Reviewer 1

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| In this manuscript, Kretzschmar et al. present a qPCR assay that is capable of enumerating Gambierdiscus lapillus from various macroalgae on which it lives as an epiphyte. The development of specific primers is certainly of value to fellow researchers. The standard curves presented were certainly of high technical competence. | Thank you. |
| However, it is of the opinion of this reviewer that the manuscript in its current form is simply a methods paper that does not rise to the standard of publication in PlosOne. The journal guidelines state: “The tool must be of use to the community and must present a proven advantage over existing alternatives.” As the primary author has previously demonstrated the ability to differentiate G. lapillus microscopically (Kretzschmar et al, 2017) this reviewer is not convinced that the additional qPCR method adds substantially to the previous method, so as to be a stand-alone paper. The greatest value of qPCR is in those situations in which either it is difficult to differentiate species, or the species is of such low concentration that an amplification of the signal is necessary for detection. Neither of these criteria seem to be warranted in this particular situation. As such a 1 or 2-figure research communication is probably more appropriate, or as an alternative the technique can be used in the context of another manuscript that characterizes the full suite of toxins in G. lapillus (a question explored but not fully answered in the 2017 Journal of Phycology paper). Alternatively, the authors could have extracted DNA directly from macroalgae and then done a comparison of the qPCR signal between dislodged G. lapillus, and those that adhered. This would have given a better idea of the ecology of the system, and probably a more accurate rendering of the population size. As microscopy of adherent microbiota on the macroalgae is much more challenging than free in suspension, it also would have given stronger justification for publication in this journal, as presumably no assay exists to quantify adherent cells. | The species description of G. lapillus clearly states that there is intra-species morphological variation which makes this species difficult to identify microscopically. This has also been observed for other Gambierdiscus species, which is why it is important to support identification with molecular techniques.  The necessity for qPCR assays for G. lapillus, as with any Gambierdiscus species, lies in the potential for toxin production. While the toxin profile has not been exhaustively characterized for this species, G. lapillus displayed ciguatoxin-like activity in bioassays (Larsson et al. 2018 [Toxicology of Gambierdiscus spp.(Dinophyceae) from tropical and temperate Australian waters](https://www.mdpi.com/1660-3397/16/1/7)). It is part of the ciguateric web around Heron Island, and as such enumerating it’s cell numbers, beyond simply identifying the species, has merit for monitoring purposes.  The environmental samples from this study do constitute characterization of adherent cells. The macroalgal samples were vigorously shaken to dislodge the community of protists attached to their surface, and then the G. lapillus fraction of the community was queried. Amended methodology to make this clearer. |
| In considering the contents of this particular manuscript, there were also some other concerns/criticisms that are important to make. First, there were a number of grammatical errors which hinted at a lack of thorough proofreading. For example, line 50 …”which is then either passes” is grammatically incorrect. Again line 70 should read “assessing” not “assess.” There are more, but this illustrates the point. | Proof read and the examples given have been fixed. |
| The intro rambled a bit, and lost focus from the main point, which is that the authors wanted to develop a specific assay for one species. | The authors couldn’t identify the specifics of this comment as the introduction, from our point of view, delivers the necessary background to the genus and method. Please supply specific points of contention and we’ll address these. |
| Discussion of the toxins from other species, [such as in lines 72-92] really had no place in this manuscript, as the question of toxins and their production was not what was being addressed. | The toxicity of these species is key to the necessity for monitoring Gambierdiscus species numbers, hence the authors have left the toxin section is as essential background. |
| I also thought the Tables were in some instances too large or unnecessary. Table 5 was unnecessary, as the text states exactly what the reader needs to know. At best this would be a supplementary figure. | Inclusion of information from table 5 is in line with previous Gambierdiscus qPCR assay development publications (see Nishimura et al. 2016 Quantitative PCR assay for detection and enumeration of ciguatera-causing dinoflagellate Gambierdiscus spp. (Gonyaulacales) in coastal areas of Japan).  Moved table 6 into supplementary. |
