Dear Editor,

Please find below our point-by-point replies to the referee's comments and suggestions including all changes made to the manuscript as well as a marked-up version of our revised manuscript 'Technical note: Sampling and processing of mesocosm sediment trap material for quantitative biogeochemical analysis'. Changes made in the text are indicated in blue.

Yours sincerely,

Tim Boxhammer

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Response to Reviewer #1 (Anonymous Reviewer)

We thank the reviewer for the constructive comments on this technical note. Our responses to the reviewer's comments, including modifications to the manuscript, are detailed in the following:

Comment 1 by Reviewer #1: In my opinion, a proper evaluation of the potential bias in keeping zooplankton in the sediment trap samples must be presented. Questions to answer and/or discuss: what is the proportion of swimmers in samples collected since 2010? According to Niehoff et al. (2013), most organisms collected in Svalbard sediment traps were alive (referred to as swimmers in opposition to sinkers).

Author response: The described methods were primarily developed for biogeochemical analysis of the vertical flux of organic matter inside mesocosms to allow elemental budgeting of the enclosed systems. Due to the restricted mesocosm length, the vertical organic matter flux includes vertical migrating zooplankton and settling larvae, which is why all particles and organisms collected in the sediment traps where analysed as a whole. It is generally very difficult to distinguish between swimmers and sinkers reaching the mesocosm sediment traps, as the traps are very shallow compared to open ocean sediment traps so that even dead individuals do not experience much degradation until collection. As the sample volumes are too high to screen the entire samples (see Author response to comment 2 by Reviewer #1) the best chance to calculate general mesozooplankton contribution (swimmers and sinkers) is to analyse subsamples as done for example by Niehoff et al. (2013). To get a direct impression on the sample compositions we started to take high-resolution images of the collected material with a plankton scanner in 2013. The attached Fig. 1 shows image details, highlighting the usually very low ratio of copepods (potential swimmers) to phytoplankton detritus.

Comment 2 by Reviewer #1: *How efficient and precise is this protocol to evaluate zoopk contribution based on subsamples of at most 5% of the sampling volume, especially considering the occasional "patchy" distribution of particles mentioned by the authors?*

Author response: The subsample volume of less than 5% for evaluation of zooplankton contribution was chosen to allow for time efficient estimation of zooplankton abundance in

the sediment trap samples keeping the subsampling bias on the main sample as low as possible. Screening of the entire sample volumes for zooplankton organisms is usually impossible within limited time as the samples often consist of a very dense particle suspension easily reaching volumes of several litres. Just as an extreme example: We collected 540 samples during a single study in 2013 which were adding up to 907 kg of particle suspension in total. However, Niehoff et al. (2013) have shown that even this relatively small subsample volume can be used to generate reliable zooplankton data from mesocosm sediment trap samples.

Comment 3 by Reviewer #1: The authors further mention removing "Mesozooplankton actively swimming in the liquid phase, mostly copepods, . . . together with the supernatant from the settled material" If "some" swimmers are indeed manually removed, how do you precisely evaluate swimmers contribution for subsequent biogeochemical analyses? Are there some alternatives, for instance, solutions to "repel" swimmers from sediment traps or to avoid sampling for them?

Author response: The subsamples for zooplankton contribution analysis were taken after sample collection but prior to processing of the bulk samples (page 18697, line 13 – 14). Thus the removal of the supernatant including actively swimming copepods after settling of the particles just affected the biomass of the bulk sample. However, this was only to a negligible extent, as it was a very small number of copepods (in the range of less than a hundred individuals \leq 70 µmol C) compared to the overall biomass collected (1000 – 465000 µmol C sample⁻¹). Carbon data used for this comparison originates from a long-term study in 2013 covering very low and extremely high productive phases of the plankton communities. To avoid confusion, we will remove the sentence that Reviewer #1 refers to on page 18698, line 28 to page 18699, line 2.

As the mesocosms are closed systems there is no possibility of adding toxins or other solutions to the collecting cylinders of the sediment traps without affecting the whole systems. Accumulated particles are known to be attractive to swimmers and so far we have not seen a possibility to exclude them from the samples.

Comment 4 by Reviewer #1: Sampling of the mesocosms: Although I do really see the advantage of a surface sampling (avoiding frequent diving in cold areas), I wonder whether the system, used for several years, has been occasionally blocked (a 1 cm inner diameter hose

seems small to me) or prone to malfunctions.

Author response: The vacuum sampling method keeps the mesocosms entirely closed while sampling the sediment traps, but involves a small risk of malfunctions at depth. Current related bending of the silicon tube can easily block the system but we found solutions to almost exclude this risk. The tube itself has relatively thick walls of 3 mm and is only fixed at two points, the collecting cylinder and the flotation frame to avoid potential kinks (Fig. 1a). A wire helix hose coating the first 1.5 m of the tube where it is connected to the collecting cylinder of the trap to prevent bending at depth. To make clear why we have connected the tube at only two points of the mesocosm structure, we will change page 18696, line 21 - 22 in a revised manuscript to read: 'The silicon tube itself is only connected to the bottom of the mesocosm and fixed to the floating frame above sea surface to avoid any kinks (Fig. 1a).'

The second reason for a system failure can be a blockage of the outlet of the collecting cylinder by lost parts of equipment. We have been using several methods to retrieve objects that felt into the mesocosms while keeping the mesocosms closed had highest priority. Only if retrieval with live view camera support by hooks, magnets or small nets failed we opened up the collecting cylinder. This is possible at the upper and the lower end, but involves the risk of loosing collected material. However, over six years of annual KOSMOS experiments (each lasting for several weeks or months) we only once had to sample one of the sediment traps 24 hours later than planed after the schedule.

To include this information in the revised manuscript we will add the following sentence on page 18705, line 4 to read: 'Only in case of a non-reversible blockage of the outlet of the collecting cylinder by artificial objects one can open up the cylinder at the top and the bottom.'

Comment 5 by Reviewer #1: Are you 100% sure of the efficiency of this sampling procedure (i.e. that all sinking material is collected)? A very informative evaluation would be to show average deviations in terms of collected mass between replicated mesocosms (control mesocosms for instance) during the various experiments.

Author response: The particles sinking down in the cylindrical mesocosm bags are concentrated by the sediment traps, which form the conical bottom end of the mesocosms and ensure quantitative collection. The critical steps to achieve quantitative sampling are the transfer of particles from the funnel surfaces into the collecting cylinders (please see also Authors response to comment 6 by Reviewer #2) and the emptying of the cylinders. We

frequently lowered down a camera system inside to mesocosms to monitor the material accumulating in the sediment traps. A short sequence of one of these videos can be seen in a published video of the sampling strategy to empty sediment traps of the KOSMOS setup, cited in the manuscript on page 18696, line 26 (Boxhammer et al., 2015 (video)). We have followed the suggested evaluation by Reviewer #1 for a perfectly suitable KOSMOS study, which included two sets of five replicates, one set with manipulated pCO_2 while the other set served as control/ambient mesocosms. The experiment lasted for 107 days, covering the whole winter-to-summer plankton succession in a marine fjord system. The average cumulative mass flux in terms of dry-weight was 196 ± 20 g for one set while 193 ± 25 g for the other (author's unpublished data). First the result shows how similar the mass flux was between treatments but secondly also how high the variation was between replicates. This variation is attributed to the slightly different volumes enclosed in the mesocosms (50.74 ± 2.5 t) but also to the plankton communities developing separately from each other for several months after mesocosm closure. Following our observations and the mass flux evaluation we are convinced to collect all settling particles in our mesocosm systems.

Comment 6 by Reviewer #1: Separation of particles from bulk seawater: This is a very informative section based on a proper evaluation of the efficiency of each technique. Since it leads to one of the main conclusion of this manuscript, it should be clearly highlighted in the abstract that does not provide any recommendations so far.

Author response: We thank Reviewer #1 for highlighting this point. We will adjust the abstract so that it reads in a revised manuscript (page 18694, line 10 - 12): 'The particulate matter of these samples was subsequently separated from bulk seawater by passive settling, centrifugation or flocculation with ferric chloride and we discuss the advantages and efficiencies of each approach.'

Comment 7 by Reviewer #1: I think that Figure 5 is not very informative and not easy to read as presented. What the reader wants to know and easily verify is: how many times did you observe an "unnatural" undersaturation in treated samples? A simple xy plot, Omega_ar before vs. after chemical treatment should be sufficient.

