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A New Method to Speed Up Nannofossil Picking for Monospecific Geochemical Analyses

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Abstract: Investigating the trace elements contained in the coccoliths, i.e., the carbonate exoskeleton, of unicellular marine phytoplankton called coccolithophores, is fundamental for calibrating environmental climate proxies, which are key tools for studying past and future climate changes. To date, lab-cultivated coccolithophores have been mainly used for measuring the elements retained within the coccoliths, whereas geochemical studies in fossil records have been limited by the difficulty in isolating monospecific samples from sediments containing highly diversified fossil assemblages. Since a comparison of the geochemical data collected from both fossil and living species is fundamental for calibrating the environmental proxies, an improvement of coccolith-picking methodology should be envisaged. Here, we present a significant advancement in the isolation of fossil species-specific coccolith achieved using a hydraulic micromanipulation system together with wet samples, never applied before on coccoliths. Our technique allows the picking of around 100 monospecific coccoliths per h, a number never achieved before with other isolation methodologies. This method opens up new possibilities in applying monospecific geochemical analyses to the fossil record not attainable before (e.g., the use of the mass spectrometer), leading to an increase in knowledge of environmental proxy calibration and coccolithophore element incorporation strategies.

Keywords: coccolithophores; calcareous nannofossils; monospecific picking; wet sample; micromanipulator; hydraulic control system; geochemistry

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1. Introduction

Coccolithophores are a group of unicellular marine phytoplankton, very abundant and widespread in both past [1] and modern oceans [2,3]. Their exoskeleton, composed of many calcite plates called coccoliths, constitutes up to 50% of the calcium carbonate ($CaCO_3$) accumulated into oceanic sediments [4,5]. As coccolithophores are strongly affected by climate changes e.g., [2,6], the study of their abundance and distribution is ideal for paleoenvironmental and paleoclimate reconstructions e.g., [7–9]. To better interpret the evidence extrapolated from fossil assemblages, it is essential to compare them with specific environmental proxies achievable through geochemical analyses. The ratio between minor elements and calcium (Ca) measured within the carbonate produced by certain marine organisms, such as the foraminifera, has been widely used to reconstruct specific (paleo)environmental conditions (e.g., paleoproductivity, sea surface temperature, pH, and sea level variations) [10,11], as their shells are built in stoichiometric equilibrium with the seawater [12]. Coccolithophores, in contrast, produce their calcite plates intracellularly [13,14]; thus, the coccoliths are generated more frequently outside the isotopic equilibrium and with a larger vital effect compared to foraminifera e.g., [15,16]. Recent studies have provided a better constraint of the vital effect in some coccolithophore species [17–20], highlighting

the high potential retained by coccolithophores for geochemical analyses and for determining specific environmental proxies [21–23]. So far, geochemical studies on coccoliths have been mainly conducted on specific species of cultivated coccolithophores [24–28]. To date, the most well-established coccolith-derived proxy is the Sr/Ca ratio which has been demonstrated to be indicative of primary productivity variations both in cultivated species [24,26,27] and fossil remains [29–31], so-called calcareous nannofossils.

Coccoliths from monospecific cultivation have been used more than fossil coccoliths for geochemical studies. This is related to important issues that have to be faced when dealing with polyspecific fossil assemblages: (1) it is very hard and time-consuming to obtain monospecific samples from marine core or bulk sediments; (2) the sediment fine fraction is mainly composed of coccoliths (1–20 µm size range), and it comprises several species making impossible to disentangle the species-specific contribution to the final geochemical signal; (3) it is difficult to obtain a fraction exclusively constituting coccoliths. In fact, the mechanical separation of the fine fraction may be easily affected by mesh size errors, which increase with the lowering of the mesh size, and clogging, leading to a poor size separation, and thus, to the presence of pieces belonging to other marine biota such as foraminifera or diatoms [32,33].

To overcome these issues and to achieve a better selection of different coccolith size fractions within the fossil assemblages, various separation techniques have been developed. These techniques include: specific settling velocities [32,34], filtration [33], flow cytometry [35], and repeated centrifugation [36]. Despite the significant improvements in precision and velocity gained by these methodologies, completely monospecific samples cannot be achieved due to the variegated size ranges and morphologies of coccolithophores, comprising nowadays more than 200 living species [37,38], with hundreds of others having evolved since their first appearance at 225 Ma [1,38].

A precise selection of monospecific samples is attainable only through the use of high-tech micromanipulators which allow the picking of each single specific coccolith [39,40]. More recently, Suchéras-Marx et al. [41] designed a hand-picking method which isolates single coccoliths without using specialized micromanipulators. Those techniques, using the principle of electrostatic forces to pick the coccoliths, allow isolating from 5 to 20 coccoliths per h. For the aims of those studies, a high number of coccoliths was not required, since they used high-resolution techniques, such as secondary ion mass spectrometry [39,42] or synchrotron-based X-ray fluorescence [31,41,43], to analyze a specific limited number of coccoliths. However, these kinds of high-tech instrumentations are not routinely used and are often difficult to access compared to other methods employed for element analyses that are more commonly used and well-established, such as the inductively-coupled mass spectrometer (ICP-MS). A disadvantage of the ICP-MS is the amount of material required for the measurements, which is only easily achievable using coccolithophore cultures. For this reason, it is extremely difficult to apply the ICP-MS to fossil-derived monospecific samples. In fact, so far geochemical investigations on nannofossils have been focused mainly on the <20 µm fraction [21,44,45] or specific size ranges of nannofossils [22,24,29].

