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# Fullerol as a Potential Pathway for Mineralization of Fullerene Nanoparticles in Biosolid-Amended Soils

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S *Supporting Information*

ABSTRACT: Although fullerenes could reach, and potentially adversely affect, the terrestrial environment via the reuse of biosolids, their fate in soil has not been well studied. This study examined the mineralization of fullerenes with drastically different physicochemical properties (C_{60} and fullerol, C_{60}-OH) in biosolid-amended soils. Using radiolabeled C_{60} and C_{60}-OH, mineralization of <0.025% C_{60} and up to 3% C_{60}-OH was observed in three contrasting soils after incubation for 55 days. This difference in observed mineralization highlights C_{60}-OH's greater bioavailability compared to that of its parent compound C_{60} in biosolid-amended soils. Glucose-induced respiration tests on C_{60}-/C_{60}-OH-contaminated soils suggest that overall microbial activity was not compromised by exposure to these contaminant species. These results indicate that transformation of certain fullerenes, such as fullerol, could provide a pathway for their mineralization, but such a transformation would be expected to be slow. This study is also the first work to examine the fate of fullerol in soils.
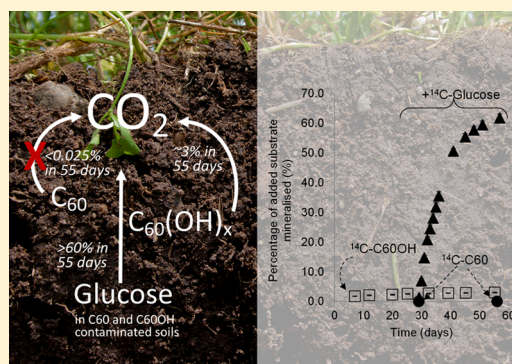
1. INTRODUCTION

Fullerenes (e.g., C_{60}, C_{70}, etc.) and its hydroxylated form (fullerols) are currently used in a wide variety of applications, which include cosmetics, medical treatments, and photovoltaics.[1−4] These carbon-based nanomaterials are already produced in large quantities, as much as 1500 t/year.[5] One of the most notable characteristics of fullerenes (including derivatives) is their strong photochemical reactivity.[6,7] Because fullerenes are generally hydrophobic (C_{60} water solubility of <10^{-9} mg/L),[8] several efforts have been made to render it soluble and/or stable in water. For example, the most commonly used fullerene, C_{60}, is prepared as suspensions (nC_{60}) or converted to fullerols, $C_{60}(OH)_x(ONa)_y$, by hydroxylation. While water solubility or stability is important for biological studies and medical applications, it has also brought about concerns that these photochemically reactive compounds may have adverse effects on the environment.

The reuse and application of treated sludge (biosolids) has been identified as an important pathway for nanomaterials, including fullerenes, to enter the terrestrial environment.[9−11] On the basis of simulated wastewater treatment studies, in which removal of up to 83−97% of C_{60} and >90% of $C_{60}(OH)_{24−y}Na_y$ (fullerol) from the wastewater can be achieved, fullerenes will accumulate in the biosolids.[9] The latest modeling study published by Sun et al. predicted a small number of micrograms of C_{60} per kilogram of biosolids.[12] Currently, there are no robust methods of analysis of fullerols and no reports of their detection in soils or biosolids. The fate of fullerenes as they enter the terrestrial environment via

biosolids has not been well studied. Our previous work, which investigated the release of C_{60} from biosolid-amended soils, revealed strong retention of C_{60} incubated in different systems that include soil and biosolids.[13] Though C_{60} transformation was not the focus of that study, some observations, including poor C_{60} mass recovery at the end of the experiment, suggested that this may be occurring.

Studies performed in aqueous solutions provide some insights into the possible transformation of fullerenes in soils. C_{60} is susceptible to oxidative transformations/breakdown mediated by light and oxygen[14−16] or by ozone,[17−19] resulting in fullerene species that contain a variety of oxidized functionalities such as vinyl ethers, epoxides, and carbonyl, carboxyl, and hydroxyl groups. A study by Hou et al. demonstrated that ≥400 nm wavelength light is important in the phototransformation of C_{60}.[14] Mineralization (complete degradation to CO_2), however, does not appear to be a direct pathway for C_{60}. Its poor degradability has been demonstrated in microbial cultures,[20] activated sludge media,[21] and soils (dark exposure conditions).[22] Compared to C_{60}, its hydroxylated form (fullerol) appears to be more susceptible to abiotic and biotic degradation. The level of mineralization of fullerols can reach up to 47% after extensive irradiation for 48 h with simulated solar irradiation in solution[23] and up to 3% upon

