An interesting case study of metabarcoding revealing unexpected diet in an endangered marine bird

Dear Marina Querejeta and colleagues,

Your article has now been reviewed for the second time by two referees and myself. All acknowledge the large improvements made on the MS, including a spectacular improvement in data analysis and results thanks to the referee's advise. Nevertheless, both referee still have a couple of concern I believe deserve to be seriously accounted for.
Following referee 1 advise, please pay attention to the use of terms such as read abundance and biomass, and necessary details in the description of what has been done: for example manual curation of data requires detailed information on the strategy adopted, the rationale behind it, and the steps leading from raw to exploited data (and associated information on taxa discarded). Discarding arbitrarily OTUs that are considered as contaminant because they are not potential prey, to the best of your knowledge, is not acceptable. You may want to use a specific package such as decontam to make an objective work and discuss uncertainties about remaining OTUs. Referee 1 also ask a set of relevant technical questions in need for an answer and more detailed & clearer explanation of the steps followed, keeping in mind that anybody with the article in hands would be able to repeat exactly the work starting with raw data and the material and method section.
Referee 2 still has two very important comments, beyond other relevant minor comments: the first one is the uncertainty around the assignment to Talitrid, the second is the interpretation (would the assignment be ascertained) in terms of dependence to fisheries and cascading impacts of the change in diet for petrels. First of all, I suggest the authors to carefully revise the manuscript replacing the taxa name 'xxx' by 'assigned to xxx', particularly when the homology is low and the assignment to the genera or family level, which is clearly the case for Talitrids. This phrasing helps to keep in mind the uncertainties we are dealing with. Second, the authors may think through the hypothesis and suggestions made by referee2, to improve data analysis and revise part of the discussion. The authors may for example consider extracting the fasta sequences of these ASVs and align them against a homemade database for amphipods, in order to ascertain manually the level of homology and the closest possible relative. Such homemade database may as much as possible strongly favour sequences of high-level confidence taxonomic inference, such as holotypes, in order to avoid badly assigned sequences in the public databases. Given the 78-86% homology that is low, it is in fact impossible to ascertain these OTUs really belong to Talidrid rather than any other closely related family absent from 16S reference databases. Would the uncertainty remain the same, a leveraged and more cautious discussion (with a lighter mention to the possibility of Talidrid dominance in the diet) would be advisable. Would the uncertainty be much lower, and the Talidrid assignment be confirmed with a much higher level of certainty, the discussion may include the path suggested by referee 2.
I will finish with a first general advice, following a first submission of the wrong file and a second submission with a track changes files that contains only a subset of the changes made since the primary submission, and personal comments exchanged among coauthors. This

reflects badly on the carefulness of the prime author and on the attention dedicated to the review process.
I thus urge you to carefully account for the comments of referee, and to prepare a carefully checked last version with very clear track changes compared to this one. A simple option is to compare the very last version to the very first one with Word, allowing a clean and complete file with track changes without omitting important ones and including personal comments.

Soiphie Arnaud-Haond

Reply to editor:
Dear Dr. Arnaud-Haond,
Thank you for this second round of useful comments and we are sorry for the last revision. Track changes are now in the manuscript (only the ones from this last revision).
Regarding the advice of referee 1, we have discarded in the manuscript the mention of Relative Read Abundance (RRA) as a proxy of food biomass. We understand that it can be confusing and we have followed her suggestions. Concerning the discard of OTUs, this is not arbitrary. However, we understand that it was not very well explained in the first manuscript. We have fixed that by stating that we discarded sequences from prokaryotes, fungi, insects, mammals and the Westland petrel itself. We cannot use a decontamination software, as this is strictly based on the biology of these taxa. We strongly believe that this is now thoroughly explained. Finally, we have done our best to answer the technical questions of referee 1.
In the case of referee 2, we have acknowledged his suggestion of using Talitroidea instead of Talitridae, which we think is a wise suggestion. Moreover, we have included his suggestions in the manuscript, including the need of further research on the diet of the Westland petrel, but using a food web approach.
We have changed the manuscript, especially, the discussion following the suggestions of both referees.

