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Title: Lessons Learned from the Calculation of One-Dimensional
                Potentials of Mean Force [Article v1.0]

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Comments of the authors to the comments of the reviewers on the manuscript:

Reviewer 1:

Reviewer's comments:
To me, this paper reads more like a best practice paper than a Lessons Learned paper.

Authors' reply:
When writing up the manuscript we were not really sure about the appropriate category. After discussion with the lead editors of the Best Practices and Lessons Learned section it turned out that it would best fit to the lessons learned category.

Reviewer's comments:
I think the authors would be better served to use the term "free-energy curve" as opposed to "potential of mean force" when talking about the output of umbrella sampling. As the authors are well aware (they explain it clearly, when they talk about the importance of the restraints), the difference between the two is the presence of the Jacobian – by definition, the PMF does not include it, while the free energy curve does. Umbrella sampling yields a free-energy curve, because the Jacobian is built in.

Authors' reply:
We are very grateful that the reviewer has pointed out this nomenclature issue. The two terms "PMF" and "free-energy profile" or "free-energy curve" are indeed sometimes written as synonyms in the literature or even with an opposite interpretation of the meaning of "presence of the Jacobian", see e.g. JCP **128**, 04106. Unfortunately, we also did not clearly distinguish the two quantities in our manuscript. We agree with the referee that the outcome of an umbrella sampling simulation in 3D delivers a free energy curve including the Jacobian. For two non-interacting particles, the Jacobian is in fact the only contribution. In the 1D case, the Jacobian is equal to 1 and, consequently, free-energy curve and PMF are equal. When revising the manuscript, we took care of the difference between free-energy curve and PMF. On page 3 of the revised version the difference between the two is now explicitly pointed out while stating that - at same time - there is no practical difference for the set-up studied here. We hope that we have removed every inconsistent use of the two terms in the revised version of the manuscript. Since we have extensively used Eq. (1) to calculate binding free enthalpies we prefer to use the term PMF for the one-dimensional curves once the difference between PMF and free-energy curve has been made clear.

Reviewer's comments:
It might be valuable to attach MDP files, plumed configs, and equivalent gromos file (or even better, all of the files). They would easily fit in the github repo, and would help someone who wanted to do similar things. In particular, I'm thinking of the way the various restraints were implemented, which I'd imagine is a pretty error-prone process.

Authors' reply:
We agree that this would be very useful and uploaded two complete simulation examples (one for GROMOS and one for GROMACS/PLUMED) to the github repository

along with a detailed explanation to which test case they belong and how to use them.

Reviewer's comments:
When talking about the sampling problem, it might be valuable to reference and discuss the work of Neale et al. and Pomes in Biophysical Journal (2014). This talks about binding of a peptide to a membrane, and demonstrates a) how extreme the sampling problem can be; b) the challenges of a suboptimal reaction coordinate.

Authors' reply:
We are grateful that the referee has pointed out another relevant example about sampling issues in the context of membrane simulations. The paper is referenced in the right column of page 2.

Reviewer's comments:
The term "free enthalpy" is correct but rather uncommon, at least in the community I live in (I had to look it up). I have no objections to its use, but I think it would be helpful to mention that it's also referred to as the Gibbs Free Energy the first time it's used.

Authors' reply:
We agree that the term "free energy" is often used for that quantity. However, the use of the term "free enthalpy" for the thermodynamic function G = U + PV − TS stems from the IUPAP convention of the year 1978, see page 19 of the Report on Symbols, Units and Nomenclature in Physics of IUPAP. The IUPAC report of 1988 calls this function the "Gibbs energy", while it does not mention "Gibbs free energy". We have added both references in the second sentence of the introduction.

Reviewer's comments:
I don't think umbrella sampling should be capitalized.

Authors' reply:
We agree and adjusted the text accordingly.

Reviewer's comments:
In section one metadynamics should be mentioned as a technique for getting free energy curves, since it's very common.

Authors' reply:
The reviewer is right that metadynamics is a widely used method. In the previous version of the manuscript we only mentioned its predecessor, i.e. local elevation. In the revised version we mention metadynamics as well.

Reviewer's comments:
In section one, I think the phrase should be "low molecular-weight substances" rather than "low-molecular substances".

Authors' reply:
This is correct.

