Response to reviewer 1

This is a very interesting and worthy contribution to Biogeosciences.

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

May I just suggest a few minor comments below: Page 1 Line 9: Are these species only marine?

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).

We have now included this information in the introduction and methods.

Page 1 Line 10: you provide a range in diatom fractionation factors between -0.5 per mille to 2 per mille in your abstract but in the introduction (page 2, line 10) only one estimation is provided (de la Rocha's). Please could you elaborate on this, to reflect your abstract.

This information is explained later in the paragraph: the range in fractionation factors was later discovered as a result of culture experiments involving different species of diatoms.

Page 2, line 24: could the authors please just clarify what they mean by their statement "that the strongest fractionation in sponges (most negative value of ε)". I assume that they mean that the most negative published ε values are derived from sponges? However, it could also be interpreted as referring to polymerisation only occurring in the most negative of the fractionation factors occurring in sponges (e.g. not all sponges).

We have clarified this statement that the strongest component of sponge silicon isotope fractionation occurs during polymerisation (as opposed to influx or efflux).

We have rephrased that sentence accordingly: "In contrast, biological models based on Michaelis-Menten kinetics and empirical data from growth rate experiments suggest that the largest contribution towards isotopic fractionation in sponges occurs during the polymerisation step rather than during influx or efflux of DSi…"

Table 1: can you change the capitalisation of Numbers please (if you wish to keep the existing comma).

The comma has now been replaced with a period.

Could you also be specific that your growth medium refer to the seawater compositions you report.

The word "seawater" has been replaced with "growth medium" in Table 1.

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.

The silicon isotopic composition of choanoflagellates: implications for a mechanistic understanding of isotopic fractionation during biosilicification

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Abstract.

The marine silicon cycle is intrinsically linked with carbon cycling in the oceans via biological production of silica by a wide range of organisms. The stable silicon isotopic composition (denoted by δ^{30}Si) of siliceous microfossils extracted from sediment cores can be used as an archive of past oceanic silicon cycling. However, the silicon isotopic composition of biogenic silica has only been measured in diatoms, sponges and radiolarians, and isotopic fractionation relative to seawater is entirely unknown for many other silicifiers. Furthermore, the biochemical pathways and mechanisms that determine isotopic fractionation during biosilicification remain poorly understood. Here, we present the first measurements of the silicon isotopic fractionation during biosilicification by loricate choanoflagellates, a group of protists closely related to animals. We cultured two species of choanoflagellates, *Diaphanoeca grandis* and *Stephanoeca diplocostata*, which showed consistently greater isotopic fractionation (approximately -5 to -7 ‰) than cultured diatoms (-0.5 to -2.1 ‰). Instead, choanoflagellate silicon isotopic fractionation appears to be more similar to sponges grown under similar DSi concentrations. Our results highlight that there is a taxonomic component to silicon isotope fractionation during biosilicification, possibly via a shared or related biochemical transport pathway. These findings have implications for the use of biogenic silica δ^{30}Si produced by different silicifiers as proxies for past oceanic change.

1 Introduction

The global silicon cycle is coupled directly to the carbon cycle in part because silica is used in the biology of various organisms. A prime example of this occurs in the case of the diatoms, siliceous algae that play a key role in the cycling of organic matter in oceans, being responsible for approximately half of the export of organic carbon from surface waters to the deep (Tréguer et al., 2018). 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 (Darley and Volcani, 1969; Hildebrand, 2008; Sapriel et al., 2009). Because of this direct link between biosilicification and primary production, quantifying the supply and uptake of DSi through time is important for understanding historical changes in the marine carbon cycle arising from fluctuations in climate (Tréguer and

De La Rocha, 2013). This is determined by studying the stable silicon isotopic composition (δ^{30}Si) of biogenic silica within marine sediments, which act as a geochemical archive of the silicon cycle (Sutton et al., 2018).