| In some cases the Tables and legends were even confusing. For example, it is stated on lines 160-161 that unique sites were determined manually. Actually you show primers in the absence of alignment. I would have preferred to see the actual alignment to see how different these were from other members of the genus. | Table 4 contains the primer sequences for G. lapillus for easy access to readers. The alignments aren’t necessary here as the work following on shows that these primers do not cross react to closely related species. The authors do take your point that some people might be interested in the alignment, so have included that this is available on request. |
| Finally, one thing that was lacking in the materials and methods was the source of cells for the standardization. Were these from strains grown in culture? I assume so, but the authors don’t state as such. | Please see the Clonal strains and culturing conditions section at the start of the Methods section. |

Reviewer 2

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| Kretzschmar et al present the development of an assay to quantify the contribution of Gambierdiscus lapillus. The microalga has been implicated in producing toxins that could lead to ciguatera fish poisoning in Australia. The experiment is well designed and they have shown the assays specificity against closely-related species and also its utility in the environment. The authors also estimate the rRNA gene copy per strain to take the gene abundance and convert it to cell numbers. The authors have made all data available. The manuscript is presented well. Some edits were suggested (minor edits), and mainly focus on grammar and organization. | Thank you! |
| “Absolute” quantification based on rRNA gene copy numbers is difficult. The authors use one method, cell counts and quantification using a gene standard. The authors should address how DNA extraction efficiency effects their estimate of rRNA gene copy number. | Added in the discussion. |
| Also, the method of DNA extraction from the environmental samples is missing. If the environmental samples are not extracted in the same manner as the cultures providing the rRNA gene copy number, how accurate is the rRNA gene copy number for that extraction method? Some additional clarification of the method for the gene copy per cell and environmental DNA extraction is needed. | Added the extraction method. To account for the difference in efficiency between DNA extraction methods, we used the same method as the cell extraction for the cell standard. |
| Line 72-92. Is Gambierdiscus similar to toxin production in Pseudo-nitzicha where the organism although present and capable, may not be expressing the toxin? Is there a consensus on the “actionable” abundance of Gambierdiscus cells in the environment from a marine fisheries/environmental monitoring perspective? | Indeed, there is not – the toxin profiles, and toxicity, for most Gambierdiscus species has not yet been determined. Some congeners are much more toxic than others, so determining the species present is important. Further to that, environmental presence does not guarantee that these cells end up in the food chain. With all these unknowns at play, there is no actionable abundance determined to date. |
| Line 106. Why would users need a reference culture for positive identification? In qPCR, the template for the standard curve could be used as the reference. This could be a plasmid, gBlock, or DNA from target organism. Is the need to verify with the melt curve using the Darius et al 2017 assay because of cross reactivity between species or primer dimer amplification? | Gambierdiscus have a large variation in rDNA gene copies. |
| Line 120-121. Was one of the recommendations put forward by Farrell et al 2017 the ability to detect and quantify organisms implicated in producing CFP toxins? Would be a good transition for you to include in your justification. | Alas she does not. It purely deals with ciguatoxins in fish and associated management for recreational and commercial fishers, not the source. |
| Line 140. The aim of this study was to develop a qPCR assay to exclusively amplify G. lapillus without the need to rely on melt curve analysis for confirmation. Your study adds to the suite of qPCR assays available to quantify organisms that contribute to CFP. | Changed, thank you. |
| Line 144. How were the strains of G. lapillus and G. polynesiensis isolated in the study identified? | Phylogenetically. Added relevant IDs GenBank Accession numbers. |
