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Technical Note: Reactivity of C1 and C2 organohalogens 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 ($CHCl_3$) dichloro-acetonitrile ($CHCl_2CN$), chloral hydrate ($CCl_3CH(OH)_2$) 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, we found increasing reactivity for nitrogenous organohalogen yield 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 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 organohalogens (Gribble, 2010). For example, the chloroform flux through the environment is estimated at $660 \pm 220 \times 10^9\,\mathrm{g\,yr^{-1}}$ with 90 % of natural origin (McCulloch, 2003), and that of bromoform at $\sim 220 \times 10^9\,\mathrm{g\,yr^{-1}}$ with 70 % from macroalgae (Carpenter and Liss, 2000). Abiotically, aliphatic volatile organohalogens were released from senescent and dead leaves (Hamilton et al., 2003), Fe-oxyhydroxide 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 from savannah fires, volcanoes, hydrothermal sources, and salt mines (Weissflog et al., 2005; Gribble, 2010). Biotically, enzymes like haloperoxidases and halogenases widespread in soils and oceans are thought to contribute the major sources of environmental organohalogens (Nightingale et al., 1995; Hoekstra et al., 1998; Öberg, 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), there is great uncertainty associated with source inventories and fluxes for C1/C2 organohalogens, with few taking bacteria precursors into account.

Bacteria may constitute one of the largest precursors for organohalogens that has been overlooked, assuming that bacterial materials can be halogenated like other natural organic matter. As reported, there exist approximately 4–6 $\times$ 10^{30} bacterial cells (or 350–550 $\times$ 10^{15} g C) on Earth, with a biomass at least comparable to global plant biomass (Hogan, 2010; Whitman et al., 1998). Bacteria have been widely found in terrestrial water (Jones, 1971), as well as in the ocean (Ducklow et al., 2000; Jiao et al., 2010). A recent study also suggested that continuous cell generation enables microbial necromass to comprise the majority of soil organic matter in terrestrial systems, amounting to 40 times higher estimates than previously reporteed (Liang and Balser, 2011). During 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 decomposers, bacteria that continuously incorporate external NOMs into bacterial cellular materials (Benner, 2011; Liang and Balser, 2011) are expected to play an 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, the 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 plants. As we hypothesize, the ubiquitous bacterial cellular materials can be a significant natural precursor of organohalogens contributing to environmental organohalogen burdens, and has rather different halogenating reactivity compared to plant materials.

Among different natural halogenation routes, halogenation of organic matter by 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 organohalogens by direct halogenation (using NaOCl solution as in Albers et al., 2011). This study focused on three types of C1 and C2 organohalogens: haloforms, halo-acetonitriles, and chloral hydrate, representing the major carbonous, nitrogenous and oxygenous organohalogens formation, respectively. Also, we compared the halogenating reactivity of four fresh and four decomposed plant litter materials with that of bacterial materials, to explore how the natural organic matter transformation from plant litter to bacteria may impact organohalogen formation.

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 resuspended 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 (R^2: 0.95–1.00), 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).

To better understand the precursors in bacteria at the molecular level, solutions of nine monomers (five amino acids: glycine, threonine, asparagine, tyrosine, and tryptophan; four nucleic acids: uracil, thymine, cytimidine and adenine) were selected for halogenation. In addition, extracts from fresh litter and decomposed litter (1–5 yr decomposed litter) of different plant species (blue oak (*Quercus douglasii*), live oak (*Quercus 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 samples and methods as in Chow et al. (2011). Briefly, approximately 300 g dry weight of each fresh and decomposed leaf materials were placed in triplicate in high-density, polyethylene (HDPE) trays (54 cm $\times$ 43 cm $\times$ 13 cm) with 2-mm mesh stainless steel screen on top and were exposed to natural conditions for 6 months. A polyethylene tube connected the tray to a 50-L HDPE carboy under each tray. Leachates were collected after each storm event and filtered through a 0.2 µm membrane filter (Millipore polycarbonate) before chlorination.

