Technical Note: Enhanced reactivity of nitrogenous organohalogen formation from plant litter to bacteria

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Abstract

C1/C2 organohalogens (organohalogens with one or two carbon atoms) can have significant environmental toxicity and ecological impact, such as carcinogenesis, ozone depletion and global warming. Natural halogenation processes have been identified for a wide range of natural organic matter, including soils, plant and animal debris, algae, and fungi. Yet, few have considered these organohalogens generated from the ubiquitous bacteria, one of the largest biomass pools on Earth. Here, we report and confirm the formation of chloroform (CHCl₃) dichloro-acetonitrile (CHCl₂CN), chloral hydrate (CCl₃CH(OH)₂) and their brominated analogues by direct halogenation of seven strains of common bacteria and nine cellular monomers. Comparing different major C stocks during litter decomposition stages in terrestrial ecosystems, from plant litter, decomposed litter, to bacteria, increasing reactivity for nitrogenous organohalogen yield was observed with decreasing C/N ratio. Our results raise the possibility that natural halogenation of bacteria represents a significant and overlooked contribution to global organohalogen burdens. As bacteria are decomposers that alter the C quality by transforming organic matter pools from high to low C/N ratio and constitute a large organic N pool, the bacterial activity is expected to affect the C, N, and halogen cycling through natural halogenation reactions.

1 Introduction

Several C1/C2 organohalogens have been documented to be carcinogenic and toxic (USEPA, 1999), whereas some volatile species pose threats to the ozone depletion (Anderson et al., 1991; Read et al., 2008) or serve as greenhouse gases (Lashof and Ahuja, 1990; Montzka et al., 2011). Natural halogenation processes, promoted either by thermal (Hamilton et al., 2003; Weissflog et al., 2005), enzymatic (Hoekstra et al., 1998; Blasiak and Drennan, 2009), or Fenton/Fenton-like reactions (Fahimi et al., 2003; Huber et al., 2009), contribute significantly to the global budget of these hazardous
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For example, the chloroform flux through the environment is estimated at $660 \pm 220 \times 10^9$ gyr$^{-1}$ with 90% of natural origin (McCulloch, 2003), and that of bromoform at $\sim 220 \times 10^9$ gyr$^{-1}$ with 70% from macroalgae (Carpenter and Liss, 2000). Abiotically, aliphatic volatile organohalogens were yielded from senescent and dead leaves (Hamilton et al., 2003), Fe-oxhydroxide and halide-containing soil or sediment (Keppler et al., 2000) (or with $H_2O_2$; Fahimi et al., 2003; Huber et al., 2009), and emissions of savannah fires, volcanoes, hydrothermal sources, and salt mines (Weissflog et al., 2005; Gribble, 2010). Biotically, enzymes like haloperoxidases and halogenases widespreadly found in soils and oceans are thought to contribute the major sources of environmental organohalogens (Nightingale et al., 1995; Hoekstra et al., 1998; Oberg, 2002; Reddy et al., 2002; Ortiz-Bermudez et al., 2007; Blasiak and Drennan, 2009; Wagner et al., 2009). Although many efforts have identified a wide range of natural organic matters (NOMs) from soils, plant and animal debris, algae, and fungi as precursors of organohalogens (Nightingale et al., 1995; Hoekstra et al., 1998; Keppler et al., 2000; Myneni, 2002; Fahimi et al., 2003; Hamilton et al., 2003; Huber et al., 2009), the source inventories and fluxes for C1/C2 organohalogens were still far from well quantified, with few taking the bacteria into account.

However, bacteria may constitute one of the largest precursors for organohalogens that has been overlooked if the bacterial materials can be halogenated like other natural organic matters. As reported, there exist approximately $4\sim6 \times 10^{30}$ bacterial cells (or $350\sim550 \times 10^{15}$ g C) on Earth, with a biomass at least comparable to global plant biomass (Hogan, 2010; Whitman et al., 1998). A recent study also suggested that continuous cell generation enables microbial necromass to be the majority of soil organic matter in terrestrial systems, amounting to 40 times higher than previously thought (Liang and Balser, 2011). During the litter decomposition and humus formation, most inorganic halide ions in litter are gradually transferred into organic form (Flodin et al., 1997; Myneni, 2002), in particular, with up to 95% of Cl, 91% of Br, and 81% of I in organic form in peat (Biester et al., 2004). As the decomposers, bacteria that continuously incorporate external NOMs into bacterial cellular materials (Benner, 2011; Liang...
and Balser, 2011) are expected to play important role in humification. Although bacteria have some similar components (e.g., carbohydrate and protein) with plant materials that have activities to be halogenated, whether or to what degree this secondary production can be halogenated are poorly known. Furthermore, bacterial biomass pool contains about 10-fold more N than do plants on Earth (Whitman et al., 1998) and has a much lower C/N ratio (commonly 3–6) than plant. As we hypothesize, the ubiquitous bacterial cellular materials can be a significant natural precursor of organohalogenes contributing to environmental organohalogen burdens, and has rather different halogenating reactivity compared to the plant materials.