incubation (16 weeks) in agar medium containing white-rot basidiomycete fungi.[24] The difference in the level of mineralization between C_{60} and fullerols is not surprising, as the double bonds (C=C, sp^2) that contribute to C_{60} stability would have been compromised in fullerols; the carbon bonding state changes from sp^2 to sp^3 upon addition of OH groups.[25] Furthermore, fullerol's greater water solubility (>6 g/L)[26] also makes it more bioavailable for microbial and enzymatic transformation pathways. Whether similar mineralization could be observed for fullerols in soils has yet to be demonstrated.

In this study, the objective was to assess the mineralization potential of C_{60} and its hydroxylated form (i.e., fullerol) in biosolid-amended soils. It builds on the work presented by Avanasi et al. that reported the first data indicating negligible mineralization (no radioactivity above background) of C_{60} in soils in the absence of light.[22] Further, it addresses some issues of experimental design in that study such as the ability to detect a very low level of mineralization that may have occurred and the overall microbial activity. To mimic a relevant scenario, fullerenes were also introduced into the soil via biosolids. Results from this study will contribute to our understanding of the environmental fate of C_{60} and fullerol, fullerenes with drastically different physicochemical properties, once they reach soils via biosolids.

2. MATERIALS AND METHODS

Isotopically labeled (^{14}C) C_{60} in toluene (9.25 MBq, 1.5392 MBq/mL, 425.5 MBq/mmol, 99.6% radiochemical purity) was obtained via custom synthesis from the Research Triangle Institute (Research Triangle Park, NC). Transformed [^{14}C]C_{60} (C_{60}-OH) in water (0.0529 MBq/mL) was prepared following the hydroxylation procedure proposed by Li et al.[27] This method was chosen because of the similarities between the final product and the commercially available fullerol. Synthesis involved mixing C_{60} (in toluene, Sigma-Aldrich) with a concentrated solution of NaOH in the presence of tetrabutyl ammonium hydroxide (Sigma) continuously for 5 days. The product was washed in methanol (Sigma-Aldrich), to remove methanol-soluble products, and filtered through a 3 kDa membrane (to remove excess salts). Up to 64% of the initial activity used was recovered in the final product. See the Supporting Information for details about the synthesis. D-[^{14}C]Glucose (9.25 MBq, 10.4 GBq/mmol) used in the control experiments was purchased from GE Healthcare. D-Glucose (Analar) was from VWR Chemicals. Sodium hydroxide pellets used to prepare the solution used for traps and hydrophilic C_{60} synthesis were from Ajax Finechem. Ultrapure deionized (MQ) water was from a Millipore Milli-Q Plus water purification system (18.2 MΩ cm resistivity) and was used without further sterilization.

Three sandy loam soils with a range of organic carbon contents and pHs (Table S1) were chosen from the group of 10 soils used in our previous study[13] and were collected fresh from three areas in South Australia (Kalangadoo, Mintaro, and Mt Shank). The biosolids were obtained from stabilized sludge from a local wastewater treatment plant (Bolivar, Australia). The different biosolid/soil combinations are noted as SB1−SB3.

Mineralization of fullerenes was followed using [^{14}C]-substrate-induced respiration (SIR), based on OECD Guideline 307.[28] Briefly, 50 g of soil (in clear polycarbonate vials) hydrated up to 50% of the maximal water holding capacity

