We would like to thank both reviewers for their valuable comments on our paper entitled: "Technical Note: Enhanced reactivity of nitrogenous organohalogen formation from plant litter to bacteria". We included all the requested modifications in the revised version of our manuscript and answered all questions (see below).

**Anonymous Referee #1**
Received and published: 17 July 2012
Review of “Enhanced reactivity of nitrogenous organohalogen formation from plant litter to bacteria” by J.-J. Wang, T.W. Ng, Q. Zhang, X.-B. Yang, R.A. Dahlgren, A.T. Chow, and P.K. Wong

General Comments
The paper by Wang et al. deals with natural halogenation processes of bacteria and the release of C1/C2 organohalogens. The authors suggest that bacteria may constitute an important precursor material for naturally produced organohalogens. In this review I will not comment on the quality of the bacterial culture experiments because my knowledge in this field is rather limited. I will comment on the paper from an environmental chemist’s perspective. The study is of interest because it adds information about possible new pathways of organohalogen formation in the environment. I found the manuscript for most parts to be well structured and clearly presented. In particular the introduction adequately details the current state of our knowledge about organohalogen formation in soils. Figure 4 shows a conceptual model that nicely summarizes the results of this work. Furthermore, the study by Wang et al. raises some interesting questions which certainly need to be answered in the future. I support publication of the manuscript, however, I have several comments which I hope the authors might consider in their revised manuscript. Below, in no particular importance order, are my specific comments:

Specific comments
1. Page 6780, line 18, Introduction: The applied concentration of 50 mmol is very high. Please explain and justify why 50 mmol per liter NaOCl solution was used for all experiments. Why were experiments not conducted at different concentrations of NaOCl?

**RESPONSE:** In the original manuscript it may be too vague so that we have misled the reviewer. The ~50 mmol/L NaOCl was the concentrated and stock solution. It has been diluted to 0.028-0.31 mmol/L NaOCl, i.e., 1.0-11 mg-Cl/L (depending on concentrations of dissolved organic carbon concentration (0-0.5 mmol/L) in the
solution) before its reaction with bacterial materials to guarantee the free chlorine residual within \(0.028\pm0.011\) mmol/L (1.0±0.4 mg/L) after 24 hr incubation. Chlorine residual within 1.0±0.4 mg/L was the only criterion to determine the NaOCl dose we used instead of using different concentrations of NaOCl, so as to make sure all bacterial carbon have completely reacted with reactive C. In Albers et al.’s (2011) paper, they used 50 mmol/L NaOCl to make dilution to 0.5 mmol/L for chlorination to conduct the simulation study, whereas our concentration used here was relatively lower. Another reason why we used this criterion (chlorine residual within 1.0±0.4 mg/L) was to make consistence with our previous study (Chow et al., 2011) so that we can compare the reactivity of litter materials and the bacterial materials.

Although different concentrations of NaOCl were not tested in this study, here we did test organohalogens formation from different bacterial C concentrations (also different C/OCl\(^{-1}\) ratios), which had supported the haloforms, haloacetonitriles, and chloral hydrate formation from bacterial carbon source.

In a separated study, we examined organohalogens formation from \(E.\ coli\) (2 mg/L carbon) with 0, 0.028, 0.056, and 0.084 mmol/L NaOCl solution (0, 1, 2, and 3 mg/L) within 1 hr reaction, and the result showed an increasing trend of haloform formation with increasing NaOCl concentration. The main focus of this study is to confirm the formation of organohalogens from bacterial carbon. Therefore, we used excessive NaOCl (chlorine residual =1.0±0.4 mg/L) but not use different NaOCl concentrations in the present study to avoid unnecessary confusion.

In the revised manuscript, we have made the revision below to make our point clearer so that the future readers would not misunderstand:

a) Original Page 6780, Line 18, “~50 mmol l\(^{-1}\)” was deleted.

b) Original Page 6781, Line 20, the original sentence has been revised to “In the chlorination test, ~50 mmol l\(^{-1}\) NaOCl stock solution was diluted to 0.028-0.31 mmol/L and used as the halogenating agent.”

2. Page 6781, line 24/25, Material and methods: “EPA Method 551 was adopted for the organohalogen quantification.” A short description of the method should be added. Please give also details of the analytical system that was used for separation and analysis of the organohalogen compounds. How were the different organohalogen compounds identified? Only using GC-ECD? Did the authors confirm the eluted compounds using a dissimilar column or by the use of GC-MS. Did the authors also search for formation of polar organohalogen compounds such as halogenated acetic acids?