Author response: We agree with Reviewer #1 that Figure 5 is not the easiest to read but we think that this figure includes important information. With this figure we want to illustrate two different messages. First, using constant ratios of ferric chloride (FeCl₃) for particle

precipitation and sodium hydroxide (NaOH) for pH compensation has lead to both positive and negative shifts in seawater pH of the samples. This is most likely the case due to different particle densities and characteristics of the sampled material as mentioned in the manuscript. The important information for the reader is that each sample has to be titrated with NaOH individually when using this method. Secondly, undersaturation with respect to aragonite was detected in samples that experienced a negative shift in pH as well as in samples where pH was increased after precipitation. We wanted to show this to illustrate that some of the samples were already undersaturated before any chemical treatment, while in fact the number of undersaturated samples was decreased after chemical treatment (page 18701, line 24 - 26). Using this method as recommended in Sect. 3.2. has the positive side effect of eliminating potential undersaturation of CaCO₃ in the samples as a consequence of CO₂ release by microbial degradation processes inside the collecting cylinders. To highlight this second point in the manuscript we will add the following sentence to page 18701, line 26 in a revised version: 'This method can therefor also be used to eliminate undersaturation of CaCO₃ in the samples as a consequence of CO₂ released by microbial degradation of the collected organic matter.'

Comment 8 by Reviewer #1: Furthermore, did you check whether these pH decreases leading to stronger undersaturations resulted in significant carbonate dissolution during the flocculation process (how long did it last? maybe I missed it)?

Author response: We did not quantify calcium carbonate (CaCO₃) dissolution during the flocculation process, while calcifying organisms were not present in sufficient numbers inside the mesocosms to build up a considerable amount of CaCO₃ ending up in the sediment traps. After adding FeCl₃ and NaOH to the well-stirred samples we allowed the flocks to settle for one hour (page 18701, line 6 - 9). Depending on the amount of concentrated particles, centrifugation of the material took one to five hours before deep-freezing the material.

To avoid Undersaturation of $CaCO_3$ when using $FeCl_3$ we highly recommend in the manuscript to adjust the pH with NaOH in each sample individually.

Comment 9 by Reviewer #1: Efficiency of grinding process: Table 2. Please provide results for N measurements as well, and if available for 13C and 15N. Showing CV% for C that represents 20-25% of the organic matter is ok. Providing similar estimates for N that is potentially 10-20 times lower in mass would be even better!

Author response: As suggested by Reviewer #1 we will add the results of nitrogen measurements to Table 2. The caption of Table 2, the description of the analysis of the ground material (Sect. 2.2) and the results of material homogeneity (Sect. 2.4) will be adjusted accordingly. In a revised manuscript these parts will than read:

Page 18714, line 1 - 2: 'Results from replicated carbon and nitrogen measurements of ground sediment trap material in order to test its homogeneity.'

Page 18698, line 13 - 15: 'Carbon and nitrogen content of the concentrated and subsequently dried and ground bulk material (processing procedure described in Sects. 2.3 and 2.4) was analyzed from subsamples of 2±0.25 mg in tin capsules (5 x 9 mm, Hekatech).'

Page 18703, line 20 - 27 and page 18704, line 1 - 2: 'We evaluated the homogeneity of finely ground sediment traps samples by five repetitive carbon and nitrogen measurements of samples collected during experiments in different ocean regions between 2010 and 2014 (Table 2). [...] The CV% estimates demonstrated that carbon (CV% = 0.15–0.99) and nitrogen (CV% = 0.28–1.86) measurements of the ground samples were at least equally reproducible as measurements of the two calibration standards acetanilide and soil standard with a CV% of 0.34 and 4.17 for carbon and 0.97 and 1.55 for nitrogen, respectively (Table 2).'

Replicated measurements of samples including ¹³C and ¹⁵N have not been done.

Comment 10 by Reviewer #1: *Figure 6 should be moved to the supplementary material.*

Author response: In accordance with the suggestion by Reviewer #1, we will move Figure 6 to the supplementary material in a revised version of the manuscript.

Comment 11 by Reviewer #1: Several cryogenic grinding systems are commercially available, providing (according to the technical specs) a powder of ~5 microns. One could ask what is the originality of your system. Is there a step forward compared to these commercial units (e.g. Cryomill from Retsch or others) that I do not see?

Author response: Our custom made ball-mill for cryogenic grinding of samples operates on the same principles of impact and friction at temperatures close to -196°C as commercial units, e.g. the CryoMill from Retsch. We developed our own system based on two reasons. First we needed larger volumes of the grinding jars/spheres, than commercially available.

Even with our maximum volume of 65.5 mL we sometimes have to split up the samples into multiple spheres to homogenise them after the grinding process (page 18703, line 4 – 5). As mesocosm studies produce several hundred of sediment trap samples it is critical to minimize the number of grinding operations. Secondly we wanted to develop an easy to adapt procedure using standard lab equipment for the sample processing to keep the costs relatively low. While a commercial cryogenic ball-mill costs about 20000€ including accessories our system was comparably cheap with less than 1000€ to equip a standard cell-mill. However, commercial systems as the CryoMill of Retch will also be perfectly suitable to grind freeze-dried sediment trap samples.

References:

Boxhammer, T., Bach, L. T., Czerny, J., Nicolai, M., Posman, K., Sswat, M. and Riebesell, U.: Video of the sampling strategy to empty sediment traps of the "Kiel Off-Shore Mesocosms for future Ocean Simulations" (KOSMOS), doi:10.3289/KOSMOS_SEDIMENT_TRAP_SAMPLING, 2015.

Niehoff, B., Schmithüsen, T., Knüppel, N., Daase, M., Czerny, J. and Boxhammer, T.: Mesozooplankton community development at elevated CO2 concentrations: results from a mesocosm experiment in an Arctic fjord, Biogeosciences, 10(3), 1391–1406, doi:10.5194/bg-10-1391-2013, 2013.

Fig. 1 High resolutions images of collected sediment trap material during a mesocosm experiment in Gullmar Fjord, 2013. While image A is dominated by aggregates of various detrital phytoplankton, image B shows mostly cells of *Coscinodiscus* spp. (diameter of about 200 µm). Red circles highlight adult copepods.



Response to Reviewer #2 (Anonymous Reviewer)

We thank the reviewer for the constructive comments on this technical note, which were very helpful to refine the manuscript. Our responses to reviewer comments, including modifications to the manuscript, are detailed in the following:

Comment 1 by Reviewer #2: The first and most obvious issue is how the authors have dealt with growth on the sides of the mesocosms? This is not mentioned in the manuscript and may have a large impact on the estimates of export from the mesocosms. Were the sides cleaned or the material left to grow – did the authors estimate that growth at the end of the experiments or were any measures taken to avoid such growth?

Author response: As the reviewer correctly points out, wall growth is be a big issue in mesocosm experiments leading to biomass build-up not represented in water column or vertical flux measurements. To prevent biofilm formation on the inside walls of the cylindrical KOSMOS mesocosms we developed a ring-shaped, double-bladed wiper used since 2011 for regular cleaning as once per week (Riebesell et al., 2013). To provide a clear reference for the reader we will rephrase page 18696, line 15 – 18 to read in revised manuscript: 'The sediment trap design of KOSMOS used since 2011 consists of a flexible thermoplastic polyurethane (TPU) funnel of 2 m in diameter, connected to the cylindrical mesocosm bag by a silicon-rubber-sealed glass fibre flange (Fig. 1a). A detailed description of the KOSMOS setup and maintenance requirements such as wall cleaning can be found in Riebesell et al. (2013).'

Comment 2 by Reviewer #2: Have the authors made any tests of the oxygen consumption of material captured in the collection cylinders at the bottom of the mesocosm? Would this material go anoxic before sampling on either daily or every second day? Anoxic conditions could have important implications for the biogeochemical measurements of the settled material, e.g. a build-up of CO2 could cause dissolution of calcium carbonate and other nutrient cycling could take place (e.g. anoxic steps of the nitrogen cycle).

Author response: So far we have not measured the oxygen level or consumption rate inside the collection cylinders. Sampling the sediment traps in a 24 or 48 hours routine we only once

observed anoxic conditions by the smell of hydrogen sulfide in collected samples from a single KOSMOS unit. The outlet of the collection cylinder of this specific unit was partly blocked for several days by a Plexiglass® pipe that got lost from a manipulation device. This pipe had the effect of a partial bypass inside the collection cylinder. Surprisingly no change in the carbon to nitrogen to phosphorus ratio of the collected material was found in comparison to the other nine mesocosm units used in that specific study (author's unpublished data). However, we clearly agree with Reviewer #2 that the formation of anoxic conditions could potentially alter the collected particle stoichiometry and therefor has to be avoided by high sampling frequency. To account for this we will change page 18705 (line 6 - 8) and page 18707 (line 1 - 2) in the revised manuscript to read: 'Sampling intervals of the traps should be kept short – two days or less – to limit bacterial- and zooplankton-mediated remineralisation of the settled material and to avoid or minimize the time of possible carbonate undersaturation or anoxia in the traps.'.

Comment 3 by Reviewer #2: These are issues of particles sticking to the sides of the funnel as they slide down to the collecting cylinder at the bottom of the mesocosm and if the flow rate of the water in the silicone tube connecting the collecting cylinder to the sample bottle is high enough to ensure collection of particles with high sinking velocities (see specific comments).