In the chlorination test, $\sim$ 50 mmol l^{-1} NaOCl stock solution was diluted to 0.028–0.31 mmol l^{-1} and used as the

halogenating agent. All pure bacterial suspensions, monomer solutions, fresh litter and partially decomposed 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) free residual chlorine (in the form of Cl_2, OCl^-, and HOCl) after 24 h incubation: 0.028 ± 0.011 mmol l^{-1} (i.e., 1.0 ± 0.4 mg l^{-1}). In parallel to the pure chlorination test, bromide at different levels (0, 6.25, 12.5, 25, and 50 µmol l^{-1} of KBr) was added before the aforementioned chlorination processes for bacteria treatments on B5, B6, and B7 in order to examine the effect of bromide on organohalogen formation. A 0.1 mol l^{-1} bromide stock solution was prepared from reagent grade potassium bromide with Milli-Q water (18.2 MΩ) and a suitable amount of stock solution was added into the bacterial mixture before chlorination. As a preliminary investigation, a pH of 8.0 was selected for the chlorination study in order to examine the organohalogen formation in bacteria containing surface waters such as river water, reservoirs, estuaries, and sea water, which commonly have a neutral or slightly alkaline pH range from 7 to 9. In addition, chlorination at pH = 8 has been tested for various sources of organic matter (e.g., Diaz et al., 2008; Zhang et al., 2009; Chow et al., 2011). The same reaction conditions were selected such that the reactivity of bacterial C in forming halocarbon could be compared to previous studies.

Nine halocarbon species, including chloroform ($CHCl_3$), bromodichloromethane ($CHCl_2Br$), chlorodibromomethane ($CHClBr_2$), bromoform ($CHBr_3$), dichloro-acetonitrile ($CHCl_2CN$), trichloro-acetonitrile (CCl_3CN), bromochloro-acetonitrile (CHBrClCN), dibromochloro-acetonitrile ($CHBr_2CN$), and chloral hydrate ($CCl_3CH(OH)_2$) were analyzed according to the USEPA method 551.1. Briefly, aqueous organohalogens were extracted with methyl tert-butyl ether and quantified using Gas Chromatography-Electron Capture Detector (HP 6890). A 0.25 mm ID × 30 m DB-1 capillary column was used to separate the organohalogens following the programmed oven temperature: an initial temperature of 35 °C was held for 22 min, then increased at a rate of 10 °C min^{-1} to 145 °C for 5 min, at 20 °C min^{-1} to 225 °C for 15 min, and at 10 °C min^{-1} to 260 °C for 30 min. The temperatures of the injector and ECD were set at 200 °C and 290 °C, respectively.

3 Results and discussion

After 24 h halogenating reaction, chloroform ($CHCl_3$), dichloro-acetonitrile ($CHCl_2CN$) and chloral hydrate ($CCl_3CH(OH)_2$) were generated from all tested bacterial strains, with average reactivity of 0.57–3.66 mmol-$CHCl_3$ mol-C^{-1}, 0.31–1.72 mmol-$CHCl_2CN$ mol-C^{-1} and 47.1–147.9 µmol-$CCl_3CH(OH)_2$ mol-C^{-1}, respectively (Fig. 1a). Trace amount of dichloro-bromomethane ($CHCl_2Br$) (25.6–88.3 µmol-CCl_3CN mol-C^{-1}) and

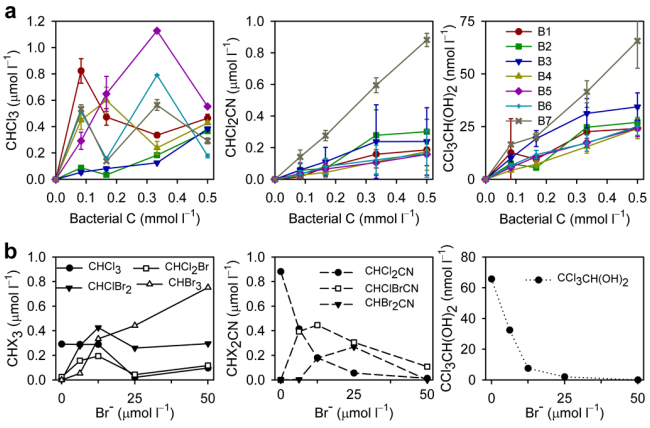

Fig. 1. Formation of some C1/C2 organohalogens by halogenation of bacteria. **(a)** effects of bacterial C concentrations (at 0.0, 0.083, 0.167, 0.333, and 0.50 mmol l^{-1}). Error bars show SD from 3 replicates. **(b)** effects of bromide (at 0, 6.25, 12.5, 25, and 50 µmol l^{-1} from KBr) taking *Staphylococcus 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*.

