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Priming Effect of Substrate Addition in Soil-Based Biodegradation Tests†

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To test whether substrate addition changes background CO₂ evolution of soil, we measured both ¹⁴CO₂ and net CO₂ evolution from various test compounds. Glucose caused a priming effect, defined as substrate-stimulated soil organic matter mineralization. Formate, benzoate, n-hexadecane, and bis(2-ethylhexyl)phthalate caused no priming, and phenol caused only a transient one. The priming effect of glucose appears to be unusual and does not require a general rejection of net CO₂ evolution measurements in biodegradability testing.

Mineralization (conversion to CO₂), most often measured in biometer flasks (1), is the basis of biodegradability test protocols by the U.S. Food and Drug Administration (12) and the U.S. Environmental Protection Agency (4). While radiolabeled test substrates are preferred, they are not always obtainable, and therefore some biodegradability screening tests must be based on net CO₂ rather than ¹⁴CO₂ evolution. A previous study in this laboratory (7) started to examine some aspects of this test, including whether background CO₂ evolution is altered by the addition of the test material. This previous work was limited by the facts that only one of the test materials was radiolabeled and that test material concentrations were not normalized as to their carbon content. Nevertheless, this work revealed that glucose, a common positive control substance in biodegradation tests, strongly stimulated the background CO₂ evolution from soil, raising concerns about the general validity of test data obtained by the use of nonradiolabeled materials. The follow-up work presented here employed diverse radiolabeled test compounds with their carbon contents normalized to 0.4 mg g⁻¹ of soil. The relationship between ¹⁴CO₂ evolution and net CO₂ evolution was measured and quantified by developing a “priming index” (PI).

Test compounds. The test compounds were selected to cover a range of water solubilities, energy contents, toxicities, and biodegradation rates. Availability in ¹⁴C-labeled form for a range of water solubilities, energy contents, toxicities, and "priming index" (PI).

Calculation of the priming index. When the soil microbial community metabolizes a substrate, some of the substrate carbon is oxidized to CO₂, some is incorporated into microbial biomass, and some may be humified. In short-term experiments, a 50% metabolic efficiency is assumed, and therefore, a 50% conversion of substrate carbon to CO₂ signifies complete or near-complete biodegradation. Unfortunately, metabolic ef-

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Efficacy varies with substrate energy content and experimental conditions within wide limits, and “short term” and “long term” are not easily defined. Therefore, when an unlabeled substrate is used, only net CO₂ evolution that exceeds 100% of the added substrate carbon constitutes clear-cut evidence of a priming effect. Usually, incubation times of 3 to 5 weeks are necessary to reach this point (7).

We have developed the PI to monitor the ratio of substrate carbon and soil organic matter (SOM) mineralization on a continuous and quantitative basis, calculated as net CO₂ evolution (percent of theoretical maximum)/14CO₂ evolution (percent of total radiocarbon added).

If substrate addition does not increase or decrease the mineralization of SOM, the PI equals 1.0. If the added substrate stimulates SOM degradation, the PI will significantly exceed 1.0. If the added substrate inhibits SOM degradation, the PI value will drop below 1.0. This calculation allows the determination of substrate effects on SOM degradation in short-term experiments and makes it possible to monitor changes with time in the direction and intensity of the effect.

Net CO₂ and 14CO₂ evolution of six substrates. In fresh Nixon Sandy Loam (texture: sand, 50%; silt, 21%; clay, 29%; SOM, 5%; pH, 5.5 to 6.0; WHC, 0.65 g/g of dry soil) the priming effect of glucose reported earlier (7) was readily reproduced (Fig. 1). While net CO₂ evolution (Fig. 1A) exceeded 100%, 14CO₂ evolution reached only 50% in 75 days (Fig. 1B), resulting in a PI of approximately 2, which remained fairly constant during the experiment (Fig. 1C). The similarly water-soluble and rapidly degraded benzoate did not produce this effect; both net CO₂ evolution and 14CO₂ evolution leveled off around 70% carbon conversion, and the PI was close to 1 throughout the experiment. Initially, n-hexadecane somewhat inhibited SOM degradation, resulting in a PI lower than 1, but a stable PI around 1 was reached after 25 days. Both net CO₂ and 14CO₂ evolutions showed an approximate 50% mineralization in 75 days. Net CO₂ evolution from bis(2-ethylhexyl)phthalate was only around 20% in 75 days, while the evolution of 14CO₂ reached 40%, indicating a negative interference of this compound on SOM mineralization. The PI pattern for this compound was omitted from Fig. 1C, because it was confusing and represented an artifact of the 14C labeling of bis(2-ethylhexyl)phthalate. This compound was not uniformly labeled but was labeled only in the aromatic portion of the molecule. Apparently, only the aliphatic side chains were initially mineralized and not the labeled portion of the molecule, resulting in a complex and fluctuating PI that did not reflect the true effect of bis(2-ethylhexyl)phthalate on SOM mineralization.