Here, we present a simple but very effective implementation of the micromanipulation technique without the electrostatic forces used so far that allows isolation of a large number of coccoliths compared to previous works. This novel approach, employing wet samples instead of dried ones, is applicable to all size ranges of nannofossil species and, thanks to its velocity and efficiency, it opens up new horizons in coccolithophore geochemical analyses at monospecific level.

2. Materials and Methods

For testing our picking technique on nannofossils, we used an unconsolidated sediment sample from the Ocean Drilling Program (ODP) Site 1209B (32°39′ N and 158°30′ E; NW Pacific Ocean; 2387 m water depth) [46]. The selected sample was collected at 1.4 m below sea floor (mbsf), and dated 118,000 years ago [44]. The deep-sea sediments at this site are mainly composed of nannofossil oozes with foraminifera, diatoms, radiolaria, and

clay as minor components [46]. The nannofossil assemblage is well preserved, and the size range of the species varies from 2.5 to 16 µm [44,47].

Here, we describe the step-by-step protocol—including all the required instrumentations, tools, and microscope settings necessary to isolate single coccoliths from sediments in order to obtain monospecific samples. This technique requires: (1) borosilicate capillary (10 cm length, outer diameter ⦰ 1 mm, inner ⦰ 0.75 mm), (2) Flaming/Brown micropipette puller Model P-97 Sutter Instrument Co., (3) microforge Narishige MF-9, (4) Petri dish (⦰ 50 mm), (5) inverted microscope Olympus IX71, (6) hydraulic micromanipulator Narishige IM-98, (7) Milli-Q water buffered with ammonia (pH > 12), (8) sample holder to deposit the coccoliths, which may vary according to the type of the required analyses (e.g., Mylar film, TEM grids, SEM cover slip, Eppendorf, Hamburg, Germany).

Firstly, it is necessary to carefully prepare a capillary with the desired diameter. A capillary of borosilicate glass tube is placed in a micropipette puller which stretches the capillary so as to make its diameter thinner and to divide it in two parts (Figure 1a). Then, one piece of the capillary is placed inside a microforge (Figure 1b) that has been modified with a tungsten filament and a glass microbead (Figure 1c). The filament overheats the borosilicate capillary until it breaks at the desired diameter. We recommend selecting a capillary slightly larger than the average size of the selected nannofossil species to make the picking easier. For our purposes, we prepared two type of capillaries with a diameter of 10 and 20 µm (Figure 1c,d), because the two targeted nannofossil species—*Helicosphaera carteri* and *Gephyrocapsa oceanica*—have a coccolith size of 8–11 µm and 3–5 µm, respectively [38]. With the above-mentioned instruments, it is also possible to make thinner capillaries down to 2–3 µm according to the size of the target species. After breaking the capillary at the selected diameter, the microforge is used to slightly bend the capillary in order to make the picking process more efficient.

Once ready, the capillary is mounted on a dedicated inox holder of a micromanipulator controlled by a hydraulic circuit; the micromanipulator is connected with an inverted microscope (Figure 2a). This kind of microscope–micromanipulator setting is usually employed in cellular biology for cytoplasm, chromosomes, and DNA transfer e.g., [48,49]. To make the picking even faster and easier, we preliminarily treated the sample with the settling technique described by Stoll and Ziveri [34] in order to divide two different size fractions and to concentrate those species, such as *H. carteri*, that have a low relative abundance, ca. 5% in the case of the studied sample [44]. Through differential settling velocities, we first removed the >20 µm fraction to retain only the nannofossils; then, we divided a larger (20–6 µm) and a smaller (<6 µm) fraction so as to obtain two samples enriched in *H. carteri* and *G. oceanica*, respectively. Specifically, we applied the following settling times: (1) after mixing the suspension of buffered Milli-Q water and sediment (30 mL = 65 mm suspension column height) in a 50 mL Falcon tube with a Pasteur pipette, we let it settle for 12 min, and then the >20 µm fraction was removed from the Falcon bottom; (2) then, after adding buffered Milli-Q water up to 30 mL and mixing, the suspension was settled for 130 min in order to let the particles >6 µm settle at the Falcon bottom; (3) finally, we separated the supernatant, retaining the <6 µm fraction (i.e., enriched with *G. oceanica*) from the fraction sedimented at the bottom with size range 6 < and > 20 µm (i.e., enriched with *H. carteri*). The obtained suspension containing nannofossils and buffered Milli-Q water is poured in the center of a Petri dish (Figure 2b) which is then placed under the inverted microscope ready for the picking (Figure 2c). Before starting the picking, the particles in the suspension need a few minutes' settling to be deposited at the bottom of the Petri dish. The dilution of the suspension has to be adjusted in order to operate in optimal conditions, i.e., avoiding too dense concentrations that may clog the capillary or too diluted suspensions which may slow down the capture process.