RESPONSE: As the reviewer suggested, we added a description about the USEPA 551.1 in the method section. Since USEPA 551.1 is a well-developed method that has been widely used for our targeted compounds (i.e., chloroform (CHCl\(_3\)), bromodichloromethane (CHCl\(_2\)Br), chlorodibromomethane (CHClBr\(_2\)), bromoform (CHBr\(_3\)), dichloro-acetonitrile (CHCl\(_2\)CN), trichloro-acetonitrile (CCl\(_3\)CN), bromochloro-acetonitrile (CHBrClCN), dibromochloro-acetonitrile (CHBr\(_2\)CN), and chloral hydrate (CCl\(_3\)CH(OH)\(_2\))), only GC-ECD was used to identify the
organohalogens in the solutions. We did use chemical standards to confirm the peaks at specific elution time for specific compounds.

Halo-acetic acid is another interesting group of organohalogens that we expect its existence after chlorination with bacteria. However, due to its solubility, different analytical technique involved methylation is required for halo-acetic acid analysis. We did not examine halo-acetic acid in this study.

3. Page 6784, line 18 “...contributed to global C1/C2 organohalogen budget...”. Please be more specific and refer to the compounds that were actually measured in this study. These results might be not applicable to other C1/C2 compounds such as monohalogenated alkanes.

RESPONSE: We sincerely appreciate the reviewer for pointing out the imprecise expression in the Page 6784, line 18. It has been revised to “...the bacterial material may have at least comparably contributed to the global budgets of haloforms and haloacetonitriles through ...” accordingly.

4. Page 6785, line 2, suggest to modify sentence “...and the presence of different halides...” into “...and the presence of halide ions at different concentrations...”. I would like to add that the pH value might be also a crucial parameter affecting formation of organohalogen formation from bacteria. The authors should mention the potential role of the pH value in the Result and discussion section.

RESPONSE: We have changed the sentence according to the reviewer's suggest. Also, the potential role of the pH value is now added in the discussion section: “pH always affects the distribution of reactive chlorine species (Cl₂, 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).”

5. Page 6789: Figure 1. No error bars are shown for the lower panel (b). Why? Give the number of replicates for results shown in (b) in the legend.

RESPONSE: Instead of conducting replication on the same bacteria, we examined bromide effects on three bacteria (B5, B6, and B7). Results are shown in the Figure R1 below. As seen, the three tested bacteria showed the same trends in CHX₃, CHX₂CN, and CCl₃CH(OH)₂ formation with an increase of bromide level. Since the tests were performed on different bacteria, average plus standard deviation (error bar) is not suitable to express the data in Figure 1 in the original manuscript. Thus, bromide effect on one bacterium (B7) without error bars is shown in the manuscript.
Figure R1. Bromide effects on the formations of haloform, haloacetonitrile, and chloral hydrate on three different bacteria.

Technical corrections:
6. Page 6781, line 10: “bromide’s” not “briomide’s
   RESPONSE: Revised accordingly.

7. Page 6784, line 21: “caused” not “casued”
   RESPONSE: Revised accordingly.
The paper “Enhanced reactivity of nitrogenous organohalogen formation from plant litter to bacteria,” by Wang et al., presents a refreshing take on the natural formation of organohalogenes, focusing on halogenation of bacterial cultures and monomers. The hypothesis is novel, the data are clearly presented and discussed, and the results are compelling. After reading the manuscript, I have a few lingering questions, which are enumerated below.

Comments on Methods:
1. The Methods section skims on details. I would appreciate a very brief outline of the EPA method used to analyze the samples, as well as some details on how the plant litter extracts were made. Also, the Methods section does not describe the conditions for the bromination studies (although we can infer the KBr concentrations from the Figure 1 caption).

RESPONSE: We sincerely appreciate the reviewer for pointing out the lacking in detail in method section. In the updated manuscript, we added the descriptions about the USEPA method 551.1, the procedure for plant leachate collection, and bromination conditions as fellows:

Procedure for plant leachate collection in the 2nd paragraph of Method section:
“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 × 43 cm × 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 events and filtered through 0.2 μm membrane filter (Millipore polycarbonate) before chlorination.”