It is now clear that silicon isotopes are fractionated during biosilicification, and that this phenomenon provides important information on past DSi concentrations in seawater. For example, diatom frustules are enriched in the lighter isotope, ^{28}Si, relative to the heavier isotopes, ^{29}Si and ^{30}Si (De La Rocha et al., 1997). During periods of intense productivity, this makes the Si isotopic composition of the ambient seawater increasingly heavy; in turn, this altered environment drives the newly formed biogenic silica towards heavier isotopic compositions. By making assumptions about the composition of seawater and the fractionation factor (ε), it is possible to calculate past surface DSi utilisation (and so, diatom productivity) from the isotopic composition of the frustule. The value of ε for the diatoms is generally taken as -1.1 ‰, and this assumption appears to be robust across different regimes of temperature, pH, and nutrients from a range of culture, mesocosm and field experiments (De La Rocha et al., 1997; Milligan et al., 2004; Varela et al., 2004; Beucher et al., 2008; Egan et al., 2012; Sun et al., 2014; Meyerink et al., 2019). However, culture experiments have highlighted that diatoms may exhibit some species-dependent differences in isotopic behaviour (Sutton et al., 2013). In contrast, apparent fractionation in benthic sponge spicules shows considerable variability, with an empirical non-linear relationship to ambient DSi concentrations (Hendry et al., 2010; Wille et al., 2010). These data are derived from geochemical approaches that have been applied in a range of palaeoceanographic settings, and we do not yet have a full understanding of the mechanism or the biochemical pathway(s) responsible for Si isotopic fractionation (Hendry et al., 2019). A fuller understanding of this phenomenon will require systematic comparative studies across diverse silicifiers.

There are several phases during biosilicification where isotopic fractionation could occur. It has been helpful to conceive this complex process as three interrelated stages: (i) DSi influx across the plasmalemma into the cytoplasm, followed by accumulation in a cellular compartment known as the silica deposition vesicle (SDV), (ii) net incorporation of Si via polymerisation within this cellular compartment, and (iii) regulatory efflux of excess DSi from the cell. Laboratory culture experiments indicate that DSi influx, rather than polymerisation or efflux, is probably the key step in determining ε in diatoms (Milligan et al., 2004). In contrast, biological models based on Michaelis-Menten kinetics and empirical data from growth rate experiments suggest that the largest contribution towards isotopic fractionation in sponges occurs during the polymerisation step rather than during influx or efflux of DSi. Fractionation values are estimated as approximately -1.5 ‰ and -5.3 ‰ for influx and net polymerisation-efflux respectively during sponge biomineralisation (Hendry and Robinson, 2012; Wille et al., 2010). However, there are considerable uncertainties with both laboratory and model-based approaches, the latter of which are based largely on empirical observations as opposed to a mechanistic understanding.

Given that there are currently no estimates of Si isotopic fractionation from culture or water column studies of other silicifiers, placing isotopic fractionation in a phylogenetic framework is not possible at present. A wide diversity of species biomineralize silica to some extent, with siliceous organisms being found right across the various eukaryotic supergroups (Marron et al., 2016; Hendry et al., 2018) despite these groups diverging over 2.1Ga (Parfrey et al., 2011). The evolutionary history of eukaryotic biosilicification is largely opaque; though there is good evidence for the independent evolution of biosilicification in some taxa (Grégoire et al., 2012; Shimizu et al., 2015; Marron et al., 2016; Ratcliffe et al., 2017), there is support for a common

origin, deep in the eukaryotic phylogeny, of several of the genes and biochemical mechanisms involved in silicon transport and polymerization (e.g. the role of long-chain polyamines (Sumper and Kröger, 2004; Matsunaga et al., 2007; Durak et al., 2016)). Understanding the links between evolutionary relationships and silicon isotope fractionation could provide insight into the mechanisms underpinning biosilicification and isotopic fractionation. Furthermore, evidence from other elemental proxies

5   (e.g. δ^{13}C) suggests that there are phylogenetically-specific effects on their isotopic fractionation (Edgar et al., 2017), and it is therefore important to discover if such biases need to be corrected for when using measurements of silicon isotopes.

   Here, we present the first measurements of silicon isotopic fractionation in laboratory cultures of choanoflagellates. Choanoflagellates are aquatic (marine and freshwater) unicellular or colonial eukaryotes typically <10 μm in size, characterised by a single apical flagellum surrounded at its base by a collar of microvilli (Nitsche, 2014; Paul, 2012; Richter and Nitsche, 2016).