(MWHC) was preincubated in the dark at 20−23 °C for 5 weeks. After this incubation period, the soil moisture was adjusted to 60% MWHC. Biosolids (2.5 g), with no measurable C_{60} in them, were mixed with the soil at a rate equivalent to that of biosolid application of 50 t/ha (5 times the typical application rate used in Australia). Biosolids were spiked with ^{14}C-labeled C_{60} (in toluene) at two rates to achieve final concentrations in the soil/biosolid systems of 200 μg/kg (118.2 Bq/g, low treatment, four replicates) and 2000 μg/kg (1181.9 Bq/g, high treatment, eight replicates). Because of the limited availability of the [^{14}C]C_{60}-OH, only one spiking rate was tested. [^{14}C]C_{60}-OH was introduced to achieve 118.2 Bq/g [equivalent to at least 350 μg/kg; assuming specific activity is maintained and a theoretical composition of $C_{60}(OH)_x(ONa)_y$, where $x + y = 24−26$] in the soil/biosolid systems (eight replicates). These spiking concentrations were chosen to be as low as reasonably achievable to allow detection of at least 0.25% mineralization to $^{14}CO_2$ (see the Supporting Information for calculations). The high (C_{60}) treatment was prepared to provide greater detection ability (0.025% mineralization). Each spiked system was immediately transferred into a clear polypropylene jar that contained a CO_2 trap (5 mL of 1 M NaOH) and then enclosed. The volume of NaOH used was calculated on the basis of a 100% mineralization of C_{60} and contribution from the soil basal respiration (see the Supporting Information). The NaOH traps for the [^{14}C]C_{60} systems were not collected until day 28. For [^{14}C]C_{60}-OH, the traps were collected approximately every 7 days. Several control treatments were prepared alongside these treatments.

Glucose-supplemented systems were prepared to demonstrate microbial activity in all the soils. Glucose is commonly used as an easily utilizable C substrate[29] and was expected to induce the maximal respiratory response from the soil microbial biomass. It was introduced into the soil as a mixture of ^{14}C-labeled glucose (125 Bq/g of soil) and ^{12}C-labeled glucose (12.5 mg/g of soil). Because microbial respiration was expected to be significantly stimulated, several trap changes were conducted for these setups. NaOH traps (10 mL of 1 M NaOH) were collected at regular time intervals, every day up to 7 days and then every 2−3 days up to 28 days. The NaOH placed in each setup was calculated to be sufficient in trapping any CO_2 produced from mineralization and basal respiration (see the Supporting Information). Treatments such as toluene with glucose and synthesis matrix with glucose supplemented systems were used to determine the effect of toluene and the C_{60}-OH synthesis matrix on soil respiration. The synthesis matrix may contain sodium hydroxide and tert-butyl ammonium hydroxide inadequately removed by the washing steps. The volume added was equivalent to the spiking volumes used (40 μL for [^{14}C]C_{60}; 100 μL for [^{14}C]C_{60}-OH), which was kept to a minimum to limit the potential effect of the solvent/solution matrix on microbial activity. [^{14}C]Glucose was introduced 1 h after C_{60} or C_{60}-OH had been added. Other treatments included negative controls (with no fullerene or fullerol, solvent, or glucose) and systems without soil or biosolids (to determine the mineralization potential in the absence of the soil/biosolid matrix). For solution controls (without soil or biosolids), equivalent concentrations of C_{60} and C_{60}-OH were prepared in unsterile water (10 mL), without the addition of inoculum. The spiked water solution was placed inside a closed container with a NaOH trap, the same as was the case for the treatments with soil and biosolids. Maximal activity in the treatments was confirmed by directly adding
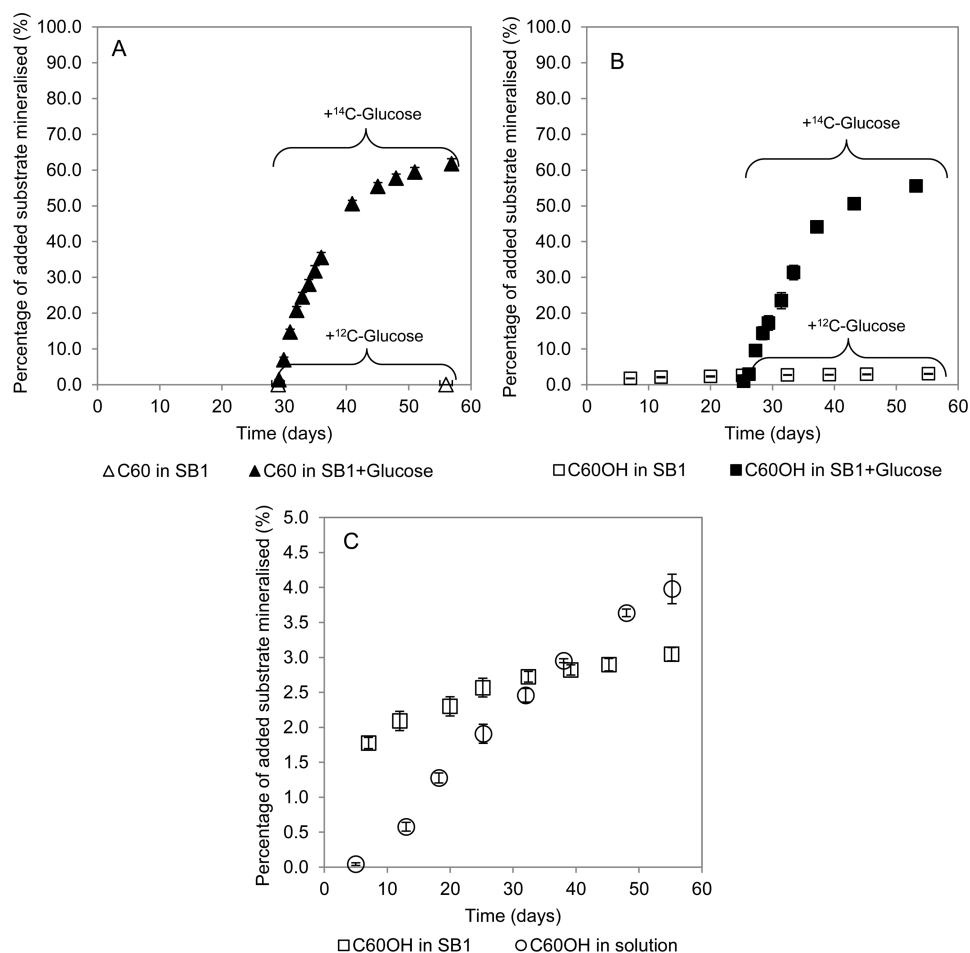
Figure 1. Mineralization of (A) 2000 μg of $[^{14}C]C_{60}$/kg (C60) and (B) 350 μg of $[^{14}C]C_{60}$-OH/kg (C60OH) in soil 1 amended with biosolids (SB1). The graphs show mineralization of the added substrate(s) over 55 days. Glucose was introduced into these treatments after 28 days: for each treatment, four replicates were spiked with $[^{14}C]$glucose, while the four other replicates were spiked with $[^{12}C]$glucose. A comparison of the mineralization of C_{60}-OH in SB1 and in solution (water only) is shown in panel C. Error bars correspond to the standard deviation of four to eight measurements.

