

## Impact of Fullerene (C<sub>60</sub>) on a Soil Microbial Community

ZHONGHUA TONG,<sup>†</sup>  
 MARIANNE BISCHOFF,<sup>†</sup> LORING NIES,<sup>‡</sup>  
 BRUCE APPELGATE,<sup>§</sup> AND  
 RONALD F. TURCO\*<sup>†</sup>

College of Agriculture – Laboratory for Soil Microbiology,  
 School of Civil Engineering – Ecological Engineering Science  
 Group, and Department of Food Science, Purdue University,  
 West Lafayette, Indiana 47907

The nascent state of the nanoparticle 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<sub>60</sub> g<sup>-1</sup> soil in aqueous suspension (nC<sub>60</sub>) or 1000 μg C<sub>60</sub> g<sup>-1</sup> soil in granular form, a control containing equivalent tetrahydrofuran residues as generated during nC<sub>60</sub> 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 effects 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<sub>60</sub> or nC<sub>60</sub>, 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<sub>60</sub>-I<sub>h</sub>)[5,6]fullerene] or C<sub>60</sub> 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<sub>60</sub>, 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<sub>60</sub> 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<sub>60</sub>.

Fullerenes are an ideal model material for environmental studies as recent work has shown that C<sub>60</sub> 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<sub>60</sub> (a water suspension), and these studies have shown C<sub>60</sub>, 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<sub>60</sub> and nC<sub>60</sub> 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<sub>60</sub> (as a granular and as an aqueous suspension nC<sub>60</sub>) 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<sub>60</sub>, 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<sub>60</sub> 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<sub>2</sub> 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

\* Corresponding author phone: (765)494 8077; fax: (765)496 2926; e-mail: rturco@purdue.edu.

<sup>†</sup> College of Agriculture – Laboratory for Soil Microbiology.

<sup>‡</sup> School of Civil Engineering – Ecological Engineering Science Group.

<sup>§</sup> Department of Food Science.

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  $\mu\text{m}$  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  $\mu\text{m}$ , 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  $\mu\text{g mL}^{-1}$ .

**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  $\mu\text{g g}^{-1}$  soil),  $nC_{60}$  (1  $\mu\text{g g}^{-1}$  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- $^{14}\text{C}$ , specific activity 264 mCi  $\text{mmol}^{-1}$ , 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  $\mu\text{Ci } ^{14}\text{C}$ -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  $^{14}\text{C}$ -treated sample to capture evolved  $^{14}\text{C}$ - $CO_2$ , 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.

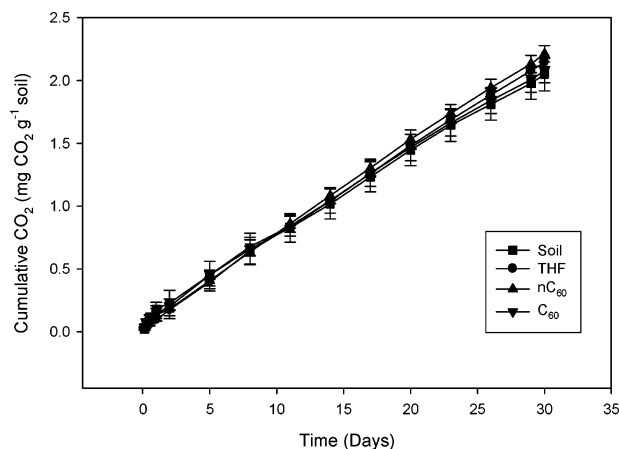
After 30 and 180 days, subsamples taken from the microcosms were lyophilized and stored frozen until analysis. Soil samples (2–5 g dry weight) were extracted to determine biomass size using phospholipid phosphate (PL- $PO_4$ ) and to generate phospholipid-fatty acid (PLFA) profiles to characterize microbial 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:Y $\omega$ Z”, 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  $\mu\text{M}$  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  $\mu\text{M}$  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,  $\beta$ -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<sub>2</sub> release from a control (■) or soil treated with THF-C (●), nC<sub>60</sub> (▲), and C<sub>60</sub> (▼) 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<sup>d</sup> after a 30- or 180-Day Incubation