Author response: Please see Author response to specific Comment 6 and 9 by Reviewer #2.

Comment 4 by Reviewer #2: Finally, why did you decide not to poison the sampled material during the sedimentation and centrifugation procedure, would this not have limited further degradation and allowed for longer sedimentation periods?

Author response: Several reasons convinced us not to poison the sampled material. As we point out on page 18695, line 26 - 29, the number of samples recovered per day and their volume can be very high depending on the experimental mesocosm setup. Including ten KOSMOS units, which are sampled on a daily basis for a two-month period, one has to process up to 30 L per day and easily more than 1000 L in total. First, the limited time for sample processing in the field, restricted by the sampling frequency, limits the need of poisoning of the samples. Second issue would be the storage of such big sample volumes and

the disposal of the toxic water after particle concentration. Finally we wanted to avoid the exposure to toxins during the grinding process.

Comment 5 by Reviewer #2: *Page 2, Line 4: With "these two processes", I guess you refer to particle flux and particle formation, maybe write the processes out to avoid confusion.*

Author response: We thank the reviewer for pointing this out. To avoid confusion we will change the sentence in a revised version of this manuscript to read (page 18694, line 3 - 4): 'However, the spatial decoupling between particle formation in the surface ocean and the collection in sediment traps often handicaps reconciliation even within the euphotic zone.'

Comment 6 by Reviewer #2: Page 4, Line 18-19: Often marine snow and other aggregates are very 'sticky' and adhere to surfaces, did you test if the aggregates did slide down the funnel surface.

Author response: We frequently lowered down a camera system inside the mesocosms to get a snapshot of the material accumulating in the sediment traps. A short sequence of one of these videos can be seen in a published video of the sampling strategy to empty sediment traps of the KOSMOS setup, cited in the manuscript on page 18696, line 26 (Boxhammer et al., (2015)). The funnel of the sediment traps is made of a flexible 1 mm thick thermoplastic polyurethane foil where the particles can slide down in a 63° angle as shown in Figure 1b of the manuscript. The vertical movement of the mesocosms due to wave action also generates slight movement of the funnel, which seems to promote the movement of particles on the funnel surface. From our frequent observations with camera systems we can state that particle aggregates do slide down the funnel surface.

Comment 7 by Reviewer #2: *Page 4, Line 20: Do you mean the tip or the bottom of the collecting cylinder?*

Author response: The collecting cylinder has a conical bottom end (Fig. 1b) with a hose connector at its tip to attach the silicon tube for sampling. This detail will be added to page 18696, line 20 - 23 in the revised manuscript so that its reads: 'A silicon tube of 1 cm inner diameter reaches down to the collecting cylinder outside of the mesocosm bag (Fig. 1a). A hose connector links the silicon tube to the conical bottom end of the collector while a wire

helix hose coating the first 1.5 m prevents current related bending of the tube (Fig. 1b).'

Comment 8 by Reviewer #2: Page 4, Line 23: How long was the tube that connected the bottom of the mesocosm to the Schott Duran glass bottle? The KOSMOS mesocosms vary in depth between 15 and 25 m, is that including the funnel below the mesocosm? If the tube was 25 m plus a bit extra so likely around 30 m long? This means that there was around 3 L of seawater in the tube itself. In addition, the collecting cylinder contained 3.1 L of seawater, which means that the total water volume in the tube and in the collecting cylinder made up 6.1 L while the Duran Schott bottle only collected 5 L of water. Was this enough to ensure that all aggregates were collected?

Author response: The KOSMOS mesocosms deployed so far reached a water depth of 15 to 25 m including the funnel of the sediment traps. The silicon tubes used for vacuum sampling of the traps were indeed up to 30 m long resulting in a maximum volume of about 2.4 L. To keep the sample volume low and not to dilute the relatively dense particle suspensions originating from the collecting cylinders we separated and discarded the water originating from the tubes if clear, as described on page 18697, line 3 - 5.

Only in the case of particles being present in the water originating from inside the tubes in combination with a collapsing phytoplankton bloom in the mesocosms one could exceed the volume of 5 L, but this was usually not the case. To ensure that we sampled all collected aggregates from the collecting cylinders we visually observed the fluent passing through the Plexiglas® pipe. The sampling procedure was only terminated when no more particle were visible in a water volume of about half a litre passing through the pipe (see Author response to Comment 13 by Reviewer #2).

Comment 9 by Reviewer #2: Page 5, Line 1-3: Have you calculated or measured the water flow in the tube? This needs to be more than the settling velocity of the collected aggregates. These can potentially sink with several hundred meters per day. Some ballasted aggregates and fecal pellets have quite high sinking velocities (e.g. Bruland and Silver 1981, Iversen and Robert 2015, Ploug et al. 2008), though most are likely around 100 m d⁻¹. Did you calculate what your theoretical flow rate was and have you considered if any potential boundary effects potentially would make you lose some particles? Author response: Unfortunately we have never measured the water flow inside the silicon tube during the sampling process. Assuming a realistic water flow rate of 0.5 L per minute at the given inner diameter of the silicon tube of 1 cm, we calculate a water flow velocity of 9167.32 m d⁻¹ according to equation (1).

$$\nu = \frac{Q}{3600 * \pi * \left(\frac{d}{2}\right)^2}$$
(1)

v = water flow velocity (m s⁻¹)

Q = water flow rate (m³ h⁻¹)

d = silicon tube inner diameter (m)

As the water flow velocity exceeds the maximum sinking velocity of even dense particles by far, we are sure to be able to recover all settled material from the sediment traps. This also correlates with our observations during sampling. Particles in the collecting cylinder are stratified after their density and grain size which becomes obvious when observing the particle suspension passing through the Plexiglas® pipe, please see Boxhammer et al., (2015). Even small screws that got lost from sampling devises inside the mesocosms were brought up within the dense particle suspensions.

Comment 10 by Reviewer #2: *Page 5, Line 3-5: Did you typically discard the volume contained within the silicon tube before sampling, e.g. 3 L for a 30 m long tube?*

Author response: Please see Author response to specific Comment 8 by Reviewer #2.

Comment 11 by Reviewer #2: Page 5, Line 7-10: It seems unlikely that the integrity of the particles were preserved during the sampling. First you collected the particles in the collection cylinder where they would land on top of each other after rolling down the sides of the funnel. Already here you have changed their size and structure. Thereafter they are pumped up a long tube and finally flushed into a Duran Schott bottle. Even if this is gently done, it will still affect the aggregates, especially marine snow, fecal pellets might survive the procedure. However, it is not important for your study to preserve the size, shape, and

structure of the aggregates, since you are interested in chemical analysis, so I would suggest to remove this sentence from the manuscript.

Author response: We agree with this statement of Reviewer #2 and will remove this sentence (page 18697, line 7 - 10) in a revised manuscript.

Comment 12 by Reviewer #2: Page 5, Line 11-14: There would be several issues by using the particles collected in this way to measure the particle sinking velocity and microbial respiration rates if you assume that the particles are the same as those formed and settling within the mesocosm. This would need some direct comparisons of aggregates collected within the mesocosm to the ones collected with the method described in this manuscript. However, as long as we are aware of the differences and changes made to the particles collected here, there is still much valuable information to be made from measurements of the particles collected here, as long as they are well characterized at each sampling point in terms of composition and type for instance.

Author response: This is an important point that Reviewer #2 has highlighted here. The particles that were sinking in-situ passed several steps of potential disintegration and reaggregation when (1) accumulating in the sediment traps, (2) being sampled through the silicon tube and (3) being subsampled in the lab. We agree that the aggregates in the subsamples used for measurements of sinking velocity and microbial respiration rate were not exactly the same as those settling in the mesocosms, but they consisted of the same primary building blocks. The potential of sinking velocity measurements from particles originating from KOSMOS mesocosm sediment traps and its limitations are discussed in (Bach et al., 2012). The detailed information about particle stoichiometry (e.g. inorganic carbon or biogenic silica load) gained by following the protocol described in the present paper can than be combined with measurements as particle sinking velocity or microbial respiration measurements.

Comment 13 by Reviewer #2: Page 5, Line 15: When did you decide that you had collected all the aggregates? 1-4 L of particle suspensions seems rather low for the 1 L, but maybe you stopped when no more particles were observed after a certain time or a certain water volume?

Author response: We thank the reviewer for pointing out this missing information. The

samples that have been pumped up to the sea surface usually had a very high particle density. During phases of low vertical particle flux the sample volume (not including the separated clear water originating from the silicon tube) can easily go down to about 0.5 L. We permanently observed the water passing through the Plexiglas® pipe and terminated the sampling procedure after about 0.5 L of clear water being sampled after the dense particle suspension. We will add this information so that it reads on page 18697, line 5 - 8: 'The dense particle suspensions originating from the collecting cylinders were then vacuum-pumped into the sampling flasks until no more particles were passing through the Plexiglas® pipe in a sampled extra volume of about 0.5 L (Boxhammer et al., 2015 (video)).'

