April 25th, 2011

Professor Eric Weitz
Senior Editor
The Journal of Physical Chemistry B

Dear Professor Weitz,

We have electronically submitted a revised manuscript (jp-2010-121307) that addresses the comments of the reviewers. We have separated our Results and Discussion into two separate sections so that we can discuss our findings in much more detail and also compare them to other recent studies involving TMAO’s role as an osmolyte. We highlight how our study is unique in that by probing TMAO’s properties spectroscopically, the local water structure around it is revealed. We show through good agreement between experiment and theory TMAO dictates the hydrogen bonded water network around it and that this structural motif involves at least three water molecules directly hydrogen bonded to TMAO. We discuss how this network is centered around TMAO’s oxygen atom and how this result compares with other recent findings involving TMAO and with the hydrophobic effect in general. This is direct spectroscopic evidence for the hydrogen bonded water network that TMAO induces and we feel is a significant contribution. With these modifications we feel that this contribution is now suitable for publication in the Journal of Physical Chemistry B. The rest of this letter provides detailed responses to the reviews, including the suitability of the material for the Journal of Physical Chemistry B.

Response to comments from Reviewer 1

1. Comment: TMAO has attracted considerable attention in recent years because of its stabilizing effect on proteins. As is mentioned by the authors in their introduction, a number of studies have investigated the effect of TMAO on the structure of water as well as on the hydrophobic effect. After reading the introduction of this manuscript it comes as somewhat of a surprise that the authors do not place their results in this broader perspective. This is especially unfortunate since a large part of the manuscript deals with the interpretation of the Raman spectrum, which is mostly a re-examination of previous results (as mentioned by the authors themselves).
**Response:** We thank the reviewer for pointing out that a broader discussion putting our results in perspective with the previous literature is desirable. We have added such a detailed discussion to the manuscript. The interpretation of the Raman spectrum, however, is key to our overall conclusions with regards to how TMAO and water interact at the molecular level. We have added these details to our separate Discussion section which starts on Page 19. Our detailed discussion of how our results fit into the hydrophobic effect can be found on Page 21.

2. **Comment:** The present interpretation of the spectrum differs on two points with previously published work, but these points are minor. Firstly, the authors have observed the lowest-frequency normal mode of TMAO ($v_{24}$), which had previously only been predicted. Secondly, a double peak at 1465 cm$^{-1}$ is now interpreted as the normal mode $v_{17}$ split into a doublet, where the two peaks where previously assigned to the normal modes $v_{16}$ and $v_{17}$.

**Response:** The reviewer is correct that we do not radically change the relative positions of TMAO’s normal modes. However, our experimental Raman spectra serve as the most detailed and comprehensive to date and serve to correct the absolute positions of earlier assignments. We took great care to record the solid state TMAO Raman spectrum in an inert atmosphere so that shifting due to microhydration did not occur. The reported locations of many of the modes in previous studies clearly were either blue- or red-shifted by the presence of water or the authors actually studied the dihydrate. In addition, by comparing our experimental results to the highest level of theory yet employed to study TMAO, our analysis should serve as the best available in the literature.

3. **Comment:** The novel result described in this manuscript is the finding that in solution TMAO forms on average three hydrogen bonds with water. While this result could be of interest to researchers who study the osmolytic properties of TMAO, I am not sure whether this finding alone warrants publication in the *Journal of Physical Chemistry*. In its present form the article is probably better suited for a more specialized journal.

**Response:** As pointed out by earlier by Reviewer 1, this contribution deals with a topic that has “attracted considerable attention in recent years.” On that same note, Reviewer 2 points out that our introduction serves as a “very good” review of this popular topic. Our manuscript serves as the most comprehensive contribution involving both spectroscopy and computational modeling to study the effects of hydration on TMAO. Our experimental Raman spectra are the most detailed and comprehensive to date and our micro-hydrated clusters are the most complex yet investigated and have been performed at the highest level of theory so far (triple-$\zeta$ basis set) on this important system. Together, we are not only able to confirm, re-assign, and correct the location of spectral features, but we are also able to connect structural motifs at the molecular level to bulk observables and the hydrophobic effect in general. This represents important spectroscopic evidence for the molecular level structure that exists in nature. Spectroscopic studies involving TMAO (or other osmolytes such as urea, tert-butyl alcohol, or guanadine chloride) and water have appeared and continue to appear in the *Journal of Physical Chemistry* and comparable peer-reviewed journals. We cite in our manuscript 16 articles studying TMAO from the *Journal of Physical Chemistry* alone and at the end of this letter we include a number of recent articles from *JPCB* that describe intermolecular interactions of osmolytes. Many of these do not present as significant of results as are in our current contribution. Yes, it is true
that our work will be of keen interest to researchers that follow efforts at explaining TMAO’s stabilizing effects on proteins. However, theoretical chemists and experimentalists alike should be very interested in the broader features of our study such as the hydrogen bonded networks that TMAO induces on water and the very good agreement between experiment and theory in this important system as studied by our collaborative team. The *Journal of Physical Chemistry* is home to many such studies and we feel that our current contribution is most definitely appropriate for *JPCB*.