Among different natural halogenation routes, halogenation of organic matter by the reactive halogen species (e.g., OCl− generated by chloroperoxidase) is the most common proposed route for natural organohalogen generation (Öberg, 2002; Ortiz-Bermudez et al., 2007; Wagner et al., 2009). In this study, seven strains of common bacteria (B1: Acinetobacter junii; B2: Aeromonas hydrophila; B3: Bacillus cereus; B4: Bacillus subtilis; B5: Escherichia coli; B6: Shigella sonnei; and B7: Staphylococcus sciuri) at stationary phase and nine monomers of bacterial materials were selected to explore the maximum potential to form organohalogenes by direct halogenation (using ∼ 50 mmol L−1 NaOCl solution as in Albers et al., 2011). Also, their halogenating reactivity were compared with those of four fresh and decomposed plant litter materials to explore how the natural organic matter transformation may impact on the organohalogen formation potential.

2 Material and methods

After a pre-incubation test to determine the time span of the stationary phase for each bacterial species, all seven strains of bacteria were inoculated into nutrient broth (10 % of the instructed dosage) and grown overnight (16–20 h) to reach the stationary phase. Bacterial cells were collected by centrifugation (11 600 g, 5 min) of aliquots of bacterial cultures, and then washed three times with saline solution (0.9 % NaCl; purity: 99.5 %)
to remove the nutrient broth (Shang and Blatchley, 2001). The cell pellets were re-
suspended in deionized water in duran bottles to obtain a cell density in the range of
$10^4$–$10^7$ colony forming units per millilitre (CFU ml$^{-1}$) for the chlorination test. Because
of the strong correlations between bacterial count and bacterial total organic carbon
(TOC) concentration (data not shown), all pure bacterial suspensions were diluted to
different bacterial carbon concentrations for better comparison. Bacterial total organic
carbon (TOC) analysis was performed using a TOC analyzer (Shimadzu) combined
with the SSM-500A (Solid Sample Module). In parallel to the pure chlorination test, bromide at different levels was added to before chlorination of B5, B6, and B7 to ex-
amine the bromide’s effects on organohalogen formation.

To better understand the precursors in bacteria at the molecular level, solutions of
nine monomers (five amino acids: glycine, threonine, asparagine, tyrosine, and trypt-
ophan; four nucleic acids: uracil, thymine, cytimidine and adenine) were selected for
halogenation. In addition, extracts from fresh litter and decomposed litter (1–5 yr de-
composed litter) of different plant species (blue oak (Quercus douglasii), live oak (Quer-
cus wislizeni), foothill pine (Pinus sabiniana), and annual grasses (Bromus diandrus
and Avena fatua)) from the Sierra Foothill Research Extension Center in Yuba County,
CA (39° 15’ 4” N, 121° 18’ 47” W) were prepared for halogenation using the same sam-
ples and methods as in Chow et al. (2011).

In the chlorination test, $\sim 50$ mmol$\cdot$l$^{-1}$ NaOCl solution was used as the halogenating
agent. All pure bacterial suspensions, monomer solutions, fresh litter and partially de-
composed litter extracts with different carbon concentrations were chlorinated under
the following conditions: (i) pH: 8.0 ± 0.2; (ii) temperature: 20.0 ± 1.0 °C; (iii) incubation
time: 24 ± 1 h; and (iv) Cl residual: 0.028 ± 0.011 mmol$\cdot$l$^{-1}$ (i.e., 1.0 ± 0.4 mg l$^{-1}$). EPA
Method 551 was adopted for the organohalogen quantification.
3 Results and discussion