Comment 14 by Reviewer #2: Page 5, Line 16-18: Consider to point out that this subsampling is not the one used to do the biogeochemical parameters, but subsampling for other measurements and that you are keeping this low in order to be able to have reliable chemical measurements from the total flux of particles. Did you measure the precise volume of the 'pre-subsamples'?

Author response: As suggested by Reviewer #2 and in addition to the statement that the bulk sample is used for biogeochemical analysis (page 18697, line 14) we will emphasise this point in line 16 - 18 (page 18697) so that it reads in the revised manuscript: 'Total volume of all subsamples should be kept low (ideally below 5 %) in order to limit the subsampling bias on the remaining sample that is processed for the quantitative biogeochemical analysis.'

We determined the quantities of each sample ('pre-subsample') and all subsamples gravimetrically with an accuracy of 0.1 g for individual share calculations. We will add this information (page 18697, line 21 - 22) in a revised version of the manuscript to read: 'Quantities of the main sample and all subsamples were gravimetrically determined with an accuracy of 0.1 g for individual share calculations.'

Comment 15 by Reviewer #2: *Page 6, Line 4: Why do you use the term total particulate carbon? Was this because you did not remove inorganic carbon (calcium carbonate) with hydrochloric acid?*

Author response: We used total particulate carbon as a proxy as this parameter was measured of all samples we used for analysis while particulate organic carbon was only measured of samples from mesocosm studies which enclosed a substantial amount of calcifying organisms in the water columns, e.g. coccolithophores.

Comment 16 by Reviewer #2: Page 6, Line 9-10: How did you know that the copepods were alive if they were on the filter? Did you do this step immediately after filtration or after freezing?

Author response: We thank Reviewer #2 for pointing this out. We will change page 18698, line 9 - 10 in the revised manuscript to read: 'Copepods, which could occasionally be found in the liquid, were carefully removed from the filters right after filtration.'

Comment 17 by Reviewer #2: Page 6, Line 22: Did you calculate what the slowest sinking velocity would be for the settling particles reaching the bottom of the bottle? If the bottle was 20 cm tall, then particles sinking with velocities slower than 2.4 m d-1 would not make it from the top of the bottle to the bottom within 2 hours, assuming that the bottle was full. Try to calculate this and see what the slowest velocity would be, some single phytoplankton cells settle with around 1 m d⁻¹. This might give you an idea of what the carbon sources for the supernatant could be.

Author response: It is an interesting point that Reviewer #2 has highlighted here. With about 4 L of particle suspension being sampled and the glass bottles stored in a 60° angle, we measured a maximum settling distance for particles of 0.18 m. Thus particles with a sinking velocity of less than 2.16 m d⁻¹ would not have been able to reach the bottom of the sampling bottles within 2 hours. Sinking velocities of marine particles are determined by their size and density, which is a result of their individual origin. To enable even single cells to settle down to the bottom of the glass bottles, settling times of grater than 48 h would be required, which is not practicable at high sampling frequencies of a set of mesocosms and would require poisoning of the samples to inhibit microbial degradation of the organic matter.

To include this information in the revised manuscript we will add the following sentence to section 2.2.1 (page 18699, line 13): 'To increase the concentration efficiency of passive settling, longer sedimentation periods of up to 48 hours e.g. for single plankton cells would be required. However, this is not practical at high sampling frequencies of a set of several mesocosms and would require poisoning of the samples to inhibit microbial degradation of organic matter.'

Comment 18 by Reviewer #2: Page 9, Line 27-29: Do you think the improved concentration efficiency of the $FeCl_3$ in comparison to the passive settling and the centrifugation was due to loss of $CaCO_3$ from both the sediment and the supernatant?

Author response: A loss of calcium carbonate (CaCO₃) would most likely decrease the concentration efficiency, as CaCO₃ should contribute more to sedimented carbon than to residual carbon in the supernatant even after a relatively short time of sedimentation (1 h). During the study where FeCl₃ was used for particle concentration there was only a negligible number of calcifying organisms present inside the mesocosms not able to build up a considerable amount of CaCO₃. Additionally, the number of undersaturated samples after precipitation with FeCl₃ was reduced by 2 and 6 samples with respect to aragonite and calcite (page 18701, line 24 - 26), why we do not think that the sample pH had an effect on particle concentration. The efficiency of chemical precipitation of particles with FeCl₃ is visualised in the supplementary video S1.

Comment 19 by Reviewer #2: *Page 12, Line 8: Change to "… macroscopic structures were visible after. …"*.

Author response: We thank the reviewer for pointing this out and will change the time to past tense for p. 18704, line 8 in the revised manuscript.

Comment 20 by Reviewer #2: Section 2.4: How did you do the quantitative measurements of the chemical parameters of the grounded material? Did you weigh the total mass of all the grounded material before taking subsamples from it?

Author response: We weighed the freeze-dried samples before and after the grinding procedure to determine the dry-weight of each sample. The dry-weight was than corrected for the subsamples taken previously to the concentration procedure. The quantitative values of biogeochemical parameters were than calculated from the individual subsample weight used for analysis and the corrected dry-weight of the main samples.

Comment 21 by Reviewer #2: *Page 12, Line 9: Change to: ". . .diatom frustules became detectable. . . ".*

Author response: We thank the reviewer for pointing this out and the time will be changed to past tense for p. 18704, line 9 in the revised manuscript.

Comment 22 by Reviewer #2: Page 12, Line 15-18: It still remains to show that particles are not stuck to the sides of the funnel when they are sliding down inside the mesocosm. In addition, it would be good to estimate the flow rate of the water within the tube leading from the bottom of the mesocosm to the collection Duran Schott bottle at the water surface and test if there are shear or boundary effects affecting the transport of particles through the silicon tube. Finally, was the water volume collected in the Duran bottle enough to sample all the particles in the collecting cylinder at the bottom of the mesocosm?

Author response: Concerning the issue of particles interacting with the funnel of the sediment traps please see Author response to comment 6 by Reviewer #2. The water flow rate has never been measured, but we estimate a realistic rate of 0.5 L per minute, which leads to a corresponding water flow velocity of 9167.33 m d⁻¹ inside the silicon tube of 1 cm inner diameter. For details please see Author response to comment 9 by Reviewer #2. We have no doubt that even particles of high density are transported in the water flow inside the silicon tube during sampling, but it is likely that shear or boundary effects modify the particle's size and structure. To clarify any concerns about the sampled water volume please see Author response to comment 8 by Reviewer #2.

Comment 23 by Reviewer #2: *Page 13, Line 17-18: Would the simplest method to use in the field not be the passive settling? It seems that a longer settling period would increase the efficiency of collecting the settling material at the bottom of the bottle?*

Author response: Passive settling would be the simplest method to use, if time and space would not be limiting factors and if samples would be poisoned to stop microbial degradation of the organic matter. As whole sample centrifugation speeds up the gravitational settling of particles we recommend using this method in the field to deal with a realistic sample volume of up to 30 L per day (10 mesocosm units, daily sampling frequency, \leq 3 L of individual sample volume). To speed up the process or in case of larger sample volumes, FeCl₃ can be used as a pre-treatment.

Comment 24 by Reviewer #2: *Page 13, Line 25-26: Do you have a reference or some tests showing that the precipitation of phosphate to particulate phosphorous is negligible?*

Author response: In fact it is likely that most of the inorganic phosphate being present in the water fraction of the samples (particle suspensions) has been precipitated in the form of ferric phosphate (FePO₄). However, the amount of potentially precipitated phosphate is negligible compared to the phosphorus incorporated into the biogenic particles in the samples.

Assuming 100% of the taken sample volumes to be particle free seawater and using the phosphate concentrations measured in the mesocosm water columns we calculated a theoretical contribution of precipitated phosphate of in average 0.5%. At high phosphate concentrations of about 0.8 μ mol L⁻¹ and very low particle flux (pre-bloom phase of the phytoplankton) we got only 4 out of 477 samples (0.8%) with a potentially higher contribution than 5% to particulate phosphorus in the samples.

A significant contribution of precipitated phosphate to particulate phosphorus in samples with low organically bound phosphorus would also be visible in the ratio of nitrogen to phosphorus, which was not the case in 540 samples that were precipitated with ferric chloride (unpublished data by authors).

Comment 25 by Reviewer #2: Page 14, Line 3: For me it seems that there are many issues with the addition of $FeCl_3$ to the sediment sample? Decrease of pH, precipitation of phosphate, addition of iron, and interference with spectrophotometric analysis?!? Would this method not be best to avoid?!?

Author response: The pH of sediment trap samples from mesocosm systems is generally a critical point as the traps cannot be poisoned and CO_2 released by microbial degradation can decrease the sample pH. Using FeCl₃ for particle precipitation the individual sample pH needs to be adjusted with sodium hydroxide (NaOH) also allowing for compensation of the biologically driven pH reduction. Thus this can also be seen as an advantage of using this method.