4. **Comment:** Since the finding that TMAO forms three hydrogen bonds with water is the main result of this paper, it would be interesting to have a discussion that provides a comparison with the results of studies that have used different techniques (such as NMR, dielectric relaxation spectroscopy, infrared spectroscopy). In addition the authors could discuss their result in a broader context. For example, the fact that TMAO can form three hydrogen bonds with water may provide information on how TMAO affects the structure of water. Would it be possible to say anything about the nature of TMAO as an osmolyte? Such a discussion is expected after reading the introduction and would in any case make the article interesting for a broader audience.

**Response:** We thank the reviewer for pointing out that a such a discussion will improve our contribution. Yes, we agree that the fact that TMAO likely forms three hydrogen bonds on average with water does illustrate how TMAO affects the structure of water. To this end we have included this in our newly expanded Discussion section starting on Page 19 and have compared our findings to a number of recent joint experimental/theoretical studies involving TMAO’s properties starting on Page 21. We specifically include the references that the reviewer is eluding to.

5. **Comment:** It would be interesting if the authors could provide a molecular interpretation of the changes in the spectrum that occur upon hydration of TMAO (Figure 4). As mentioned by the authors certain features are related to fact that the multiplet splitting observed in the crystal disappears upon hydration. Other features, however, are likely to be directly related to the effects of hydration; for example, the blue shift of $\nu_{18}$. Is this caused by the weak hydrogen bonds to the CH groups that are present in some of the simulated clusters? What is going on in the CH-stretch region; that is, what is the origin of the increased intensity of the modes $\nu_{15}$ and $\nu_2$?

**Response:** We have added a more thorough discussion in the text with regards to the molecular level interpretation of the blue- and red-shifting of some of the normal modes. This Discussion section starts on Page 19. It turns out that this discussion is perhaps the most intriguing result of this work and we thank the reviewer for requesting it.

6. **Comment:** It would be useful to have the peaks in Figure 3 labeled with the assigned normal modes, so that one does not always have look these up Table 1. In addition, as Table 1 does not list the predicted Raman intensities one is forced to switch continuously between Figure 3 and Table 1.

**Response:** We thank the reviewer for pointing this out and have added the appropriate labels to Figure 3.
7. **Comment:** Figure 5: How have these spectra been normalized?

**Response:** Since we are not interested in absolute intensities, but rather in the qualitative evolution of the spectral features, for clarity Figure 5 has not been normalized but rather each spectrum is scaled to the maximum feature in each. Using an absolute scale would make it very difficult to interpret.

8. **Comment:** Page 14, lines 50-51: This sentence is a bit confusing. On first reading it seems to suggest that it is specifically the C$_3$v symmetry that is destroyed by the tilted water molecule. Presumably what is meant is that the mere presence of a single water molecule lowers the C$_3$v symmetry, while the subsequent tilt destroys all remaining symmetry.

**Response:** Yes, the reviewer is correct. Our wording was to convey that although a water molecule added to a C$_3$v structure could still have C$_s$ symmetry, our manuscript states that it is the tilt that destroys all of the symmetry.

9. **Comment:** Page 18, lines 4-8: This sentence is difficult to understand. It is not immediately clear that the word “latter” refers to one of the two hydrogen-bonded water molecules. In addition it is not obvious from the figure that the NO bond lies in the planes of both hydrogen-bonded water molecules.

**Response:** In this sentence, the word “latter” refers to the two water molecules hydrogen-bonded to the central oxygen of TMAO as opposed to the water molecule placed between them. We agree with the reviewer that the planarity is not completely obvious from the figure. That is why we talk about its structure in the text.

10. **Comment:** The authors mention that the assignment of all normal modes in the spectrum is made possible by using the gradual evolution of the spectrum upon hydration. It is, however, not completely clear for which normal modes this is absolutely necessary. At the bottom of page 18 it is stated that the gradual evolution is used for the normal modes in both the CH bending and stretching modes. The CH bending region, however, had already been completely assigned before considering any of the hydrated spectra, so that this statement is somewhat confusing. In this regard it would be useful if the authors would guide the reader a bit more in the assignment of the normal modes.