| Line 204. The cell-based calibration curve is integral to estimating cell numbers. Were the counts using the Sedgwick Rafter done multiple times. Were multiple samples collected and processed for DNA extraction from the cell count or are the copy number per cell based on a single DNA extraction that was serially diluted? The estimation based on a single dilution curve from a single count for each species could be suspect. | The samples were only counted once, then DNA extracted and diluted. The possible uncertainty arising from this has been added in the discussion along with a recommendation that multiple cell counts are performed in future studies. |
| Line 229. How was DNA extracted from 10ml RNAlater preserved samples? You introduced four methods: CTAB, PowerSoil, DNeasy Plant Mini Kit or FastDNA spin kit. The method of extraction and kit has an impact on extraction efficiency and estimating rRNA gene copy number per cell. | Modified CTAB, added for clarification. We kept the extraction method the same between the cell standard and the environmental samples. |
| Line 230. Were gene or cell-based standards used to generate standard curves for the environmental samples? How were cell numbers calculated? | Expanded upon. |
| Line 241. Only three strains of G. lapillus are listed in Table 3, but five in table 5. A negative result could also be due to inhibition. Was the DNA extracted from the non-target species amplifiable with other primer sets (e.g. Eukaryotic or Gambierdiscus general PCR)? | HG1 and HG26 had died before the start of this study. However, I still had genomic DNA frozen to test the qPCR primer sets on, but no cell counts for qPCR which is why these strains were not listed in table 3. However, HG6 was also not used to generate a cell based qPCR standard, so it has been removed from table 3.  Good point about inhibition. I did verify that the genomic DNA amplifies for each species, and that the species identification was correct. Added this in the methods and another column in table 5. |
| Line 250. Perhaps include a sentence on the melt curve analysis for the standards and then the environmental samples. Did the authors observe a single peak and no primer-dimer signal? Were the melt curves for the two strains of G. lapillus identical? | Single peak in the melt curve and no primer dimers or unspecific amplification. Added in the relevant results section. |
| Figure 3. & Lines 268-269 Could the difference in HG4 and HG7 cell number curves also be attributed to extraction efficiency or accurate cell counts? Were the environmental samples extracted in the same way as the cultures used to make estimates on the copy number? Could the authors address the effect extraction efficiency would have on their estimates of cell number? | Addressed these questions in the discussion. |
| Line 343-350. It appears that the qPCR assay is quite sensitive. You are able to detect less than once cell per gram of algal wet weight. Did any of the sites have “actionable” concentrations of G. lapillus from a human health/fisheries perspective? | We don’t quite know what an actionable concentration is at this point. It is highly dependent on the toxicity of the species, which still in question for G. lapillus. Another factor is which macroalgae they are found on as the consumption of the macroalgae by herbivores is going to determine whether the toxins from those cells end up in the food chain. |
| Page 1 line 20-21 Delete “in triplicate” | Deleted. |
| Page 2 line 33-34 move “as a matter of urgency” after the words “was developed” | Moved. |
| Page 3 line 50 change “which is then either” to “which then either” | Changed. |
| Page 3 line 59. Change inhabit to inhabits | Changed. |
| Line 66-68. Reports of Gambierdiscus spp. …. I’m not clear as to what this means: Is the identification based on morphology for some Gambierdiscus spp suspect? | Yes, it is. The Litaker et al. study extensively revised the genus, some species are morphologically highly similar and can even display characteristics used to identify other species. A lot of studies before the genus expansion don’t show enough morphological detail to make a confident identification, and/or don’t include molecular analysis for species ID. |
| Line 70 change assess to assessing | Changed. |
| Line 73-75. While any of these can contribute to toxicity, the toxin profile of many Gambierdiscus species is not well understood and its only CTX that has been clearing lined to CFP in humans. Many assays have been used … | Changed. |
| Line 84. Change to: Extracts from other species, G. lapillus (Larsson 2018) show CTX-like activity, but their LC-MS/MS profiles show an uncharacteristic peak in the CTX phase and none of the typical CTX congeners. Therefore…. | Changed. |
| Line 91-92 Therefore, although bioassays provide a strong indicator for toxin production progress in determining the toxin profiles produced … | Changed. |
| Line 100 change DNA samples to DNA | Changed. |
| Line 120 delete since then. Change if recommendations to of recommendations. | Changed. |