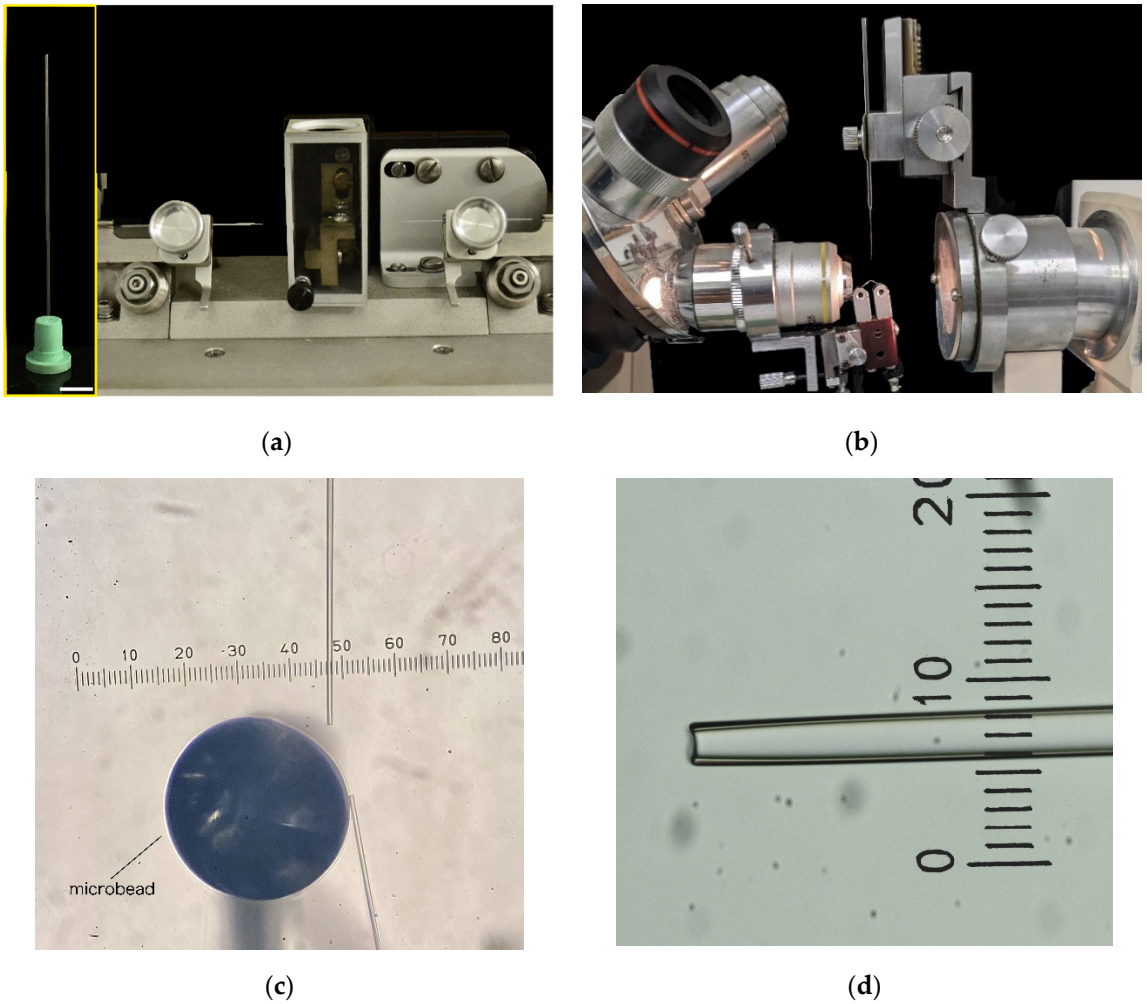
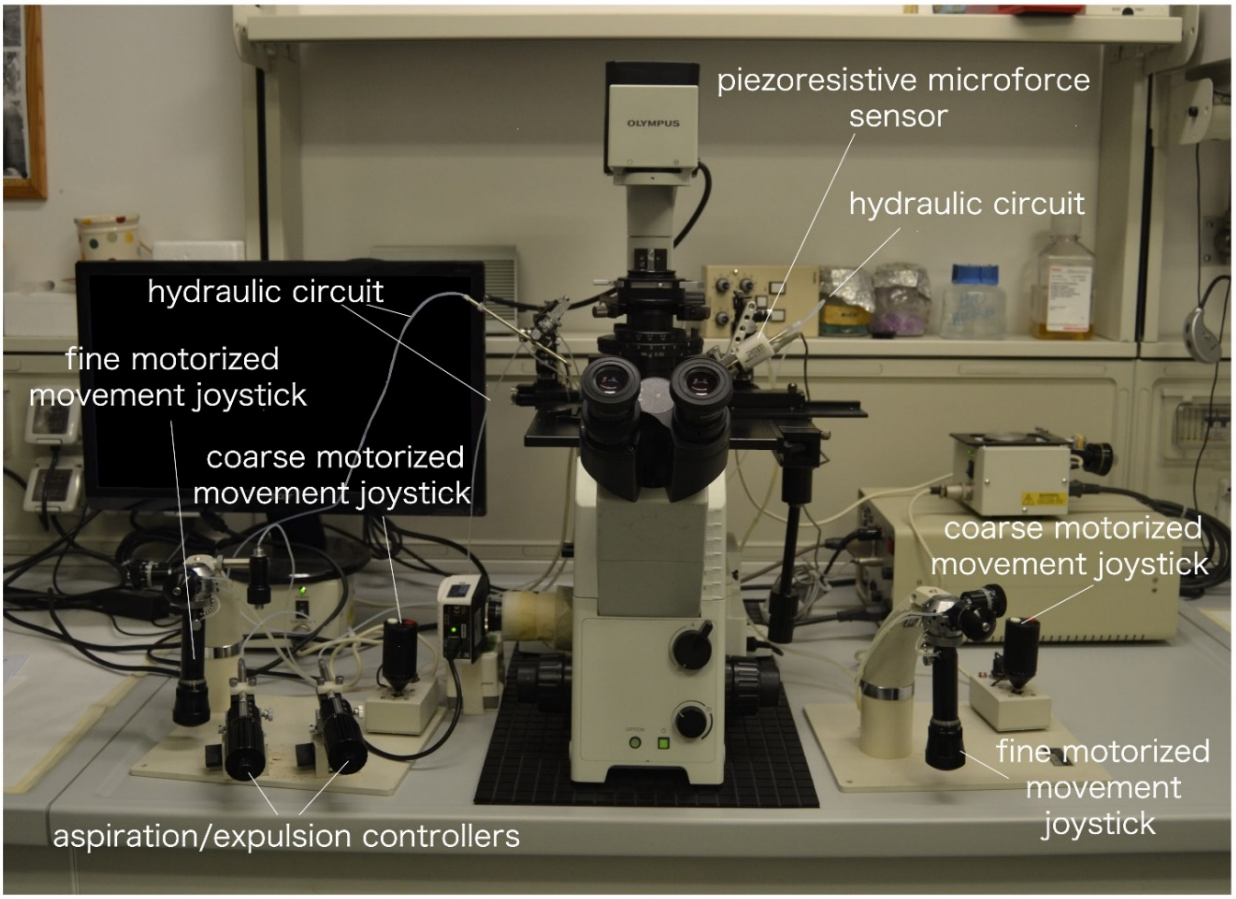
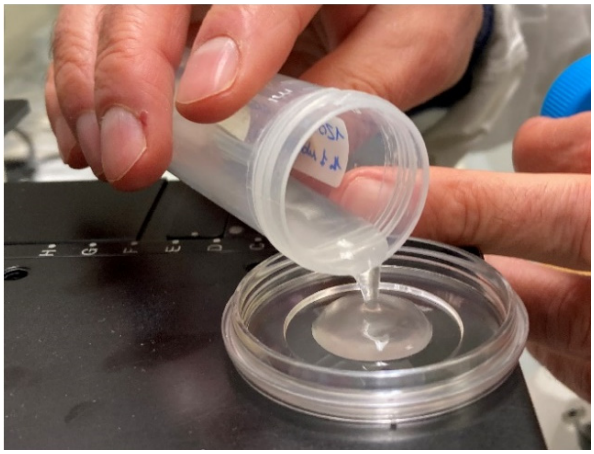


