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Effects of Trifluoroacetate, an Atmospheric Breakdown Product of Hydrofluorocarbon Refrigerants, on Acetate Metabolism by Freshwater Benthic Microbial Communities

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Hydrofluorocarbon (HFC) and hydrochlorofluorocarbon (HCFC) compounds, in use as replacements for chlorofluorocarbon (CFC) refrigerants, undergo oxidative, hydrolytic, and photolytic reactions in the atmosphere that result in the formation of trifluoroacetate (TFA) (Wallington, et al. 1994). TFA is highly water soluble and is expected to become widely dispersed in the biosphere. Initial predictions were that concentrations of refrigerant-derived TFA in precipitation might be 17.5 nM (2 µg/L). Recent computer models indicate that by 2010 a global average concentration of 1.40 nM (0.16 µg/L) is more likely and that in specialized habitats where evapotranspiration is high and other losses may be low (e.g., vernal pools), concentrations >877 nM (100 µg/L) might be attained (Tromp *et al.* 1995). In 1995, concentrations of TFA measured in precipitation were reported to average 100 ng/L (0.87 nM); levels in lake, lotic, and coastal marine locations in Europe had concentrations in the range of 0.52 - 2.28 nM (0.06-0.26 µg/L); and concentrations in the Dead Sea, Israel, already were 56.1 nM (6.4 µg/L) (Frank *et al.* 1996). These concentrations, if verified, may reflect contributions from other sources.

The expected widespread distribution of TFA has generated concern regarding potential environmental effects. Mammalian toxicity tests indicate that TFA is not acutely toxic (Rusch 1994), in contrast to monofluoroacetate (Marais 1944, Peters 1972). TFA toxicity to duckweed (*Lemna gibba*) and to four of five algal species was minimal; the exception was *Selenastrum capricornutum* for which the EC_{50} was 42 µM (4.8 mg/L), a concentration considerably greater than those expected in the environment (Thompson 1994). While monofluoroacetate utilizing bacteria have been isolated (Meyer *et al.* 1990), there are no reports of aerobic bacteria capable of using TFA as a sole carbon and energy source. However, because TFA possesses a chemical structure similar to acetate, which is an important metabolic substrate for both aerobic and anaerobic microbes, we performed experiments to assess whether TFA would be toxic to microbes utilizing acetate or affect the metabolism of acetate. We focused our studies on freshwater microbial communities in the sediments of shallow streams, habitats where elevated TFA concentrations might be expected.

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MATERIALS AND METHODS

Sediment samples for experiments were collected from White Clay Creek (WCC, Chester Co., PA) and from stream mesocosms allowing continuous experimental exposure of the stream biota to TFA. Each mesocosm (2.23 m long x 0.20 m wide x 0.13 m deep) contained stones, gravel and sediment from WCC, simulating a range of benthic habitats, and held 35 L of stream water, which was recirculated. WCC water (1 L/min) was added continuously to replace the entire water volume every 35 min. Water depth averaged 1.0 cm and water velocities ranged between 9.6 and 17.2 cm/sec. The mesocosms were immersed in water jackets with once-through passage of WCC water to maintain near-ambient stream water temperatures and were located in a greenhouse. Natural communities of algae, floating and rooted plants and invertebrates developed in each system. TFA was metered continuously into two mesocosms from a concentrated stock solution held in a refrigerator next to the mesocosms; two other mesocosms were used as controls.

TFA and acetate as the sodium salts were used in all work. Samples for analysis were collected in muffled (500°C) glassware and stored frozen or refrigerated. TFA and acetate concentrations were determined by Du Pont Environmental Remediation Services (Glasgow, DE) using ion exclusion chromatography (HPICE-ASI column, Dionex 2000i) with a conductivity detector, 1 mM HCl mobile phase, and an anion micromembrane suppresser with 5 mM tetrabutylammonium hydroxide as a regenerant. The detection limit was ~175 nM (20 µg/L). TFA concentrations averaged 272 ± 78 nM (31.0 ± 8.9 µg/L, $\overline{x}$ ± SD, n=22 samples between September l8, 1992 and June 4, 1993) in one experimental mesocosm and 282 ± 64 nM (32.2 ± 7.3 µg/L, $\overline{x}$ ± SD, n=5 spot check samples) in the other. TFA was never detected in samples from the Control mesocosms.