10   Choanoflagellates are the closest living sister group to the animals (King et al., 2008; Ruiz-Trillo et al., 2008; Nitsche et al., 2011) and possess cytological features often observed as being similar to sponge collar cells (Mah et al., 2014). This is noteworthy as sponges are believed to represent the most basal, or at the very least an early-diverging, clade within the animals (Simion et al., 2017). However, there is no known microfossil record for choanoflagellates (Leadbeater, 2015). One family of choanoflagellates, the Acanthoecidae, produce an extracellular lorica composed of multiple silicified costal strips. The siliceous

15   costal strips are formed inside the cell before being exocytosed to the cell exterior (Leadbeater, 2015). Molecular studies suggest that this process is mediated by biochemical pathways that are broadly comparable to other silicifiers; for example, the presence of Silicon Transporter (SIT) genes (Hildebrand et al., 1997; Durak et al., 2016; Marron et al., 2016), the role of the cytoskeleton in shaping the forming costal strip (Froesler and Leadbeater, 2009; Tesson and Hildebrand, 2010; Nomura and Ishida, 2016; Durak et al., 2017) and the involvement of glycoproteins in biosilica polymerization (Ludwig et al., 1996;

20   Gong et al., 2010). Understanding Si isotopic fractionation in choanoflagellates therefore has implications for the evolution of biosilicification pathways in animals and across the eukaryotes, and for the use of Si isotopic archives as environmental proxies.

2   Material and Methods

Silicon isotope variations are denoted throughout by δ^{30}Si, reported relative to a known standard (NIST quartz standard NBS28

25   RM8546) according to Equation 1:

$$\delta^{30}\text{Si}(\text{‰}) = \left(\frac{\left(\frac{^{30}\text{Si}}{^{28}\text{Si}} \right)_{\text{Sample}}}{\left(\frac{^{30}\text{Si}}{^{28}\text{Si}} \right)_{\text{NBS28}}} - 1 \right) \times 1000 \tag{1}$$

   Two species of marine choanoflagellates, *Diaphanoeca grandis* and *Stephanoeca diplocostata* (Family Acanthoecidae, Nitsche et al. (2011)) were cultured in artificial seawater (ASW) made from 36.5 g/l of a single batch of Marin Salts (Dr.

Biener Aquarientechnik, Wartenberg Germany) in double-distilled (dd) H_2O. ASW was vacuum-filtered through a 0.22 μm Steriop GP Express Plus filter (Millipore, MA) into a sterile 1l screw-top glass bottle (Schott Duran) and sterilized by autoclaving. Organic enrichment medium (4 g/l Proteose Peptone [Sigma], 0.8 g/l Yeast Extract [Fluka Biochemika] in ddHO, autoclaved sterile) was added to new cultures at a concentration of 7.5 μl/ml ASW to provide nutrition for prey bacteria.

5 Cultures of both species were grown in 50ml polystyrene triangular flasks (Corning) at 13.5°C and split every four to eight weeks. Several litres of culture were required to obtain sufficient siliceous material for analysis. Cultures were growing in the culture medium for a minimum of two months (for *Diaphanoeca grandis*) and seven months (for *Stephanoeca diplocostata*) before harvesting. It should be noted that there is no evidence that loricate choanoflagellates store reserves of silicon or DSi (Leadbeater, 1987, 1989; Leadbeater and Cheng, 2010).

10 Cells were harvested by centrifugation at 4000g for 60 minutes at 4°C. The pellet was washed in 10ml ddH$_2$O, resuspended by vortexing, sonicated for 5 minutes and centrifuged again at 4000g for 90 minutes at 4°C. The washed pellet was oven-dried overnight at 50°C.

Organic matter was removed from the pelleted loricae by heating in 30% hydrogen peroxide at 80°C for an hour. The cleaned loricae were washed once in 18 MΩ.cm deionised water, resuspended in a small amount of water in a clean Teflon vial and

15 dried under vacuum. This pellet was resuspended in 100 μl of distilled concentrated nitric acid and dried again. The silica from the loricae was solubilised in 1ml 0.4M sodium hydroxide solution (Titrapur) at 100°C overnight. The resulting solution was acidified with 50 μl of in-house distilled 8M nitric acid and diluted with 1ml of water before the dissolved silica was purified by cation exchange chromatography (Georg et al., 2006; Hendry et al., 2019).