Figure 1. Preparation of the capillary before the picking procedure: (**a**) Borosilicate capillary before the cut and Flaming/Brown micropipette puller holding the cut capillary; (**b**) Cutting and bending of the capillary through a microforge; (**c**) Cut capillary of 10 μm inner diameter; (**d**) Cut capillary of 20 μm inner diameter. Scale bars are also reported.

Before sinking the capillary into the suspension drop, it is important to trap some air in it in order to keep the suspension separated from the Milli-Q water necessary to make the hydraulic system function. Then, the capillary is carefully inserted into the suspension drop. Once the nannofossil of interest has been identified, the operator moves the capillary as close as possible to it (Figure 3a,d). If necessary for a better isolation of the coccolith from other surrounding particles, it is possible to use rapid fluid movements or slight vibrations produced by the piezoresistive microforce sensor supporting the capillary. Though useful to speed up the process, the use of the piezoresistive microforce is not strictly necessary for coccolith isolation, allowing reduced cost of the instrumentation since the piezoresistive sensor is one of the most expensive tools of the micromanipulation system. Once the capillary is placed just above the isolated coccolith (Figure 3b,e), it captures it through suction controlled by the hydraulic manipulation system (Figure 3c,f). This operation can be repeated many times in order to collect a substantial number of coccoliths. All the selected coccoliths remain inside the capillary (Figure 3c,f) until the moment of their release onto the proper sample holder used later for geochemical analyses (e.g., TEM grids, Mylar film, SEM slide, Eppendorf). We suggest placing a drop of buffered Milli-Q water on the sample holder before depositing the coccoliths to avoid dispersion, especially if the holder is large.