|                             | phospholipid (% of total phospholipid) |       |                  |                 |
|-----------------------------|--|-------|------------------|-----------------|
|                             | soil                                   | THF-C | nC <sub>60</sub> | C <sub>60</sub> |
| bacterial PLFA <sup>a</sup> |  |       |                  |                 |
| 30 days                     | 62                                     | 62    | 62               | 63              |
| 180 days                    | 63                                     | 64    | 63               | 64              |
| Gram-PLFA <sup>b</sup>      |  |       |                  |                 |
| 30 days                     | 21                                     | 22    | 22               | 22              |
| 180 days                    | 30                                     | 31    | 30               | 31              |
| Gram + PLFA <sup>c</sup>    |  |       |                  |                 |
| 30 days                     | 28                                     | 26    | 26               | 26              |
| 180 days                    | 22                                     | 21    | 22               | 22              |

|          | total phospholipid (nmol PO <sub>4</sub> -P g <sup>-1</sup> dry soil) |             |                  |                 |
|----------|---|-------------|------------------|-----------------|
|          | soil  | THF-C       | nC <sub>60</sub> | C <sub>60</sub> |
| 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      |

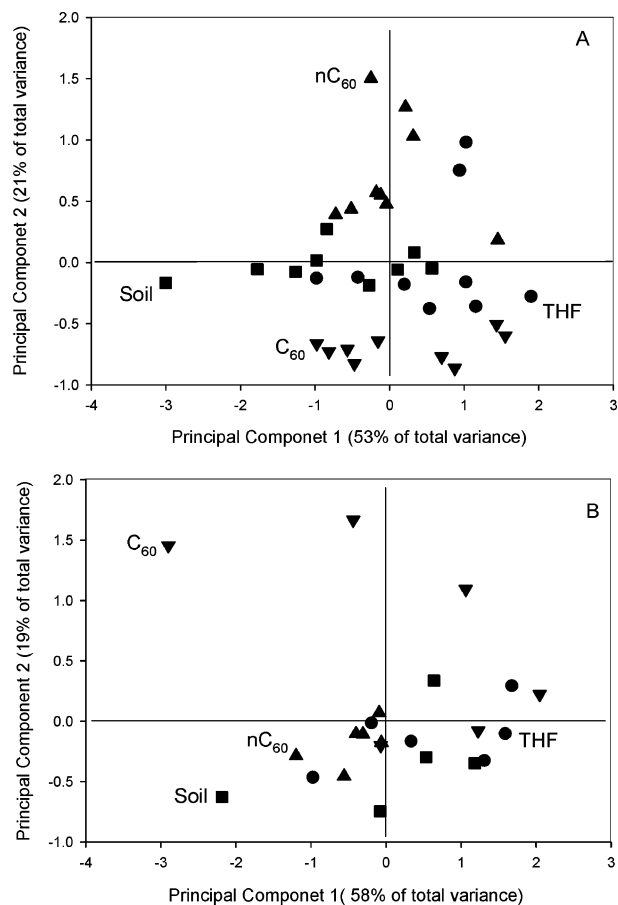
<sup>a</sup> Contribution from PLFA: i15:0, a15:0, 15:0, i16:0, i17:0, a17:0, cy17:0, 17:0, 18:1 $\omega$ 9c/t9,12, 18:1 $\omega$ 9t, and cy19:0c11,12, only. <sup>b</sup> Contribution from PLFA: cy17:0, 18:1 $\omega$ 9c/t9,12 and cy19:0c11,12, only. <sup>c</sup> Contribution from PLFA: i15:0, a15:0, i16:0, i17:0, and a17:0, only. <sup>d</sup> All values are means  $\pm$  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

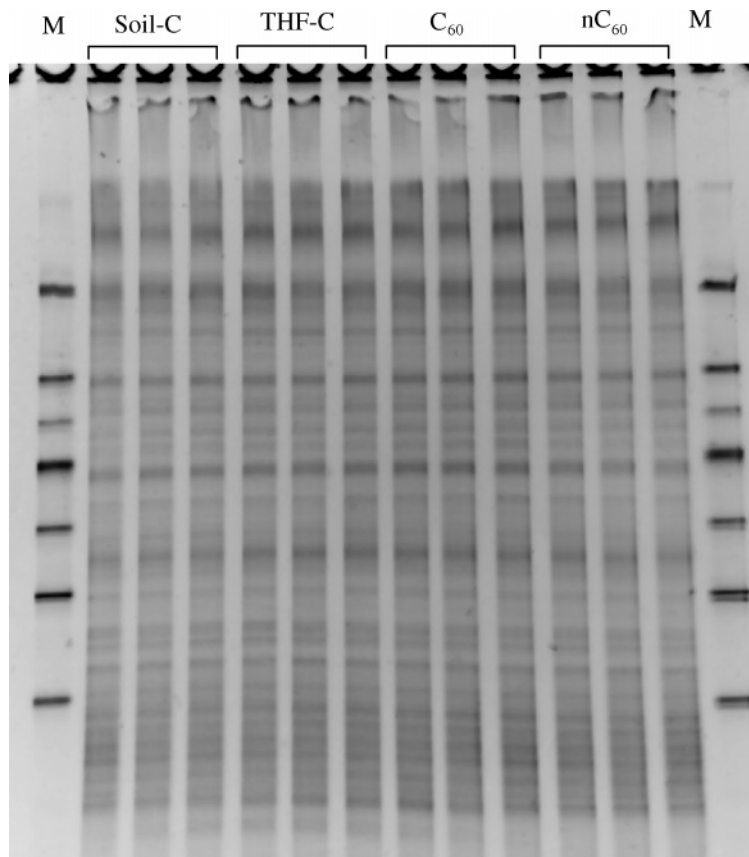
**Soil Respiration.** In this study soil respiration was monitored 14 times over 30 days following treatment with either distilled water, C<sub>60</sub>, nC<sub>60</sub>, or THF-C and for simplicity is reported as cumulative CO<sub>2</sub> production (Figure 1). A reduction in the



