Response to reviewer 2

The manuscript "The silicon isotopic composition of choanoflagellates: implications for a mechanistic understanding of isotopic fractionation during biosilicification" authored by Marron et al. investigates the silicate [DSi] uptake fractionation factor for two choanoflagellate species. As this is important work to better understand the marine Si cycle and a first step in using choanoflagellates as a paleoproxy for past [DSi] utilization, I recommend its publication in Biogeosciences. However, I am not convinced about the way, the authors calculated the fractionation factor (ε), which deviates from previous culture studies in diatoms and therefore is not comparable. Furthermore, clarifications in terms of the experimental set-up are necessary. A re-calculation of the fractionation factor might have great importance on the discussion, I consider this manuscript as major revisions. Please see detailed comments below

We thank the reviewer for her constructive and positive comments, and are happy to address the issues raised.

Major comments:

1) Calculation of the fractionation factor (ε):
The equation (3) is adapted from Varela et al., (2004), which is a field study estimating ε via a Raleigh system approach. Even though this is not necessarily incorrect, the authors should use the same equation, that was used in previous diatom culture studies (e.g. de LaRocha et al., 1997; Sutton et al., 2013) in order to make it comparable to the diatom fractionation factor. This will shift the fractionation factor to significantly lower values, which has a strong influence on the discussion and comparison with diatoms and sponges.

We chose to use the simple approach of assuming ε is equivalent to the apparent fractionation (i.e. difference between growth medium and choanoflagellates, equation 2 in the text) because the DSi utilisation in the cultures was so low. We wanted to compare this to the Varela et al. equation (equation 3 in the text) as it gave the most conservative estimate of the error associated with taking the more simplistic approach of equation 2 to calculate ε.

However, we have taken the advice of the reviewer and used the alternative equation from Sutton et al., 2013 for consistency.

In several parts, the authors use e for the fractionation factor in sponges. Isn't that only defined as _ (apparent fractionation factor)? " only equals e in steady-state systems or in case δ30BSi represents the instantaneous BSi in the Rayleigh model approach. This has to be considered comparing " in diatoms with ε in sponges. This part is very important, as this is the first study on fractionation factors in choanoflagellates and it is necessary to calculate the fractionation factor correct and comparable to other systems!

This has been corrected throughout to "apparent fractionation factor", and clarified in the caption to Fig. 1.

2) Experiment set-up.
How long were the Choanoflagellates adapted to their new growth "medium" or were they grown in the same medium before the start of the experiment (with the same d30Dsi composition?)

Diaphanoeca grandis cultures were set up a minimum of 2 months before harvesting, while Stephanoeca diplocostata cultures were set up a minimum 7 months before harvesting. All cultures were grown in culture media prepared from the same batch of artificial seawater salts, and the seed cultures that were used to start the experimental cultures had been growing in ASW made from this batch of salts for approximately 3 years.
The methods have now been changed to include this information.

How are growth rates? According to your study, the choanoflagellates only took up 4% of the DSi, which is more or less within the error of your DSi measurements. As this is a fairly low amount, I was wondering, if they might use up previously-stored DSi (not sure, if they do that) for biosilification!

There is no evidence that loricate choanoflagellates store silicon: the silicon starvation and replenishment experiments done by Leadbeater and colleagues on these species (Leadbeater BSC, Cheng R. 2010. Costal strip production and lorica assembly in the large tectiform choanoflagellate Diaphanoeca grandis Ellis. European Journal of Protistology 46:96–110; Leadbeater BSC. 1989. Developmental studies on the loricate choanoflagellate Stephanoeca diplocostata Ellis VI. Effects of silica replenishment on silica impoverished cells. Protoplasma 153:71–84.; Leadbeater BSC. 1985. Developmental studies on the loricate choanoflagellate Stephanoeca diplocostata Ellis. IV. Effects of silica deprivation on growth and loricate production. Protoplasma 127:171–179.) would argue against there being any (significant) store. The long period of culture, using the same media batch, would also argue against the isotopic signature being a legacy of a previous culture environment.

