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Impact of Fullerene (C60) on a Soil Microbial Community

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The nascent state of the nanoproduct industry calls for important early assessment of environmental impacts before significant releases have occurred. Clearly, the impact of manufactured nanomaterials on key soil processes must be addressed so that an unbiased discussion concerning the environmental consequences of nanotechnology can take place. In this study, soils were treated with either 1 μg C_{60} g^{-1} soil in aqueous suspension (nC_{60}) or 1000 μg C_{60} g^{-1} soil in granular form, a control containing equivalent tetrahydrofuran residues as generated during nC_{60} formation process or water and incubated for up to 180 days. Treatment effects on soil respiration, both basal and glucose-induced, were evaluated. The effects on the soil microbial community size was evaluated using total phospholipid derived phosphate. The impact on community structure was evaluated using both fatty acid profiles and following extraction of total genomic DNA, by DGGE after PCR amplification of total genomic DNA using bacterial variable V3 region targeted primers. In addition, treatment affects on soil enzymatic activities for β-glucosidase, acid-phosphatase, dehydrogenase, and urease were followed. Our observations show that the introduction of fullerene, as either C_{60} or nC_{60}, has little impact on the structure and function of the soil microbial community and microbial processes.

Introduction

Since being discovered in 1985 (*1*), buckminsterfullerene [$(C_{60}-I_h)$[5,6]fullerene] or C_{60} has received considerable attention due to its unique characteristics and numerous potential applications. As the development of the nanotechnology industry continues, large amounts of carbon nanoparticles, exemplified by C_{60}, will be produced, used, and possibly released into the environment (*2, 3*). Concerns over their potential environmental and health effects have been raised, and to date few studies on the environmental impact of manufactured nanomaterials have been published (*4*). Using C_{60} as a model, we provide the first report on the impact of manufactured nanomaterials on the microbial aspects of a soil; this is a key first step in establishing an understanding of the environmental impact of C_{60}.

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Fullerenes are an ideal model material for environmental studies as recent work has shown that C_{60} has either harmful (*5, 6*) or neutral biological consequences (*7−10*). The antibacterial activities of fullerenes have been investigated using water soluble fullerene derivatives (*11−13*) or nC_{60} (a water suspension), and these studies have shown C_{60}, when prepared under specific low salt conditions, to be toxic to bacteria (*14−16*). Clearly the impact of manufactured nanomaterials on microbial function in natural soil must be addressed. Manufactured nanomaterials could enter soil along with biosolids originating from waste treatment programs that utilize land application or they could enter soil following a spill or manufacturing error.

Based on the suggested antibacterial activities of fullerenes, this study was designed to investigate the effects of C_{60} and nC_{60} on aerobic soil microbial community structure and key biological activities. Our approach follows two pathways: we describe the responses of bacterial community size and composition (structure) to C_{60} (as a granular and as an aqueous suspension nC60) and to tetrahydrofuran (THF) residues, and we also describe the impacts on soil microbial activity (function). The solvent THF is commonly used in the preparation of C_{60}, and little is known about its environmental impact. To our knowledge this is the first reported investigation of impact of manufactured nanomaterials on soil processes.

For this effort we used total soil phospholipids to define the effects of fullerene on the size of the microbial biomass (*17−20*) and then used phospholipid fatty acid analysis (PLFA) profiles to understand the structure of the soil microbial community (*17, 21, 22*) as it responds to C_{60} or the THF residues. Work in our laboratory and other locations has shown this approach to be a sensitive method for assessing microbial changes caused by contamination or land management (*18, 21, 23*). Additional information on the impact to the structure of microbial communities was obtained from analysis of DGGE banding patterns following PCR amplification using universal primers for the bacterial variable V3 region following the extraction of total soil genomic DNA. The band pattern differences have been demonstrated as being useful in assessing the consequences of introduced chemicals on the soil microbial community structure (*24−26*).

The impact of manufactured nanomaterials on soil microbial function was estimated using basal CO_2 production (*27, 28*) as it is an established method to determine the effects of anthropogenic chemicals on aerobic soil activity (*29, 30*) and has been applied widely in studies of pollutants in soil (*31*). The implications of nanomaterials on the ability of the soil microbial community to respond to introduced substrates were evaluated using a modified substrate response protocol (*30, 32, 33*) following soil incubation with nanomaterials. The effects of nanomaterials on general soil processes and nutrient cycling were evaluated using four different enzyme activities. These enzyme activities are excellent indicators of soil microbial function, are key components in nutrient cycling (*23, 34, 35*), and have been applied in studies assessing soil quality and management (*36*). Soil acid phosphatase is an important enzyme in the catalysis of organic phosphate esters into inorganic phosphate, which is important in phosphorus mineralization and processing (*37, 38*). β-Glucosidase is an important first step in the transformation of complex forms of carbon (*39, 40*). Dehydrogenase activity was assessed as a general indicator of oxidative capacity of soil microorganisms (*41, 42*). Urease was assessed because of its role in the hydrolysis of urea, the major organic form

of nitrogen, and its implications in nitrogen uptake and cycling (*43*, *44*).

In general we are following a protocol where we treat aerobic soil microcosms with C_{60} and then assess biomass size and structure, soil respiration, and enzymatic activities for up to 6 months. The results of this study will help to define the soil microbial impact portion of the discussion framework concerning the environmental consequences of nanomaterials.

Materials and Methods

Soil Collection. Surface soil (Drummer, silty clay loam, 4% organic matter, pH 6.9) was collected from continuous corn no-till plots at Purdue Agriculture Research and Education Center located northwest of the Purdue campus. This soil was chosen because it is commonly encountered in the Midwest and widely used in land application programs. An aggregate sample was generated by collecting soil from ten separate locations across the field. Soil was well mixed upon arrival at the laboratory. Pebbles, large plant residues, and macrobiota were removed, and the samples were sieved to 4 mm. This process resulted in a homogeneous mixture of soil shown by other studies in the lab.