USEPA method 551.1 in the 4th paragraph of Method section:
“Nine halocarbon species, including chloroform (CHCl3), bromodichloromethane (CHCl2Br), chlorodibromomethane (CHClBr2), bromoform (CHBr3), dichloro-acetonitrile (CHCl2CN), trichloro-acetonitrile (CCl3CN), bromochloro-acetonitrile (CHBrClCN), dibromochloro-acetonitrile (CHBr2CN), and chloral hydrate (CCl3CH(OH)2) were analyzed according to the USEPA method 551.1 (USEPA 1995). Briefly, organohalogenes from water solutions 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 organohalogenes 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.”
Bromination conditions in the 3rd paragraph of Method section:

“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 Cl₂, OCl⁻, and HOCl) after 24 hour 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 B5, B6, and B7 in order to examine the bromide’s effects 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 suitable amount of stock solution was added into bacterial mixture before chlorination.”

2. Why were these reactions performed at basic pH (8.0 +/- 0.2)? I imagine this must have to do with the added NaOCl, which is very basic. However, terrestrial soils are generally more acidic. It would be good for the authors at least to justify their selection of this high pH value and comment on its relevance to actual environmental systems.

RESPONSE: This statement has been included in the 3rd paragraph of the Method section:

“As a preliminary investigation, 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 neutral or slightly alkaline pH ranges from 7 to 9. In addition, chlorination for pH at 8 has been tested for various sources of organic matters (e.g., Diaz et al., 2008; Zhang et al., 2009; Chow et al., 2011). Same reaction condition is selected such that the reactivity of bacterial C in forming halocarbon can be compared to previous studies.”

Also, as reviewer suggested, bacteria can be distributed and halogenated in rather different pH environment, especially in the large terrestrial soil pool. Thus, we discussed the limits of our study in the Discussion section as follows:

“Moreover, multiple factors such as the ambient environmental conditions (e.g. pH (Huber et al., 2009), temperature (Hamilton et al., 2003), sunlight radiation (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 (Cl₂, 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 (Nikolaou et al., 2004; Dabrowska and Nawrocki, 2009). 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 organohalogenes.”

3. What is the “Cl residual” mentioned in the Methods in line 24? Please define this term.

RESPONSE: It has been change to a more official term “free residual chlorine” with note in parentheses. It means the remaining reactive chlorine in the forms of Cl₂, OCI⁻ and HOCI after chlorination reaction.

Comments on Results and Discussion:

4. Although the data are nice, the underlying motivation for the bromination studies remains unclear. We would expect bromide added to a NaOCl solution to become oxidized to hypobromite, a reactive brominating species, through the equilibrium NaOCl + Br⁻ = NaOCl + Cl⁻. Why, then, is the formation of brominated C1/C2 compounds surprising or interesting? The authors should expand the discussion to explain the meaning and importance of the bromination studies.

RESPONSE: Yes, the OBr⁻ and HOBr are expected after reaction between NaOCl and Br⁻. In general, reactivity in forming organobromine is greater than that of organochlorine (Chow et al., 2007; Shan et al., 2012) and greater amount of organohalogen can be formed when bromide exists. In addition, some C1/C2 organobromines are much toxic and greater ozone depletion potential than their chlorinated analogues (Richardson et al., 2007; WMO (World Meteorological Organization), 2007). As the reviewer suggested, we added more information in the discussion to explain the environmental implication of the bromination studies. Please read the second paragraph in the section of results and discussion.

5. In Figure 1b, in the middle graph, the concentrations of mono- and dibromoacetonitriles plummet at high [Br-]. The authors mention that this could be due to formation of other species, e.g., cyanogens halides. Why does this only apply for the nitrile compounds and not the chloro/bromoforms or hydrates? Was tribromoacetonitrile measured? Also, how repeatable were the results presented in the Figure 1b graphs across all the bacterial species examined?

RESPONSE: We thank for the valuable comment from reviewer. We reanalyzed several possible reasons carefully and considered that the formation of tribromoacetonitrile may be a more possible reason than the original one leading to the variation trends of mono- and di-bromoacetonitriles. In our original thought, higher bromide led to higher level of OBr⁻ that significantly changed the speciation of nitrogenous organohalogenes like in Le Roux et al. (2012). Yet, how haloacetonitriles change in this process is still unknown. But as brominated acetonitriles have been proved to be more stable than the chlorinated acetonitriles (Glezer et al., 1999), the total bromine in the halo-acetonitrile should have increased. We thus speculate that maybe the transformation of tribromo-acetonitrile from mono- and
di-bromoacetonitrile was the real reason to the variation trends of haloacetonitrile with increasing bromide. We do wish to measure tribromoacetonitrile but we did not have the method to measure it in our original experiment. That is why we can only speculate on the reason.