**FIGURE 2.** A. Principal component analysis of PLFA profiles obtained from the control (■) or soil treated with THF-C (●), nC<sub>60</sub> (▲), and C<sub>60</sub> (▼) 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<sub>2</sub> production from soils treated with subtoxic levels of Pb, Cr, and hydrocarbons (30). The impact of the C<sub>60</sub> 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 <sup>14</sup>C-labeled glucose in subsamples of soil, after 30- or 180-day incubation. At 30 days, the level of <sup>14</sup>C-CO<sub>2</sub> 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<sub>60</sub>, and C<sub>60</sub> after 180 days of incubation. M is the DGGE marker.**

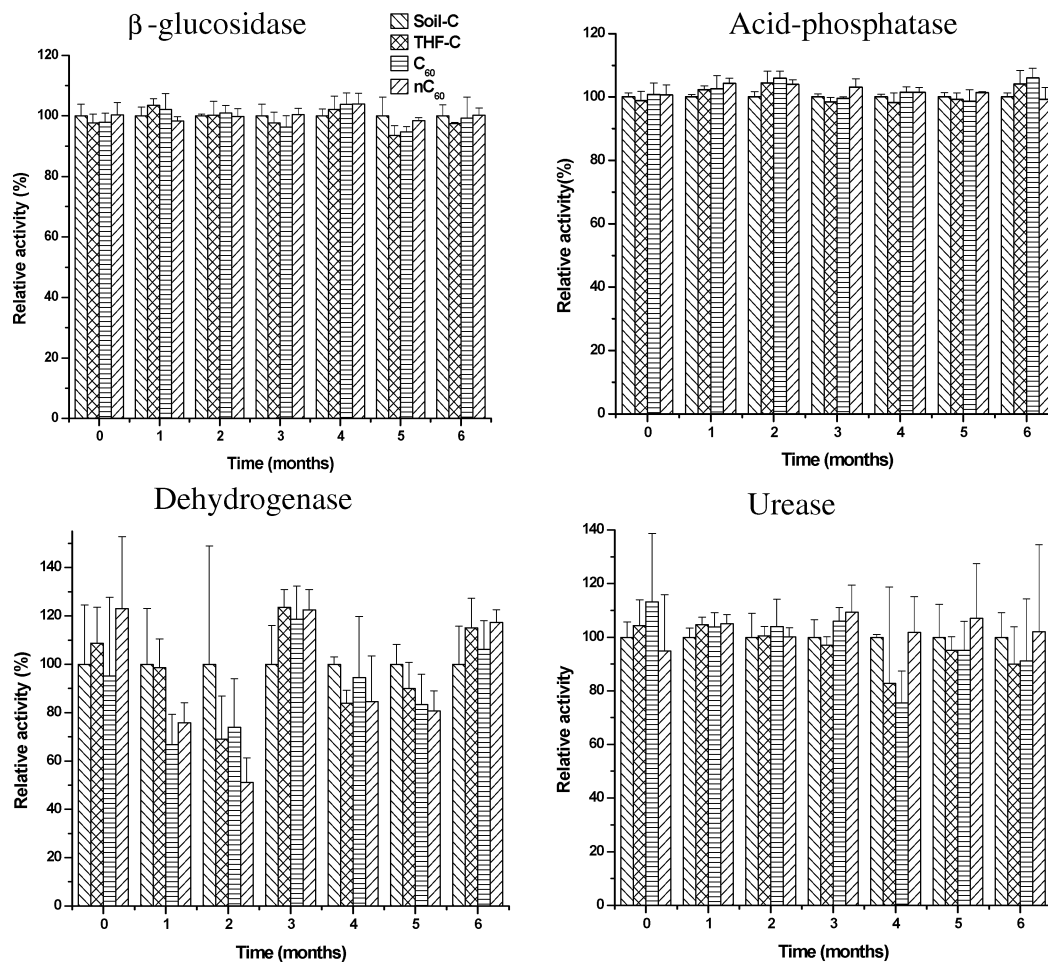
56 nmol PL-PO<sub>4</sub> g<sup>-1</sup> dry soil (Table 1). At the end of the 180-day incubation, the total biomass had declined to approximately one-half the starting level ranging from 26 to 28 nmol PL-PO<sub>4</sub> g<sup>-1</sup> dry soil. However, no significant ( $P > 0.05$ ) difference in the 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 (16:1 $\omega$ 9c, 18:1 $\omega$ 9c/t9,12, 18:1 $\omega$ 9t), a polyunsaturated PLFA (18:2 $\omega$ 9), 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, 18:1 $\omega$ 9c/t9,12, 18:1 $\omega$ 9t, 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, 18:1 $\omega$ 9c/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<sub>60</sub>, THF-C, and the control soils were distributed along principal component 1 (53% of the total variance), and the effects of nC<sub>60</sub> and C<sub>60</sub> were separated along component 2 (21% of the total variance) (Figure 2). The analysis indicates that both THF-C and C<sub>60</sub> 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 18:1 $\omega$ 9c/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<sub>60</sub> treated samples as compared to nC<sub>60</sub> and soil. PCA analysis of the PLFA patterns for the 180-day samples showed the effects of nC<sub>60</sub> and THF residues were distributed along component 1 (58% of the total variance), while the effects of C<sub>60</sub> were distributed on component 2 (19% of the variance). Most of the affect of C<sub>60</sub> 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<sub>60</sub> 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  $\beta$ -glucosidase (A), acid-phosphatase (B), dehydrogenase (C), and urease (D) of samples treated with THF-C, nC<sub>60</sub>, and C<sub>60</sub> 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<sub>60</sub>, THF residues, and C<sub>60</sub> 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  $\beta$ -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 dehydrogenase, 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<sub>60</sub> 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<sub>60</sub> on a soil microbial community. Two forms of fullerene, granular C<sub>60</sub>, and an