Referee 1. Babett Günther

Dear Authors and Editor,
tanks for the possibly to review this paper again and for using dDNA I will include that in my future work.
The authors reanalyzed the data with an improved up to date bioinformatics pipeline and included most of the mentioned points. The strait forward and easy to understand methods presenting nice results. However I still have some questions regarding analyzing the final data and their interpretation, essential for the discussion. Including missing arguments and changing given statements would made this study eligible for publication. Congrats to your work.
Kind regards, Babett Günther
General short remark
There was a misunderstanding what a document with track changes are, there should be all, not only from the last corrections of the coauthors. I hardly see the changes done mentioned in the text, majority is not shown, that makes it much harder to review. You have to use using the comparing function in words. Happened to me before, so I am not judging, but please be sure you working on the correct version for the revision. I was hiding all changes and comments, so please consider this for the line numbers to review. Figures belong to the end not within the text. Lot of spaces and volatility errors, can it be that per accident the wrong version was send? Only to make sure there is no confusion with the further corrections
Major points
1. There seems to be a big misunderstanding of read abundance and their interpretation, at the end of the discussion it is stated as "read abundance (food biomass)". This is not correct; the current literature is showing across the fields and ecosystems simply an indication of a link or even trend between biomass and read abundance. However, read abundance should not be interpreted as biomass, without intense testing of the set up. Has crustacean biomass/ or volume of tissue the same amount of targeted DNA than octopus or fish; have the cells the same size, weight and density? Are they degrading differently fast via digestion based on different skeletons? We simply do not know. Relative read abundance is a useful tool to compare relatively between detection within families or even phyla, however between phyla it has to be taken with caution.
Please read Elbrecht, V., & Leese, F (see below). The PCR bias and the Primer bias are essential to understand your data. A logical point has to be, that higher ratios of Arthropods indicating the primer easier bound to arthropods than to others. It is possible that you actually have a majority of crustacean DNA in the samples; however, we simply cannot determine it with this set up. I recommend including this in the discussion to avoid any further confusion.
Elbrecht, V., & Leese, F. (2015). Can DNA-based ecosystem assessments quantify species abundance? Testing primer bias and biomass—sequence relationships with an innovative metabarcoding protocol. PloS one, 10(7), e0130324. This based on macoinvertbrates but it
2. What Metabarcoding can clearly say id the detection of diversity. I am wondering about the deleting of OTU unique to one sample, this can have a major impact on the alpha diversity. I wonder how much this is affecting your comparison between the seasons and populations.
In addition, the "manual filtering" of OTU, of "possible not prey" without further explanation is not fulfilling scientific standards. In this logic, amphipods based on their size should be excluded

as prey. Latter in the discussion it is distinguished correctly between active, passive, primary and secondary prey, but after potential prey was deleted, because it could not be prey… these logic is not fulfilling. See below in more details
3. Technical flaws of the protocol are not degrading this good study, but have to be acknowledged. The mock was for bacteria and could not be used at all, so it should be simply be deleted from the text. Secondly, the PCR products where mixed between two Primer set ups, and probably as a consequence, one fragment was sequenced insufficient. The statements made in the discussion need to be corrected urgently. See in detail below
4. Including the first three point, you could think about restructure your discussion for a better reading flow. Your comparison of literature is deep and omnibus leading to really interesting points, but they get a little lost. By avoiding your methodological limitations, it seems sometimes forced to find ecological explanations for the results. There are some singled out paragraphs for potential explanations, which than later seen as proven facts. Better is to understand what the data are able to tell and explain the indications for the ecological behavior of the birds.

Dear Dr. Gunther,
We strongly appreciate this second round of useful suggestions and we apologize for the misunderstanding in the document in the latest version. As suggested, we have accepted the previous changes and left only the ones from this last revision to avoid any confusion. Figures are now after the references, as required.
Concerning the relationship between "food biomass" and Relative Read Abundance (RRA), we agree that indeed there is bias as bigger species have shown to produce more sequence reads in some studies. It is a "hot" topic in continuous discussion (Evans et al., 2016; Neby et al., 2021; Rytkönen et al., 2019; Schenk et al., 2019; Zamora-Terol et al., 2020). But, it could be also true that if a prey is bigger, it will be reflected in the number of reads, but also, in the biomass. The predator would potentially eat more grams of this species. But this is only a hypothesis and we agree that could lead to confusion and that dedicated studies would be needed to draw conclusions on prey biomass. Thus, although RRA has been used as a proxy of abundance in several publications, we have removed any reference to biomass from this latest version of the manuscript. We only use RRA for comparisons and descriptions. Also, please note that we always present RRA together with Frequency Of Occurrence (FOO) as this other metric is more conservative (even if other biases exist). Together, RRA and FOO have been proven to be the best and least bias manner to characterize diets from metabarcoding studies. We also agree that there could be a primer bias that overestimates the number of talitrids (arthropods) and we now acknowledge that in our discussion.
Regarding your second major point about removing singletons, we strongly believe that, in our case, it is a correct measure of filtering. After processing metabarcoding data, the aim should be to obtain a set of biological sequences that are the nearest possible of what is really present in the target community (diet of Westland petrel here). Our main concern is not so much that singletons could be contaminants but more importantly that they could be artifacts (Brown et al., 2015; Majaneva et al., 2015). Usually, singletons are known to affect in a minimal way community composition (Shade et al., 2012) or multivariate analyses (Gobet et al., 2010; Lindahl et al., 2013). These are just arguments from the literature, but in our case, we only had