[$_{14}$C]acetate metabolism was measured in the presence of either TFA or non-radioactive acetate added over a wide concentration range (between 0.02 and 200 µg/L in tenfold increments; 0.18 - 1754 nM for TFA; 0.34 - 3400 nM for acetate) and in unamended samples. In one experiment amendments ranged from 39 to 3,859 µM and from 102 to 6,270 µM for TFA and acetate, respectively. Sediments were stirred and aliquots were transferred to sterilized vials containing autoclaved groundwater. Non-radioactive compound was added followed by 2-[$_{14}$C]acetate ([^{14}C]H_3COOH, sp. act. 55 mCi/mmole, New England Nuclear, Boston, MA) to provide a final concentration of 0.1 µCi in 10 ml (254 nM, 15 µg/L). From 3 to 5 replicates and either 1 or 2 controls (killed by adding 0.1 ml formalin/vial 10 - 15 min. before the [^{14}C]acetate) were used at each concentration. Each vial was closed with a serum stopper with an attached CO_2 trap and samples were incubated for 2 h at a temperature in the environmental range (12 - 20°C). Incorporation was stopped by adding 0.2 ml 2 N H_2SO_4 to the vial. Phenethylamine (PEA, 0.2 ml) was added to a glass fiber filter wick in the CO_2 trap to collect CO_2 released by the acidification step while vials were shaken for 2 h on ice. Sediments with attached microbes were recovered by centrifugation

(12,000 x g for 10 min. at 4°C), washed twice in streamwater to remove unincorporated radiolabel, and dried at 60°C. Aliquots were weighed and cornbusted in a sample oxidizer (Model 306, Packard Instruments, Naperville, IL). Radioactivity recovered in CO_2 traps and associated with sediments was determined using liquid scintillation counting.

Data for amended samples were compared with the unamended control using a Dunnett's test. Comparison of the effect of TFA and non-radioactive acetate at a similar order of magnitude concentration, and comparisons between the Control and TFA mesocosms at similar concentrations were made using *t*-tests. A (log+l) transformation was used before analyses. Statistical procedures were performed using SAS software (Version 6.08, SAS Institute, Cary, NC) on a Digital MicroVAX 3100 computer.

Bacterial densities were determined by sonicating three formalin fixed samples for 45 set at 30 W in 0.1 M sodium pyrophosphate to release cells from particles, and centrifuging in 30% glycerol (412 x g for 5 min at 4°C) to remove sediment particles from the cell suspension (Bolt and Kaplan 1993). Aliquots of cell suspension were stained with propidium iodide (200 µg/ml final concentration) and counts of stained bacteria were performed using a Zeiss SL- 16 epifluorescence microscope equipped with a Zeiss 487715 filter set (excitation wavelength of 546 nm and emission band pass at >590 nm).

RESULTS AND DISCUSSION

The effect of TFA on [^{14}C]acetate metabolism (incorporation plus respiration) by streambed microbial communities was studied using sediments collected from a natural stream and from mesocosms in which pre-exposure to TFA for 5 months allowed for potential chronic effects. Comparison of responses to amendments with either TFA or non-radioactive acetate allowed for detection of both overt toxicity and molecular substitution for [^{14}C]acetate.