(**a**)



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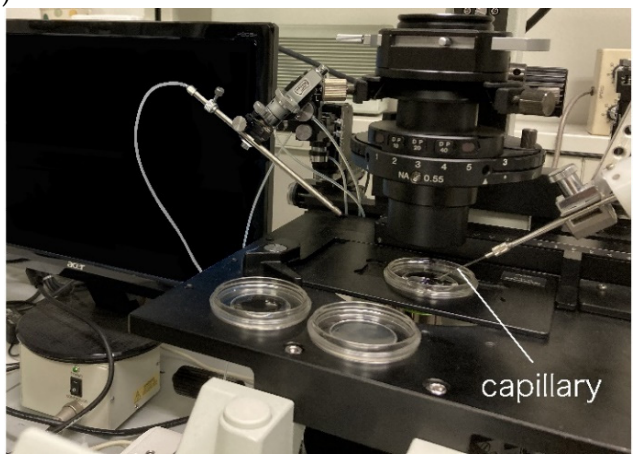

(**c**)

Figure 2. Micromanipulator and microscope settings together with sample placement before the picking: (**a**) Micromanipulator Narishige MF-9 maneuvered with a hydraulic circuit and inverted microscope Olympus IX71. The key parts of the instrumentation are specified; (**b**) Pouring of the drop of suspension containing diluted sediment from ODP Site 1209B into the Petri dish; (**c**) Details of the micromanipulator and microscope settings showing the allocation of the capillary and Petri dish with the drop of sediment suspension.

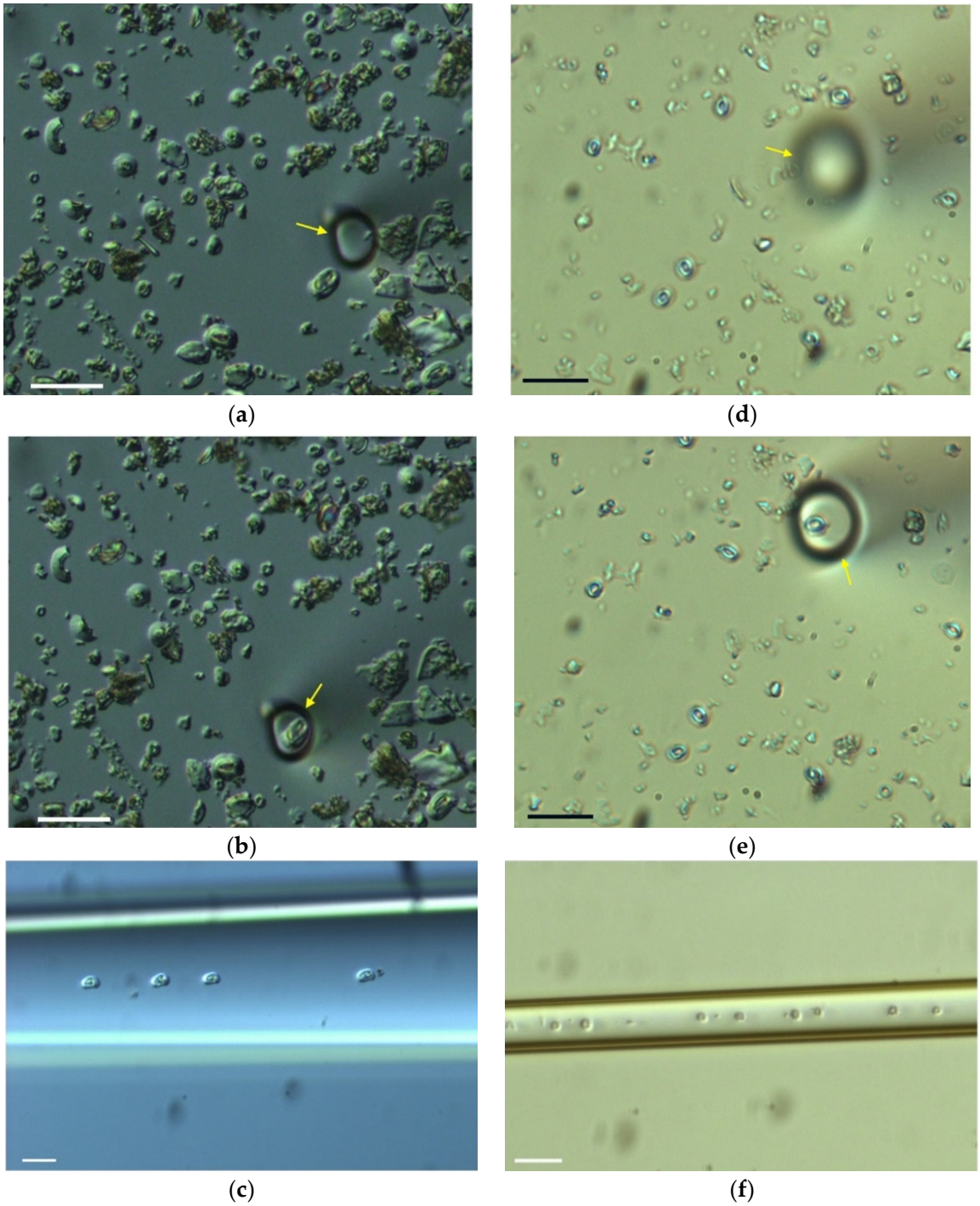

Figure 3. Images of the coccolith capture procedure of two nannofossil species with different size ranges, *Helicosphaera carteri* and *Gephyrocapsa oceanica*: (**a**) Microscope view of the selected *H. carteri* coccolith with a cross section of the capillary (yellow arrow) before the capture moment; (**b**) *H. carteri* before being aspirated inside the capillary; (**c**) nannofossils of *H. carteri* retained inside the capillary; (**d**–**f**) Images of the capture procedure and (**f**) of the nannofossils retained within the capillary for the species *G. oceanica*. Scale bars are 20 µm.