After 24 h halogenating reaction, chloroform (CHCl\textsubscript{3}), dichloro-acetonitrile (CHCl\textsubscript{2}CN) and chloral hydrate (CCl\textsubscript{3}CH(OH)\textsubscript{2}) were generated from all tested bacterial strains, with average reactivity of 0.567–3.66 mmol-CHCl\textsubscript{3} mol\textsuperscript{-1}-C, 0.311–1.72 mmol-CHCl\textsubscript{2}CN mol\textsuperscript{-1}-C and 47.09–147.9 µmol-CCl\textsubscript{3}CH(OH)\textsubscript{2} mol\textsuperscript{-1}-C, respectively (Fig. 1a). Trace amount of dichloro-bromomethane (CHCl\textsubscript{2}Br) (25.6–88.3 µmol-CCl\textsubscript{3}CN mol\textsuperscript{-1}-C) and trichloro-acetonitrile (CCl\textsubscript{3}CN) (3.26–27.4 µmol-CCl\textsubscript{3}CN mol\textsuperscript{-1}-C) were also detected for all seven bacteria but were commonly less than 5 % of CHCl\textsubscript{3} and 5 % of CHCl\textsubscript{2}CN, respectively. These results confirmed our hypothesis that pure bacterial materials may serve as precursors of C1/C2 organohalogens. With increasing bacterial carbon concentration, the CHCl\textsubscript{2}CN and CCl\textsubscript{3}CH(OH)\textsubscript{2} concentrations were enhanced linearly ($R^2$: 0.912–0.999, $n = 15$, $P < 0.001$ for CHCl\textsubscript{2}CN; $R^2$: 0.842–0.998, $n = 15$, $P < 0.001$ for CCl\textsubscript{3}CH(OH)\textsubscript{2}). Dissimilarly, the yield of CHCl\textsubscript{3} did not consistently show linear increase with the bacterial C concentration.

As bromide is ubiquitous in marine and coastal environments (~65 ppm in seawater), it is expected to participate in the natural process of bacterial halogenation and contribute to the yield of hazardous organobromine (Nightingale et al., 1995; Keppler et al., 2000; Leri and Myneni, 2012). Thus, we added different amounts of bromide to B5 (Escherichia coli), B6 (Shigella sonnei) and B7 (Staphylococcus sciuri) during chlorination to determine the effects of Br. All three bacteria displayed the same trends of organohalogen concentrations with increasing Br concentration. Taking B7 as an example, the effects of Br can be inferred from Fig. 1b. The pure chlorinated species (CHCl\textsubscript{3}, CHCl\textsubscript{2}CN, and CCl\textsubscript{3}CH(OH)\textsubscript{2}) generally decreased with increasing Br concentration, whereas brominated analogues were generated in the presence of Br. Mono- and di-brominated methanes both peaked at 12.5 µmol l\textsuperscript{-1} Br, whereas bromoform dominated all halogenated methanes (also all 8 tested C1/C2 organohalogens) at Br concentrations ≥25 µmol l\textsuperscript{-1}. Brominated acetonitrile was found with peak concentrations at 12.5 or 25 µmol l\textsuperscript{-1} Br concentrations, but all halogenated acetonitriles
dropped to undetectable level when the Br level increased to >50 µmol l \(^{-1}\), probably resulting from the formation of more reactive halogenating species (OBr\(^{-}\)), which promoted the yield of cyanogen halide (CNX) and other N-organohalogen species rather than haloacetonitriles (Heller-Grossman et al., 1999; Hua et al., 2006; Le Roux et al., 2012).

The reaction reactivity of different bio-molecules (Fig. 2) confirmed the possibility of abiotic reaction so that large amount of bacterial necromass (Liang and Balser, 2011) can also be organohalogen precursor as long as halogenating reagent exists. Still, these monomers had highly different reactivity to generate different C1/C2 organohalogens dependent on the molecular structure. All tested monomers showed reactivity to generate both CHCl\(_3\) and CCl\(_3\)CH(OH)\(_2\) (except glycine for CCl\(_3\)CH(OH)\(_2\) generation), with tyrosine, tryptophan and uracil having the highest reactivity. Three out of five amino acids but no nucleic acid were able to yield CHCl\(_2\)CN, with reactivity following tyrosine > tryptophan > asparagine (t-test). The structure-activity relationship showed that the phenol containing compound (tyrosine) had the strongest reactivity to generate all three target organohalogens as previous studies suggested (Keppler et al., 2000; Fahimi et al., 2003; Huber et al., 2009), whereas the heterocyclic compounds did not necessarily have high reactivity and were highly influenced by the functional groups attaching to the ring (e.g., enhanced reactivity by carbonyl comparing uracil with cytididine; weakened by methyl comparing uracil with thymine).