0.027% of singleton reads, these reads did not affect our final results and we did not have to exclude any rare taxa (according to preliminary analyses performed with singletons). Moreover, our aim is to describe the diet of the Westland petrel, to find general patterns rather than detecting rare species. In short, we strongly believe that this approach is conservative and necessary in our case as it allows us to conserve important data but to filter out potential artifacts and contaminants. We have included the related references in this revision of the manuscript.
The manual filtering was really precise and repeatable, we discarded sequences classified as prokaryote, fungi, insects, mammals and the Westland petrel itself, according to the previous knowledge regarding the diet of the petrel (Freeman, 1998; Imber, 1976) (and to any seabird). Nothing that could potentially be a prey was discarded without discussion.
Following your suggestion, we have deleted the reference to the mock community from the latest version of the manuscript. We agree that a variation in the protocol of the two primer sets could have maybe shown better results and we have modified the text making clear that this is the case. However, in our experience dividing the libraries per primer set (if possible) has shown better performance and more reliable results. That being said, these methodological considerations go beyond the scope of the current manuscript. Thus, we have modified the text accordingly and we appreciate your comment.
And, finally, we have re-structured the discussion and we hope that now is more fluid and understandable. We also took care of all suggested minor points.

Minor points
Introduction
L 89 "Selecting the correct experimental design…. " is true for everything, and correct/accurate sounds a little over the top. My suggestion delete the first part of the sentence and keep "challenging based on rare direct observations". Which also true for most animals.
Changed.
L 105 rewrite potential biases, as it is not clear. Define the biases, probably you want to indicate that the species identification is often not possible, and soft prey are overseen in biomass calculations.
Rewritten and changed.
L115 "(and specifically dDNA) "is nice, but don't belong to this sentence and in front of this publication; delete
Deleted.
L 144 I would delete the first part of the sentence, because based on you introduction this unnecessary, even if, this studies are old and not reflecting the current fishery impact. "The composition of….."
Changed accordingly.

At the end of your introduction, instead of using questions and statements, better define research aims I,II,III….
Corrected.
Methods
Based on you answer to the mock communities, your answer was:

The inclusion of a mock community was standard practice by the sequencing company, but this mock community is designed for microbial studies. Un surpisingly, this mock community did not produce any reads, which is why we did not give any further information about it.
Than delete it out of the manuscript, because it is misleading.
Done.
190 -198? Mentioned that the primers are tagged for the two step PCR
Done.
203 not specific enough, "manufacturer's standard protocol", there are several. Please include more details about the concentrations
Done.
204 I don't think this was the best time to pool, can you give a explanation why you pooled there, if not its kay, but please include if you equalized the PCR products.
Done. Samples were indeed equalized and pooled here, this was a requirement of the sequencing platform.
231 picking?
Changed.
234 "mapped a by-sample reads to OTUs" makes no sense.
Changed.
236 is the fasta including the sequence of the OTU or the asv per OTU?
OTU sequences. Changed for clarity.
238 the sentence is not really clear, please rewrite. What did you download, or did you blast by uploading the sequences to the internet? If you did blast on your computer, server, please give the exact release of the database
Rewritten. In this case, we retrieved it from the web interface.
239-242 can't follow, you say the r script does "assign a taxonomic classification", but this is done by blast n, what do you use the r script for?
Rewritten. We used a script to retrieve the best hit and all the parameters that we need in a .csv. We are aware that *BLASTn* does that. However, the custom script was more efficient and useful for us in that case.
246 you don't "classify" OTU's , please change
Changed.
248 "the" taxonomic assignment, discarded are OTU's……
Changed.
249 be more specific with subtracted. Means all was deleted, or the number of sequences? When the OTU included 100 sequences in the negative and a sample 1500 , is the whole OTU deleted, or are 500 left at the sample.
Rewritten. Exactly, it is the number of reads what we substracted.
251 singletons among samples and OTUS, makes no sense at this step anymore? You mean OTU only present in one OTU? Could it be a rare prey, only one bird catch, as you compare different population it would be interesting to see the diversity. In combination of making an presence absences FOO, you definition is a Prey has to be found by at least 2 individuals? Why? Simply contamination is not enough argumentation therefore.
We actually mean OTU represented by single reads only. As explained in the general answer before the minor comments, we did not take them out only because of they could be a

contaminant but more importantly, they could be artifacts. We now explain this in the text, we also include references supporting this (e.g. Brown et al. 2015). Given that our aim was to describe the diet of the species, we believe this conservative approach is the best option especially given that these OTU represent a very small percentage of reads.