Aqueous C_{60} Preparation. Aqueous dispersion of C_{60} (denoted as nC_{60}) was prepared as described by Fortner et al. (*16*). Briefly, C_{60} (99.5%, Sigma-Aldrich, St. Louis, MO) and freshly distilled tetrahydrofuran (THF, Fisher Scientific, Itasca, IL) were placed in a bottle on a gyratory shaker at 125 rpm overnight at room temperature. Excess C_{60} was removed with a 0.2 μm nylon membrane filter. The resulting solution was then added to an equal amount of water. The THF was purged using N_2 gas passed through a cartridge filter (Whatman Polycap TF, 0.2 μm, VWR, Batavia, IL). A THF residue control (denoted as THF-C) was generated as above without adding C_{60}. Concentration of nC_{60} was measured spectrophotometrically at 330 nm following the method developed by Fortner et al. (*16*) with some modifications. The average diameter of the aggregates in aqueous suspension was 85 nm determined by dynamic light scattering (DynaPro99, Protein Solutions, Lakewood, NJ). Concentration of the resulting stock solution was 40 μg mL^{-1}.

Soil Basal Respiration. The basal soil respiration study was conducted using biometer flask microcosms (*46*). Soil samples (50 g dry weight equivalent) were weighed into 250 mL biometer flasks and preincubated at 23 °C for at least 3 days. Soil moisture content was adjusted to −0.03 MPa. Granular C_{60} (1000 μg g^{-1} soil), nC_{60} (1 μg g^{-1} soil), or THF-C was added to microcosms and thoroughly mixed with soils. Soil controls with water only were also included as well as a no-soil blank. All treatments were done in triplicate, and the experiment was repeated at least once. The CO_2 released from the incubated soils was trapped in 10 mL of 1 M KOH and determined by titration with HCl. After 30 days of basal respiration observations, a microbial activity assessment, phospholipid fatty acid assay, and community profile by DGGE were conducted on subsamples taken from the microcosms.

Microbial Activity Assessment. Glucose-induced respiration was determined by treating subsamples from each microcosm with radiolabeled glucose (D-glucose-UL-^{14}C, specific activity 264 mCi mmol^{-1}, Sigma-Aldrich, St. Louis, MO). A subsample (10 g dry weight equivalent) was removed from each treatment, transferred to a screw-top jar, mixed with 2.5 mg of nonlabeled glucose, and supplemented with 0.148 μCi ^{14}C-labeled glucose after 30 or 180 days of incubation. This amount of glucose was determined to be that which gave maximal respiration in a preliminary experiment. Preliminary experiments indicated a 3-h incubation time represented the maximum level for the soils standing microbial biomass to respond to the added sub-strate, and this is consistent with other results (*18*). A vial containing 10 mL of 1 M KOH was placed into each jar with the ^{14}C-treated sample to capture evolved ^{14}C-CO_2, and the jars were incubated at 23 °C. KOH trap solutions were sampled at 3 h by removing a 1 mL aliquot to a 22 mL scintillation vial, which was mixed with 15 mL of scintillation cocktail (Econosafe, Research Products International, Mt. Prospect, IL). Vials were stored in the dark at room temperature overnight prior to liquid scintillation counting using a Packard 1600 TR liquid scintillation analyzer (Perkin-Elmer, Shelton, CT) with external standard quench correction.

Microbial Biomass and Phospholipid Fatty Acid Analysis. After 30 and 180 days, subsamples taken from the microcosms were lyophilized and stored frozen until analysis. Soil samples (2−5 g dry weight) were extracted to determine biomass size using phospholipid phosphate (PL-PO$_4$) and to generate phospholipid-fatty acid (PLFA) profiles to characterize microbial community structure. Phospholipids were determined by the method of Acosta-Martínez et al. (*23*), except that the phospholipid extracts were resuspended in a 95:5 chloroform:methanol solution prior to silicic acid column chromatography. Fatty acid is denoted in the form of "*X*: *YωZ*", where *X* is the total number of carbon atoms, *Y* is the number of double bonds, and *Z* is the position of the double bond from the distal end of the molecule. The suffixes c and t indicate cis and trans isomers, and the prefixes i and a refer to iso- and antiiso-branching, respectively. Cy and 10Me indicate cyclopropyl group and a methyl group on the tenth carbon atom, respectively.

DNA Extraction and PCR-DGGE. After 30 or 180 days, total genomic DNA was extracted from microcosm soil samples (0.5 g dry equivalent weight) with the FastDNA SPIN kit for soil (MP Biomedical, Solon, OH). Extracted DNA was visualized prior to amplification by electrophoresis in 0.7% ethidium bromide stained agarose gels and quantified using Kodak 1D v.3.6.1 (4Science Park, New Haven, CT).

PCR was performed with the Mastercycler (Eppendorf North America, Westbury, NY) using universal primers for bacteria F338GC and R534 (*47*), which amplify the variable V3 region of 16S rDNA. PCR mixture consisted 1X PCR buffer (Promega, Madison, WI), 2.0 mM MgCl$_2$, 0.8 mM dNTP's, 0.1% bovine serum albumin, 0.375 μM of primer F338 (5'-ACT CCT ACG GGA GGC AGC AG-3') with a GC-clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') attached to the 5' end, 0.375 μM of primer R534 (5'-ATT ACC GCG GCT GCT GG-3'), 5U of Taq polymerase, and 1−10 ng of template DNA (*48*). Amplification was performed using an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 30 s each at 92 °C, 55 °C, and 72 °C, with a final extension of 15 min at 72 °C. The resulting PCR products were analyzed for their molecular weight and the yield on a 1% agarose gel using ethidium bromide staining.

PCR products from each sample were run on a 8% polyacrylamide gel using a D-Code system (BioRad Laboratories, Hercules, CA). The gel had a denaturing gradient ranging from 35 to 55% (where the 100% denaturant contains 7 M urea and 40% (vol/vol) formamide). The gel was subjected to electrophoresis for 5.5 h at 60 °C and 200 V. The gel was removed from the D-Code, stained for 20 min in 1X TAE containing a 1:5000 dilution of SYBR green I dye (Cambrex Bioscience, Walkersville, MD), and photographed immediately using the Kodak Imaging Station (Eastman Kodak Co., Rochester, NY). The DNA amplification and DGGE profiles were replicated to ensure consistent results.

