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Dear Dr Urlich

Review of "Pochon, X., Keeley, N. and Wood, S. (2017) Development of a multi-trophic level metabarcoding tool for benthic monitoring of aquaculture farms. Prepared for Seafood Innovation Ltd, New Zealand King Salmon Ltd, Ngāi Tahu Seafood, the Ministry for Primary Industries, Waikato Regional Council, and the Marlborough District Council. Cawthron Report No. 2980. 48 p. plus appendices"

As requested, I have reviewed the Cawthorn report to assess whether the report provides a robust empirical basis for using metabarcoding in environmental monitoring of benthic effects from aquaculture farms. I have recorded my observations in this letter, including a brief background to show how this work fits into the current regulatory environment. I have made some general comments on the report, before going into some aspects in more detail.

Background

The aquaculture sector is growing rapidly and now accounts for >50% of global fish-supplies. Salmon is one of several high-value aquaculture species and is grown in several centres globally including New Zealand. Salmon culture has a number of impacts on the receiving environment that, in the NZ context, are primarily attributable to farm-derived organic material (faeces) and the loss of feed. This organic material sinks to the seabed where it accumulates to various degrees as a function of current exposure and farm-proximity. Seabed-accumulated organic material is associated with changes that vary from modest enhancement of benthic richness and biomass to complete macrobenthic extirpation.

Most jurisdictions, including NZ, require benthic monitoring around aquaculture sites to inform the sustainable management of the industry. In common with many countries, NZ benthic monitoring includes the routine grabbing of sediment samples, washing the material through a 1mm sieve and identifying the retained macrobenthos. Patterns of macrobenthos, for example the dominance by opportunistic species, together with physical measurements (e.g. organic matter and redox) are used to derive an 'Enrichment stage'. This index is used in the assessment and management of the industry.

Macrobenthic sampling and analysis, particularly species-level identification, is both costly and dependent on a declining pool of suitably qualified taxonomists. Relatively recently, technological advances are enabling the identification of fauna based on the presence of their DNA and/or RNA extracted from sediments. Specific genes within extracted DNA/RNA are subject to amplification via PCR to generate a 'library'. These genes allow varying degree of taxonomic identification. The

sample-library is then subject to sequencing using high-throughput sequencers (HTS) and unique DNA/RNA sequences are linked individual taxa. Various technological innovations allow the combining of numerous samples within a single library and these are sequenced automatically. The potential for a substantial per-sample cost reduction using HTS is considerable.

General comments

The work reviewed here aimed to demonstrate that the information that could be derived from a sample's 'molecular-based' signature (multi-trophic Metabarcoding Biotic Index, mt-MBI) was equivalent to that derived from the traditional macrobenthic approach. The study was based around 3 farms only, and therefore the results should only be considered within this sampling context.

The reported approach does not attempt to reproduce current macrobenthic monitoring by limiting the molecular identification to macrobenthos. The adopted approach is truly multi-trophic (covering community components from bacteria to megabenthos) and this confers a considerable advantage, particularly in assessing change in organisms that may respond at different temporal-scales to farm-related pressures. In addition, the focus on macrobenthic eDNA, as adopted by some researchers, faces the problem of adequate sampling given the very small volumes of sediment taken for molecular analysis. The approach adopted here does not suffer this disadvantage.

The reported approach is highly empirical and based on pattern matching. This is entirely appropriate within the context of the questions being asked of the research but it does limit the technique to the farms/sea-areas that were assessed. This is fully acknowledged by the authors, hence their recommendation that the technique be developed on further sites, both in terms of testing current indicator taxa and identifying further indicator taxa. The apparent site-independence of the bacteria-based MBI is very encouraging (but needs further development, as is acknowledged).

The proof-of-concept in relation to the logistics of high-throughput-sequencing and multiplexing is entirely appropriate; the research makes a valuable contribution to this aspect of technique development. The research indicates limited applicability of foraminifera (forams) as biomarkers; this is of considerable interest as excluding this group will not lose significant discriminatory power and will reduce costs (forams require a separate PCR step).

The research indicates that analyses based on DNA and RNA give equivalent results. The authors conclude that RNA analysis may not necessary – this would offer a substantial cost saving because RNA analysis is considerably more technically challenging compared to DNA analysis. This is a very worthwhile contribution to the technique.

The analysis presented here is based on a presence-absence transformation of the reads data. This severe transformation has the benefit of normalising across each taxonomic group making the analysis more straightforward. This approach offers a valuable saving in analytical time and the simplification will make the technique more broadly applicable.

The research presented was conducted thoroughly and is excellently reported. In terms of the main conclusions drawn from the work (Section 4 and 5) my only slight concerns relates to the use of confidence intervals (Figure 11). My recommendation is to replace these with prediction intervals,

based on a given sample size (see Sokal and Rohf, 1995, Biometry, 3rd Ed). The use of prediction intervals would more appropriately reflect the objective of matching known Enrichment Stage with the predicted mtMBI-based ES.

The specific comments below indicate where additional detail and explanation would assist future interpretation.

Specific comments

1. The development of the multi-trophic index is clearly weighted for those species/ groups that are associated with Eco-group V i.e. associated with highly enriched sediments. This empirically derived weighting does not detract from the findings but it suggests that the mt-MBI is largely determined by the presence of opportunistic species. This indicates that the technique might be less able to distinguish between samples that are considered to be 'intermediate' in terms of traditionally derived enrichment stages.
2. I would be interested to see, in table form, the degree to which the OUTs ('molecular species') are unassigned against, for example, phyla/class and Enrichment Stage. For example, there are >6,000 bacterial OTUs (Table 5 and Section 3.1.2) – most of which were not assigned to a particular species (I assume). Better understanding of this would help in interpreting the data.
3. The minimum and maximum number of clean-reads per sample (Table 5) should be provided to aid interpretation – perhaps some samples had very low number of clean-reads? If so, this should be explained. Plotting the Enrichment Stage vs number of clean-reads would also have been interesting to see.
4. Figures 5A – D are particularly useful in data interpretation. However, in order to better determine the utility of the technique it would be helpful to see the outcome of the triplicate samples. I assume that the MDS-points on the ordinations represent the mean of the three replicates (replication as indicated in Table 2). Understanding the variability between grabs, at the same stations, using the molecular approach is essential in determining the most useful sampling approach. Understanding temporal and spatial variability in mt-MBI is a necessary prerequisite to fully utilising this technique (this includes within the grab, including as a function of sediment depth i.e. compare 0-2 cm and 2-4 cm).
5. Figure 11 shows the functional relationship between the determined ES and the associated MBI. In the context of this research should the predicted parameter (ES) be on the Y axis and the predictor (MBI) be on the X axis? Also, confidence intervals are given but, in this context, prediction intervals would be more appropriate given a prediction from one (MBI) to the other (ES) is the objective.

Conclusions

The work reported is of high quality. We concur with their findings contained within 'Section 5. Conclusions and future recommendations' and would urge the relevant authorities to facilitate the research necessary to commercialise this technology.

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