Environmental Pollution 242 (2018) 769–777

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Unraveling microbial turnover and non-extractable residues of bromoxynil in soil microcosms with ^{13}C-isotope probing☆

Karolina M. Nowak [a, b, *], Markus Telscher [c], Erika Seidel [c], Anja Miltner [b]

[a] Chair of Geobiotechnology, Technische Universität Berlin, Ackerstraße 76, 13355 Berlin, Germany
[b] Helmholtz-Centre for Environmental Research – UFZ, Department of Environmental Biotechnology, Permoserstr. 15, 04318 Leipzig, Germany
[c] Bayer CropScience AG, Alfred-Nobel-Str. 50, 40789 Monheim am Rhein, Germany

ARTICLE INFO

Article history:
Received 27 April 2018
Received in revised form
1 July 2018
Accepted 11 July 2018
Available online 17 July 2018

Keywords:
Herbicide
Microbial activity
Biodegradation
Bound residues
Biogenic residues

ABSTRACT

Bromoxynil is a widely used nitrile herbicide applied to maize and other cereals in many countries. To date, still little is known about bromoxynil turnover and the structural identity of bromoxynil non-extractable residues (NER) which are reported to occur in high amounts. Therefore, we investigated the microbial turnover of ^{13}C-labeled bromoxynil for 32 days. A focus was laid on the estimation of biogenic NER based on the turnover of ^{13}C into amino acids (AA). At the end, 25% of $^{13}C_6$-bromoxynil equivalents were mineralized, 2% assigned to extractable residues and 72.5% to NER. Based on 12% in the ^{13}C-total AA and an assumed share of AA of 50% in microbial biomass we arrived at 24% of total ^{13}C-biogenic NER. About 33% of the total ^{13}C-NER could thus be explained by ^{13}C-biogenic NER; 67% was unknown and by definition xenobiotic NER with potential for toxicity. The ^{13}C label from $^{13}C_6$-bromoxynil was mainly detected in the humic acids (28.5%), but significant amounts were also found in non-humics (17.6%), fulvic acids (13.2%) and humins (12.7%). The ^{13}C-total amino acids hydrolyzed from humic acids, humins and fulvic acids amounted to 5.2%, 6.1% and 1.2% of $^{13}C_6$-bromoxynil equivalents, respectively, corresponding to total ^{13}C-biogenic NER amounts of 10.4%, 12.2% and 2.4%. The humins contained mostly ^{13}C-biogenic NER, whereas the humic and fulvic acids may be dominated by the xenobiotic NER. Due to the high proportion of unknown ^{13}C-NER and particularly in the humic and fulvic acids, future studies should focus on the detailed characterization of these fractions.

1. Introduction

Bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) is the most widespread nitrile herbicide applied to maize and other cereals in Germany and other countries (Scheunert et al., 2001; Cai et al., 2011). Bromoxynil dissipates quickly in soils with a half-life (DT_{50}) of a few days (Scheunert et al., 2001; Zablotowicz et al., 2009). High or moderate mineralization of bromoxynil (20–60%) was observed in various agricultural soils (Collins, 1973; Poßberg et al., 2016; Scheunert et al., 2001; Smith, 1971; Zablotowicz et al., 2009). Studies with pure cultures of bacteria (*Flavobacterium* sp., *Rhodococcus rhodochrous* or *Klebsiella pneumoniae*) revealed that bromoxynil is converted to 3-oxoadipate via the intermediate products 3,5-dibromo-4-hydroxybenzamide or 2,6-dibromohydroquinone (McBride et al., 1986; Stalker et al., 1988; Topp et al., 1992; Vokounová et al., 1992; see also degradation pathway in Supplementary Information Fig. S1). The 3-oxoadipate is transformed to succinyl-CoA and acetyl-CoA which are the potential carbon inflow pathways into the tricarboxylic acid cycle (TCA) for bromoxynil. However, to date, detailed knowledge on microbial turnover and carbon assimilation pathways via these 3-oxoadipate intermediates from bromoxynil in soil is still missing.