3. Results

3.1. Efficiency of the Picking Process

Our study aims to identify the best settings and precautions to be taken in order to isolate the highest number of coccoliths per h using a hydraulically controlled microma-

nipulator. It was not necessary to substantially modify the original instrument settings designed for biological purposes, but attention needed to be paid during the capillary preparation since it is pivotal for the hydraulic system to work efficiently. It is worth dedicating more time to the capillary shaping because it makes the picking process smoother and faster. The only difference to biological micromanipulation is that mercury is employed to keep the biological material separated from the water of the hydraulic system, whereas in our case we used the air trapped in the capillary. With optimal instrument and tool settings, we observed that it is possible to collect from 20 up to 40 coccoliths during one single capture–release phase that on average lasts for 12–13 min (Figure 4a,b and Table 1). Since we aimed to analyze single coccoliths of nannofossils applying highly resolved synchrotron-based techniques, we deposited the *H. carteri* coccoliths on a Mylar film (Figure 4c) and on a TEM grid with a Formvar layer (Figure 4c,d) [50], but any kind of sample holder can be easily used.

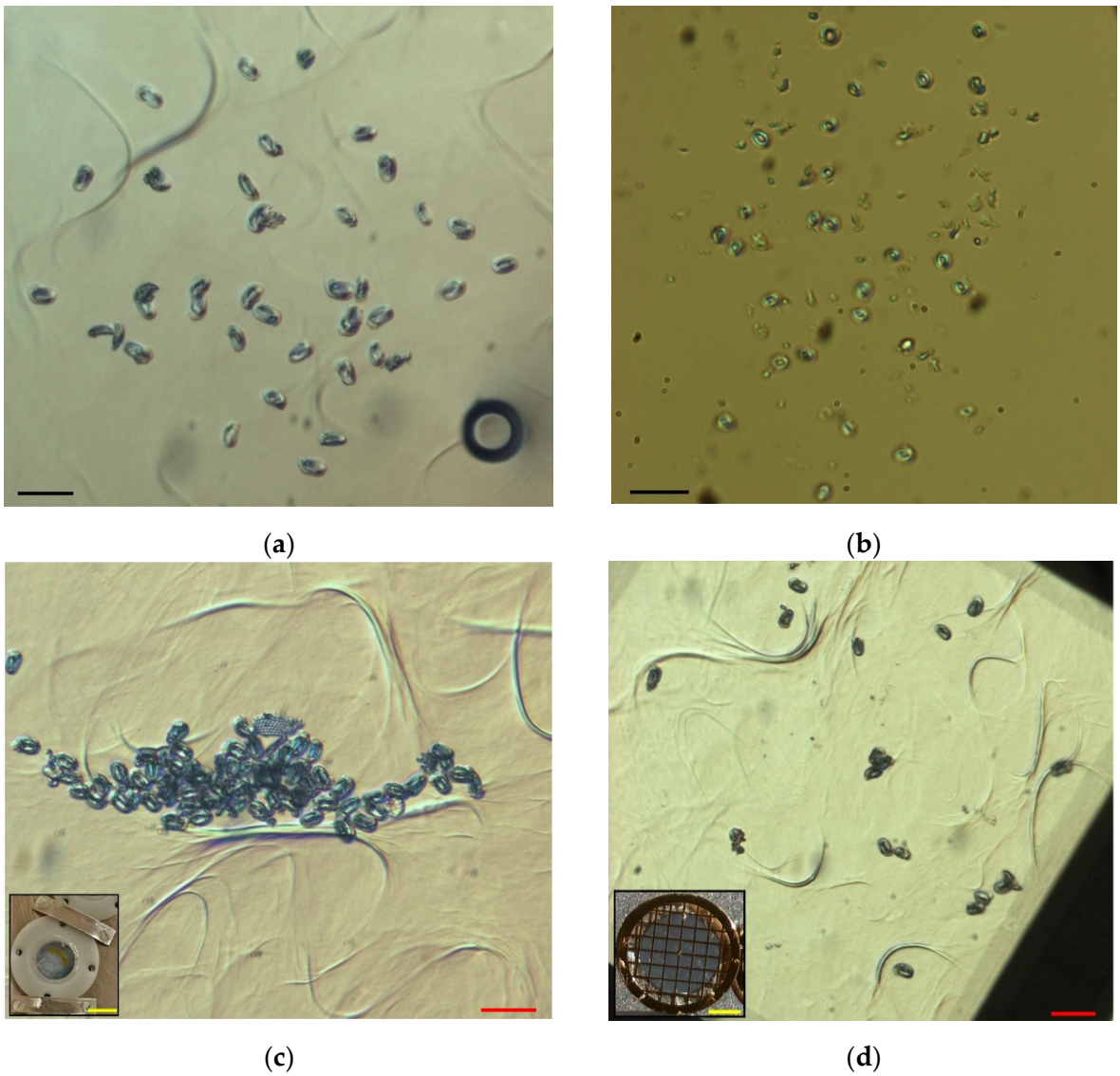

Figure 4. Nannofossils isolated in a single capture–release procedure and deposited on a Petri dish for the species *H. carteri* (**a**) and *G. oceanica* (**b**), scale bars 10 µm; *H. carteri* coccoliths isolated for synchrotron-based analyses and deposited on (**c**) Mylar film sealed with a Delrin interlocking ring, scale bars are 1 cm (yellow) and 20 µm (red), and on (**d**) an Au TEM grid with a Formvar layer scale bars are 1 mm (yellow) and 20 µm (red).

Table 1. Data collected to evaluate the efficiency of the hydraulic micromanipulation system in picking monospecific nannofossils of *H. carteri* (H.c.) and *G. oceanica* (G.o.). Reported in the table are: the number of attempts of a complete capture–release procedure; the number of picked coccoliths during the capture phase; the ones lost in the process of moving the capillary out from the suspension before deposition; the ones deposited on the selected sample holder; the time necessary for the entire procedure (min); the efficiency of the methodology expressed as number of coccoliths deposited versus time (h). Hashtag (#) means "numbers".

#Attempt	#Picked Coccoliths	#Lost Coccoliths	#Deposited Coccoliths	Min	Efficiency (#Coccoliths/h)
1 H.c.	30	5	25	10	150
2 H.c.	16	2	14	7	120
3 H.c.	20	5	15	11	81
4 H.c.	36	6	30	15	120
5 H.c.	23	4	19	18	66
6 H.c.	33	3	30	16	114
7 H.c.	18	1	17	10	102
1 G.o.	37	8	29	11	156
2 G.o.	40	6	34	17	120
3 G.o.	31	5	26	13	120
4 G.o.	27	4	23	9	150
5 G.o.	23	2	21	8	156
6 G.o.	32	5	27	14	114