The contribution of precipitated inorganic phosphate (FePO₄) to particulate phosphorus is negligible as shown in the Author response to comment 24 by Reviewer #2.

As we stated on page 18705, line 26 and following, iron ions have the potential to interfere with spectrophotometric analysis but require very high concentrations. According to Hansen and Koroleff, (2007), the colour intensity when measuring phosphate is only increased by

about 1% with 180 μ mol L⁻¹ of iron ions being present in a measured sample. Even though each of our 540 samples contained varying amounts of organic material and iron ions we have not observed any influence on measurements, which should be visible in the elemental stoichiometry (increased silicate or phosphorus values, relative to carbon or nitrogen).

Being aware of the advantages and pitfalls of this method we recommend using FeCl₃ for particle precipitation when enclosing highly productive ecosystems in pelagic mesocosms with sediment trap samples of larger than three litres.

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Technical note: Sampling and processing of mesocosm sediment trap material for quantitative biogeochemical analysis

4

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9

10 Abstract

11 Sediment traps are the most common tool to investigate vertical particle flux in the marine 12 realm. However, the spatial decoupling between particle formation in the surface ocean and 13 the collection in sediment traps often handicaps reconciliation even within the euphotic zone. Pelagic mesocosms have the advantage of being closed systems and are therefore ideally 14 15 suited to study how processes in natural plankton communities influence particle formation 16 and settling in the ocean's surface. We therefore developed a protocol for efficient sample 17 recovery and processing of quantitatively collected pelagic mesocosm sediment trap samples for biogeochemical analysis. Sedimented material was recovered by pumping it under gentle 18 19 vacuum through a silicon tube to the sea surface. The particulate matter of these samples was 20 subsequently separated from bulk seawater by passive settling, centrifugation or flocculation 21 with ferric chloride and we discuss the advantages and efficiencies of each approach. After 22 concentration, samples were freeze-dried and ground with an easy to adapt procedure using 23 standard lab equipment. Grain size of the finely ground samples ranges from fine to coarse 24 silt $(2-63 \mu m)$, which guarantees homogeneity for representative subsampling, a widespread 25 problem in sediment trap research. Subsamples of the ground material were perfectly suitable 26 for a variety of biogeochemical measurements and even at very low particle fluxes we were 27 able to get a detailed insight on various parameters characterizing the sinking particles. The 28 methods and recommendations described here are a key improvement for sediment trap applications in mesocosms, as they facilitate processing of large amounts of samples and
 allow for high-quality biogeochemical flux data.

3

4 **1** Introduction

5 Sediment traps of various designs are the most common tool to study vertical particle flux in 6 the oceans since mid of the last century (Bloesch and Burns, 1980). During this period, the 7 impact of anthropogenic pollution and climate change on marine biogeochemical cycles has grown steadily (Doney, 2010). Pelagic mesocosm systems enclose natural plankton 8 9 communities in a controlled environment (Lalli, 1990; Riebesell et al., 2011) and allow us to 10 investigate how changing environmental factors influence elemental cycling in the ocean's 11 surface. The closed nature of these systems makes them particularly useful to investigate plankton community processes that quantitatively and qualitatively determine particle 12 13 formation and settling. Cylindrical or funnel shaped particle traps were suspended inside various pelagic mesocosm designs (Schulz et al., 2008; Svensen et al., 2001; Vadstein et al., 14 15 2012; von Bröckel, 1982). Covering only a small share of the mesocosm's diameter they were 16 prone to potential collection bias also well-known from oceanic particle traps in particular in 17 the upper-ocean (Buesseler, 1991).

18 To study vertical particle flux in mesocosms it is essential to achieve collection of all particles 19 settling to the bottom. This improves not only the measurement accuracy but also drains the material from the pelagic system, as it is the case in a naturally stratified water body. 20 21 Different pelagic mesocosm designs like the "Controlled Ecosystem Enclosures" (CEE, 22 (Menzel and Case, 1977), the "Large Clean Mesocosms" (Guieu et al., 2010) or the "Kiel 23 Off-Shore Mesocosms for future Ocean Simulations" (KOSMOS, Riebesell et al., 2013) 24 achieved quantitative collection of settling particles through a cone-shaped bottom of the 25 columnar enclosures. Two different techniques were generally used to sample collected 26 material of these sediment traps: (1) through replaceable collection cups or polyethylene 27 bottles, regularly exchanged by divers (Gamble et al., 1977; Guieu et al., 2010), (2) by means 28 of an extraction tube reaching down to the particle collector (Jinping et al., 1992; Menzel and 29 Case, 1977; Riebesell et al., 2013).

The key difficulty of sediment trap applications in pelagic mesocosms is the sample processing after recovery. Depending on the setup (number of enclosures, trap design, sampling frequency, experiment duration), samples are high in number, relatively large in volume (up to several liters) and can reach extremely high particle densities during
 aggregation events.

3 In the past the collected material was usually only partly characterized to answer specific questions (e.g. Harrison and Davies, 1977; Huasheng et al., 1992; Olsen et al., 2007) while 4 the full potential of the samples remained unexplored and the methodology of sample 5 6 processing was commonly described in little detail. To fill this gap and to facilitate a broader 7 biogeochemical analysis of the collected material, we refined methods for efficient sampling, 8 particle concentrating and processing of quantitatively collected mesocosm sediment trap 9 samples. Our primary objective was the development of an efficient and easy to adopt 10 protocol, which enables a comprehensive and accurate characterization of the vertical particle flux within pelagic mesocosms. The methods described in this paper were developed and 11 12 applied during KOSMOS studies from 2010 until spring 2014 covering five different marine ecosystems at diverse stages in the succession of the enclosed plankton communities. 13

14

15 **2** Protocol for sampling and processing

16 **2.1 Sampling strategy**

The sediment trap design of KOSMOS used since 2011 consists of a flexible thermoplastic 17 18 polyurethane (TPU) funnel of 2 m in diameter, connected to the cylindrical mesocosm bag by a silicon-rubber-sealed glass fibre flange (Fig. 1a). A detailed description of the KOSMOS 19 20 setup and maintenance requirements such as wall cleaning can be found in Riebesell et al. (2013). Settling particles are quantitatively collected on the 7 m^2 funnel surface, where they 21 slide down in a 63° angle into the collecting cylinder of 3.1 L volume (Fig. 1b). A silicon tube 22 23 of 1 cm inner diameter reaches down to the collecting cylinder outside of the mesocosm bag 24 (Fig. 1a). A hose connector links the silicon tube to the conical bottom end of the collector 25 while a wire helix hose coating the first 1.5 m prevents current related bending of the tube 26 (Fig. 1b). The silicon tube itself is only connected to the bottom of the mesocosm and fixed to the floating frame above sea surface to avoid any kinks (Fig. 1a). To empty the collecting 27 cylinders, we connected 5 L Schott Duran[®] glass bottles via a Plexiglas[®] pipe to the silicon 28 29 tubes attached at the floating mesocosm frames (Fig. 1b; Boxhammer et al., 2015 [video]). A 30 slight vacuum of ~300 mbar was built up in the glass bottles by means of a manual kite surf 31 pump, for gentle suction of the water inside the silicon tubes (step 1 in Fig. 2). When first

particles showed up in the Plexiglas® pipe the sampling process was briefly interrupted, 1 2 seawater in the bottles screened for particles and only discarded if clear. The dense particle suspensions originating from the collecting cylinders were then vacuum-pumped into the 3 4 sampling flasks until no more particles were passing through the Plexiglas[®] pipe in a sampled 5 extra volume of about 0.5 L (Boxhammer et al., 2015 [video]). 6 Subsamples of sediment trap material for measurements such as zooplankton contribution 7 (Niehoff et al., 2013), particle sinking velocity (Bach et al., 2012) or respiration rates of 8 particle colonizing bacteria were taken with a pipette after sample collection but prior to 9 processing of the bulk sample for biogeochemical analysis. For this the particle suspension (~1-4 L) was gently mixed and subsample volumes withdrawn immediately before re-10 suspended particles were able to settle down. Total volume of all subsamples should be kept 11 12 low (ideally below 5 %) in order to limit the subsampling bias on the remaining sample that is processed for the quantitative biogeochemical analysis. We occasionally noticed a patchy 13 14 distribution of particles within the sampling bottles despite the mixing but we consider this 15 subsampling bias to be rather small because subsample volume was usually large enough to 16 tolerate a certain degree of sample heterogeneity. Quantities of the main sample and all

17 <u>subsamples were gravimetrically determined with an accuracy of 0.1 g for individual share</u>

18 <u>calculations.</u>

19 2.2 Separating particles from bulk seawater

20 Particulate material recovered from the mesocosm sediment traps and transferred into 21 sampling flasks needs to be separated from bulk seawater collected during the sampling 22 procedure. In this section we describe three different methods for separating particles from 23 bulk seawater, as this was the most critical and time-intense step in the sampling procedure.