Following this experiment, we also explored the patterns produced by a highly toxic substrate, phenol (2a), and by one with an extremely low energy content, formate (Fig. 2). Phenol at 0.4 mg/g of soil produced an initial burst of net CO₂ evolution (Fig. 2A) that, in the light of some air drying and fumigation experiments (8), we interpret as the mineralization of killed microbial biomass by more resistant surviving microorganisms. Clearly, it was not matched by 14CO₂ evolution from phenol (Fig. 2B). After 1 week, cumulative net CO₂ evolution started to decline in comparison with that in the control soil that did not receive phenol (Fig. 2A), even though some of the phenol was being mineralized (Fig. 2B). This was reflected also by the unusual pattern of the PI (Fig. 2C), which was positive at 2.5 and subsequently declined to 1.2 by day 25. To aid our interpretation of this priming effect, we also tested phenol at additional concentrations of 0.025, 0.1, and 0.2 mg/g of soil. At 0.2 mg/g (result not shown), the temporal PI pattern resembled the one caused by 0.4 mg/g, but the PI maximum reached only

<table>
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<th>TABLE 1. Radiocarbon-labeled compounds used</th>
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<tr>
<td><strong>Compound</strong></td>
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<tr>
<td>---------------</td>
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<tr>
<td>UL d-glucose</td>
</tr>
<tr>
<td>n-1-Hexadecane</td>
</tr>
<tr>
<td>Formate</td>
</tr>
<tr>
<td>Ring-UL benzoate</td>
</tr>
<tr>
<td>UL phenol</td>
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<tr>
<td>Ring-UL bis(2-ethylhexyl)phthalate</td>
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| a Radiochemical purity, as determined by the manufacturer by using high-pressure liquid chromatography, was 98% or higher, and the compounds were used without further purification. UL, uniformly labeled.
To summarize these experiments, a positive PI, which may make an unlabeled test substrate appear to degrade more extensively than it actually does, was evident only in the case of glucose. Toxic substances may give a transient positive PI, but this is too brief to be confused with extensive biodegradation of the test compound. There is evidence showing that biodegradation of some hydrophobic substances such as n-hexadecane and bis(2-ethylhexyl)phthalate, as measured by net CO$_2$ evolution, may be somewhat underestimated, as they seem to suppress to some extent the degradation of SOM.

Historically, the stimulation of mineralization by added extraneous substrate was reported by Broadbent and Norman in 1946 (2), who then coined the term “priming effect” to describe it. The focus of subsequent papers, too numerous to review here, was degradation of agricultural plant residues (green manures) in soil. Uneven radiolabeling of the plant material components made these experiments hard to interpret. However, some authors also noted the priming effect of glucose (3, 10), and one may assume the presence of glucose and glucose polymers also in plant materials. The priming effect was reported to be capricious, being present and absent in the same series of experiments without a clear reason (9).

No new research on the subject was published after the 1970s, and Tate (11) in his 1987 book on SOM regarded the whole priming-effect phenomenon as an experimental artifact. Our research on biodegradability tests has revived the subject in a different context.

We are somewhat at a loss to explain why glucose has the unusual priming effect evident in these experiments. Benzoate, which degrades even faster and has a higher energy content than glucose, does not show this effect. Because of its anomalous behavior, glucose is not an appropriate positive control compound in soil biodegradation studies, but benzoate is suitable. The question of whether compounds unrelated to glucose can give strong positive priming effects cannot be answered conclusively at this time. So far, the behavior of glucose appears to be unusual and does not warrant a general rejection of the net CO$_2$ measurement approach.

**REFERENCES**