Enzymatic Activities. Enzyme activities for dehydrogenase (*49*), phosphatase, β-glucosidase (*50*), and urease (*50*) in soil were evaluated using four treatments described above, and separate subsamples were collected after 0, 1, 2, 3, 4, 5, and 6 months of incubation. The assay method for urease activity was modified from Gianfreda et al. (*50*). Soil (1 g) was mixed
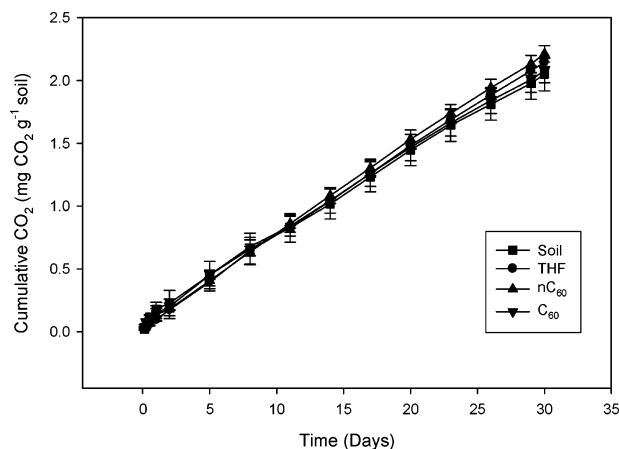
FIGURE 1. Cumulative CO_2 release from a control (■) or soil treated with THF-C (●), nC_{60} (▲), and C_{60} (▼) over a 30-day incubation.

TABLE 1. Phospholipid Fatty Acid Levels for Bacteria, Gram-negative, and Gram-positive Bacteria and Phospholipid Phosphate as Total Biomass[d] after a 30- or 180-Day Incubation

	phospholipid (% of total phospholipid)			
	soil	THF-C	nC_{60}	C_{60}
bacterial PLFA[a]				
30 days	62	62	62	63
180 days	63	64	63	64
Gram-PLFA[b]				
30 days	21	22	22	22
180 days	30	31	30	31
Gram + PLFA[c]				
30 days	28	26	26	26
180 days	22	21	22	22

	total phospholipid (nmol PO_4-P g^{-1} dry soil)			
	soil	THF-C	nC_{60}	C_{60}
30 days	56.1 ± 3.16	49.8 ± 7.93	54.3 ± 1.08	55.1 ± 3.15
180 days	27.8 ± 1.67	26.7 ± 1.97	26.7 ± 6.7	26.8 ± 2.0

[a] Contribution from PLFA: i15:0, a15:0, 15:0, i16:0, i17:0, a17:0, cy17:0, 17:0, 18:1ω9c/t9,12, 18:1ω9t, and cy19:0c11,12, only. [b] Contribution from PLFA: cy17:0, 18:1ω9c/t9,12 and cy19:0c11,12, only. [c] Contribution from PLFA: i15:0, a15:0, i16:0, i17:0, and a17:0, only. [d] All values are means ± one standard deviation ($n = 9$ at 30 days and $n = 6$ at 180 days).

with 4 mL of 0.1 M phosphate buffer (pH 7.1) and 1 mL of 0.2 M urea and incubated at 37 °C for 1 h. After incubating, 10 mL of 2 M KCl was added, and the mixtures were kept at 4 °C for 10 min to stop the reaction. The mixture was filtered through a Whatman no. 2 filter paper. Ammonia concentration in an aliquot (1 mL) of the filtrate was determined by the hypochlorite-alkaline phenol method (51).

Data Analysis. Treatment effects and significant differences were compared using one-way Analysis of Variance (ANOVA) with SAS (v 9.1, Cary, NC) at $\alpha = 0.05$ for microbial biomass, soil respiration, and enzyme activities or in Excel (Microsoft, Redmond, WA) for fatty acids. Principal component analysis (PCA) was applied to a set of 16 PLFAs, occurring at high mole fraction using Unscrambler (CAMO Software, Oslo, Norway.)

Results and Discussion

Soil Respiration. In this study soil respiration was monitored 14 times over 30 days following treatment with either distilled water, C_{60}, nC_{60}, or THF-C and for simplicity is reported as cumulative CO_2 production (Figure 1). A reduction in the
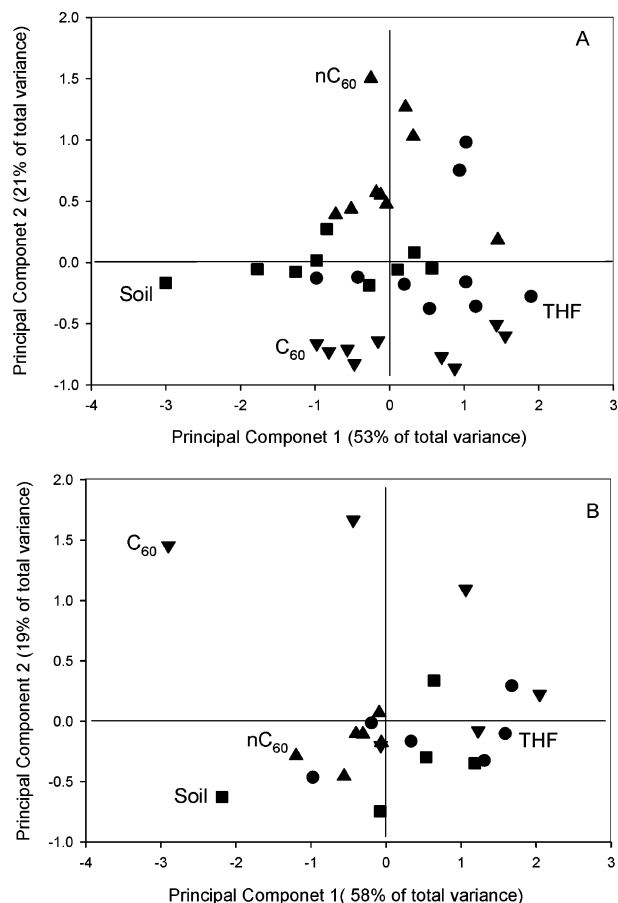

FIGURE 2. A. Principal component analysis of PLFA profiles obtained from the control (■) or soil treated with THF-C (●), nC_{60} (▲), and C_{60} (▼) after a 30-day incubation. B. Principal component analysis of PLFA after 180 days of incubation.