| Lines 154-156 on DNA extraction. Move to the next section, Evaluation of primer specificity. | Left the extraction method where it is but specified which strains this was referring to. The primer specificity section covers a lot of DNA extractions from samples sent to us, which is why we chose to separate those sections. |
| Line 190-193 Delete “For curves …. were generated. Cell-based …. cell concentrations.  That information should be in the section below. In some cases the information was repeated. | Deleted. |
| Line 197-199 Total gBlock fragment size? Region? | Changed and added for clarity. |
| Line 234. Do the dashed lines indicate the location of the surrounding reefs? | Yes, added to figure legend. |
| Line 246. Table 5 unnecessary. Add the strain names in parenthesis in the methods. | The authors feel table 5 is necessary to summarize that DNA extractions were successful and that the qPCR assay is specific. There is precedence for this in table 2 in Nishimura et al. 2018 Quantitative PCR assay for detection and enumeration of  ciguatera-causing dinoflagellate Gambierdiscus spp. (Gonyaulacales) in coastal areas of Japan |
| Lines 259-262. Methods say gene curves were from 10^3-10^8, that’s only 5 orders of magnitude. Figure 4 only shows amplification down to ~ 5x10^4 and up to 4x10^9. | Changed to five orders for both standards. |
| Line 272. Add from macroalgae around Heron Island. | Added. |
| Line 333 change to rRNA gene copy number. | Changed throughout the text. |
| Table 6. Add units to column G. labillus cell number per…. List both copy number and an estimated cell number range using both strains in Table 6. By using HG7 standard you would tend to overestimate the cell number. | HG7 would be the conservative estimate. Fig 3 shows that it takes more qPCR cycles for the same cell numbers of HG4 to be detected, hence HG7 has fewer copies of the SSU gene fragment. HG7 was chosen as the reference because it is the type strain for the species, and because we thought it prudent to go with the conservative estimate. |
| Figure 5. Label stations or combine with figure 1. One has to have 3 figures visible to determine the distribution with figures 5 & 6. Consider changing from using red and green. Those that are colorblind will have a difficult time interpreting the data. | Stations labelled with numbers and red changed to purple to be visible for the colour blind, thank you for picking that up. |
| Line 279. Change spatial replicates to samples. | Changed. |
| Lind 380. First names for Dr Adachi and Dr. Nishimura are missing. | Changed, thank you for picking up. |

Reviewer 3

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| This manuscript describes the development of a useful molecular tool for the detection of a potentially toxic species of Gambierdiscus. The use of this tool is especially important in Australia, as the known agent of ciguatoxins has not been identified in this region. Overall the paper is well-written and presented. It also develops a relatively novel technique (use of gBlocks) for the confirmation of positive results and quantification. | Thank you. |
| However, the paper does not emphasise the importance of molecular tools for these taxa which are extremely difficult to monitor using traditional techniques. Below I have highlighted some areas where this can be improved. | Thank you. |
| Additionally, the authors do not describe in detail the gBlocks technique which would be very useful for readers who are not familiar with this approach. | Technique expanded upon and references supplied. |
| Abstract – Would be good to include a little bit of general information here. Assumes a lot of prior knowledge of CFP and Gambierdiscus in Australia. | Information added. |
| Line 22 – Change to: “…determine the presence and patchiness of this species across samples from Chnoospora sp.,..” | Changed. |
| Line 27 and 35 – I would change algae/microalgae to dinoflagellate or protist. | Changed |
| Line 32 – UNESCO should in written out in full. | Changed. |
| Figure 1 – This figure is probably not required for this manuscript and would be more suitable for a review paper of CFP. I would recommend it is removed. Please refer to appropriate references instead. | The authors feel that this figure should stay in as a visual explanation to readers why the elucidation of Gambierdiscus species and cell numbers at the base of the food chain is important. |
| Line 56 – Mention that in extreme cases CFP may result in death. | Included. |
| Line 58 – I would remove mention of MTX here. Gambierdiscus species produce many compounds and you explain this further on in the paper. | Removed and references updated. |
| Line 60 – Change to: “….toxins can bioaccumulate in herbivorous fish…” | Changed. |