Full biological triplicates were available for *S. diplocostata*; material from only one sample of pelleted loricae was available

20 for *D. grandis*, but this was split into three aliquots to make analytical triplicates. Samples of the growth medium used in the cultures were analysed in duplicate for DSi and isotopic composition. The DSi concentrations of the fresh medium, post-culture medium (*D. grandis* only), and organic enrichment medium were measured using a molybdate blue spectrophotometric method (Hach Lange), with an estimated precision of 3% based on replicate measurements of a SiO_2 standard (<0.1% alkali fluorosilicate). The artificial seawater medium was prepared in duplicate for isotopic analysis using a magnesium co-precipitation

25 method (Cassarino et al., 2018): Si was pre-concentrated by the addition of 1.2% v/v 1M sodium hydroxide (NaOH), followed by 1% v/v 1M NaOH after 24 hours. The precipitate was rinsed with 1mM NaOH before dissolution in 6N in-house distilled HCl, dilution in Milli-Q water, and purification using cation exchange resins.

Silicon isotopes were measured using Multi-Collector Inductively Coupled Plasma Mass Spectrometry (MC-ICP-MS Thermo Neptune, Bristol Isotope Group). Mass bias corrections were carried out offline using both standard-sample bracketing with

30 standard NBS28 (RM8546), and magnesium isotope doping (Cardinal et al., 2003). All standards and samples were doped with a magnesium solution, intensity matched with ^{28}Si. Seawater samples (and associated bracketing standards) were additionally doped with 0.05M HCl and 0.003M H_2SO_4 to account for matrix effects (Hughes et al., 2011).

Sample ^{29}Si and ^{30}Si values were related linearly, with a gradient of 0.522 (SE 0.007, $R^2 > 0.99$), which is within the range of theoretical mass dependent fractionation. Rock reference standard Diatomite (Reynolds et al., 2007) and sponge standard

35 LMG08 (Hendry et al., 2011) are used to assess long-term reproducibility, yielding δ^{30}Si values of +1.23 ± 0.15 ‰ and -3.44

$\pm\,0.16\,\text{‰}$ (2SD) respectively (Cassarino et al., 2018). As an additional quality check, the new seawater standard ALOHA deep was analysed and give a δ^{30}Si values of $+1.11 \pm 0.18\,\text{‰}$ (2SD). All standards fell well within error of published values (Grasse et al., 2017).

3   Results

3.1   DSi concentrations in growth media

For cultures of *D. grandis* we determined that ASW culture media had DSi concentrations of 94 and 89 μM respectively. Measuring the same media after culturing determined that 94% ($\pm$ 4%) of the available Si remained in solution after the choanoflagellate growth. The concentrated organic enrichment medium contained 44 μM DSi, but was only added to a working concentration of 0.75% (v/v). The change in Si isotopic composition of the ASW from the organic enrichment medium is thus considered negligible.

3.2   Isotopic compositions of choanoflagellates and growth media

The δ^{30}Si composition of ASW growth medium was $+3.33 \pm 0.05\,\text{‰}$. The δ^{30}Si composition of choanoflagellate loricate silica was $-3.32 \pm 0.07\,\text{‰}$ for *D. grandis* (analytical triplicates, 1SE) and $-2.38 \pm 0.07\,\text{‰}$ for *S. diplocostata* (biological triplicates, 1SE; table 1). Given that the overall drawdown of DSi was so low, the fractionation factor, ε, was assumed to approximate the apparent fractination Δ^{30}Si calculated using Equation 2 (Figure 1):

$$\varepsilon \approx \Delta^{30}\text{Si} = \delta^{30}\text{Si}_{choano} - \delta^{30}\text{Si}_{ASW} \tag{2}$$

Figure 1 compares Δ^{30}Si from the current study to previous literature values for other marine silicifiers.