rate of respiration compared to the control would be expected if soil populations were subjected to a highly toxic material (29−31). Noncumulative respiration data collected at each time point were subjected to Tukey's test for the treatments effects and showed no significant differences ($P > 0.05$) between any treatments and the control. These results are similar to other respiration results from our laboratory where we have assessed CO_2 production from soils treated with subtoxic levels of Pb, Cr, and hydrocarbons (30). The impact of the C_{60} on the microbial community's ability to respond to added nutrients was estimated using a substrate response protocol (30). Again, reductions in respiratory response would be expected if the system had been exposed to a toxic material or was in a suppressed state as a result of exposure to toxic material. The respiratory response was estimated from the mineralization of ^{14}C-labeled glucose in subsamples of soil, after 30- or 180-day incubation. At 30 days, the level of ^{14}C-CO_2 production in 3 h ranged from 16.5 to 18% of the applied glucose with no significant ($P > 0.05$) differences between treatments. At 180 days the rate of mineralization as compared to 1 month was reduced by 3%, but no significant differences ($P > 0.05$) between treatments were noted. In either case, the magnitudes of the response were consistent with other results from our laboratory (53). These results are similar to our findings with metal contaminated soil (18) and to the work of Engelen et al. (54) where little impact on the response to substrate addition following chemical treatment was observed.

Microbial Biomass and PLFA Analysis. At the end of the 30-day incubation, the total microbial biomass levels as determined by phospholipid phosphate ranged from 50 to
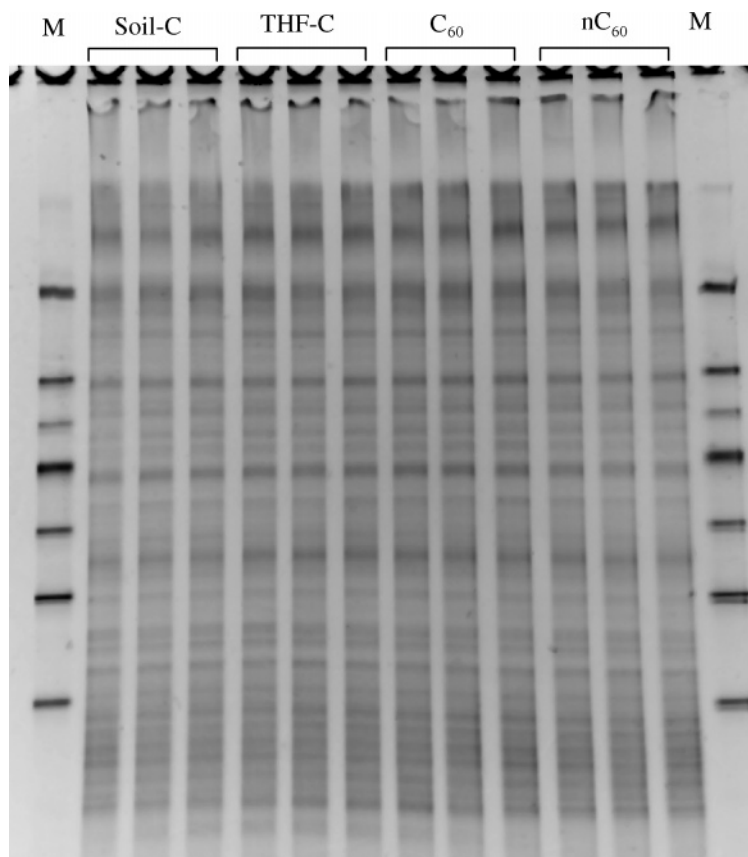
FIGURE 3. DGGE profiles of 16S rRNA fragment amplified for triplicate samples from the control soil and soils treated with THF-C, nC_{60}, and C_{60} after 180 days of incubation. M is the DGGE marker.

56 nmol $PL-PO_4$ g^{-1} dry soil (Table 1). At the end of the 180-day incubation, the total biomass had declined to approximately one-half the starting level ranging from 26 to 28 nmol $PL-PO_4$ g^{-1} dry soil. However, no significant ($P > 0.05$) difference in the size of the microbial biomass was observed between the control samples and the treated soil for either time period. This level of biomass was similar to the level obtained in other labs (55, 56) for fresh samples, and declines in biomass size for controls during laboratory incubations at 25 °C have been reported by others (57, 58).

Sixteen PLFAs with high mole fractions in all soil treatments were used to evaluate microbial community structure changes. These included six branched-chain (i15:0, a15:0, i16:0, i17:0, a17:0), five saturated PLFAs (15:0, 16:0, 17:0, 18:0), four monounsaturated PLFAs (16:1ω9c, 18:1ω9c/t9,12, 18:1ω9t), a polyunsaturated PLFA (18:2ω9)), two methyl branching PLFAs (10Me 16:0, 10Me 17:0), and two cyclopropane PLFAs (cy17:0 and cy19:0c11,12). The fatty acids i15:0, a15:0, 15:0, i16:0, i17:0, a17:0, cy17:0, 17:0, 18:1ω9c/t9,12, 18:1ω9t, and cy19:0c11,12 were used as an indicator of total bacterial PLFAs (23, 56); while the amount of Gram-negative bacteria were indicated by cy17:0, 18:1ω9c/t9,12, and cy19:0 and Gram-positive bacteria were indicated by i15:0, a15:0, i16:0, i17:0, and a17:0. Regardless of sampling time, bacteria fatty acids constituted greater than half of the fatty acids in all of the samples (Table 1). When PLFA analysis in this study was restricted to the PLFA levels for Gram-negative and -positive bacteria, the levels were also similar for all treatments (Table 1).

PCA analysis of the PLFA patterns for the 1 month samples showed that the effects of C_{60}, THF-C, and the control soils were distributed along principal component 1 (53% of the total variance), and the effects of nC_{60} and C_{60} were separated along component 2 (21% of the total variance) (Figure 2). The analysis indicates that both THF-C and C_{60} treated soil

were slightly distant from the control, but the response in the control soil was somewhat variable. For the 30-day samples, negative loading on component 1 was controlled by the levels of fatty acids i15:0, i17:0, a15:0, and C18:0. Positive loading was controlled by fatty acids 18:1ω9c/t9,12, and cy19:0c11,12. Separation on component 2 was controlled by differences in the level of C16:0 which was significantly reduced ($P > 0.05$) in C_{60} treated samples as compared to nC60 and soil. PCA analysis of the PLFA patterns for the 180-day samples showed the effects of nC_{60} and THF residues were distributed along component 1 (58% of the total variance), while the effects of C_{60} were distributed on component 2 (19% of the variance). Most of the affect of C_{60} could be associated with increased levels of C18:0; while differences were graphically apparent, changes in the levels of fatty acids were not significant ($P > 0.05$). These data suggest a limited impact of fullerenes on the overall community signature as only minor separation of the clusters was evident especially when compared to other studies (17, 21).