24 The particle concentration efficiency (%) of the three methods (subsections 2.2.1 - 2.2.3) was 25 determined as the percentage of total particulate carbon (TPC) concentrated in the processed 26 samples in relation to the sum of concentrated and residual TPC in the remaining bulk water. 27 Residual TPC in the bulk water was determined of subsamples that were filtered on combusted GF/F filters (Whatman, 0.7 µm pore size, 450°C, 6 h) with gentle vacuum 28 (< 200 mbar) and stored in combusted glass petri dishes (450°C, 6 h) at -20°C. Copepods, 29 which could occasionally be found in the liquid, were carefully removed from the filters right 30 31 after filtration. The filters were oven-dried at 60°C over night, packed into tin foil and stored 2 included as blanks and measured alongside with the sample filters. Carbon and nitrogen content of the concentrated and subsequently dried and ground bulk material (processing 3 4 procedure described in sections 2.3 and 2.4) was analyzed from subsamples of 2 ± 0.25 mg in 5 tin capsules (5*9 mm, Hekatech). For this subsamples were directly transferred into the tin 6 capsules and weight determined on a microbalance (M2P, Satorius) with an accuracy of 7 0.001 mg. All samples were measured with an elemental analyzer (Euro EA-CN, Hekatech), 8 which was calibrated with acetanilide (C_8H_9NO) and soil standard (Hekatech, Catalogue no. 9 HE33860101) prior to each measurement run.

in a desiccator until analysis. Combusted GF/F filters without filtered supernatant were

10 2.2.1 Separating particles from bulk seawater by passive settling

1

11 Particles were allowed to settle down for two hours in 5 L glass bottles in darkness at in-situ 12 water temperature before separating the supernatant liquid. After this sedimentation period the 13 supernatant was removed and transferred into separate vacuum bottles by means of a 10 mL pipette connected to a vacuum pump (Czerny et al., 2013; Gamble et al., 1977). We found 14 removal of the supernatant to be most efficient when glass bottles were stored in a 60° angle 15 16 so that particles could accumulate in the bottom edge of the bottles (step 2 in Fig. 2). The 17 dense particle suspension at the bottom of the glass bottles was concentrated in 110 mL tubes by centrifugation for 10 minutes at 5039 * g (3K12 centrifuge, Sigma) to form compact 18 sediment pellets (step 3 in Fig. 2). These pellets were then frozen at -30°C. A cable tie with its 19 tip bent in a 90° angle was stuck into each sample before freezing in order to enable easy 20 21 recovery of the material from the centrifugation tubes. The frozen samples were transferred to 22 plastic screw cap jars (40 - 80 mL) for preservation and storage in the dark at -30°C before 23 freeze-drying (sect. 2.3).

24 Separating particulate material from the liquid by passive gravitational settling resulted in a median concentration efficiency of 92.9%. The relatively wide range of scores (99.3 - 86.8%) 25 reflects a non-ideal reproducibility of this particle concentration method (Fig. 3, green). The 26 27 applied sedimentation period of 2 hours was occasionally not long enough for small or lowdensity particles to settle. To increase the concentration efficiency of passive settling, longer 28 29 sedimentation periods of up to 48 hours e.g. for single plankton cells would be required. 30 However, this is not practical at high sampling frequencies of a set of several mesocosms and 31 would require poisoning of the samples to inhibit microbial degradation of organic matter.

2.2.2 Separating particles from bulk seawater by whole sample centrifugation

2 Centrifuging the entire sample volume, which is usually between 1 - 4 L, can considerably enhance gravitational separation of particles from bulk seawater. This procedure requires a 3 4 large-volume centrifuge that is not necessarily standard lab equipment and difficult to take out 5 into the field due to its high weight. For this approach we transferred particle suspensions 6 originating from the sediment traps directly from the 5 L sampling flasks into 800 mL 7 centrifuge beakers. Separation of particulate material was achieved within 10 minutes at 8 5236 * g using a 6-16KS centrifuge (Sigma), followed by slow deceleration to avoid re-9 suspension of particles (step 3 in Fig. 2). The supernatant was then carefully decanted and collected for filtration, while the sample pellets were transferred into 110 mL centrifuge 10 tubes. This procedure was repeated until the 5 L sampling flasks were emptied. In a second 11 step of centrifugation for 10 minutes at 5039 * g in the small tubes (3K12, Sigma) samples 12 were compressed into compact sediment pellets which can be frozen and stored in plastic 13 14 screw cap jars as described in section 2.2.1.

Whole sample centrifugation resulted in a high concentration efficiency of particles with a
median of 98.9% and a low variability (98.1 – 99.6%), indicating the high reproducibility of
this method (Fig. 3, blue).

18 **2.2.3** Concentrating samples by flocculation and coagulation of particles

19 Ferric chloride (FeCl₃) is well known as a flocculant and coagulant in sewage treatment 20 (Amokrane et al., 1997; Renou et al., 2008), but can also be used for concentrating marine 21 viruses (John et al., 2011) or microalgae (Knuckey et al., 2006; Sukenik et al., 1988). The iron 22 ions form a series of metal hydrolysis species aggregating to tridimensional polymeric 23 structures (sweeping flock formation) and enhance the adsorption characteristics of colloidal 24 compounds by reducing or neutralizing their electrostatic charges (coagulation). Best precipitation results at salinity of 29.6 were obtained by addition of 300 µL of 2.4 molar 25 26 FeCl₃ solution per liter of well-stirred particle suspension, resulting in a very clear supernatant. The disadvantage of particle precipitation with FeCl₃, however, is that FeCl₃ is a 27 28 fairly strong Lewis acid and therefore reduces the pH upon addition to a seawater sample. A 29 pH decline in sediment trap samples needs to be avoided in order to prevent dissolution of 30 collected calcium carbonate (CaCO₃).

To quantify the FeCl₃ related pH reduction we added $FeCl_3$ to (1) a seawater sample 1 2 originating from mesocosms deployed in Gullmar Fjord (Sweden 2013) and (2) to a seawater 3 sample of the same origin in which we re-suspended sediment trap material. This test was 4 carried out in 500 mL beakers at 25°C using a stationary pH meter (NBS scale, 713, 5 METROHM) to monitor changes of the seawater pH (Fig. 4). As expected, addition of 6 150 µL FeCl₃ (2.4 M) solution resulted in a distinct drop in seawater pH of about 3 units in 7 the absence of particles (Fig. 4, blue, full boxes) and 1.3 units in the presence of re-suspended 8 particles (Fig. 4, red, empty boxes). The pH decrease was compensated by stepwise titration 9 with three molar NaOH reaching the initial seawater pH after addition of ~330 µL NaOH both 10 in absence and presence of particles. In both cases the calculated aragonite saturation state, 11 representing the more soluble form of biogenic CaCO₃, was well above $\Omega = 1$ (Fig. 4, grev 12 dashed line), as calculated with CO2SYS MS Excel Macro (Pierrot et al., 2006) at 25°C, 13 0 dbar, salinity = 29.62 and total alkalinity (TA) = 2206.1 (Bach et al. 2016) with constants of 14 Mehrbach et al., 1973, refitted by Dickson and Millero, 1987.

15 According to the test, 660 µL NaOH (3 M) were simultaneously added with 300 µL FeCl₃ 16 (2.4 M) to each liter of particle suspension to stabilize the sample pH and to achieve optimal 17 particle precipitation (S1 [video]). The formation of dense and rapidly settling flocks allowed 18 separation of the supernatant and concentration of the deposit as described in section 2.2.1 19 after only one hour of sedimentation. Even though buffering the samples with NaOH, we still 20 observed shifts in seawater pH. Delta pH (Δ pH) was calculated from 50 pH measurements 21 before and after addition of FeCl₃ and NaOH to sediment trap samples (pH meter, 3310 22 WTW; InLab Routine Pt1000 electrode, Mettler Toledo). The resulting ΔpH (Fig. 5) differed 23 between individual samples of the same day as well as between sampling days over the 107 days of experiment. A maximum spread of 0.46 pH units was observed on day 63 while the 24 25 minimum difference of 0.15 units occurred on day 103. We did not detect a trend towards a 26 positive or negative shift in pH as the variation in the data lead to an average ΔpH of -0.01. It 27 is likely that differences in the amount and composition of particles in the samples led to the 28 observed pattern. Aragonite and calcite saturation states of the samples after precipitation 29 (Fig. 5) were calculated as described above using in situ storage temperature, pH 30 measurements of the samples and TA values from mesocosm water column measurements (Bach et al. 2016). Undersaturation of both carbonate species already occurred in several 31 samples prior to FeCl₃ addition as ocean acidification scenarios were established inside the 32 33 mesocosm bags and CO₂ released by biomass degradation likely further reduced seawater pH. 1 In fact the number of undersaturated samples after precipitation was reduced by 2 and 6

- 2 samples with respect to aragonite and calcite. This method can therefor also be used to
- 3 eliminate undersaturation of $CaCO_3$ in the samples as a consequence of CO_2 released by
- 4 microbial degradation of the collected organic matter.