**Response:** We thank the reviewer for pointing out this confusion. Yes, modes in the CH bending region can be assigned through comparison with theory. However, monitoring the blue- or red-shifting of modes as water molecules are added both experimentally and theoretically confirm these assignments and aid in the assignment of modes where the original assignment is not clear base on comparison with theory alone. In addition, the locations of most peaks have been in question due to the presence of water in earlier spectra. Here, we present solid phase spectra in an inert atmosphere. We have clarified this in the text on Page 12 that shifts induced by the presence of water help confirm these assignments later on in the text.

11. **Comment:** Regarding the region above 2800 cm$^{-1}$, it is not clear what the peak positions quoted in Table 1 (under “current Raman”) refer to. Do these refer to the solid TMAO spectrum or to one of the hydrated spectra?

**Response:** Table 1 only refers to the solid TMAO spectrum. We have clarified this in the text.
12. **Comment:** Finally the authors state that the broad peak at 3040 cm$^{-1}$ is composed of six overlapped normal modes. How should one understand this? It has previously been stated that there are only 5 normal modes in this whole region (2800-3200 cm$^{-1}$), leaving only three unassigned modes after the assignment of $\nu_2$ and $\nu_{15}$. Does this have anything to do with the fact that the degeneracy of some modes is lifted by the presence of the solvent? A remark about this is the text would be helpful.

**Response:** We thank the reviewer for pointing out this confusion and have clarified it in the text. This figure shows the theoretical spectrum of TMAO with five water molecules. The additional water molecules interacting with the methyl groups on TMAO increase the number of possible C-H stretches. Yes, only six normal modes exist in neat TMAO (we only were able to assign 5), but with the addition of hydrogen bonded water molecules there are additional modes in this new molecular cluster system that lead to broadening in the condensed phase.

13. **Comment:** page 6, line 49: the symbol $\chi_{TMAO}$ is introduced without a definition.

**Response:** We have changed $\chi_{TMAO}$ to read “$\chi_2$” for simplicity and have added a definition of $\chi_2$ next to its introduction.

14. **Comment:** page 8, line 33: Figure 2 should be Figure 3

**Response:** We thank the reviewer for pointing this out and have made this correction.

15. **Comment:** Figure 3: there is a typo, “Solid” instead of “Soild”

**Response:** We thank the reviewer for pointing this out and have made this correction.

16. **Comment:** The table of contents graphic has the wrong caption.

**Response:** We thank the reviewer for pointing this out and have made this correction.

**Response to comments from Reviewer 2**

1. **Comment:** The description of the theoretical method has unnecessary detail. B3LYP/aug-cc-pvttz is a standard and widely used method.

**Response:** While the method and basis set are relatively standard and widely used, we find that non-theoreticians rarely have any idea about these details even if they have used the method and or basis set for some of their own work. We agree that these details are superfluous if we were to publish the theoretical results alone in a journal read only by theoreticians. Since this article is expected to have wide appeal among experimental biophysical chemists, we feel that the details, while maybe not completely necessary, are appropriate.

2. **Comment:** It would be worthwhile for the authors to briefly describe how the water clusters were chosen. Is it possible that some lower energy structures were not investigated?

**Response:** See response to (3) below.
3. **Comment:** No structures were calculated when water is adjacent to the methyl groups. There are certainly water molecules there which, even if weekly bound, could affect the methyl group vibrations.

**Response:** The structures were chosen by considering numerous starting geometries, many of which converged to the same final structure. Many of these initial geometries had water molecules near the methyl groups; they just didn't stay near the methyls during the optimization process. We agree with the reviewer that this is an important point and so we have added these details to the manuscript in the Theoretical Methods section. Only structures 5W-A, 7W-B, and 8W-B converged to geometries in which some water molecules are still near the methyls interacting through weak CH…O hydrogen bonds. In the latter one (8W-B), there are no direct interactions with the waters, but they are still relatively close. While no lower energy structures were found, we do not claim that are no other structures which might have lower energy than some or all of our structures for a given number of water molecules. However, we would be very surprised if a lower energy structure were found for any of the systems up to at least four waters.

**Suitability for the Journal of Physical Chemistry**

Over the past couple of years, the *Journal of Physical Chemistry B* has included a number of articles concerned with the interactions and activity of osmolytes. This is especially true of studies interested in their effects on hydrogen bonded water networks. Many of these are also joint spectroscopic and theoretical studies similar to our contribution here. A few examples are provided below:


Sincerely:

\[\text{\textit{Nathan Hammer}}\]

Dr. Nathan I. Hammer
Assistant Professor