DGGE Patterns. The limited impact of the C_{60} on bacterial diversity was confirmed using whole soil DNA recovery followed by PCR application of the V3 region using general microbial primers followed by denaturing gradient gel electrophoresis. This approach has been demonstrated as a useful method to assess the consequences of introduced chemicals (24, 25). DGGE profile for PCR-amplified 16S rDNA fragment from soil collected at 180 days showed a large number of indistinguishable bands with only a few major bands (Figure 3) regardless of the treatment. These results were similar for soil recovered at 30 days (data not shown). Previous work in our laboratory as well as other laboratories has shown a similar pattern as observed for uncontaminated agriculture soil as well as treated soils that maintained robust community function (25, 30). In general, we have found
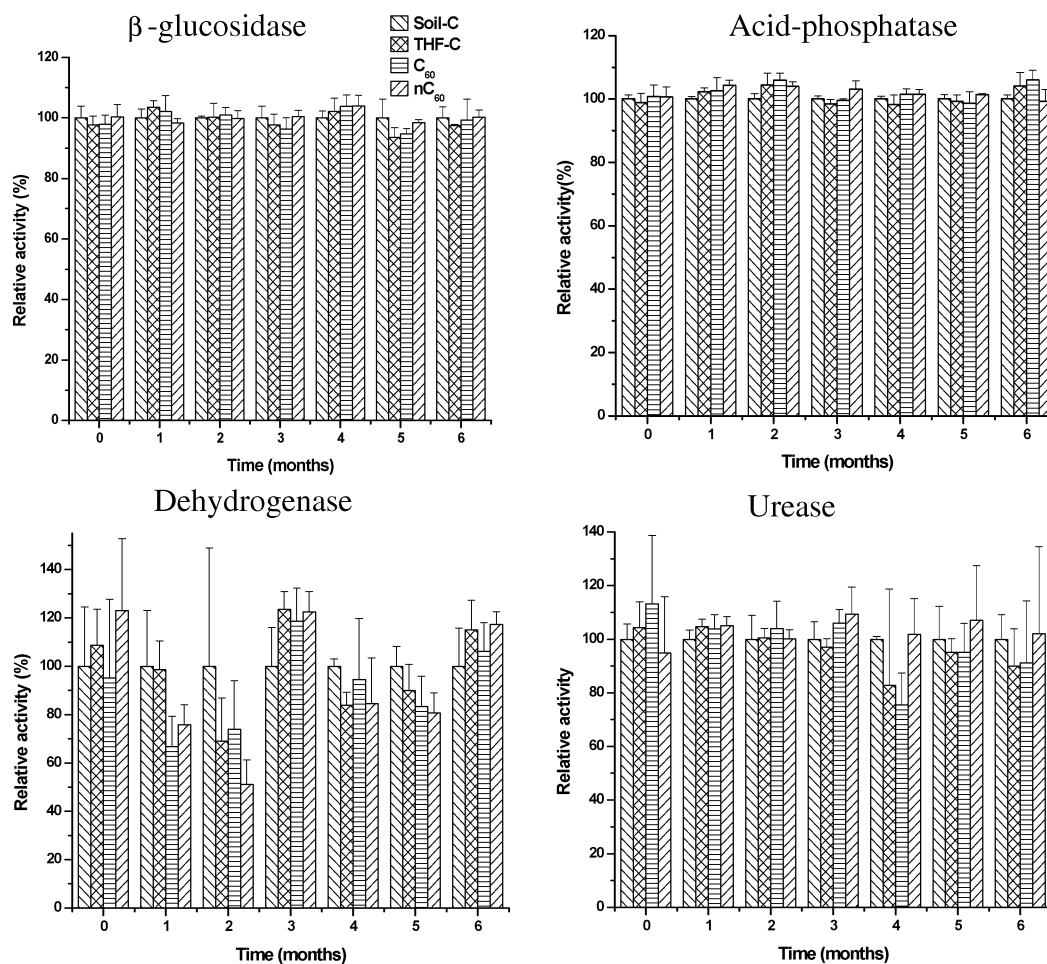
FIGURE 4. Relative activities of β-glucosidase (A), acid-phosphatase (B), dehydrogenase (C), and urease (D) of samples treated with THF-C, nC_{60}, and C_{60} compared with soil controls (set to 100%) at times up to 6 months (180 days). Bars indicated the standard deviation of the mean.

distressed soils to have a simpler banding pattern indicative of low diversity due to selection or enrichment of tolerant or dominant species (*25, 30*). The control soils as well as the treated soils exhibit a complex and diverse pattern indicating the introduction of nanomaterials had little impact on the community structure (Figure 3).

Soil Enzymatic Activities. Data in Figure 4 summarize the results of enzyme assays for soils incubated in the presences of nC_{60}, THF residues, and C_{60} for up to 180 days. Like the respiration response, reduction in enzyme activity would be expected in a system exposed to a toxic or interfering material. Enzyme assays have been previously applied in studies assessing soil quality and management (*34, 59*); we assessed enzyme activity related to nutrient cycling of C, N, and P using β-glucosidase, urease, and soil acid phosphatase, respectively (*37–40*). We also included dehydrogenase as it is an indicator of oxidative capacity of soil microorganisms and has been correlated with biomass size (*44*). There were some variations, especially with dehdyrogenase, but when compared to the soil controls, we find that only during the first month is there a significant increase between the phosphatase activity in the soil treated with nC_{60} and the control ($P = 0.005$). A reduction in enzyme activities would be the expected response to an acutely toxic chemical. Enzyme activities are sensitive indicators of stress, and these results suggest the enzyme activity in the soil system has not been adversely affected (*23, 54*).