The impact of DSi utilisation on calculation of ε can be assessed for the *D. grandis* culture, given that the DSi concentration of the media was measured before and after cell growth. Assuming a closed system, ε can be calculated according to Equation 3.

$$\varepsilon \approx 1000\,(\alpha - 1) \tag{3}$$

Where α is defined by Equation 4 (Sutton et al., 2013).

$$\alpha = \frac{ln\left(1 - \frac{\left(1 + \delta^{30}\text{Si}_{choano}/1000\right)\left(1 - f\right)}{1 + \delta^{30}\text{Si}_{ASW}/1000}\right)}{inf} \tag{4}$$

Where f is the fraction of DSi remaining in solution. This calculation suggests that a 6% utilisation may increase the calculated fractionation by approximately 0.2 ‰, which is the same as a conservative estimate of uncertainty (1SD).

4 Discussion

4.1 Interspecies differences in choanoflagellate silicon isotopic fractionation

There is a significant 1 ‰ offset between the two species of choanoflagellate. Whilst further work is required to understand the offset between the species, we can speculate on potential origins of this offset. Firstly, the difference may be a result of differences in growth rates between the two species (Andersen, 1988; Geider and Leadbeater, 1988; Eccleston-Parry and Leadbeater, 1994). However, the observed growth rates were highly variable and did not show systematic differences between the two species. Secondly, the difference in fractionation could be due to the difference in the degree of silicification between the two species, with *Stephanoeca* lorica being composed of a greater number of silicified costal strips than *Diaphanoeca* lorica.

4.2 Implications for evolution of silicon isotopic fractionation by biosilicifiers

We show here that two silicifying choanoflagellate species both exhibit Si fractionation that is more similar to sponges and markedly different from diatoms grown under equivalent culture conditions. This is consistent with the phylogenetic relationships of these organisms, with choanoflagellates being more closely related phylogenetically to the sponges than either of these are to the diatoms (Adl et al., 2019). These results have implications for the mechanistic basis of fractionation. One possibility is that silicon isotopic fractionation occurs at the precipitation stage. This could explain the differences observed between sponges and diatoms, which use different biomolecules to drive silica formation. Though some features of silicification are shared between sponges and diatoms, e.g. long-chain polyamines, (Sumper and Kröger, 2004; Matsunaga et al., 2007), sponges lack many the molecules that are key features of diatom biosilica formation, such as silaffins (Kroger et al., 2002) and silacidins (Wenzl et al., 2008). However, our results argue against silica precipitation being a key driver for isotopic fractionation. Sponge silica spicules are deposited around a core protein filament (e.g. silicatein, glassin) and such proteins are apparently absent from analyses of choanoflagellate costal strip proteins or transcriptomes (Gong et al., 2010; Richter et al., 2018). Without molecular evidence for common biosilica formation pathways, it is difficult to envisage how comparable

values could occur between sponges and choanoflagellates if specific mineral-forming mechanisms were responsible for fractionation. To place this observation in a broader context, different sponges can use very different proteins during silicification yet have consistent isotopic fractionation across clades (Hendry et al., 2019). Although we cannot rule out that the different polymerisation pathways all fortuitously give rise to the same degree of fractionation, this seems unlikely.

Another possibility is that fractionation occurs during silica uptake into the cell. Available evidence indicates that the cellular influx of DSi is generally governed by active or passive membrane transport proteins under physiological conditions (Maldonado et al., 2011; Marron et al., 2016; Thamatrakoln and Hildebrand, 2007; Thamatrakoln and Kustka, 2009). Candidates for mediating this active transport include the SITs and related SIT-L transporter proteins, which occur in a wide variety of silicifiers such as diatoms, haptophytes, radiolarians and choanoflagellates. A separate group of active transporters known as the Lsi2-like family were first identified in plants (Ma and Yamaji, 2015) and have now been found throughout the eukaryotes (see Figure 2), including sponges, as well as in archeans and eubacteria (Marron et al., 2016). Examples of passive transporters are modified aquaporins like the plant Lsi1s (Ma and Yamaji, 2015), which have specialized channel pore sizes and selectivity filters that make them permeable to DSi (Mitani-Ueno et al., 2011).