Three experiments were conducted with WCC sediments. The data were analyzed together since temperatures (13.4°C, 11.6°C, and 15.0°C) and bacterial cell densities ($8.80 \pm 2.93 \times 10^9$, $1.90 \pm 0.56 \times 10^{10}$, and $7.26 \pm 2.12 \times 10^9$cells /g dry sediment, respectively, ± SD, n=3) were similar between experiments. Cell specific [14C]acetate metabolism in the amended samples was never significantly different from the unamended control (Dunnett's tests, $p>0.05$) (Fig. 1). Furthermore, none of the differences in metabolism at the same order of magnitude amendment were statistically significant (*t* tests, $p>0.05$). Given that the concentration of added [$_{14}$C]acetate was approximately 254 nM, isotopic dilution was expected only at the higher amendments of non-radioactive acetate. Between 85 - 90% of the [^{14}C]acetate was found in the biomass under every exposure condition, and none of the differences in incorporated radioactivity (data not shown) were statistically significant. Clearly, TFA was not overtly toxic to these communities.

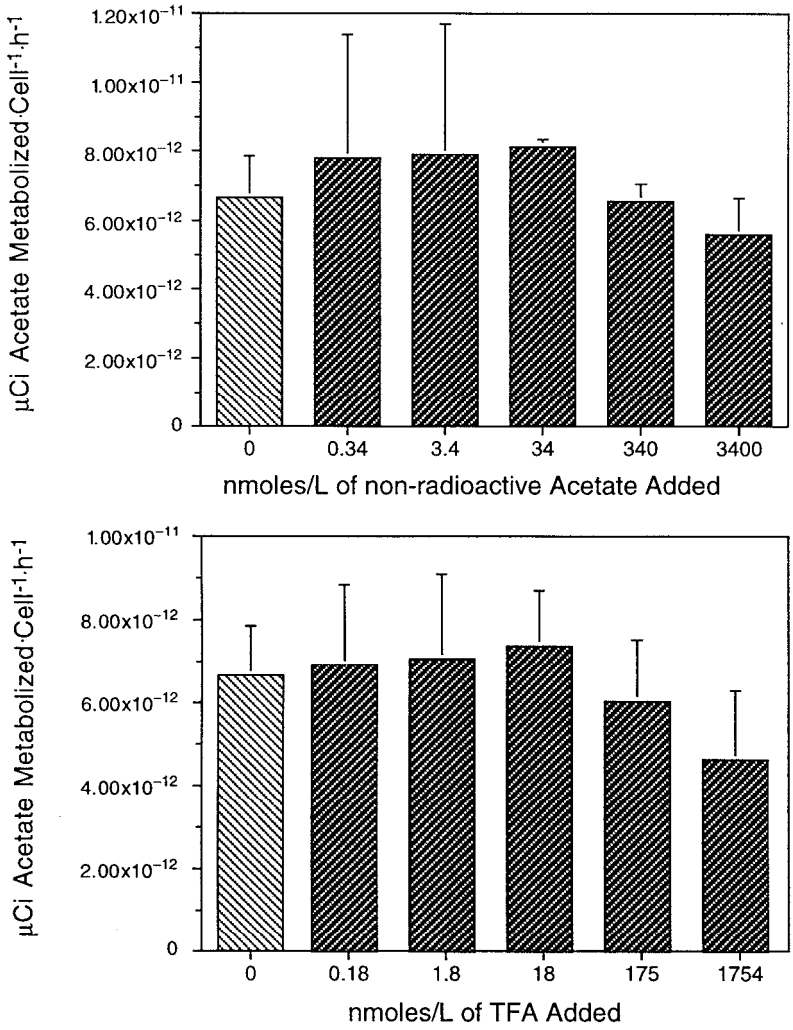

Figure 1. Cell - specific metabolism of [^{14}C] acetate by WCC sediment communities exposed to increasing concentrations of non - radioactive acetate (top panel) or TFA (bottom panel) ($\overline{x}$ ± SD, n = 3 experiments).