radiolabeled compounds to NaOH or to scintillant. Four replicates were prepared for these controls. All incubations were conducted at 20−23 °C under fluorescent lighting.

Soil microbial activity was also determined post-C_{60} and -C_{60}-OH treatment using glucose. Glucose was introduced into the soils treated with $[^{14}C]C_{60}$ (high treatment) and $[^{14}C]C_{60}$-OH (low treatment) as a $[^{14}C]$glucose/$[^{12}C]$glucose mixture. To determine whether $^{14}CO_2$ captured by the NaOH trap originated from glucose or C_{60}, four other replicates were spiked with $[^{12}C]$glucose. This also helped determine whether mineralization was stimulated by the addition of the glucose substrate.

Radioactivity was measured using a PerkinElmer Tri Carb 3110 TR liquid scintillation analyzer. Samples for radiochemical analyses were prepared by mixing an aliquot of the sample with scintillant (PerkinElmer Optiphase Hisafe 3) for scintillation counting (10 min counting time).

Mineralization is expressed as the percentage of added substrate mineralized or the percentage of CO_2 produced from the added substrate. Rates are expressed as percent per day.

3. RESULTS AND DISCUSSION

Determining the mineralization potential of fullerenes in biosolid-amended soils is essential in assessing their fate, as

fullerenes are likely to reach the soil environment via biosolids. The highly relevant study by Avanasi et al.,[22] which was the first report of the negligible mineralization of C_{60} in unamended soils, had a number of uncertainties in the design of experiments that we have addressed in the study presented here. The low concentration of C_{60} used in this study was confirmed to be sufficient to detect at least 0.25% mineralization of the spiked C_{60} through calculations described in the Supporting Information. This addressed the concern that the concentration and/or activity used in the previous study[22] might have been too low to see detectable mineralization and/or activity in the traps. Second, as demonstrated in our calculations (see the Supporting Information), we ensured that the amounts of NaOH in the CO_2 traps used were sufficient to capture CO_2 from C_{60} mineralization and from soil basal respiration. This was a concern in the previous study, as a CO_2 trap kept for several months could have been completely saturated by CO_2 from the soil's basal respiration and therefore unable to trap CO_2 potentially released from C_{60}. Other concerns from the previous study that were addressed include the microbial activity of the soils used before and after exposure to C_{60}. Control treatments using glucose were prepared as a positive control to demonstrate that the soils used in the experiment were indeed microbially active. Glucose was also

added to systems that have been initially incubated with C_{60} to determine whether C_{60} had any impact on soil microbial activity. Furthermore, mineralization was evaluated for a potential transformation product of C_{60} (C_{60}-OH). To the best of our knowledge, this is the first study of the fate of C_{60}-OH in soils.