| Line 60 – Please remove the reference to filter feeders here. You are discussing the accumulation of CTXs from the ingestion of macroalgae which is not relevant to filter feeders. As far as I know the accumulation of CTXs in filter feeders has not been confirmed under natural conditions. Also references 7 and 8 don’t seem appropriate support for this statement. | References updated. |
| Line 62 – Please change identified to described. | Changed. |
| Line 64 – Please change to “…16 described species and 4 currently undescribed species or types..” | Changed. |
| Lines 62-70 – It would be good to focus here on the molecular aspect of Gambierdiscus taxonomy. The number of described Gambierdiscus species has risen drastically in the last 5 years due to these tools and species ID has been limited in the past by the difficulties in morphological identification. This also has huge implications for monitoring programmes – which is why the assay in this paper was developed and is important – and I think this can be emphasised more in the introduction. | Emphasized. |
| Line 78 – LC-MS/MS needs to be in full here for the first reference. | Acronym introduced. |
| Line 83-84 – This shows the high variability in compound production within Gambierdiscus species. Can you stress this point here? | Mentioned. |
| Line 85 – This is the first reference to G. lapillus in the paper so an introduction to the species and why it is the focus of this research would be good before launching into the toxin profile. | Introduced. |
| Line 87 – What kind of bioassay? | Included. |
| Table 1 – Gambierdiscus type 2 is now described as G. jejuensis. Reference 40 does not describe an assay for Fukuyoa cf. yasumotoi. | Changed and reference fixed. |
| Line 109 and Table 1 – Fukuyoa is misspelled. | Changed. |
| Line 120 – Change to: “….put forward a series of recommendations to manage the…” | Changed. |
| Line 128 – Spell out LSU in full for first reference | Acronym deleted as it does not re-appear in the text. |
| Line 129 – Is this identification of G. toxicus certain? As outlined in the text above, Gambierdiscus identifications as toxicus are uncertain before 2009. | **Shauna – can you comment on this? I do not have the book.** |
| Change new to undescribed. | Changed. |
| The Fukuyoa species detected in Australia was originally called Gambierdiscus yasumotoi but has since been described as Fukuyoa paulensis (Gomez et al. 2015). | Changed, thank you for picking up. |
| Line 140 – Change to: “The assay was then..” | Changed. |
| Line 141 – GBR should be written in full for the first reference. | Changed. |
| Line 145 – Were these strains isolated as part of this study or as part of the study in reference 19? Why were these three species targeted? | These were isolated as part of study 19. These species were targeted because, frankly, that is what we could isolate. |
| Tables 3 and 5 – These tables can be condensed by grouping the strains to a single line, i.e. one line for each species. | Changed for table 3, however the authors wanted to show that all species were successfully amplified in table 5 so each strain remains with their own row. |
| Line 154 – CTAB in full here. | Included. |
| Line 157 – Change to: “…small subunit ribosomal DNA (SSU rDNA). | Changed. |
| Line 158 – Change to: “…GenBank reference database (accession numbers KU558929-33).” | Changed. |
| Line 177 – Should be Qiagen. | Changed. |
| Line 179 – It is also useful to test for cross-reactivity with species from outside the genus, e.g. Ostreopsis spp, Coolia spp, Alexandrium spp. etc. Was this done? | **Shauna – input please** |
| Line 180 – Please be more specific about what “appropriate positive and negative controls” were applied. | Removed. |
| Line 182 – It not clear to me how the authors tested primer sensitivity here. Can you please include more explanation? | Expanded upon. |
| Line 188 – What is the synthetic gene approach? Is this the gBlocks? | Yes indeed, added for clarification |
| Line 189 – Change to: “…also to quantify species presence..” | Changed. |
| Line 196 – Use of gBlocks: This is a relatively unique aspect of this research/assay but it hasn’t been explained in much detail. Readers may not be aware of this technique. In the introduction the authors allude to the use of gBlocks and how they may allow users to run the assays without a genomic DNA sample of the target species. It would be useful to include in the introduction some more information around the use of gBlocks. | Explained. **Shauna, Gurjeet and Arjun, could you proof read this addition? Not sure if too simplistic or rambling.** |
| Line 198 – This sentence is not clear. | Explanation added. |
| Line 200 – What is this copy number referring too? Is this the number of copies of the gBlock fragments in a 1 ng/uL solution? | It is indeed. Re-worded for clarity. |
| .Line 204 – Its not clear in this paragraph how the extracts for the standard curves were created. Did you extract from the samples that were counted directly or did you count a subsample of the culture from which a known volume of culture was then extracted? Did you do replicates? | Re-worded for clarity. The dilution series used for the gene based calibration curve was used, whose cells were counted before DNA extraction, then diluted. |