The first experiment with mesocosm stream communities was performed after approximately 3 months of exposure to TFA. Acetate concentrations were higher than expected for the two highest additions (5640 and 515 nM rather than 3400 and 340 nM) and matched the desired levels at 34 and 3.4 nmole/L. The lowest non-radioactive acetate amendment was eliminated here because it was negligible compared to the [^{14}C]acetate addition. TFA concentrations approximated the desired levels at 1770, 177, and 21 nM but were below detection at the lower concentrations. On a sediment dry weight basis, acetate metabolism in the TFA mesocosm was nearly twice that in the Control mesocosm (Fig. 2 A vs. B,

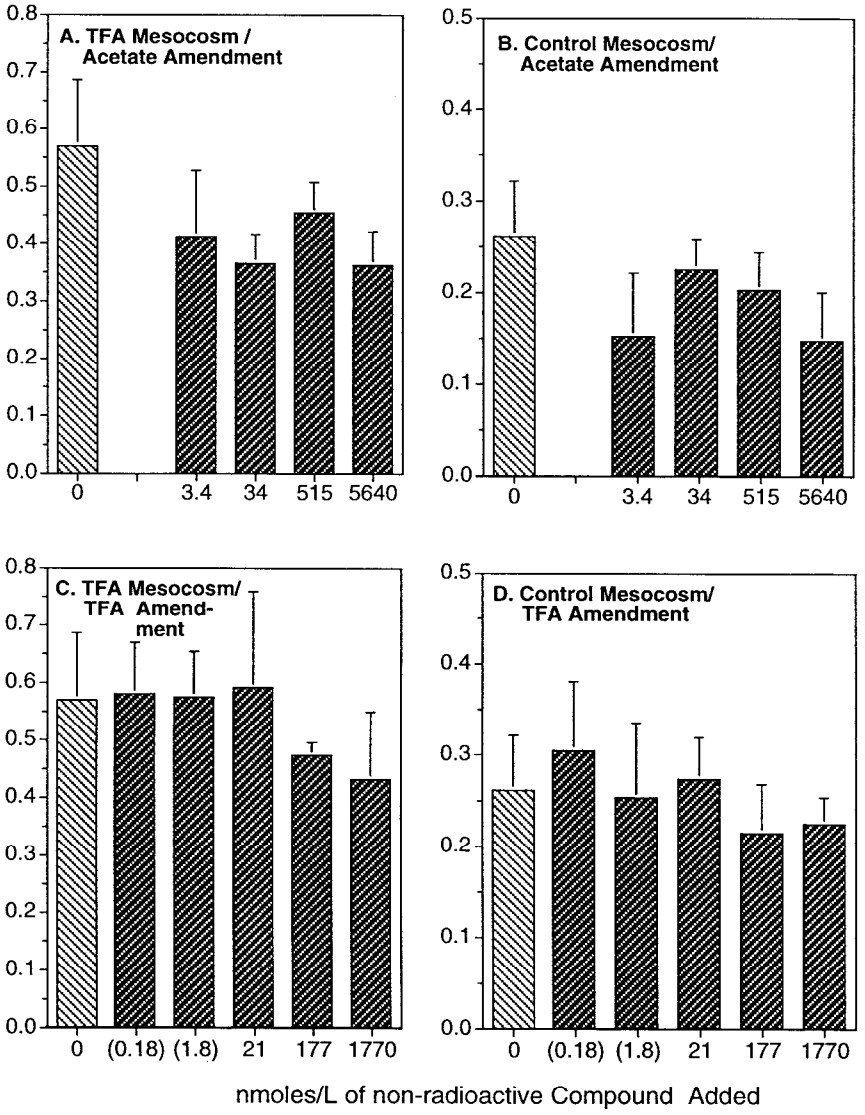

Figure 2. Metabolism of [^{14}C]acetate by mesocosm sediment communities ($\bar{x}$ ± SD, n = 5) exposed to increasing concentrations of non - radioactive acetate or TFA. TFA concentrations in parentheses are target concentrations not verified by ion chromatography.