The strong linear relationship in dichloro-acetonitrile and chloral hydrate but fluctuation in CHCl\(_3\) yielded with bacterial C (Fig. 1a) may be explained by reactivity of cellular monomers. Within these nine monomers, the ranking of standard error of reactivity was CHCl\(_2\)CN (0.54 mmol mol\(^{-1}\) – C) < CCl\(_3\)CH(OH)\(_2\) (1.75 mmol mol\(^{-1}\) – C) < CHCl\(_3\) (5.23 mmol mol\(^{-1}\) – C), which was consistent with the ranking of the significance of regressions between organohalogen concentration and bacterial C (CHCl\(_2\)CN > CCl\(_3\)CH(OH)\(_2\) > CHCl\(_3\)). This evidence supports the possibility that bacteria metabolism accompanied by shifting relative abundance of certain molecules would have led to high variation of CHCl\(_3\) formation.
Unlike most biomass from primary production, bacterial biomass is commonly considered as secondary production that has a net effect of moving organic matter and inorganic nutrients from the external environment to bacterial cells (Ducklow, 2000), and thus constitutes the large C (350–550 × 10^{15} g) and N pools (85–130 × 10^{15} g) (Whitman et al., 1998). Although no difference in formation reactivity of CHCl_3 or CCl_3CH(OH)_2 between fresh litter and bacteria was observed (both P > 0.1; based on t-test), the difference in CHCl_2CN formation reactivity between fresh litter and bacteria was highly significant (P < 0.001). With lower C/N ratio, bacteria showed enhanced reactivity for CHCl_2CN formation compared with plant litters and their partially decomposed materials under the same halogenating condition (Fig. 3). Previous studies indicated that the total organic halide increased during the humification process as C/N ratio decreased (Myneni, 2002; Keppler and Biester, 2003; Keppler et al., 2003). In this study, we showed that the essential role of the bacteria as decomposer may enhance the fraction of nitrogenous organohalogen in the total organic halide, thereby influencing C, N and halogen cycling via natural halogenation processes (Fig. 4). Supposing that the bacterial materials and plant litter materials have the same opportunity to be halogenated by the environmental OCl^−, the bacterial material may have at least comparably contributed to global C1/C2 organohalogen budget (at least for the three groups of organohalogens here) through this direct halogenation pathway because its large biomass on Earth as claimed (Whitman et al., 1998; Hogan, 2010) and ubiquitous necromass caused by fast turnover rate (Benner, 2011; Liang and Balser, 2011).

Although some bacteria are considered as sinks for organohalogens via their ability to utilize or remove organohalogens by biotic dehalogenation processes (Suflita et al., 1982; McAnulla et al., 2001), our results confirm that bacterial materials can be a significant source for organohalogens in the presence of halogenating reagents (like OCl^− generated from enzymes). While bacteria transfer organic carbon from the primary production into the secondary production, their halogenated products can be shifted with the changing C/N ratio. However, the magnitude of global organohalogen productions from bacteria is still difficult to predict limited by the single and direct halogenating
approach we adopted among many. Moreover, multiple factors such as the ambient environmental conditions, bacterial C quality, and the presence of different halides will also cause uncertainty based on our results. Further studies exploring halogenating processes for bacterial materials and field observations of organohalogens yields associated with bacterial biomass in different biomes will help us better understand a more quantitative contribution of bacterial-derived organohalogens.

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References


Fig. 1. Formation of some C1/C2 organohalogens by halogenation of bacteria. (a) Effects of bacterial C concentrations (at 0.0, 0.083, 0.167, 0.333, and 0.50 mmol L\(^{-1}\)). Error bars show SD from 3 replicates. (b) Effects of bromide (at 0, 6.25, 12.5, 25, and 50 µmol L\(^{-1}\) from KBr) taking *Staphylococcus sciuri* at 0.5 mmol-C L\(^{-1}\) as example. B1: *Acinetobacter junii*; B2: *Aeromonas hydrophila*; B3: *Bacillus cereus*; B4: *Bacillus subtilis*; B5: *Escherichia coli*; B6: *Shigella sonnei*; and B7: *Staphylococcus sciuri*.
Fig. 2. Halogenation reactivity of 9 bacterial cellular monomers. Error bars show standard deviation from totally 10 replicates (two for each C concentration: 0.083, 0.167, 0.250, 0.333, and 0.5 mmol-C l⁻¹).
Fig. 3. Reactivity of dichloro-acetonitrile formation for extracts of fresh litter (n = 32), decomposed litter (n = 30) and bacterial suspensions (n = 28) along a C/N ratio gradient. B1–B7 refer to the same seven different bacterial strains in Fig. 1.
Fig. 4. Conceptual model for the impact of bacterial activity on C and N cycling during halogenation.