetate µM | Propionate µM | Butyrate µM | Formate µM | Fumarate[a] µM | Oxalate[a] µM |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Non-irradiated | 0.74 ± 0.02 | 0.53 ± 0.02 | n.d. | 0.40 ± 0.01 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 0.5 Gy $h^{-1}$ | 0.70 ± 0.02 | 0.29 ± 0.03 | 0.01 ± 0.02 | 0.40 ± 0.00 | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| 30 Gy $h^{-1}$ | 0.81 ± 0.03 | 0.13 ± 0.01 | 0.16 ± 0.01 | 0.42 ± 0.01 | 3.5 ± 3.0 | 57.2 ± 70.0 | 3.9 ± 0.4 | 1.1 ± 0.9 | 32.7 ± 18.1 | 45.5 ± 13.7 | 59.5 ± 17.9 |

n.d. = not detected.
[a] Fumarate and oxalate both have identical retention times on the chromatography system used. Concentrations have been determined for each based on their respective molecular mass.

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