Diatoms and choanoflagellates both possess SIT genes, whereas sponges do not. This implies that if the SITs were solely responsible for silicon isotopic fractionation then values for choanoflagellates and diatoms should be similar, and distinct from the sponges. Our data argue against this, showing instead that choanoflagellate fractionation is much closer to the sponges under the conditions tested. We speculate that if fractionation does occur during influx and assimilation, it could be mediated instead by Lsi2-type transporters. While sponges, diatoms and choanoflagellates all contain Lsi2-like proteins, the sponge and choanoflagellate genes are more closely related to each other than to the diatom sequences (Figure 2, also see Marron et al. (2016)). Lsi2 genes are also responsible for silicon transport in plants, and studies have implicated them in isotopic fractionation (Opfergelt et al., 2006). Within the Lsi2-like phylogeny the land plant Lsi2 clade is distinct from the 'sponge + choanoflagellate + eumetazoan' Lsi2-branch and from the diatom sequences (Marron et al., 2016). This is in line with the much lower fractionation values observed for plant biosilica, with the caveat that the siliceous plants studied were grown under different conditions (reviewed in Frings et al. (2016)).

Finally, it is also plausible that fractionation is influenced by silica efflux from the cell. However, the molecular basis for this process remains so ill-defined that it is difficult to draw conclusions on this from the data presented here.

5 Conclusions and outlook

Choanoflagellates grown in culture exhibit strong silicon isotopic fractionation during biosilicification, comparable to that of sponges grown under equivalent DSi concentrations in natural conditions. This similarity between choanoflagellates and sponges indicates that the biochemical pathway responsible for the fractionation of silicon isotopes is shared between the two sister taxa. We postulate that influx of DSi by Lsi2-type transporters play a key role, but that further testing with other silicifiers for inter- and intra-clade comparisons will provide further insight into this pathway.

The hypothesis that the fractionation of silicon isotopes during biosilicification has its basis in phylogeny will influence how we view the use of silicon isotope-based oceanic archives, as a better mechanistic understanding of such proxies allows more

30   robust interpretation of downcore records. Furthermore, this phylogenetic framework has implications for the long-term history of silicon and silicon isotope budgets in the biosphere (Tatzel et al., 2017). Whole ocean DSi concentrations are likely to have been coupled over geological timescales with the evolution of silicifiers even before the emergence of the diatoms (Conley et al., 2017; Hendry et al., 2018). If strong silicon isotopic fractionation is indeed driven largely by cellular transporters, the origin of which extends back into the Precambrian, then such coupling would also have impacted the isotopic composition of the oceans at least as far back as a billion years ago.

Author contributions. AM, PC, and KH devised the study, wrote the majority of the text and produced the figures. AM carried out the

5   cultures; LC and JH conducted the isotopic analysis.

Competing interests. The authors declare that they have no conflict of interest.