This information is now included in the introduction.

In line 17 (P5) you mention, that d30DSi was measured before and after, but I do not find these values!

Apologies – this was a typo and should have read "given that the DSi concentration of the media was measured before and after cell growth". This has now been corrected.

Minor comments:

P1, L10: it should say -0.5 to -2.1 ‰ as the fractionation factor of Chaetoceros is -2.1 (Sutton et al., 2013)

Corrected.

P1, L16: the sentence is imprecise: The Si cycle does not only describe the transport of Si into the biosphere, also vice versa (otherwise it wouldn't be a cycle). Furthermore, it also describes the geochemical cycling of DSi, e.g weathering, vs. inverse weathering. Please rephrase.

This is a fair point. We have shortened the first two sentences to: "The global silicon cycle is coupled directly to the carbon cycle in part because silica is used in the biology of various organisms."

P1, L20: Not all diatoms have "heavily" silicified shells.

We take the reviewer's point. We intended this to mean 'relative to choanoflagellates', but to avoid confusion we have changed this sentence to: "For most (although not all) diatoms, dissolved silica (DSi) is an essential nutrient since it is required for the formation of silicified cell walls, known as frustules".

P2, L 11: The study by Meyerink et al., 2019 actually shows that there might be an influence of iron on the fractionation factor.

We do not believe that these authors are reporting an impact of Fe on silicon isotopic fractionation. Whilst Fe is impacting silicon utilisation, the isotopic fractionation values that they report in the paper are not highly variable. We have added this citation to the introduction (see below).

P2, L 12: By now, there are many more culture studies about diatoms besides de LaRocha et al., 1997 and Sutton et al., 2013, eg. Milligan et al., 2004 Sun et al., 2014 and a mesocosm study by Meyerink et al., 2019

To acknowledge the body of work on silicon isotopes in diatoms, we have added additional references to the introduction.
Please note that all references used in Fig. 1 have been provided in the caption, including the Milligan et al., 2004 reference. Note also that in the figure we only used culture studies where diatoms were grown under high DSi where growth conditions could be considered comparable. The Meyerink study, for example, was carried out at low DSi concentrations (~2uM).

P2, L 13: Isn't it a linear relationship, otherwise it would not work as proxy?

The relationship between sponge silicon isotopic composition and ambient DSi is non-linear, and is instead described best by an inverse function of DSi, which makes sense if sponge silicon isotope fractionation is a function of uptake kinetics (e.g. Wille et al., 2010). As the relationship can be modelled with an equation in a statistically robust way, we would argue that the silicon isotopic composition of spicules can still be used as a proxy.

P3, L3: delete the word "vital", this is at term geologist use, if they do not know what is happening and they are confused by biology.

Done.

General: The authors do not discuss the different fractionation factor between both species, possibly growth rate?

We would like to thank the reviewer for this excellent idea. We have added a short, additional discussion section to discuss the differences in fractionation between the two species.

The published maximum growth rates for Diaphanoeca grandis are higher than Stephanoeca diplocostata (5.8 hours versus 8.8 hours), but across the cultures we used the growth rates were observed to be highly variable, both within and between species. Stephanoeca diplocostata cells have more silicified strips than Diaphanoeca grandis cells, so the difference in fractionation could be due to differences in the degree of silicification.

It would be nice to have some more information (maybe two sentences) about habitat of choanoflagellates (marine, brackish, freshwater (which species did you use); water

depth, pelagic, benthic, importance in paleo records (Where, when?)

The two species used here are marine, from temperate waters (isolated from coastal Brittany) but they are found in polar waters too. Generally loricate choanoflagellates are found waters from the tropics to the poles, though more prevalent in polar waters. Historically they were thought to be from marine and brackish water, but they have now additionally been isolated from freshwaters (Paul, 2011; Nitsche, 2014; Richter & Nitsche, 2016). They have no known microfossil record (Leadbeater, 2015), most likely due to the small size of the individual costal strips hindering their identification.
We have now included this information in the introduction and methods.

We have also attached a highlighted version of the revised manuscript.