Chemical Concentration. In this study, we present the first examination of the effects of C_{60} on a soil microbial community. Two forms of fullerene, granular C_{60}, and an aqueous dispersion nC_{60}, THF-C, and soil control were examined. We used a 1000 μg g^{-1} of granular C_{60} to generate a worst case scenario as 1000 μg g^{-1} (*60–63*). We applied nC_{60} at a concentration to achieve 1 μg g^{-1} soil. The amount of nC_{60} we could apply was limited by our ability to make a highly concentrated stock solution and the need to achieve a water content equivalent to −0.03 Mpa.

Although some researchers have shown aqueous suspension of C_{60} as being toxic to bacteria (*15, 16*), our results did not show any toxic effects from either granular C_{60} or nC_{60} in aerobic soil microcosms. We suggest our findings differ from others in terms of toxicity (*15, 16*) in that we are releasing the C_{60} into soil that contains both organic matter and salts which control the availability of C_{60} in soil which will ultimately control the exposure level and toxicity of fullerenes (*10, 11*). Work in our laboratory (data not shown) as well as others (*15, 16*) has shown C_{60} to have a limited toxicity toward Gram-negative bacteria when they are grown in cultures with concentrations of salts typical of culture media. We would also expect ionic strength to affect the physical behavior of C_{60} in aqueous systems (*11*). Combined these data suggest that bioavailability of chemicals is important in controlling their environmental impacts. On the other hand, these data do suggest that at 180 days, the C_{60} is having at least a minor effect on the fatty acids profile of the resident microorganisms. When exposed to soil, it is suggested that C_{60} partitions into soil organic matter decreasing the solution-level bioavailability. Although the surface of nC_{60} and soil are negatively charged (*9, 31*) the presence of divalent cations may work to bridge C_{60} with soil particles. We suggest, however, that

partitioning into soil organic matter is most likely the major factor controlling availability as recent work has shown log K_{oc} values to approach 7.73 (64). This level of sorption indicates the soil will retain most of the applied material, especially at the lower application levels, thus ameliorating any possible toxic effects of the C_{60} on the biota. However, work in this area is limited, and the long-term implications of the retained materials in soil remains to be assessed.

Previous studies with experimental designs that resulted in facilitated routes of exposure of C_{60} to bacteria have indicated some level of toxic response. We hypothesized that an introduction of 1000 μg g^{-1} granular C_{60} would overcome both sorption and salt effects and generate a negative effect if one actually exists. Even at these high application rates data suggest that the overall effects of the nanomaterials are limited. However, when PLFA was differentiated between Gram-positive and Gram-negative bacteria, the proportion of Gram-negative bacteria in all treated soils was slightly ($\sim$5%) higher. Ongoing work includes using more specific primers to target specific populations to investigate these results.

This study is one more example that illustrates that toxic effects of high MW hydrophobic chemicals are significantly affected by bioavailability in natural soils. Under real environmental conditions it is likely that exposure pathways will be a rate-limiting step. Therefore, long-term observations of the behavior of fullerenes and their interactions with soil microbial communities in natural soils are needed.

Acknowledgments

Literature Cited

(1) Kroto, H. W.; Heath, J. R.; Obrien, S. C.; Curl, R. F.; Smalley, R. E. C60-Buckminsterfullerene. *Nature* **1985**, *318*, 162−163.

(2) *Carbon nanotechnology: recent developments in chemistry, physics, materials science and applications*; Dai, L., Ed.; Elsevier: Boston, 2006.

(3) *Nanotechnology − Small matter, many unknows*. Risk perception. http://www.swissre.com (May 15, 2006).

(4) Colvin, V. L. The potential environmental impact of engineered nanomaterials. *Nat. Biotechnol.* **2003**, *21*, 1166−1170.

(5) Tsuchiya, T.; Oguri, I.; Yamakoshi, Y. N.; Miyata, N. Novel harmful effects of [60] fullerene on mouse embryos in vitro and in vivo. *FEBS Lett.* **1996**, *393*, 139−145.

(6) Sayes, C. M.; Fortner, J. D.; Guo, W.; Lyon, D.; Boyd, A. M.; Ausman, K. D.; Tao, Y. J.; Sitharaman, B.; Wilson, L. J.; Hughes, J. B.; West, J. L.; Colvin, V. L. The differential cytotoxicity of water-soluble fullerenes. *Nano Lett.* **2004**, *4*, 1881−1887.

(7) Scrivens, W. A.; Tour, J. M.; Creek, K. E.; Pirisi, L. Synthesis of 14C-labelled C60, its suspension in water, and its uptake by human keratinocytes. *J. Am. Chem. Soc.* **1994**, *116*, 4517−4518.

(8) Moussa, F.; Trivin, F.; Ceolin, R.; Hadchouel, M.; Sizaret, P. Y.; Greugny, V.; Fabre, C.; Rassat, A.; Szwarc, H. Early effects of C-60 administration in Swiss mice: A preliminary account for in vivo C-60 toxicity. *Fullerene Sci. Technol.* **1996**, *4*, 21−29.

(9) Jia, G.; Wang, H. F.; Yan, L.; Wang, X.; Pei, R. J.; Yan, T.; Zhao, Y. L.; Guo, X. B. Cytotoxicity of carbon nanomaterials: Single-wall nanotube, multi-wall nanotube, and fullerene. *Environ. Sci. Technol.* **2005**, *39*, 1378−1383.

(10) DaRos, T.; Prato, M.; Novello, F.; Maggini, M.; Banfi, E. Easy access to water-soluble fullerene derivatives via 1, 3-dipolar cyclo additions of azomethine ylides to C-60. *J. Org. Chem.* **1996**, *61*, 9070−9072.

(11) Bosi, S.; DaRos, T.; Castellano, S.; Banfi, E.; Prato, M. Antimicrobial activity of ionic fullerene derivatives. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1043−1045.

(12) Tsao, N.; Kanakamma, P. P.; Luh, T. Y.; Chou, C. K.; Lei, H. Y. Inhibition of *Escherichia coli*-induced meningitis by carboxyfullerene. *Antimicrob. Agents Chemother.* **1999**, *43*, 2273−2277.