Instead of conducting replication on the same bacteria, we examined bromide’s effects on three bacteria (B5, B6, and B7). Results are shown in the Figure R1, as in the responses to the Reviewer #1 above. As seen, the three tested bacteria showed the same trends in CHX₃, CHX₂CN, and CCl₃CH(OH)₂ formation with an increase of bromide level.

6. In Figure 1a, there is a good correlation between bacterial [C] with dichloroacetonitrile and chloral hydrate formation, but not much of a correlation with chloroform formation. The authors present a cogent explanation for in lines 21-29 of the Results, stating that chloroform formation is highly variable even for the studies of the bacterial monomers. This trend emerges clearly from the data in Figure 2 and resonates with the results in Figure 1a. Could the authors speculate on the molecular basis for this variable chloroform formation? What about the molecular structure of chloroform and the potential mechanisms of its formation lead to this variability?

RESPONSE: As the reviewer suggested, we added more discussion on the chloroform formation from different bio-molecules:

“Previous studies also indicated large variations in reactivity of CHCl₃ formation among biomolecules. Carbohydrate, such as glucose and maltotriose generally has a greater reactivity, ranging from 4.4 to 6.6 mmol-CHCl₃ mol-C⁻¹ (Navalon et al., 2008). Amino acid such as cysteine and glycine has a large range of reactivity, ranging from 0.004 to 14.8 mmol-CHCl₃ mol-C⁻¹ (Hong et al., 2009). Lipids such as β-Carotene, retinol, and ellagic acid have a significantly low reactivity, ranging from 1 to 84 μmol-CHCl₃ mol-C⁻³ (Joll et al., 2010). The large variation in reactivity of CHCl₃ 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 CHCl₃ formation.”

The mechanisms in forming CHCl₃ from different types of biomolecules are expected to be different. In carbohydrates, chloroform formation is involved carbon-carbon bond cleavage from α-hydroxy aldehydes and the formation a halogenated carbanion (CCl₃⁻), as proposed in Navalon et al. (2008). In lipid, haloform reaction on methyl ketone groups within many of the intermediates (Joll et al., 2010). In amino acid or protein, the chlorination of alanine generated monochloro-N-amino acid and then formed acetonitrile by decarboxylation and hydrochloric acid elimination. Acetonitrile is substituted by chlorine to generate dichloro-acetonitrile and trichloro-acetonitrile. Chloroform is formed by hydrolysis of trichloro-acetonitrile (Chu et al., 2009). However, because in this study we are not focusing on the molecular basis of the CHCl₃ formation but trying to make the paper
short, we did not include the related discussion in the updated manuscript.

7. With regard to Figure 3, the discussion of these very interesting results should be expanded. In particular, I am curious about the high variability in dichloroacetonitrile formation amongst the different bacteria. Why does it skyrocket for the B7 bacterium, despite its similar molar C/N ratio to the other species?

RESPONSE: We also noticed extremely high reactivity of *Staphylococcus sciuri* (B7) in dichloroacetonitrile (DCAN) formation. Comparing the characteristics of seven different bacterial strains (Table R1), we can exclude the large difference of dichloroacetonitrile formation between B7 and other bacteria caused by either the bacterial group (Gram-negative/Gram-positive), bacterial metabolism (aerobic/anaerobic), bacterial size, or bacterial habitat. We suspect that the differences in DCAN formation could relate to the physiological and genetic differences among bacterial cells. Research studies have shown the biochemical compositions of algal cells can have large range differences in organohalogen formation during chlorination (Hong et al., 2008; Wei et al., 2011). For example, protein, carbohydrates, and lipid of *Thalassiosira oceanica* amount to 19.2%, 9.26, and 71.6%, whereas *Skeletonema costatum* is 58.4%, 8.11%, and 33.5%, respectively. Notably, the reactivity of these bio-molecules in forming different organohalogens species are significantly different from each other, as shown in this study and other independent studies (Navalon et al., 2008; Shan et al., 2012). Furthermore, the existences of amine and other organic nitrogen could affect the formation of oxidant species (hypochlorous acid versus organo-chloramine), eventually affecting the formation of organohalogens (Le Roux et al., 2012). With the results of the present study, we do not have valid conclusion what factors causing high DCAN in B7. Further study is needed to identify the cause.
### Table R1. The characteristics of seven selected bacteria