aqueous dispersion nC<sub>60</sub>, THF-C, and soil control were examined. We used a 1000  $\mu\text{g g}^{-1}$  of granular C<sub>60</sub> to generate a worst case scenario as 1000  $\mu\text{g g}^{-1}$  (60–63). We applied nC<sub>60</sub> at a concentration to achieve 1  $\mu\text{g g}^{-1}$  soil. The amount of nC<sub>60</sub> 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<sub>60</sub> as being toxic to bacteria (15, 16), our results did not show any toxic effects from either granular C<sub>60</sub> or nC<sub>60</sub> in aerobic soil microcosms. We suggest our findings differ from others in terms of toxicity (15, 16) in that we are releasing the C<sub>60</sub> into soil that contains both organic matter and salts which control the availability of C<sub>60</sub> 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<sub>60</sub> 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<sub>60</sub> 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<sub>60</sub> 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<sub>60</sub> partitions into soil organic matter decreasing the solution-level bioavailability. Although the surface of nC<sub>60</sub> and soil are negatively charged (9, 31) the presence of divalent cations may work to bridge C<sub>60</sub> 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<sub>60</sub> 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<sub>60</sub> to bacteria have indicated some level of toxic response. We hypothesized that an introduction of 1000  $\mu\text{g g}^{-1}$  granular C<sub>60</sub> 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 (~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

The authors acknowledge support from the National Science Foundation (NSF) under Award EEC-0404006 and United States EPA STAR Grant, Award RD-83172001-0. The authors also acknowledge the contribution of Dr. Allan Konopka's lab group as well as the program support provided by Elizabeth Rulli, the Birck Nanotechnology Center, and the Center for the Environment. We also acknowledge the Environmental Sciences and Engineering Institute for supporting our initial studies. This is paper number 2007-18091 of the Purdue Agriculture Experiment station series.

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Received for review August 14, 2006. Revised manuscript received January 9, 2007. Accepted February 12, 2007.

ES061953L