Reviewer's comments:
In section one, at the bottom of the first column on page 2, the statement that the PMF goes flat should be refined a bit – real PMFs will go flat, and free energy curves where the Jacobian is 1 will go flat, but most choices of reaction coordinates won't go flat.

Authors' reply:
In our statement we refer to the real PMF and hope that this point is better understandable in the new paragraph on the left column of page 3.

Reviewer's comments:
In the second column, the explanation for offsets in reference 27 about noise accumulation strikes me as just a rephrase of the sampling problem. If I'm misunderstanding, it should be clarified.

Authors' reply:
We think the reviewer is right and rephrased that sentence.

Reviewer's comments:
In Eq. 1, I think the Jacobian(r, omega) should be included in both integrals. The authors deal with it correctly in the discussion, but I think it's worth mentioning explicitly in the equation since people get it wrong a lot.

Authors' reply:
We agree and changed the equation accordingly. Moreover, we point out that if the Jacobian appears in the equation, the W is to be the true PMF.

Reviewer's comments:
The authors state that delta W_r is the plateau value, there's some nuance there that's missing. Really you should be integrating over the unbound state, just like the bound state. I guess that the logic is that the integrating part is taken care of analytically, but I'd like to see a more detailed discussion.

Authors' reply:
Indeed, we agree that the reader will profit from a more detailed explanation. In some cases, the beginning of the unbound region might be ambiguous. Or due to numerical noise the PMF might not be completely flat. In such cases one might calculate a thermally averaged well depth by numerical quadrature as we explain at the bottom of the right column on page 3 of the revised version. In the case of a flat PMF the exponent can be taken out of the integration in Eq. (3).

Reviewer's comments:
End of section 2, I think "unnecessary" is a better word choice than "redundant", since the point is that there's no statistical error to an analytical solution.

Authors' reply:
We agree that this is the better choice.

Reviewer's comments:
In Eq. 9, I think fractions would be clearer than 0.5, 1.5 and 0.25.

Authors' reply:
We amended the equation accordingly.

Reviewer's comments:
In the second column of page 5, I think the commas should be removed to make the sentence: "… order parameters used for both the actual PMF calculation and … orthogonal movement are defined …"

Authors' reply:
We removed the commas.

Reviewer's comments:
On page 9, I think it would be good to make the point that the various methods of restraining the host don't change the answer more visible. This is a significant point, and it's buried in the middle of a paragraph – perfect color and or bold type to make it stand out?

Authors' reply:
We also think that this investigation is important. In the revised version of the manuscript a figure is added on page 10 to make this point more prominent.

Reviewer's comments:
In the section on restraining ligand lateral movement, I think "are redundant" should be replaced with "make no contribution" or "are zero by definition".

Authors' reply:
We have corrected that sentence.

Reviewer's comments:
Bottom of page 10, "incapable to rotate" -> "unable to rotate".

Authors' reply:
We have corrected this sentence and a similar sentence in the caption of Table 4.

Reviewer's comments:
In the section on treatment of electrostatics, I think the sentence should say "None of the cases included an orientational restraint".

Authors' reply:
The sentence was corrected.

Reviewer's comments:
The authors mention on the bottom of page 13 that PME converged faster than RF. Any idea why? If so (and it's simple enough to deal with in a sentence or two), it might be interesting to add it in.

Authors' reply:
We did not perform a detailed investigation of possible reasons for this observation. It has probably to do with the differences in the water structure between the two approaches. However, to avoid speculation we did not include any explanation in the text.

Reviewer's comments:
Figure 9: any idea why the error bars are bigger at positive x vs negative? Also, any idea why there's a small harmonic oscillation in the plateaus (it's particularly visible in the green and blue curves)?

Authors' reply:
We report cumulative errors as explained in the original umbrella integration paper. This is now explained in more detail on page 8 of the revised version. Regarding the small harmonic oscillations, we refer to our answer to the next comment.

Reviewer's comments:
The same oscillation is also very visible in the blue curve in Fig. 13. It's not a big deal, but it's a little weird.