(13) Tsao, N.; Luh, T. Y.; Chou, C. K.; Wu, J. J.; Lin, Y. S.; Lei, H. Y. Inhibition of group A streptococcus infection by carboxyfullerene. *Antimicrob. Agents Chemother.* **2001**, *45*, 1788−1793.

(14) Brant, J.; Lecoanet, H.; Hotze, M.; Wiesner, M. Comparison of electrokinetic properties of colloidal fullerenes (n-C-60) formed using two procedures. *Environ. Sci. Technol.* **2005**, *39*, 6343−6351.

(15) Lyon, D. Y.; Fortner, J. D.; Sayes, C. M.; Colvin, V. L.; Hughes, J. B. Bacterial cell association and antimicrobial activity of a C-60 water suspension. *Environ. Toxicol. Chem.* **2005**, *24*, 2757−2762.

(16) Fortner, J. D.; Lyon, D. Y.; Sayes, C. M.; Boyd, A. M.; Falkner, J. C.; Hotze, E. M.; Alemany, L. B.; Tao, Y. J.; Guo, W.; Ausman, K. D.; Colvin, V. L.; Hughes, J. B. C-60 in water: Nanocrystal formation and microbial response. *Environ. Sci. Technol.* **2005**, *39*, 4307−4316.

(17) Bossio, D. A.; Scow, K. W.; Gunapala, N.; Graham, K. J. Determinants of soil microbial communities: effects of agricultural management, season, and soil type on phospholipids fatty acid profiles. *Microb. Ecol.* **1998**, *36*, 1−12.

(18) Konopka, A.; Zakharova, T.; Bischoff, M.; Oliver, L.; Nakatsu, C.; Turco, R. F. Microbial biomass and activity in lead-contaminated soil. *Appl. Environ. Microbiol.* **1999**, *65*, 2256−2259.

(19) Findlay, R. H.; Watling, L. Seasonal variation in the structure of a marine benthic microbial community. *Microb. Ecol.* **1998**, *36*, 23−30.

(20) Blume, E.; Bischoff, M.; Moorman, T. B.; Turco, R. F. Degradation and binding of atrazine in surface and subsurface soils. *J. Agric. Food Chem.* **2004**, *52*, 7382−7388.

(21) Blume, E.; Bischoff, M.; Reichert, J. M.; Moorman, T.; Konopka, A.; Turco, R. F. Surface and subsurface microbial biomass, community structure and metabolic activity as a function of soil depth and season. *Appl. Soil Ecol.* **2002**, *20*, 171−181.

(22) Fraterrigo, J. M.; Balser, T. C.; Turner, M. G. Microbial community variation and its relationship with nitrogen mineralization in historically altered forests. *Ecology* **2006**, *87*, 570−579.

(23) Acosta-Martínez, V.; Reicher, Z.; Bischoff, M.; Turco, R. F. The role of tree leaf mulch and nitrogen fertilizer on turfgrass soil quality. *Biol. Fertil. Soils* **1999**, *29*, 55−61.

(24) Becker, J. M.; Parkin, T.; Nakatsu, C. H.; Wilbur, J. D.; Konopka, A. Bacterial activity, community structure, and centimeter-scale spatial heterogeneity in contaminated soil. *Microb. Ecol.* **2006**, *51*, 220−231.

(25) Sigler, W. V.; Turco, R. F. The impact of chlorothalonil application on soil bacterial and fungal populations as assessed by denaturing gradient gel electrophoresis. *Appl. Soil Ecol.* **2002**, *21*, 107−118.

(26) Sigler, W. V.; Nakatsu, C. H.; Reicher, Z. J.; Turco, R. F. Fate of the biological control agent *Pseudomonas aureofaciens* TX-1 after application to turfgrass. *Appl. Soil Ecol.* **2001**, *67*, 3542−3548.

(27) Wright, A. L.; Reddy, K. R. Heterotrophic microbial activity in northern Everglades wetland soils. *Soil Sci. Soc. Am. J.* **2001**, *65*, 1856−1864.

(28) Chow, A. T.; Tanji, K. K.; Gao, S. D.; Dahlgren, R. A. Temperature, water content and wet-dry cycle effects on DOC production and carbon mineralization in agricultural peat soils. *Soil Biol. Biochem.* **2006**, *38*, 477−488.

(29) Jones, W. J.; Ananyeva, N. D. Correlations between pesticide transformation rate and microbial respiration activity in soil of different ecosystems. *Biol. Fertil. Soils* **2001**, *33*, 477−483.

(30) Shi, W.; Becker, J.; Bischoff, M.; Turco, R. F.; Konopka, A. E. Association of microbial community composition and activity with lead, chromium, and hydrocarbon contamination. *Appl. Environ. Microbiol.* **2002**, *68*, 3859−3866.

(31) Kaufmann, K.; Chapman, S. J.; Campbell, C. D.; Harms, H.; Hohener, P. Miniaturized test system for soil respiration induced by volatile pollutants. *Environ. Pollut.* **2006**, *140*, 269−278.

(32) Margesin, R.; Zimmerbauer, A.; Schinner, F. Monitoring of bioremediation by soil biological activities. *Chemosphere* **2000**, *40*, 339−346.

(33) Ley, R. E.; Schmidt, S. K. Fungal and bacterial responses to phenolic compounds and amino acids in high altitude barren soils. *Soil Biol. Biochem.* **2002**, *34*, 989−995.

(34) Verchot, L. V.; Borelli, T. Application of para-nitrophenol (pNP) enzyme assays in degraded tropical soils. *Soil Biol. Biochem.* **2005**, *37*, 625−633.

(35) Belyaeva, O. N.; Haynes, R. J.; Birukova, O. A. Barley yield and soil microbial and enzyme activities as affected by contamination of two soils with lead, zinc or copper. *Biol. Fertil. Soils* **2005**, *41*, 85−94.

(36) Roldan, A.; Salinas-Garcia, J. R.; Alguacil, M. M.; Diaz, E.; Caravaca, F. Soil enzyme activities suggest advantages of conservation tillage practices in sorghum cultivation under subtropical conditions. *Geoderma* **2005**, *129*, 178−185.

(37) Amador, J. A.; Glucksman, A. M.; Lyons, J. B.; Gorres, J. H. Spatial distribution of soil phosphatase activity within a riparian forest. *Soil Sci.* **1997**, *162*, 808−825.