252 "manual filter…" that's a clear no no. There is no nut picking in science which data you want to use and which not. In addition, the whole process has to be able to be repeated with leading to the same result. That's scientifitc standards. You can clearly state, that every prokaryotes and certain phyla are deleted as they are unlikely to be intentional prey. But you have to clearly set the standards here. Otherwise, you have to give a clear list of all deleted taxa, with a reasoning, at the supplementary

Done. We have clearly stated which taxa were discarded. You are very right on this. Thank you very much for this suggestion.

260 this sentence is confusing, you did a taxonomic assignment already way before, what is the meaning and intention here?

This is now better explained in the text. In short, Loliginidae classification produced hits corresponding to taxa that could not be in New Zealand's waters as it is not their distribution range. That is the reason why we thought this was a problem of assignment. After checking existing sequences in the reference libraries we found that the amplicon used does not have enough resolution to resolve taxonomy at species level within this group. To be on the safe side, we stayed at the family level, which already provides useful biological information.

329-334 please give the number of OTUs

Included.

334 delete prey

Done.

334, is one of this 17 samples with an OTU, which was not in any other sample? If yes, you should really think about not excluding OTU only because they are unique to one poo sample

No, there was no rare OTU deleted, we only removed the singletons as they could potentially be artifacts .

336 correct "additionnal" to additional

Done.

344 "24.02% (19 OTUs, 195,358 reads) were identified to species level, 29.11% (23 OTUs, 222,447 reads) were identified to genus level and 100% (56 OTUs, 316,587 reads)"; this calculation makes no sense. So the 19 on species level are included within the 23 at genus level? And how can 56 OTU of 79 be 100%

Changed.

348 delete "Gobally,", and potential prey, that's judging but we still in the results. Combine it with the next sentence and simply state its phyla with the highest abundance. Moreover, delete the finally as well

Done.

392 make P.westlandica italic

Given that the subheading is in italic, the latin name has to be conspicuous with the rest of the text. Therefore, the latin name is not italicized. For clarity, we have underlined the latin name.

depending on the final formatting in the journal, the name may be written in italics if the rest of the subheading is not.

394 delete" important "
Deleted.
458 again if you delete single OTU, and then compare alpha diversity, its questionable
No significant changes. Already explained above.
475 "infer almost 90% of the prey species" this number based on what?
It is the completeness of the sampling according to the rarefraction curve and the bootstrapt estimate (Fig.S2). The bootstrap estimates that the asymptot of the rarefaction curve is 89 OTUs, yet the curve itself reaches 79 OTUs, which corresponds to 88.6% of the asymptot.
478 include more refernces and more recent ones, like Wangensteen, O. S., Palacín, C., Guardiola, M., & Turon, X. (2018). DNA metabarcoding of littoral hard-bottom communities: High diversity and database gaps revealed by two molecular markers. PeerJ, 6, e4705. doi: 10.7717/peerj.4705
Done.
479 it was definitely not "approach proved unnecessary". Having a multigene and primer approach is good practice and should be standard. However, you had PCR products, or? So the primer worked. You simply made a big mistake to pool the PCR products together incorrectly. There are ways to sequences several primers together to ensure necessary sequencing depth, you simply did not apply it. This can happen, as your other results are good, no big deal. But proven good practice, and probably good primers as a failure because of your "quick and dirty" approach did not work out, has no substance here.
Rewritten.
481-483 delete this senctence based on the comment before
Done.
509 516 you have to clearly sate that you theory is secondary predation. You only indicate it here
We have re-arranged the paragraph in order to be clearer but our theory is not that is secondary prey, we give the two hypotheses. In fact, our main theory is that is a mixed of both The main message here is that it is important within the flow of energy.
517 see major point 1, Metabarcoding as biomass, is not accepted by the scientific community. Studies show that we can use relative abundance, but these systems have been tested for that, or at least to be compared to morphological data. So your logical conclsion has to be that you genetic system cannot be used to describe abundance but for detecting diversity.
Changed.
560, also variation of populations could be acknowledged, or is it the same the same location as this papers?
These articles do not account for geographical variation and do not provide the required level of detail for such analysis. It is likely that previous authors did not investigate such variation because all Westland petrel are restricted to a relatively small area in New Zealand. In any ways, any differences between older studies and the current one is likely to reflect temporal variation in the diet (since older studies took place 20 to 30 years prior) rather than geographical variations as the nidification areas remained the same.