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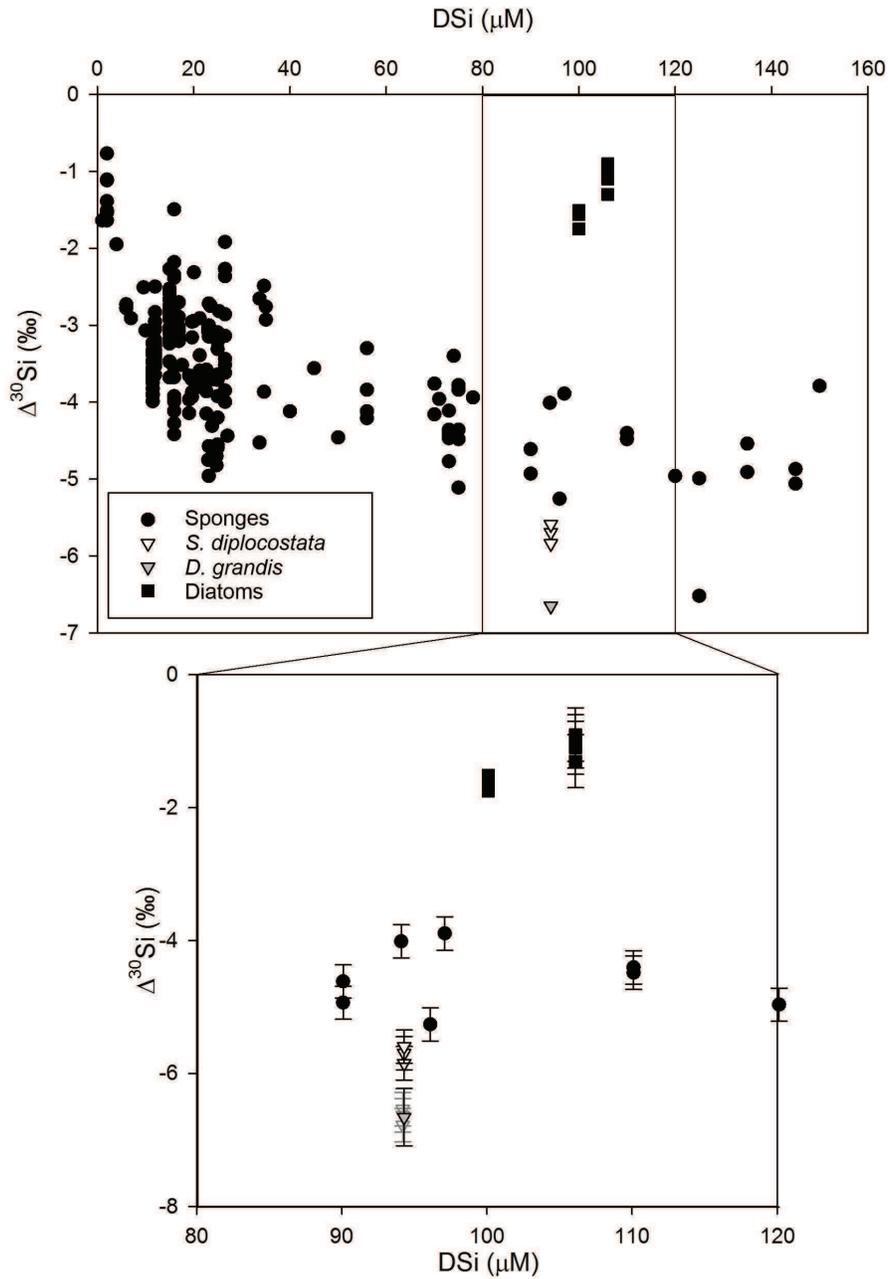
Figure 1. Apparent fractionation (Δ^{30}Si, Equation 2) of choanoflagellates (this study, triangles), sponges (circles, Hendry et al. (2010); Wille et al. (2010); Hendry and Robinson (2012); Cassarino et al. (2018)), and fractionation (ε, Equation 4) from diatom cultures (squares, De La Rocha et al. (1997); Milligan et al. (2004)). Note that for the diatoms, the only available comparative data comes from a narrow concentration range at approximately 100μM DSi. The lower panel expands the values for all organisms grown in DSi concentrations of 80 - 120μM. The error bars show 2SD errors based on replicate measurements of biogenic silica standards (see text). For *D. grandis*, the analytical replicates are shown in hollow grey triangles, mean ($\pm$ 2SD of the replicates) is shown in the solid grey triangle.
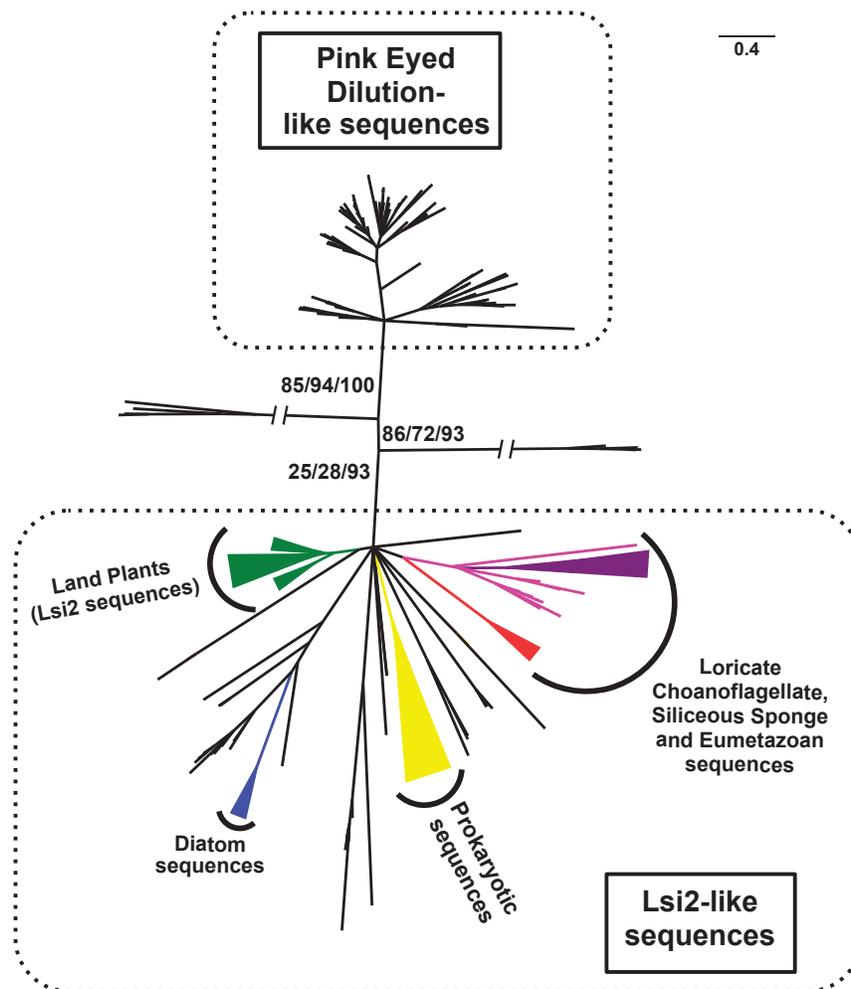
Figure 2. Simplified phylogenetic tree of Lsi2-like sequences (adapted from Marron et al. (2016)). Phylogenetic tree of sequence similar to the rice active Si transporter Low Silicon 2 (Lsi2) from a taxonomically diverse range of eukaryotes and prokaryotes. The sequences divide into two main groups: one containing Pink-Eyed Dilution P-protein sequences and the other containing the plant Lsi2s, hence being designated as Lsi2-like. Among the opisthokonts investigated only loricate choanoflagellates (red), siliceous sponges (magenta) and