trichloro-acetonitrile (CCl_3CN) (3.26–27.4 µmol-CCl_3CN mol-C^{-1}) were also detected for all seven bacteria but were commonly less than 5 % of $CHCl_3$ and 5 % of $CHCl_2CN$, respectively. These results confirmed our hypothesis that pure bacterial materials may serve as precursors of C1/C2 organohalogens. With increasing bacterial carbon concentration, the $CHCl_2CN$ and $CCl_3CH(OH)_2$ concentrations were enhanced linearly (R^2: 0.912–0.999, $n = 15$, $P < 0.001$ for $CHCl_2CN$; R^2: 0.842–0.998, $n = 15$, $P < 0.001$ for $CCl_3CH(OH)_2$). In contrast, the yield of $CHCl_3$ did not consistently show a 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 compounds (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 similar trends of organohalogen concentrations with increasing Br concentration (Fig. S1 in the Supplement). Taking B7 as an example, the effects of Br can be inferred from Fig. 1b. The pure chlorinated species ($CHCl_3$, $CHCl_2CN$, and $CCl_3CH(OH)_2$) generally decreased with increasing Br concentration, whereas brominated analogues were generated in the presence of Br. Mono- and di-brominated methanes both peaked at about 12.5 µmol l^{-1} Br, whereas bromoform dominated all halogenated methanes (also all 9 tested C1/C2 organohalogens) at Br concentrations ≥ 25 µmol l^{-1}. Mono- and di-brominated

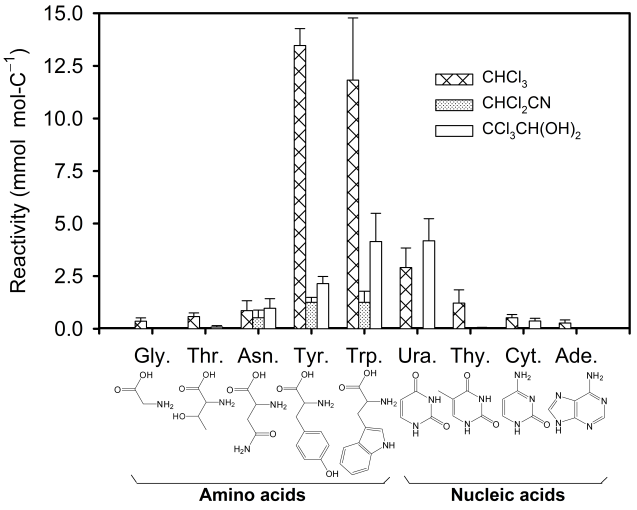

Fig. 2. Halogenation reactivity of 9 bacterial cellular monomers. Error bars show standard deviation from a total of 10 replicates (two for each C concentration: 0.083, 0.167, 0.250, 0.333, and 0.5 mmol-C l^{-1}).

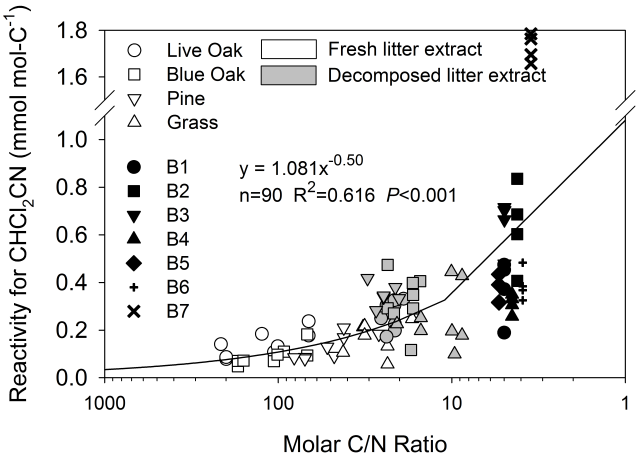

Fig. 3. Reactivity of dichloro-acetonitrile formation for extracts of fresh litter ($n = 32$), decomposed litter ($n = 30$) and bacterial suspensions ($n = 28$) along a C/N ratio gradient. B1–B7 refer to the same seven different bacterial strains in Fig. 1.

acetonitriles were found with peak concentrations at certain middle Br concentrations, but all selected halogenated acetonitriles dropped to an undetectable level (< 0.15 nmol l^{-1}) when the Br level increased to > 50 µmol l^{-1}. We speculate that the decrease of bromochloro-acetonitrile and dibromoacetonitrile may result from the formation of tribromoacetonitrile, which is similar to the transformation of haloforms. Studies have shown that the presence of bromide promotes reactivity in forming organohalogens (Chow et al., 2007; Shan et al., 2012). Therefore, greater organohalogen concentrations can be produced in water with a significant level of bromide, such as in brackish and sea waters. Importantly, some C1/C2 organobromides are much more toxic (Richardson et al., 2007) and have a greater ozone depletion potential than their chlorinated analogues (World Meteorological Organization, 2007).