SIR tests using the fullerenes reveal negligible mineralization of C_{60} that was incubated in soil for 28 days (Figure 1A) at both concentrations. The high concentrations allowed sensitive detection to 0.025% mineralization of the spiked C_{60}. On the other hand, $[^{14}C]C_{60}$-OH enhanced the mineralization potential of fullerenes, a result that is consistent with the findings from studies of aqueous solutions and with fungi (Figure 1B).[23,24] Mineralization was detected as early as 7 days (first trap collected). At this time point, ~1.7% of $[^{14}C]C_{60}$-OH had mineralized. The total percentage of C_{60}-OH mineralized was 2.6−3.1% in the three soils after incubation for 55 days. These percentages are equivalent to an overall rate of 0.05− 0.06% $[^{14}C]C_{60}$-OH mineralized per day, which is slightly higher than the results obtained by Schreiner et al. for fullerol incubated with white-rot basidiomycete fungi (overall rate of 0.01−0.02% per day; up to 3% CO_2 in 112 days).[24] The mineralization rates were similar (no statistical difference) for the three soils used, which is likely due to the retention of C_{60}-OH in the biosolids. Interestingly, mineralization of C_{60}-OH was also observed in solution (in water, without soil or biosolids), although the mineralization of C_{60}-OH was initially more rapid in soils than in solution (Figure 1C). For example, after incubation for 12 days, 1.8% of C_{60}-OH was mineralized in soil while only 0.6% was mineralized in solution. This difference is likely due to the greater microbial activity in soil than in solution. However, because the water only treatment was not inoculated, the role of photoinduced mineralization of fullerol could have played a role. Such photoinduced mineralization of fullerol in soil would be expected to be even lower than that observed in the water only treatment. Therefore, at least in soil/biosolid systems, the role of biomineralization cannot be ruled out. The amount of C_{60}-OH mineralized in soils, however, leveled off, while it continued to increase in solution. After incubation for 55 days, the %CO_2 values (from $[^{14}C]C_{60}$-OH) produced in soil and in solution were 2.6−3.1 and 4%, respectively; the differences in mineralization between soil and water were statistically significant (Student's t test; $p < 0.05$). The slowing effect of soils on mineralization of C_{60}-OH over time is suggestive of possible interactions with the solid phase such as C_{60}-OH retention. Despite being more hydrophilic than C_{60}, fullerols are moderately retained by soil (unpublished data). Retention would reduce the bioavailability of C_{60}-OH for microbial and/or enzymatic transformation pathways. In solution, where the availability of C_{60}-OH is unaffected by time, mineralization continued at a steady rate.

It is important to note that the SIR test performed using $[^{14}C]$glucose showing its rapid mineralization suggests that the soils used remained active during the experiment. As shown in Figure 2, neither the addition of toluene nor the synthesis matrix affected the overall mineralization of glucose. This suggests that the matrix by which C_{60} and C_{60}-OH were introduced into the soil did not compromise mineralization of glucose and soil microbial activity.

Considering a continuum of oxidized C_{60} species [e.g., $C_{60}(OH)_x(ONa)_y$, where $x + y = 10$−36, $C_{60}O_x$, etc.], the hydroxylated C_{60} used in this study (C_{60}-OH) represents a probable pathway for fullerene mineralization. The addition of
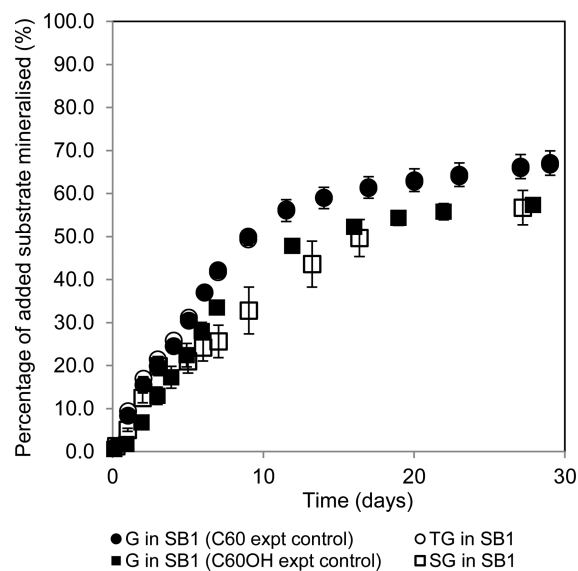