<table>
<thead>
<tr>
<th>Species</th>
<th>Acinetobacter junii (B1)</th>
<th>Aeromonas hydrophila (B2)</th>
<th>Bacillus cereus (B3)</th>
<th>Bacillus subtilis (B4)</th>
<th>Escherichia coli (B5)</th>
<th>Shigella sonnei (B6)</th>
<th>Staphylococcus sciuri (B7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
<td>Gram-negative</td>
<td>Gram-negative</td>
<td>Gram-positive</td>
<td>Gram-positive</td>
<td>Gram-negative</td>
<td>Gram-negative</td>
<td>Gram-positive</td>
</tr>
<tr>
<td><strong>Morphology</strong></td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
<td>Spherical</td>
</tr>
<tr>
<td><strong>Metabolism</strong></td>
<td>Aerobic</td>
<td>Facultative anaerobic</td>
<td>Facultative anaerobic</td>
<td>Facultative anaerobic</td>
<td>Facultative anaerobic</td>
<td>Facultative anaerobic</td>
<td>Facultative anaerobic</td>
</tr>
<tr>
<td><strong>Size (μm)</strong></td>
<td>0.9-1.6 × 1.5-2.5</td>
<td>0.3-1.0 × 1.0-3.5</td>
<td>Endospores</td>
<td>Endospores</td>
<td>1.1-1.5 × 2.0-6.0</td>
<td>0.7-1.0 × 1.0-3.0</td>
<td>0.5-1.5</td>
</tr>
<tr>
<td><strong>Habitat</strong></td>
<td>Soil, water, sewage, and food</td>
<td>Fresh and marine waters, warm-blood animals</td>
<td>Widely distributed in nature</td>
<td>Widely distributed in nature, primary in soil</td>
<td>Colon of human and warm-blood animals</td>
<td>Soil, water</td>
<td>Human and warm-blood animals; natural waters</td>
</tr>
</tbody>
</table>
8. The data in Figure 3 also made me eager to see the production of dichloroacetonitrile (as well as the other organohalogens) in extracts and bacterial cultures WITHOUT NaOCl added. To what extent do these compounds form under the natural oxidative conditions of soil organic matter decomposition?

RESPONSE: In our blank and control tests, the production of dichloroacetonitrile in both extracts and bacterial cultures without NaOCl were all below detection limit (0.010 μg/L). We understand the reviewer should be concerning about the natural occurrence of these organohalogens, but this study is mainly focusing on the reactivity of different materials with halogenating reagent rather than field monitoring. Also, the results of production of dichloroacetonitrile in extracts and bacterial cultures without NaOCl added did not reflect the field occurrence as the reviewer suggested. For example, the bacteria are pure strains cultivated in lab rather than those found in the nature environments. They did not have chance to react with the natural halogenating reagents from either enzyme (haloperoxidases and halogenases) or Fenton/Fenton-like reagents which are common in soils.

We expect the environmental occurrence of dichloroacetonitrile could be at low level and concentrating techniques such as solid phase micro-extraction should be required.

9. As a point of curiosity, why did the authors choose these three particular organochlorines to analyze? The difference in formation of the N-containing organochlorine vs. the others becomes clear at the end of the article, with the discussion of the data in Figure 3, but what was the original rationale for the selection of these three compounds?

RESPONSE: The major concerns are the relative abundance and their environmental impact. Halomethane, halo-acetonitrile, and chloral hydrate are among the categories that have the largest yield after direct chlorination (Dabrowska and Nawrocki, 2009) and can represent the major carbonous, nitrogenous and oxgenous organohalogens formation. Also, these three groups of organohalogens all showed large toxicity to human health. This information is added in the introduction. Please read the last paragraph of the introduction.

10. As a final note, I appreciate the title but don’t think it encapsulates the main thrust of the paper, which is focused more generally on bacterial production of C1 and C2 organohalogens, not just the comparatively greater formation of dichloroacetonitrile by bacteria compared with plant litter extracts. In short, the title seems too narrow, only representing a part of this paper’s contribution. Thus, it might be beneficial to formulate a broader title that better describes the overall findings of the study.

RESPONSE: We considered reviewer’s suggestion and have changed the title to “Reactivity of C1 and C2 organohalogens formation from bacteria”.
Minor editorial corrections:

11. In line 9 of the Introduction, “widespready found” would be better replaced by “are widespread.”

RESPONSE: Revised accordingly.

12. The sentence in lines 18-20 of the Introduction is awkward and should be rewritten for clarity.

RESPONSE: In the updated manuscript, we rewrote the sentence to make it clearer as the reviewer suggested.

REFERENCES:


Hong, H. C., Mazumder, A., Wong, M. H., and Liang, Y.: Yield of trihalomethanes and haloacetic acids upon chlorinating algal cells, and its prediction via algal cellular biochemical