C vs. D). However, bacterial densities in the TFA mesocosm were 1.48 times greater than in the Control mesocosm (1.79 ± 0.12 x 10^{10} vs. 1.21 ± 0.08 x 10^{10} cells/g dry mass, ($\bar{x}$ ± SD, n=3) and thus cell-specific activity differed between mesocosms by a factor of about 1.45, averaging 2.78 ± 0.52 x 10^{-11} and 1.92 ± 0.48 x 10^{-11} µCi acetate·cell^{-1}·h^{-1} over all treatments in the TFA and Control mesocosms, respectively.

In the TFA mesocosm, [^{14}C]acetate metabolism per g dry sediment in the unamended controls was significantly higher than in the 34 and 5640 nM acetate amendments, while in the Control mesocosm, activity in the unamended controls was significantly greater than in the 3.4 and 5640 nM amendments (Dunnett's tests $p<0.05$, Fig. 2A & B). None of the differences between TFA amended samples and unamended controls were statistically significant (Dunnett's tests $p>0.05$, Fig. 2C & D). Within each mesocosm, none of the differences in [^{14}C]acetate metabolism at non-radioactive acetate and TFA concentrations of the same order of magnitude were statistically significant (*t* tests, $p>0.05$, Fig. 2 A vs. C, B vs. D). The percentage of radioactivity in biomass ranged between 80 and 90% of total metabolism and, as for WCC communities, none of the differences in incorporated radioactivity were significant.

The communities from the TFA mesocosm were exposed to TFA for ~ 5 months when an experiment was performed with high (µM) additions of non-radioactive acetate and TFA. The expected isotopic dilution was striking with acetate amendments and differences from the respective unamended control were highly significant in each mesocosm (Dunnett's tests, all at $p\leq0.001$, Fig. 3 A and B). Importantly, [^{14}C]acetate metabolism also decreased with high TFA additions, although differences between amended samples and respective control were statistically significant only for the 3859 µM amendment in the Control mesocosm (Dunnett's tests, $p=0.05$, Fig. 3 C and D). [^{14}C]acetate metabolism was significantly lower with the acetate amendments than with TFA additions of a comparable order of magnitude in each mesocosm (*t* tests, $p<0.05$, Fig. 3A vs. C, B vs. D). Cell densities were 1.67 times higher in the Control mesocosm than in the TFA-mesocosm, being $1.50 \pm 0.36 \times 10^{10}$ and $8.99 \pm 0.55 \times 10^{9}$ cells/g dry sediment, respectively, ($\bar{x} \pm$ SD, n=3). Thus cell specific activity in the Control mesocosm ranged from 83 to 109% of that in the TFA mesocosm at comparable amendments, except at the highest amendment with TFA (64%). A slightly lower, but statistically significant, percentage of acetate-derived radioactivity was recovered in the biomass in the acetate amendments (77.1 ± 0.3 and 77.0 ± 2.3% for the TFA and Control mesocosms, respectively) than in the unamended controls (86.1 and 88.0%, respectively), but TFA amendment did not affect incorporated radioactivity significantly (84.1 ± 7.4 and 78.8 ± 3.9% for the TFA and Control mesocosms, respectively).

In conclusion, using acetate metabolism as an index of toxicity, TFA was not acutely toxic to these benthic microbial communities. Statistically significant effects of TFA were found only in three out of 25 incubations and at TFA concentrations far above those predicted from refrigerant sources (Tromp *et al.* 1995) or measured to date in the environment (Frank *et al.* 1996): 175 nM in one experiment with WCC sediments, 17.5 µM in an experiment (data not reported) which indicated that a short (16h) preexposure of WCC sediments to TFA did not significantly effect acetate metabolism, and 3,859 µM in the high exposure experiments. Incremental additions of non-radioactive acetate resulted in significantly lower metabolism of [^{14}C]acetate, as would be expected from