Figure 2. Mineralization of glucose in soil 1 amended with biosolids (SB1). The graphs show mineralization of glucose (G) over 28−29 days in uncontaminated soils (● and ■) and in soils spiked with toluene (T, ○) and the synthesis matrix (S, □). The two glucose mineralization curves are from the C_{60} and C_{60}-OH experiment controls. Error bars correspond to the standard deviation of four measurements. TG in SB1 response overlaps with G in SB1 (C_{60}).

hydroxyl functional groups to the C_{60} cage involves the breaking of C=C bonds, forming less stable C−C bonds, hence compromising the stability of the C_{60} closed-ring structure. Compared to other oxidized forms of C_{60}, such as C_{60} oxides ($C_{60}O$), the (poly)hydroxylated C_{60} $[[^{14}C]$-$C_{60}(OH)_x(ONa)_y$, where $x + y = 24$−26] is likely to have greater bioavailability to microorganisms and susceptibility to hydrolysis in soil because of its higher water solubility.[26] Indeed, studies of the microbial degradation of other carbon nanomaterials (e.g., nanotubes and graphene) suggest that the presence of oxygen-containing functional groups can promote the production of enzymes that can facilitate degradation of these carbon nanomaterials.[30,31]

The tests performed on systems that had been incubated with fullerenes (post-SIR) revealed that the fullerenes did not affect soil microbial activity (Figure 1). The amounts of CO_2 produced (percent mineralized from glucose) in glucose-spiked soils previously incubated with $[^{14}C]C_{60}$ were similar to those from the SIR test in which the only carbon substrate was glucose (Figure 2). Because activity was not detected in the traps of the $[^{14}C]C_{60}/[^{12}C]$glucose setups, the activity measured in the traps of the $[^{14}C]C_{60}/[^{14}C-^{12}C]$glucose setups could be confirmed to be from glucose and not C_{60} (Figure 1). It also suggests that the addition of glucose did not stimulate mineralization of C_{60} in these systems. The same is true for systems initially treated with $[^{14}C]C_{60}$-OH. These demonstrate that exposure of soil to C_{60}- or C_{60}-OH-contaminated biosolids (even at unrealistically high concentrations used to discern a smallest amount of mineralization) did not adversely affect the overall soil microbial activity responsible for the turnover of carbon. The rates of CO_2 production (from glucose) obtained throughout the experiment suggest that the soil was active in all incubations: fullerenes, toluene, and synthesis matrix. These rates are summarized in Table S2.

This study confirmed the results reported by Avanasi et al.[22] on negligible mineralization of C_{60} in soils. Our study, together

with those cited above, demonstrates that C_{60} is not directly mineralized under light-exposed or dark conditions in soils. The poor recovery in our previous study[13] was clearly not due to any mineralization loss, leaving only transformations responsible for the lack of mass balance in that study. Further, our study presents the first set of data on mineralization of hydroxylated C_{60} (C_{60}-OH) in biosolid-amended soils. Mineralization of C_{60}-OH to CO_2 reached up to 3% after incubation for 55 days, with a CO_2 conversion rate that is 100 times slower than that observed for glucose. The additional glucose also did not stimulate mineralization of either C_{60} or C_{60}-OH. Overall, these findings suggest that transformation of fullerenes, exemplified by C_{60}-OH, provides a pathway by which fullerenes are likely to be mineralized in the soil environment. Fullerol's mineralization in soil is, however, slow and appears to be affected by its retention and reduced bioavailability in soil. The precise mechanism for fullerene mineralization in soil (i.e., microbial, enzymatic, or photochemical) and whether C_{60} can transform to fullerol in soils are important subjects for future studies.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.5b00292.

> Fullerol preparation and characterization, soil properties, calculations for mineralization, and rates of C consumption (PDF)

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Notes
The authors declare no competing financial interest.

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