The reactivity of different bio-molecules (Fig. 2) confirmed the possibility of abiotic reaction so that large amounts 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 $CCl_3CH(OH)_2$ (except glycine for $CCl_3CH(OH)_2$ generation), with tyrosine, tryptophan and uracil having the highest reactivity. Three out of five amino acids but no nucleic acid were able to yield $CHCl_2CN$, with reactivity following tyrosine $\geq$ 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). In contrast, the heterocyclic compounds did not necessarily have high reactivity and were highly influenced by the functional groups attached to the ring (e.g., enhanced reactivity by carbonyl comparing uracil with cytimidine; weakened by methyl comparing uracil with thymine).

The strong linear relationship in dichloro-acetonitrile and chloral hydrate, but fluctuation in $CHCl_3$ yield with bacterial C may be explained by reactivity of cellular monomers. Within these nine monomers, the ranking of standard error of reactivity was $CHCl_2CN$ (0.54 mmol mol-C^{-1}) $< CCl_3CH(OH)_2$ (1.75 mmol mol-C^{-1}) $< CHCl_3$ (5.23 mmol mol-C^{-1}), which was consistent with the ranking of the significance of regressions between organohalogen concentration and bacterial C ($CHCl_2CN > CCl_3CH(OH)_2 > CHCl_3$). Previous studies also indicated large variations in reactivity of $CHCl_3$ formation among biomolecules. Carbohydrates such as glucose and maltotriose generally have relatively high reactivity, ranging from 4.4 to 6.6 mmol-$CHCl_3$ mol-C^{-1} (Navalon et al., 2008). Amino acids like cysteine and glycine have a large range of reactivity, ranging from 0.004 to 14.8 mmol-$CHCl_3$ mol-C^{-1} (Hong et al., 2009). Lipids including β-Carotene, retinol and ellagic acid have significantly lower reactivity, ranging from 1 to 84 µmol-$CHCl_3$ mol-C^{-1} (Joll et al., 2010). The large variation in reactivity of $CHCl_3$ formation from various biomolecules 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 the 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 decomposers may enhance the fraction of nitrogenous organohalogen in the total organic halide, thereby influencing C, N and halogen cycling via natural halogenation processes (Fig. S2 in the Supplement). 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 the global budgets of haloforms, and haloacetonitriles through this direct halogenation pathway because of its large biomass on earth (Whitman et al., 1998; Hogan, 2010) and ubiquitous necromass caused by fast turnover rates (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 primary production into secondary production, their halogenated products can be shifted with the changing C/N ratio. However, the magnitude of global organohalogen production from bacteria is still difficult to predict limited by the single and direct halogenating approach we adopted from among many. Moreover, multiple factors such as the ambient environmental conditions (e.g., pH: Huber et al., 2009; temperature: Hamilton et al., 2003; sun radiation intensity: Chow et al., 2008), bacterial C quality, and the presence of halide ions at different concentrations will also cause uncertainty and affect the yield and species of organohalogen formation. In particular, pH always affects the distribution of reactive chlorine species (Cl_2, OCl^- and HOCl with different halogenation ability) and thus alters the yield and speciation of organohalogen formation (Snoeyink and Jenkins, 1980). Also, some organohalogens can transform to other species under certain pH conditions via various reactions such as hydrolysis, substitution and elimination (Nikolaou et al., 2004; Dabrowska and Nawrocki, 2009). Further studies exploring halogenating processes for bacterial materials and field observations of organohalogen yields associated with bacterial biomass in different biomes will help us better understand a more quantitative contribution of bacterial-derived organohalogens.

Supplementary material related to this article is available online at: http://www.biogeosciences.net/9/3721/2012/bg-9-3721-2012-supplement.pdf.

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