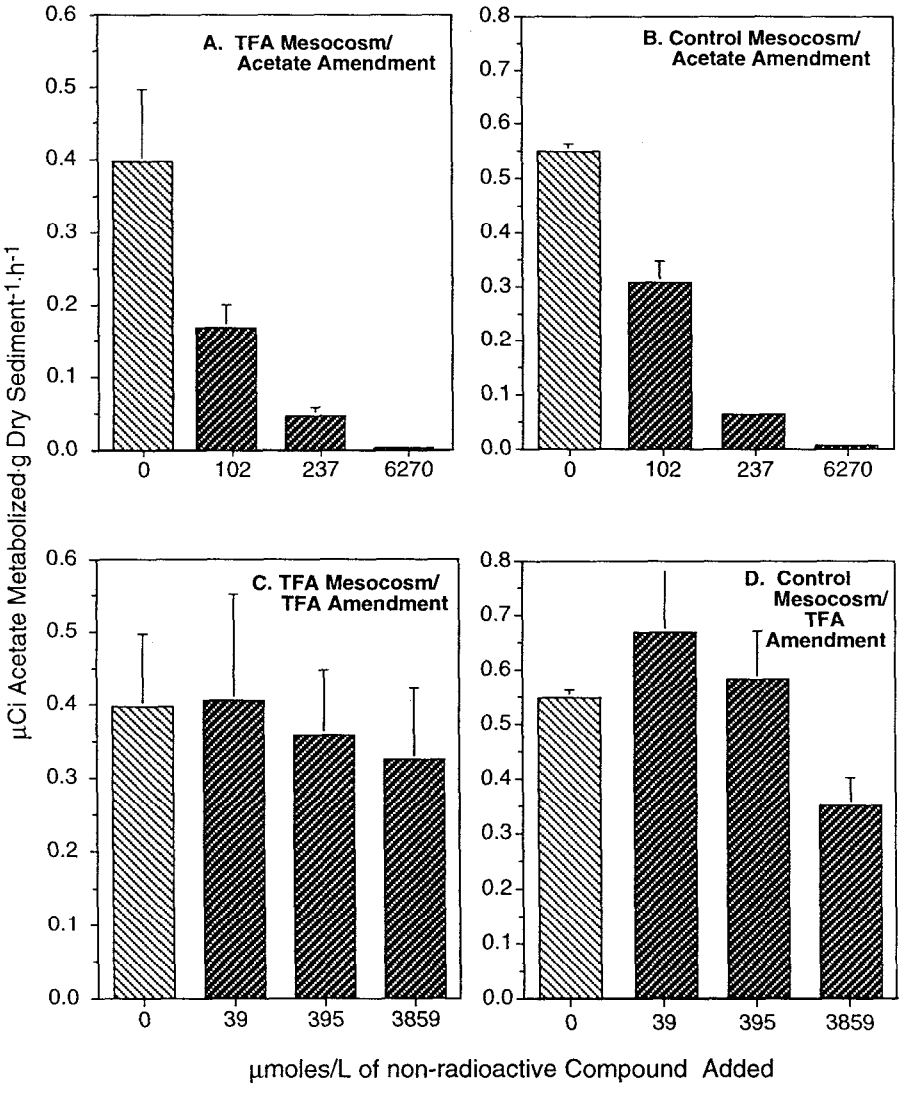

Figure 3. Metabolism of [^{14}C]acetate ($\overline{x}$ ± SD, n = 5) by mesocosm sediment communities exposed to high concentrations of non-radioactive acetate and TFA.

isotopic dilution. However, there also was a trend to reduced [^{14}C]acetate metabolism with increasing TFA concentration. The reduction was never as pronounced as that which occurred with the addition of non-radioactive acetate at a similar order of magnitude. However, [^{14}C]acetate metabolism at the higher TFA additions was never greater than in the control as would be expected if this were just random variation. This suggested that TFA, while not overtly toxic and not greatly utilized, might be actively incorporated by microorganisms, an observation confirmed in other studies (Standley and Bott, in press).

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