A high formation of non-extractable residues (NER; 50–70%) from bromoxynil is the current concern related to this herbicide turnover in soils (Poßberg et al., 2016; Scheunert et al., 2001; Zablotowicz et al., 2009). To date, it is not clear whether the NER are dominated by bromoxynil or its potentially toxic metabolites (xenobiotic NER) or by the non-toxic biogenic NER (bioNER; Kästner et al., 2014; Nowak et al., 2011). Microorganisms can use carbon derived from the pesticide to synthesize their biomass compounds and to grow; after their death the pesticide-derived carbon accumulated in their biomass is stabilized in the organic

☆ This paper has been recommended for acceptance by Dr. Chen Da.

* Corresponding author. Chair of Geobiotechnology, Technische Universität Berlin, Ackerstraße 76, 13355 Berlin, Germany
E-mail addresses: karolina.nowak@ufz.de, karolina.nowak@tu-berlin.de (K.M. Nowak).

https://doi.org/10.1016/j.envpol.2018.07.049
0269-7491/

matter (OM) forming bioNER (Nowak et al., 2011). Previous studies reported a major contribution of bioNER to the NER from $^{13}C_6$ to 2,4-D, $^{13}C_6$-ibuprofen, $^{13}C_3^{15}N$-glyphosate and $^{13}C_6$-metamitron (Nowak et al., 2011, 2013; Wang et al., 2016, 2017a; 2017b). Poßberg et al. (2016) have recently estimated that 37% of the ^{14}C-NER from ^{14}C-bromoxynil could be assigned to bioNER. Most of the ^{14}C-NER from ^{14}C-bromoxynil was recovered in the humic acids followed by the fulvic acids (Zablotowicz et al., 2009); however, the structural identity of ^{14}C-carrying compounds accumulated in these fractions is unknown. Therefore, the bromoxynil-NER are a 'black box' when assessing the fate of bromoxynil. The knowledge about identity and mechanisms of NER formation is necessary for a proper assessment of potential risks associated with the NER (Kästner et al., 2014; Nowak et al., 2011). Particular attention is paid to the sequestered xenobiotic NER with release potential (Kästner et al., 2014).

Several studies about the fate of bromoxynil in soils using radiotracers have been published (Collins, 1973; Poßberg et al., 2016; Scheunert et al., 2001; Smith, 1971; Zablotowicz et al., 2009). Radiotracers enable tracking of contaminant fate at environmental concentrations (Kästner et al., 2014), but only allow a rough bioNER quantitation in complex environmental systems (Poßberg et al., 2016). Although stable isotope tracers need to be applied at high concentrations due to the high natural abundance of these isotopes, they allow detailed investigation of compound turnover and in particular the formation of bioNER (Nowak et al., 2011, 2013; Wang et al., 2016, 2017a; 2017b). For this reason, ^{13}C-isotope labeling was implemented to study the turnover of bromoxynil in soil, including closer characterization of the NER formed from this compound.

This study thus aimed to (i) determine the microbial turnover of $^{13}C_6$-bromoxynil in natural soil, (ii) to quantify the incorporation of carbon-derived bromoxynil into microbial biomass (fatty acids and amino acids), and to (iii) assess the contribution of bioNER versus xenobiotic NER to the NER. In addition, the distribution of ^{13}C-bioNER within the humic substances fractions of the soil was also investigated and the contents of ^{13}C-bioNER in each fraction quantified.

2. Materials and methods

2.1. Chemicals

All the chemicals used in this study were obtained from Carl Roth (Karlsruhe, Germany) or VWR/Merck (Darmstadt, Germany), unless otherwise specified. Phenyl ring labeled $^{13}C_6$-bromoxynil (^{13}C isotopic purity 99 at%; chemical purity 98%) was provided by Bayer Crop Science, Monheim, Germany.

2.2. Reference soil

The reference soil was a sandy loam collected from the topsoil layer (0–20 cm) of a grassland area at Laacher Hof (N51°04.647′ E6°53.517′) in Monheim (Rhein), Germany. The soil had not received any pesticides in the last five years. It consisted of 7% clay, 17% silt and 76% sand. The contents of total organic carbon and total nitrogen were 1.9% (w/w) and 0.16% (w/w), respectively. The maximum water holding capacity (WHC_{max}) was 52%, and the pH was 5.1. The soil was sieved through a 2 mm screen prior to the incubation experiment.