Authors' reply:
We are grateful that the referee has pointed out this subtle artifact. We realized that this oscillatory behavior was caused by choosing a too large bin width (or in other words, a too small number of bins), which is why we think that this artifact is introduced in the numerical integration step of the UI approach. In Refs 92 and 93 of the revised version of the manuscript, the UI inventors explicitly hint that the integration becomes more accurate with an increasing number of bins and recommend bin numbers of 200-400. We verified that by using a smaller bin width (more bins), the oscillations disappear while the overall shape, barrier heights and sizes of error

estimates are not affected. We updated the corresponding figures Fig. 10 (former Fig. 9) and Fig. 14 (former Fig. 13) in the revised manuscript.

Reviewer 2:

Reviewer's comments:
I found several typos grammatical errors. A careful read of the manuscript is necessary to fix mistakes such as "the methods itself or wrong use of them" or "the fact that this contribution". There is also a reference to table A1 which I was unable to find.

Authors' reply:
We hope that the revised version of the manuscript does not contain any of these errors. Table A1 is located in the Appendix on the last page of the document.

Reviewer's comments:
In the article, the authors used a united atom force field. They should include a discussion pertaining the validity of the reported artifacts under this approximation. The authors touch briefly on this in the last paragraph of the Conclusions but I think this should be discussed more extensively.

Authors' reply:
We had earlier results obtained with the CHARMM force field (all-atom) for the cyclodextrin alcohol binding. However, these were generated with a slightly different set-up compared to that used for all the other simulations. Therefore, we decided to just mention that offsets appear also with other force fields. For the revised version of the manuscript we repeated the CHARMM simulations using the same methodology as used for the united atom force field and added a figure to the Appendix showing clearly that the same phenomenon occurs.

Reviewer 3:

Reviewer's comments:
The work is of significant pedantic and instructional relevance to the community performing umbrella sampling calculations of host-guest systems. It is of relevance to the targeted audience, but perhaps too targeted. It is worthy of publication but there are certain perspectives which are lacking and would/could alleviate many of the problems mentioned above.

Authors' reply:
With the manuscript we would like to sensitize especially newcomers and non-experts in the field who have for example carried out introductory MD tutorials. Moreover, especially the nomenclature issue pointed out by reviewer 1 (potential of mean force vs. free-energy curve) is indeed handled inconsistently across publications, even from leading groups in the field, making this manuscript also of interest for more experienced users. We agree that for both target groups a broader perspective would be useful. However, without having conducted actual simulations using other methods for these particular systems we are a bit hesitant to convey statements that these are free of artefacts.

Reviewer's comments:
The authors focus on 1-d umbrella sampling simulations using distance as the reaction coordinate and then highlight a series of issues that could arise from such a protocol, how these issues could be identified, and to some extent, how such issues could be at least partially alleviated. In this reviewer's opinion the essential issue is that the 1-d reaction coordinate used for sampling is missing important slow degrees of freedom, or that there are multiple independent pathways thus corresponding to the need for a higher

dimensional reaction coordinate. The authors do a good job of describing how to identify when this is the case. However, they simply do not consider the possibility of biasing along a smarter 1-d reaction coordinate, one that includes these other degrees of freedom. Methods such as Noe group TICA, Fergusons's MESA, Clementi's diffusion map directed MD and Tiwary's SGOOP could be very useful here in performing next generation of umbrella sampling simulations as well as other sampling methods (which the authors rightly mention have similar issues). And if the problem has multiple pathways, then either one could do umbrella sampling along a 2-d space. Thus, while the article is overall excellent, I felt it was missing a "21st century" discussion of the central inherent reaction coordinate problem which is the root cause of the problems discussed here.

Authors' reply:
In our opinion the essential issue is dissipation if the ligand switches its orientation immediately once it has the chance to do so. In the umbrella windows that belong to the bound state, the ligand is pretensioned and once the ligand has the freedom to turn, this tension is lost. In that sense, this issue is similar to the dissipation issue reported by Gilson and co-workers (Refs 28, 29). A two-dimensional umbrella coordinate that includes an angle, specifying the orientation in addition to the distance might indeed be a suitable solution. In the revised version of the manuscript we mention this possibility in the conclusion section and also provide some references for this methodology. Employing the set of further methods mentioned by the reviewer would be interesting but is beyond the scope of the present manuscript. However, we would be very happy if our system is revisited by employing other "21st century" methods in the future, since this would perfectly fit into the concept of the journal to publish living documents.