563 no it does not confirm, you suggest/indicate/underline, but not confirm
We guess that you meant line 555 and we changed it to take into account your comment. We rewrote also 563.
652 , but also…. This part of the sentence is an own argument and should not be part of the conclusion.
Done. (but it was line 644, right?)

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Referee 2. Francis John Burdon

Comments to the Author

I appreciate the time invested by the authors carefully considering the comments made by the reviewers and editor. As I see it, there are two problems with the amphipod result. One is if the correct assignment has been made – although the amphipods could be a marine talitrid, they could also be another closely related family (or families). The other problem is what this result means – are most of the amphipods prey of fish that the tāiko have scavenged in fisheries waste? If so, that would be an interesting result, because it would appear that the petrels are highly dependent on trophic subsidies from the fisheries industry (further confirming the study by Freeman 1998), and that the prey of fish are of consequence for the petrels. That is, changes in the diet of the fish could have cascading impacts on petrels. Although further work needs to be done to confirm this, if true it would have conservation implications.

I am mostly satisfied that the authors have addressed my concerns to the best of current knowledge. However, I think the authors could check the assignment of the amphipods and if it makes sense to use a higher level (see below). That problem notwithstanding, I expect that the present research will help stimulate future studies to help resolve some of the open questions.
I think the limitations of matching taxonomic units with the identified sequences available mean the authors must be very cautious with the assignment of the Talitridae. Whilst there are marine talitrids (e.g., Lowry and Bopiah 2012), their assignment in the present study could be the result of 1) insufficient information or 2) incorrect information resulting in a faulty match. For the latter scenario, a genus like Allorchestes (from the family Dogielinotidae) – a coastal marine amphipod that was previously associated the Talitridae (see Hurley 1957) – could mislead the authors due to a faulty match in the genetic database (so it could be worth checking this and/or considering using a higher-level grouping under the superfamily Talitroidea). The ongoing challenges in amphipodan taxonomy probably needs to be recognized, and Fenwick (2001) described Allorchestes as “another long-confused species” temporarily placing it under the Hyalidae. For more recent taxonomic information I recommend the authors refer to the WoRMS online register (e.g., AphiaID: 236962). Their exact taxonomic identity notwithstanding, I think in all probability the amphipod(s) are marine species, since the other crustaceans found in the diet of tāiko (and most likely the fish they feed on) are all coastal marine species.
At any rate, I am happy that the authors recognize the potential for the “Russian dolls” problem with regards to fish and potential prey items, having dealt with this in the revised manuscript. It could be worth highlighting that a food web approach might resolve these problems (by unequivocally describing the diet of prey fish).
I can comment that the controversy over “what is environmental DNA?” is not new: there has been considerable discussion on this matter in the literature (see Pawlowski et al. 2020, Rodriguez-Ezpeleta et al. 2021). I personally have no problem with DNA from fecal samples sourced in the environment being described as environmental DNA, but using dDNA is fine as long as it is clear that DNA from fecal samples has been metabarcoded.
Please see an annotated version of the manuscript here for some suggested changes.

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Dear Dr. Burdon,

We appreciate and thank you for the last suggestions regarding our manuscript.

Regarding the amphipods (Talitridae) problem, we understand that it is a flaw in our study but, at the same time, we agree with you that it opens a new avenue of research. We agree with you that using the higher-level classification, the superfamily Talitroidea would be more conservative and would encourage further research. Even more, when taking into account the somewhat unresolved taxonomy of amphipods. However, this manuscript does not aim at delving into the taxonomic challenges of this group and the WoRMS database cannot help resolving the DNA identification. Thus, we now use Talitroidea throughout the manuscript and explained it the first time this taxon is mentioned. We thank you for this suggestion. We also think that this has important implications from a conservation point of view, but further research, outside the scope of this study, is needed before making more conclusions.

Moreover, we have acknowledged in our manuscript that a food web approach would be convenient to shed light onto the “Russian dolls” issue and it will help us know whether Talitroidea is really direct or secondary prey, or both (which is our preferred hypothesis at the moment).

We are aware of the environmental DNA title as a “hot” topic. We have changed it to dietary DNA (dDNA) following the suggestions of the editor and other reviewer. We think that both terms are correct if they are correctly explained but I think we have explained that it comes from faecal samples (even more now with the changed title).

Finally, we thank you for the very useful track changes suggestions in the manuscript. We have included them.