2.3. Experimental setup and incubation conditions

Incubation experiments were performed according to the OECD guideline 307 (OECD, 2002). Three different treatments were prepared as follows (i) soil with ^{13}C-labeled bromoxynil, (ii) soil with unlabeled bromoxynil (control I), (iii) soil without bromoxynil (control II). The two controls were included to correct for the natural abundance of ^{13}C in the analyzed samples. All soils except for control II were spiked with bromoxynil, either unlabeled or -labeled, dissolved in acetone. To prevent killing of all microorganisms and an introduction of an additional carbon source, only 10% of the soil used for incubation was spiked with the herbicide. After evaporation of the acetone excess, the spiked soil was mixed with the non-spiked soil. The final concentration of bromoxynil in the soil was 50 mg kg^{-1}. Although this concentration is far beyond the recommended dose for agriculture, it was necessary to obtain reliable isotopic enrichment results against the natural ^{13}C background. For comparison, an additional setup at a lower dose (2 mg kg^{-1}) closer to recommended agronomic bromoxynil dose of 0.6 mg kg^{-1} was also conducted. In addition, to understand the potential effect of this high-dose application on microbial activity, respiration and microbial biomass analyses were included and the parameters were compared with that in the control soil without bromoxynil and at low concentration. This enabled the assessment of possible concentration effects on the mineralization of this herbicide. However, the detailed mass balance analysis was restricted to the setup with the higher concentration. All experiments were conducted in triplicate, in the dark and at 20 °C (±2 °C) for 32 days. Carbon dioxide derived from the mineralized bromoxynil and OM of soil was absorbed in 2 M NaOH traps placed inside each incubation vessel. The bottles were periodically flushed with humidified air to supply oxygen for soil respiration after each sampling. The NaOH solution was exchanged and measured after 2, 5, 9, 15, 23 and 32 days in all systems. The soil was sampled and analyzed after 32 days.

2.4. Mass balance

The turnover mass balance of bromoxynil in soil was determined by analyzing solvent-extractable bromoxynil and its primary transformation products as well as ^{13}C in CO_2 and total NER. This general mass balance based on the ^{13}C-labeling technique was extended by fatty acid (FA) and amino acid (AA) analyses. This allowed estimating the extent of ^{13}C label incorporation into microbial biomass and ultimately bioNER formation from $^{13}C_6$-bromoxynil.

Mineralization. The CO_2 contents in the NaOH traps, originating from soil respiration and mineralization of $^{13}C_6$-bromoxynil, were measured by means of a total organic carbon analyzer (Multi N/C 21005, Jena, Germany). The isotopic composition of CO_2 was determined by gas chromatography-isotope ratio mass spectrometry (GC-irMS; Finnigan MAT 252, Thermo Electron, Bremen, Germany, coupled to Hewlett Packard 6890 GC; Agilent Technologies, Germany), after separation from other permanent gases on a Poraabond Q-HT Plot FS column (50 m × 0.32 mm × 5 µm; Chrompack, Middleburg, Netherlands; Girardi et al., 2013). Mineralization of $^{13}C_6$-bromoxynil was calculated from the total amount of CO_2 and its isotopic composition.

Extractable bromoxynil and transformation products. Bromoxynil and its metabolites were extracted from soil in two steps. The first extraction involved 2 h shaking of soil with 0.01 M $CaCl_2$ and aimed at assessing readily available bromoxynil (Zablotowicz et al., 2009). After shaking, soil extracts were centrifuged (10 min, 5000 rpm) and the supernatant was collected. The accessible bromoxynil and metabolites associated to the soil matrix were further extracted from the soil residue in the second step using methanol/water (80%/20%) and shaking for 20 h at room temperature. Thereafter, in analogy to $CaCl_2$ extract, the soil pellets were also separated from the supernatant by centrifugation. Aliquots of the supernatants of both 0.01 M $CaCl_2$ and 80% methanol were purified separately over SPE columns (CHROMABOND® EASY, 200 mg,