(38) Knight, T. R.; Dick, R. P. Differentiating microbial and stabilized beta-glucosidase activity relative to soil quality. *Soil Biol. Biochem.* **2004**, *36*, 2089−2096.

(39) Eivazi, F.; Tabatabai, M. A. Factors affecting glucosidase and galactosidase activities in soils. *Soil Biol. Biochem.* **1990**, *22*, 891−897.

(40) Crecchio, C.; Curci, M.; Mininni, R.; Ricciuti, P.; Ruggiero, P. Short-term effects of municipal solid waste compost amendments on soil carbon and nitrogen content, some enzyme activities and genetic diversity. *Biol. Fertil. Soils* **2001**, *34*, 311−318.

(41) Fernandez, P.; Sommer, I.; Cram, S.; Rosas, I. N.; Gutierrez, M. The influence of water-soluble As(III) dehydrogenase activity in soils affected and As(V) on by mine tailings. *Sci. Total Environ.* **2005**, *348*, 231−243.

(42) Rauch-Williams, T.; Drewes, J. E. Using soil biomass as an indicator for the biological removal of effluent-derived organic carbon during soil infiltration. *Water Res.* **2006**, *40*, 961−968.

(43) Sardans, J.; Penuelas, J. Drought decreases soil enzyme activity in a Mediterranean Quercus ilex L. forest. *Soil Biol. Biochem.* **2005**, *37*, 455−461.

(44) Sukul, P. Enzymatic activities and microbial biomass in soil as influenced by metalaxyl residues. *Soil Biol. Biochem.* **2006**, *38*, 320−326.

(45) Deguchi, S.; Alargova, R. G.; Tsujii, K. Stable dispersions of fullerenes, C-60 and C-70, in water: preparation and characterization. *Langmuir* **2001**, *17*, 6013−6017.

(46) Bartha, R.; Pramer, D. Note: features of a flask and method for measuring the persistence and biological effects of pesticides in soil. *Soil Sci.* **1965**, *100*, 68−70.

(47) Pepper, I. L.; Gerba, C. P.; Brendecke, J. W. *Environmental microbiology: a laboratory manual*; Academic Press: San Diego, 1995.

(48) Ovreas, L.; Torsvik, V. Microbial diversity and community structure in two different agricultural soil communities. *Microb. Ecol.* **1998**, *36*, 303−315.

(49) Weaver, R. W.; Angle, S.; Bottomley, P.; Bezdicek, D.; Smith, S.; Tabatabai, A.; Wollum, A. *Methods of soil analysis. Part 2, Microbiological and biochemical properties*; Soil Science Society of America: Madison, WI, 1994.

(50) Gianfreda, L.; Sannino, F.; Ortega, N.; Nannipieri, P. Activity of free and immobilized urease in soil-effects of pesticides. *Soil Biol. Biochem.* **1994**, *26*, 777−784.

(51) Chaney, A. L.; Marbach, E. P. Modified reagents for determination of urea and ammonia. *Clin. Chem.* **1962**, *8*, 130−132.

(52) Anderson, J. P. E.; Domsch, K. H. Physiological method for quantitative measurement of microbial biomass in soils. *Soil Biol. Biochem.* **1978**, *10*, 215−221.

(53) Shi, W.; Bischoff, M.; Turco, R. F.; Konopka, A. Long-term effects of chromium and lead upon the activity of soil microbial communities. *Appl. Soil Ecol.* **2002**, *21*, 169−177.

(54) Engelen, B.; Meinken, K.; von Wintzintgerode, F.; Heuer, H.; Malkomes, H. P.; Backhaus, H. Monitoring impact of a pesticide treatment on bacterial soil communities by metabolic and genetic fingerprinting in addition to conventional testing procedures. *Appl. Environ. Microbiol.* **1998**, *64*, 2814−2821.

(55) Haack, S. K.; Garchow, H.; Odelson, D. A.; Forney, L. J.; Klug, M. J. Accuracy, reproducibility, and interpretation of fatty-acid methyl-ester profiles of model bacterial communities. *Appl. Environ. Microbiol.* **1994**, *60*, 2483−2493.

(56) Zelles, L. Fatty acid patterns of phospholipids and lipopolysaccharides in the characterization of microbial communities in soil: a review. *Biol. Fertil. Soils* **1999**, *29*, 111−129.

(57) Petersen, S. O.; Klug, M. Effect of sieving, storage, and incubation temperature on the phospholipid fatty acid profile of a soil microbial community. *Appl. Environ. Microbiol.* **1994**, *60*, 2412−2430.

(58) Petersen, S. O.; Henriksen, K.; Blackburn, T. H.; Klug, G. M. A comparison of phospholipid and chloroform fumigation analyses for biomass in soil: potentials and limitations. *FEMS* **1991**, *85*, 257−268.

(59) Dick, R. P.; Breakwell, D. P.; Turco, R. F. *Methods for Assessing Soil Quality*; Soil Science Society American: Madison, WI, 1996; pp 247−271.

(60) Crecchio, C.; Curci, M.; Mininni, R.; Ricciuti, P.; Ruggiero, P. Short-term effects of municipal solid waste compost amendments on soil carbon and nitrogen content, some enzyme activities and genetic diversity. *Biol. Fertil. Soils* **2001**, *34*, 311−318.

(61) Sedarati, M. R.; Keshavarz, T.; Leontievsky, A. A.; Evans, C. S. Transformation of high concentrations of chlorophenols by the white-rot basidiomycete Trametes versicolor immobilized on nylon mesh. *Electron. J. Biotechnol.* **2003**, *6*, 104−114.

(62) Pieniazek, D.; Bukowska, B.; Duda, W. Comparison of the effect of Roundup Ultra 360 SL pesticide and its active compound glyphosate on human erythrocytes. *Pestic. Biochem. Physiol.* **2004**, *79*, 58−63.

(63) Kumar, A.; Kumar, S.; Kumar, S. Biodegradation kinetics of phenol and catechol using Pseudomonas putida MTCC 1194. *Biochem. Eng. J.* **2005**, *22*, 151−159.

(64) Kulkarni, P. P.; Jafvert C. T. *Abstract 230th American Chemical Society National Meeting*; Washington, DC, 2005.