In our experiments, the picking was made easier by a preparatory coccolith size separation via settling since the species *H. carteri* has a low relative abundance in the studied sample (ca. 5%) [44]. Nevertheless, even if the sample is not preliminary size-sorted, a significant number of isolated coccoliths can be achieved. We performed some tests to detect the efficiency of our picking technique from the two size-sorted samples. The selection of *H. carteri* coccoliths shows an average efficiency (i.e., number of coccoliths selected per h) of 107 coccoliths/h, whereas the picking of *G. oceanica* seems to be more efficient, reaching up to 136 coccoliths/h (Table 1). The highest efficiency returned by the *G. oceanica*-picking compared to *H. carteri* is ascribable to the highest (10%) abundance of *G. oceanica* in the selected sample [44], which speeds up the capture phase. From our data we can infer that it is possible to isolate about 800–1000 monospecific coccoliths during one working day. We suggest incrementing the capture–release procedures with a low number of picked coccoliths (10–15) to avoid the coccoliths getting stuck inside the capillary or they may be lost in the capture–release process.

3.2. Possible Dissolution Issues

During our experiments with the species *H. carteri*, we also noticed that it is crucial to pay attention to dissolution processes that may occur after the deposition on the sample holder. We documented that when buffered Milli-Q water with a pH between 8 and 10 [51,52] is used for preparing the initial suspension, the high concentration of coccolith contained is enough to maintain the alkalinity stability and not affect the nannofossil preservation (Figure 3). On the contrary, after the coccolith release onto the sample holder, if the pH is not kept very high (>12) the alkalinity of the suspension does not compensate for the lower carbonate concentration compared to the suspension with the sediment, and thus, dissolution effects start to affect the coccoliths. Our tests show that after depositing a well-preserved population of *H. carteri* coccoliths onto a holder with Milli-Q water at pH = 9 (Figure 5a), some etching starts to appear in less than 1 h (Figure 5b). Specifically, observing the appearance of etching, the first element that begins to dissolve is the flange. As the flange becomes thinner and transparent, dissolution starts to also affect the coccolith edges with higher intensity, decreasing the coccolith size. After 74 min, the dissolution also becomes evident around the central openings, leaving only a small structure with a big

central aperture (Figure 5c). Then, after 90 min, all the picked coccoliths are completely dissolved (Figure 5d). It should be noted that during the entire dissolution process, the coccoliths move within the solution because of thickness reduction and weight loss (Figure 5). As reference, we used one coccolith of the well-resistant species *Coccolithus pelagicus*, which was dissolved after 4 h. To prevent the etching and dissolution of calcite coccoliths, we recommend adding a drop of buffered Milli-Q water with high pH (>12) on the sample holder before releasing the picked coccoliths. In this way the original preservation state of the nannofossils is maintained (Figure 4).