5 The FeCl₃ approach yielded the highest concentration efficiency among the three methods 6 with a median of 99.6% and a narrow range of scores (98.2 – 99.9%), indicating a remarkable 7 reproducibility (Fig. 3, red). The outliers seen in the boxplot are likely caused by extremely 8 high amounts of transparent exopolymer particles (TEP) in specific samples. We observed 9 TEP in the supernatant of these samples in the form of strings (Alldredge et al., 1993) likely 10 promoting buoyancy of attached particles (Azetsu-Scott and Passow, 2004) and thereby 11 explaining the slightly decreased concentration efficiency in these samples.

12 **2.3 Freeze-drying samples**

13 The water content of the frozen samples was removed by freeze-drying for up to 72 hours 14 depending on pellet size (step 4 in Fig. 2). Lyophilization is preferable to drying the material in the oven for better preservation of phytoplankton pigments (McClymont et al., 2007) and 15 significant improvement of pigment extraction (Buffan-Dubau and Carman, 2000; van 16 Leeuwe et al., 2006). Sedimentation rates within the mesocosms (expressed as collected dry-17 18 weight per unit time) were gravimetrically determined and should be corrected for sea salt content. Residual sea salt can be estimated with known loss of water during freeze-drying and 19 20 known salinity of water in the respective samples. The alternative of removing sea salt before 21 freeze-drying with ultra pure water has the downside of potential osmotic cell rupture and loss 22 of intracellular compounds and should therefore be avoided.

23 **2.4 Grinding the desiccated material**

The desiccated sediment pellets were cryogenically ground into a fine powder of homogeneous composition to guarantee representative subsampling. We therefore developed a ball-mill to grind sample sizes from 0.1 to 7.0 g dry-weight. Hollow spheres with volumes ranging from 11.5 to 65.5 mL were cut out of blocks of stainless steel (V4A/1.4571). Each hollow sphere is divided into two hemispheres of exactly the same shape only connected by two guide pins and sealed by a metal sealing (Fig. S1). The size of the grinding sphere was selected according to the dry-weight of the freeze-dried sediment pellets (Table 1). A set

number and size of grinding balls (stainless steal, 1.3541) ranging from 10-20 mm in 1 2 diameter is transferred into the hemisphere containing the sample pellet (Table 1). The second hemisphere is then put on top of the other so that the two hemispheres form a hollow sphere 3 4 with the sample and the grinding balls locked inside. Sediment pellets heavier than 7.0 g have 5 to be split up into multiple spheres and require homogenization after grinding. After loading 6 the grinding spheres we cooled them down in liquid nitrogen (step 5 in Fig. 2) until the liquid 7 stopped boiling (-196°C). We observed that deep-freezing of the samples is essential for 8 embrittlement of lipids in the organic matter and additionally protects phytoplankton 9 pigments from frictional heating during the grinding process. The deep-frozen spheres 10 (ca. -196°C) were clamped on a cell mill (Vibrogen VI 6, Edmund Bühler) shaking with 11 75 Hz for 5 min (step 6 in Fig. 2), thereby grinding the material by impact and friction. Before 12 opening the grinding spheres they needed to be warmed up to room temperature to avoid 13 condensation of air moisture on the ground sample material. This was done by means of 14 infrared light bulbs (150 W) installed in about 5 cm distance (step 7 in Fig. 2). The very finely 15 ground samples were then recovered from the opened spheres with a spoon and transferred into gas tight glass vials to protect the powder from air moisture (step 8 in Fig. 2). Samples 16 were stored in the dark at -80°C to minimize pigment degradation. All handling of the 17 samples during the grinding process was done over a mirror for complete recovery of the 18 19 ground material.

We evaluated the homogeneity of finely ground sediment traps samples by five repetitive carbon and nitrogen measurements of samples collected during experiments in different ocean regions between 2010 and 2014 (Table 2). Reproducibility of the measurements was expressed by the coefficient of variation in percent (CV%) reflecting the dispersion of measurements relative to the mean:

$$25 \qquad \text{CV\%} = \frac{\text{SD}}{\text{MEAN}} * 100$$

(1)

The CV% estimates demonstrated that carbon (CV% = 0.15-0.99) and nitrogen (CV% = 0.28-1.86) measurements of the ground samples were at least equally reproducible as measurements of the two calibration standards acetanilide and soil standard with a CV% of 0.34 and 4.17 for carbon and 0.97 and 1.55 for nitrogen, respectively (Table 2).

Homogeneity of ground samples is mainly determined by the grain size, which is therefore crucial for representative subsampling. Scanning electron microscopy (SEM) photographs of fresh sediment trap samples (Fig. 6 a, b) show that the collected material consists of a heterogeneous mixture of all kind of debris particles such as agglutinated diatom chains, fecal
pellets and macroscopic aggregates. None of these macroscopic structures were visible after
the grinding procedure (Fig. 6 c, d). Only at 2500–fold magnification, details such as pores of
former diatom frustules became detectable in tiny fragments (Fig. 6 e, f). Grain size
representing grinding quality was in the range of fine to coarse silt (2 – 63 µm, international
scale) independent of the sample origin and primary composition (Fig. 6 c, d).

7

8 **3** Conclusions and recommendations

9 3.1 Sediment trap design and sample recovery

The quantitative collection of settling particles, as realized in several pelagic mesocosm 10 designs (e.g. CEE, KOSMOS, Large Clean Mesocosms), combines the advantage of sampling 11 12 all settling particles produced by the enclosed plankton community with the removal of 13 settled organic matter from the bottom of the enclosures. Collecting all settling particles avoids the potential sampling bias of suspended particle traps in mesocosm enclosures and 14 leads to more accurate particle flux rates. Removing the accumulating material prevents re-15 16 suspension and non-quantified resupply of nutrients and other dissolved compounds released 17 by degradation back into the water column.

We applied the vacuum sampling method to allow easy sample recovery in short time 18 19 intervals and to keep the systems sealed for minimal disturbance of the enclosed water bodies. 20 Opening of the sediment traps even for a very short time can lead to water exchange due to 21 density gradients between enclosed and surrounding water. The vacuum sampling method is 22 therefore ideal to keep the mesocosm enclosures completely sealed and thereby exclude 23 introduction of plankton seed-populations and to allow for proper budgeting of elements. 24 Furthermore the extraction of the collected material from the sea surface does not require 25 diving activities. Only in case of a non-reversible blockage of the outlet of the collecting cylinder by artificial objects one can open up the cylinder at the top and the bottom. 26

Sediment traps of mesocosms can obviously not be poisoned to prevent organic matter
degradation, raising the importance of frequent sampling. <u>Sampling intervals of the traps</u>
should be kept short - two days or less - to limit bacterial- and zooplankton-mediated

30 remineralisation of the settled material and to avoid or minimize the time of possible

31 <u>carbonate undersaturation or anoxic conditions.</u>

3.2 Particle concentration

2 Centrifuging the entire sample volume (sect. 2.2.2) as well as precipitating particles with 3 FeCl₃ (sect. 2.2.3) was shown to effectively concentrate sediment trap samples containing 4 large amounts of bulk seawater without the need of separate analysis of the supernatant. In 5 contrast, particle concentration by passive settling (sect. 2.2.1) should be complemented by 6 additional measurements of material remaining in the supernatant as mean concentration 7 efficiency is much lower and more depending on particle characteristics.

The simplest method to use in the field was centrifugation of the whole sample volume. We 8 9 therefore recommend this method for sample volumes of up to three liters, as it avoids separate supernatant analysis or re-adjustment of the samples' pH and undesired enrichment 10 with iron. Concentration of samples larger than three liters can be accelerated by precipitation 11 12 of particles with FeCl₃ prior to centrifugation and is advisable during bloom and post-bloom events of high particle fluxes. If applied in the future, we strongly advise to adjust pH after 13 FeCl₃ addition with NaOH in each sample individually to ensure CaCO₃ preservation. FeCl₃ 14 is also known to precipitate dissolved inorganic phosphate (PO₄³⁻) (Jenkins et al., 1971), but 15 the relative contribution of precipitated PO_4^{3-} to particulate phosphorus in the samples is 16 likely to be negligible. The potential of iron to interfere with the spectrophotometric analysis 17 18 of biogenic silica or particulate phosphorus leading to increased absorption at very high iron concentrations (Hansen and Koroleff, 1999) can not be confirmed based on our observations 19 20 (author's unpublished data).