the biosilica-producing eumetazoans *Lottia gigantea*, *Capitella teleta*, and *Lingula anatina* (purple) possessed Lsi2-like sequences. The opisthokont sequences clearly branch distinct from the diatom sequences (in blue). The support for the eumetazoan clade is 97/97/99; for the sponge+eumetazoan clade is 93/99/100 and for the loricate choanoflagellate+siliceous sponge+eumetazoan branch is 31/36/89. Plant Lsi2 sequences (green) form a strongly supported (86/99/100) monophyletic clade, as do the diatom Lsi2-like sequences (100/100/100) and prokaryotic arsB (yellow) sequences (74/90/98). This tree was produced using RaxML maximum likelihood analysis with the best-fitting LG+G4 model from an alignment of 247 amino acid residues, analyses were also conducted with PhyML and IQ-Tree under the same model. Statistical support values are given in the form % RaxML/%PhyML/%IQ-Tree bootstrap. Nodes with <20% bootstrap support were collapsed to give a topology agreed across all methods. Scale bar indicates average number of amino acid substitutions per site; slashes indicate very long branches that were clipped for display purposes.

Table 1. Silicon isotopic composition of choanoflagellates and growth medium. Numbers in parentheses show 1SD internal errors; $\pm$ shows external error from biological triplicates.

S. diplocostata δ^{30}Si	*D. grandis* δ^{30}Si	**Growth medium** δ^{30}Si
(‰)	(‰)	(‰)
-2.36 (0.08)	-3.32 $\pm$ 0.07	3.28 (0.15)
-2.51 (0.07)		3.38 (0.12)
-2.26 (0.11)		