| Line 222 – Might be useful to include some taxonomic information for these genera? | **Shauna and Gurjeet, input please** |
| Line 226 – Should this be 120 µm mesh? | Yes indeed, thanks for picking up. |
| Line 227 – It is better to use g as a unit for centrifugation steps rather than rpm. | **Gurjeet, please comment now on line 249** |
| Line 229 – How were samples screened? Were samples also checked by light microscope for Gamberidiscus cells? I know it would be difficult to identify them accurately to species level but it would be interesting to estimate the cell abundance of Gambierdiscus using LM from these samples. | No, they were not. **Unless you did this Gurjeet?** |
| Line 243 – There is no explanation of the Gambierdiscus clades elsewhere in the text. It would be good information for the introduction to include what clade G. lapillus belongs to and subsequently why certain species were selected for cross-reactivity. | Mentioned in the introduction. |
| Table 5 – Would be useful to explain the gDNA gel band. Im assuming it was to estimate suitability for PCR amplification? | Explanation added. |
| Line 268 – Why was the number of gene copies not calculated for all available strains of G. lapillus? In table 3 three strains are listed and five are listed in table 5, yet only two were used for gene copy calculations. The more strains will give a better idea of the variation in this species. What was the error associated with these copy number calculations? | Table 3 has been changed to reflect the only 2 strains used for cell standards. Sadly, the other strains died before this study. Genomic DNA was still available, but no associated cell counts to calculate gene copy numbers. |
| Table 6 – This table may not be necessary or could be moved to supplementary material as the information is shown in figures 5 and 6. | Moved. |
| Line 316 – Again it would be good to include an explanation of gBlocks and why a genomic DNA extract is not required for a positive control. | Explanation of gBlocks added. |
| Line 330 – Please include some explanation around “ghost” cells. | Clarified. |
| Line 363 – Chnoospora sp. | Changed. |
| Line 368 – It may be useful to comment here on how these artificial substrates are useful for monitoring programmes and how they can work with molecular tools. | Expanded upon. |
| Paragraph 370 – Do the authors have any comments or recommendations on how this assay will be used for future research or monitoring programmes? | Included as part of the recommendation for using artificial substrate sampling. |
| Figures 2 and 4 – Are the x-axis log scale? | They are indeed. |

Reviewer 4

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| The manuscript described the development of a PCR assay for detection and quantification of Gambierdiscus lapillus. The sensitivity and specificity of the assay was evaluated on analytical specimens. 25 environmental samples were also tested. | Aye. |
| My main criticism is that the assay performance was not fully validated, include  1) the assay was not evaluated against a reference method (gold standard) such as sequencing; | **Shauna, Gurjeet and Arjun, I have no idea what this reviewer wants here. The cultures were sequenced, do they mean base sequencing from the DNA extracts? Input would be appreciated.** |
| 2) the lower limit of detection was not determined; | This was because the detection was for 0.04 and 0.05 cells based on the two G. lapillus strains. |
| 3) the specificity wasn’t sufficiently tested, in particular on environmental samples. | Please be clearer on how you would like this to be done beyond what is described in the manuscript. |
| Line 173-177: clarify why different extraction methods were used. | Clarified. |
| Line 184: 2ng of DNA wouldn’t be the lowest concentration that could be detectable (as mentioned in Line 261-262). Further dilution of the template should be tested to assess the sensitivity. | Disagree. This section was designed to show that the binding of the primers is to one binding site only, as can be determined from the single melt curve. The dilution of the template to test how low the detection can go is conducted in the following ‘calibration curve construction’ section. |
| Table 5 could be removed since the results were described in the text. | Table 5 was expanded upon and kept to summarize that DNA extractions were successful and that the qPCR assay is specific. There is precedence for this in table 2 in Nishimura et al. 2018 Quantitative PCR assay for detection and enumeration of  ciguatera-causing dinoflagellate Gambierdiscus spp. (Gonyaulacales) in coastal areas of Japan |
| Table 6: need to specify the unit in the table. | Units specified. |