21 3.3 Sample analyses

22 Processing of the sediment trap material to a finely ground and homogeneous powder proved 23 to be ideally suited for reproducible elemental composition analysis. So far we successfully measured content of major bioactive elements such as total/organic/inorganic carbon, 24 nitrogen, phosphorus and biogenic silica using standard methods for particulates in seawater 25 (Table 3). Isotopic tracers such as ¹³C and ¹⁵N added to the mesocosms as well as natural 26 27 isotope signals were additionally measured in settled organic matter (de Kluijver et al., 2013; Paul et al., 2015a). Furthermore phytoplankton pigments extracted from the ground samples 28 29 were analyzed revealing contribution of key phytoplankton groups to settling particle formation (Paul et al., 2015a). As only a few milligram of material are needed for these 30

analyses, measurement of further parameters such as lithogenic material or amino acids
 should be tested in the future.

3 **3.4 Recommendations**

This section highlights the most important recommendations for improving particle collection
in pelagic mesocosms along with sampling and processing of the collected material for
biogeochemical analysis.

7

Quantitative collection of settling particles with full-size funnel traps leads to accurate
 flux measurements and minimizes impact of organic matter degradation on the
 enclosed water column.

- Vacuum sampling of the sediment traps via an extraction tube allows keeping the
 mesocosms sealed, excluding seawater and organism exchange.
- High sampling frequency limits organic matter degradation and potential carbonate
 undersaturation or anoxia in the traps.
- Separation of particles and bulk seawater in the samples is highly efficient when
 achieved by centrifugation or chemical precipitation with FeCl₃.
- Freeze-drying the collected material is preferable to drying the samples in the oven to
 better preserve phytoplankton pigments.
- Grinding of the entire samples guarantees representative subsampling for
 biogeochemical analysis.
- 21

Following our successfully applied protocol (Fig. 2, sect. 2) and the above recommendations
will lead to accurate biogeochemical flux data of mesocosm sediment traps, irrespective of the
magnitude of the particle flux.

25

26 Author contribution

U. Riebesell conceived the mesocosm experiments between 2010 and spring 2014. T.
Boxhammer and J. Czerny developed the methods for sample acquisition and material
processing. T. Boxhammer carried out the practical work, while the presented data were
analyzed by T. Boxhammer and L.T. Bach. T. Boxhammer prepared the manuscript with
contributions from all co-authors.

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Table 1. Depending on the dry-weight of the freeze-dried sediment trap samples, different grinding sphere volumes and numbers of grinding balls (10 - 20 mm) are recommended to achieve optimal grinding results at a set run time of the ball mill (5 minutes). The optimal combination of the different factors was determined empirically to achieve a grain size smaller than 63 µm and to minimize frictional heating of the samples.

Hollow sphere	# of grinding balls	Run time of the	
volume	and size	ball mill	
[mL]	[mm]	[min]	
11.5	1 x 10	5	
24.4	$1 \times 15 + 2 \times 10$	5	
47.7	$2 \times 15 + 2 \times 10$	5	
65.5	1×20	5	
	Hollow sphere volume [mL] 11.5 24.4 47.7 65.5	Hollow sphere volume [mL] # of grinding balls and size [mm] 11.5 1 x 10 24.4 1 x 15 + 2 x 10 47.7 2 x 15 + 2 x 10 65.5 1 x 20	Hollow sphere volume $[mL]$ # of grinding balls and size $[mm]$ Run time of the ball mill $[min]$ 11.51 x 10524.41 x 15 + 2 x 10547.72 x 15 + 2 x 10565.51 x 205

Table 2. <u>Results from replicate carbon and nitrogen measurements of ground sediment trap material in order to test its homogeneity.</u> Powdered samples originating from different pelagic mesocosm experiments were tested and compared with commercially available standards commonly used for calibration of elemental analyzers (Soil Standard [STD], Acetanilide Standard [STD]). Homogeneity is expressed by the coefficient of variation in percent (CV%). As well presented are the number of measured aliquots, the amount of material analyzed, average carbon content, calculated standard deviation (SD) and grain size derived from scanning electron microscopy. ND = grain size not determined.

Sample origin	Measured aliquots #	Aliquot weight [mg]	Grain size [µm]	Average carbon [μmol mg ⁻¹]	SD (carbon)	CV% (carbon)	<u>Average</u> <u>nitrogen</u> [μmol mg ⁻¹]	<u>SD</u> (nitrogen)	<u>CV%</u> (nitrogen)
Soil STD C = 3.429% Acetanilide STD C = 71.089% Svalbard 2010 # SV106 Norway 2011 # NO124 Finland 2012 # F1114 Sweden 2013 # SE502 Gran Canaria 2014	5 5 5 5 5 5 5 5	4 ± 0.25 1 ± 0.15 2 ± 0.25 2 ± 0.25	ND ND ≤ 63 ≤ 63 ≤ 63 ≤ 63	2.83 58.81 22.74 19.57 22.53 29.03 17.15	0.12 0.20 0.12 0.09 0.03 0.23 0.17	4.17 0.34 0.51 0.48 0.15 0.80 0.99	0.16 7.34 3.77 2.53 3.58 1.65 0.94	0.00 0.07 0.01 0.01 0.01 0.03 0.00	1.55 0.97 0.39 0.54 0.28 1.86 0.28

Table 3. List of parameters measured from ground sediment trap samples originating from
 KOSMOS experiments. The methods / instruments applied and the corresponding references
 with data sets and detailed descriptions of the methods are furthermore provided.

Parameter	Method / Instrument	Corresponding publications
Total carbon	Elemental analyzer	Czerny et al., 2013; Paul et al., 2015b
Organic carbon	Removal of inorganic carbon by direct addition of hydrochloric acid (Bisutti et al., 2004); Elemental analyzer	<u>Riebesell et al., 2016</u>
Inorganic carbon	Calculated from total and org. carbon	Riebesell et al., 2016
Total nitrogen	Elemental analyzer	Czerny et al., 2013; Paul et al., 2015b
Phosphorus	Spectrophotometry (Hansen and Koroleff, 1999)	Czerny et al., 2013; Paul et al., 2015b
Biogenic silica	Spectrophotometry (Hansen and Koroleff, 1999)	Czerny et al., 2013; Paul et al., 2015b
Isotopic tracers (¹³ C, ¹⁵ N)	Mass spectrometry, Elemental analyzer	de Kluijver et al., 2013; Paul et al., 2015a
Phytoplankton pigments	High pressure liquid chromatography	Paul et al., 2015a



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Figure 1. (a) Technical drawing of the KOSMOS flotation frame with unfolded TPU enclosure bag and attached funnel-shaped sediment trap. **(b)** A silicon tube connects the collecting cylinder at the tip of the sediment trap with a 5 L sampling flask. A wire-reinforced hose prevents current related bending of the first 1.5 meters. Particles can be easily detected in the Plexiglass® pipe linking up the silicon tube with the sampling flask.



Figure 2. Protocol of mesocosm sediment trap sampling (1), particle concentration (2 – 3),
freeze-drying (4) and grinding (5 – 8) to convert heterogeneous sediment trap samples into
homogeneous powder for biogeochemical analysis.





3 Figure 3. Boxplot of the concentration efficiency (%) of three different methods for particle 4 concentration of mesocosm sediment trap samples. Concentration of particles by passive 5 settling (green) is compared with gravitational deposition of particulates by whole sample 6 centrifugation (blue). The third option of flocculation and coagulation with FeCl₃ for 7 enhanced particle settling is presented in red. Concentration efficiency is defined as the percentage of TPC concentrated in the processed sediment trap samples in relation to the 8 9 particulate carbon in the originally sampled suspensions (sum of concentrated and residual TPC in the bulk water). Outliers (circles) are defined as any data points below 1.5 * IQR 10 (interquartile range) of the first quartile hinge or above 1.5 * IQR of the third quartile hinge. 11



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3 Figure 4. Titration of 500 mL sea water (blue, filled box and line) and 500 mL particle 4 suspension (red, empty box and line) with 3 M NaOH after addition of 150 µL 2.4 M FeCl₃ 5 solution. The grey solid line indicates the pH of seawater before any manipulation. pH (NBS 6 scale) was measured at 25°C with a stationary pH meter (713, METROHM). Calculated aragonite saturation state of $\Omega = 1$ is represented by the grey dashed line. 7



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Figure 5. Delta pH of 50 sediment trap samples, calculated from pH measurements before and after addition of FeCl₃ (300 μ L L⁻¹, 2.4 M) and NaOH (660 μ L L⁻¹, 3 M) for precipitation of suspended particulate material. $\Omega_{ARAGONITE}$ after chemical treatment of the samples is indicated by a color gradient from red over grey to blue, representing undersaturated, saturated and oversaturated samples, respectively. $\Omega_{CALCITE} < 1$ is tagged by black edging of the colored data points.







- 3 Figure 6. Scanning electron microscopy (SEM) photographs of two sediment trap samples
- 4 before (a, b) and after grinding (c f). (c) and (d) represent the average grain size of the
- 5 ground samples, while (e) and (f) reveal details visible at 2500 fold magnification.