**Reviewer 1**

This manuscript describes a theoretical framework aimed at helping to determine the number of individuals sampled via environmental metabarcoding approaches.

I will start off by saying that I do not have the appropriate background in population genetics or mutational theory to rigorously assess the utility of this statistical framework; furthermore, this manuscript appears to focus on COI metabarcoding, which is not my background either. However I will offer some general comments based on my experience with empirical rRNA metabarcoding studies.

Are the methods in this manuscript targeted towards non-coding barcoding loci (e.g. nuclear rRNA genes, which are commonly used in environmental sequencing surveys of eukaryotes such as protists, fungi, nematodes, etc.), or protein-coding barcoding genes such as COI? The introduction mentions rRNA genes, however the experimental section and results appears to exclusively focus on haplotypes recovered from COI or other protein-coding genes (lines 99-104). Because of the differential inheritance mechanisms and mutational constraints for loci such as nuclear rRNA genes vs. COI, it would not be appropriate to use a generalized mathematical framework that broadly covers both types of loci. This should be explicitly addressed and stated up front in this manuscript.

Lines 57-64: Eukaryotic diversity estimates are also more complicated because of the existence of intragenomic variation in rRNA barcoding loci (e.g. see Bik et al. 2013 Intra-Genomic Variation in the Ribosomal Repeats of Nematodes, PLoS ONE, 8(10): e78230). This means that there is usually more than one barcode representing one individual (or eukaryotic nucleus), usually differentially abundant in the genome as “dominant” and “minor” gene copies.

**Reviewer 2**

In this manuscript, Wares and Pappalardo compare statistical methods that can be used to extract true relative abundances of species from those affected by biases in metabarcoding approaches. Addressing this issue is crucial as relative abundances are negatively affected by several method-derived biases and efficient statistics to improve sequence-derived relative abundances to real relative abundances would improve our understanding of information on community structures obtained from sequence data.

 This work is well written, and explains in an easy-to-follow way each part. One concern is that many parts (especially the beginnings of the introduction, experimental selection etc) could be shortened and more focused to the scientific information needed rather than narrative. Some more focus could be placed in the beginning of the introduction by directly explaining the relevant study question and only later introduce the example of flipping a coin. But this is a personal feeling and this suggestion could, depending on the readership, be discarded.

However, the results and especially the discussion should be extended. How do these result make sense in the light of other studies, what can we infer from it etc. E.g. in the most relevant discussion part (3.4 comparing methods), I am missing a clear statement what should be applied and why. If the gamma method is only suitable for small datasets, could this then entirely be omitted not to step into the trap of analysing a too big sample set? Can or should the sampling theory method always be applied? How have other studies applied these statistics so far and could that have lead to a different meaning/conclusions?

The conclusions part is rather long and includes a lot from what I think should be discussion. Please consider rearranging it.

Further I think that more references would be needed to explain the background and even more, related the findings to work that has been done before (e.g. on nematodes (Porazinska et al. 2009, Creer et al. 2010, Porazinska et al. 2010)). This would widen the scope and relevance of the work described here.

Other minor comments

L43-44: I agree that studying organisms in the context of climate change is important, but “more than ever” sounds a bit overstated (and is also not focus of this work)- previous analyses also were often of similarly high value.

L50: NGS approaches rarely (and, if so, mostly unreliably) identify taxa. Rather it is OTU, some of which can more or less reliably be assigned as a real taxon. This is now getting better with improving sequencing technologies, but I would appreciate a more careful wording and interpretation.

L56 onwards: if the term “microbes” are used rather than prokaryotes, some other examples of microbial organisms are needed. For instance, the issue of primer bias, differences in copy numbers and annotation difficulties have recently been shown to exist in fungi (Ryberg in press) and protists (Geisen et al. 2015). Therefore, please include those kind of examples or use “prokaryotes”.

L99-104: COI might be a haploid marker, but multiple mitochondria can be present in a cell and sometimes different variants (minor, i.e. 1 or 2 bp differences in the COI marker) can exist at least in asexual populations. Therefore, the term allele as also used later (e.g. L152) might not entirely be the correct term or should be defined more clearly.

L297 onwards: I am not sure if there is any environment from which we know the entity of taxa being present and ongoing improvements of sequencing technologies will continue to show missing taxa. Furthermore, most systems change over time and will result in an altered community. This might be mentioned here as it will change the conclusion.

L346 onwards: Most studies show that relative abundances of many groups are not reliably reflected by metabarcoding approaches (references cited in 346 and e.g. (Porazinska et al. 2009,Geisen et al. 2015)), therefore a single example mentioned showing that relative sequence amounts can reliably reflect true relative abundances of the taxon targeted seems rather odd to mention. Of course sequence data reliably reflects relative abundances of taxa in a sample but the fact that it does not work for even more is more important as the technique itself is then not reliable in case “true” relative abundances need to be determined. This should be mentioned in more detail.

Additional literature that could be cited:

Creer, S., V. G. Fonseca, D. L. Porazinska, R. M. Giblin-Davis, W. Sung, D. M. Power, M. Packer, G. R. Carvalho, M. L. Blaxter, P. J. D. Lambshead, and W. K. Thomas. 2010. Ultrasequencing of the meiofaunal biosphere: practice, pitfalls and promises. Mol. Ecol. **19**:4-20.

Geisen, S., I. Laros, A. Vizcaíno, M. Bonkowski, and G. A. de Groot. 2015. Not all are free-living: high-throughput DNA metabarcoding reveals a diverse community of protists parasitizing soil metazoa. Mol. Ecol. **24**:4556–4569.

Porazinska, D. L., R. M. Giblin-Davis, A. Esquivel, T. O. Powers, W. Sung, and W. K. Thomas. 2010. Ecometagenetics confirm high tropical rainforest nematode diversity. Mol. Ecol. **19**:5521-5530.

Porazinska, D. L., R. M. Giblin-Davis, L. Faller, W. Farmerie, N. Kanzaki, K. Morris, T. O. Powers, A. E. Tucker, W. A. Y. Sung, and W. K. Thomas. 2009. Evaluating high-throughput sequencing as a method for metagenomic analysis of nematode diversity. Mol. Ecol. Res. **9**:1439-1450.

Ryberg, M. in press. Molecular operational taxonomic units as approximations of species in the light of evolutionary models and empirical data from Fungi. Mol. Ecol.