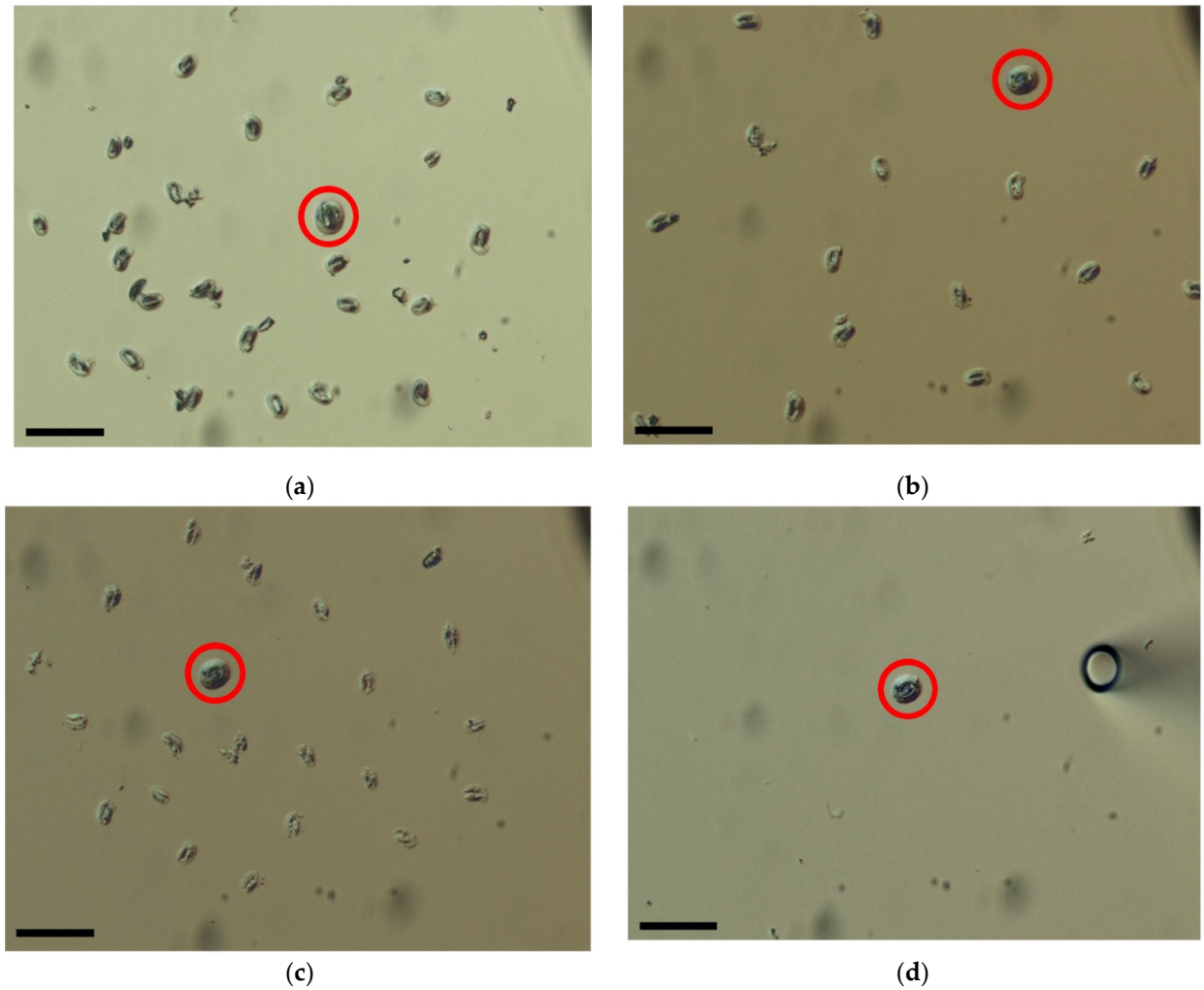

Figure 5. Etching and dissolution of picked coccoliths of *H. carteri* deposited in a solution with pH = 9. For reference, one coccolith of the species *Coccolithus braarudii* highly resistant to dissolution has been placed in the field of view (red circle). (**a**) Freshly picked coccoliths in well-preserved state; (**b**) Coccolith etching after 55 min; (**c**) Coccolith dissolution after 74 min; (**d**) Coccoliths completely dissolved after 90 min. Scale bars are 20 µm.

4. Discussion and Conclusions

In this work, we present for the first time a significant improvement in the isolation of monospecific nannofossils from deep-sea sediment using wet samples together with a hydraulically controlled micromanipulation system. The novel use of applying the suction of coccolith from a suspension (i.e., sediment fraction suspended in buffered Milli-Q water) allows the entire picking process to be sped up compared to previous methods (Table 2). In particular, we documented that up to 100 monospecific coccoliths can be isolated in 1 h with a hydraulic micromanipulator system (Table 2), paying attention to preliminarily

size-sorting the sample, especially in the case of low-abundance species or samples with highly variable size of the fossil content, e.g., if diatoms (20–200 µm), foraminifera (100 µm on average), or radiolaria (30 µm–2 mm) are also present.

Applying the suction and expulsion principle to isolate the coccolith instead of the electrostatic forces used by previous works (Table 2) also makes the picking of small and fragile species such as *G. oceanica* or *Emiliania huxleyi* easier. Stoll et al. [39], who worked with dry samples and a micromanipulated tungsten needle as their picking tool, stated that during the picking of *E. huxleyi,* the contact with the needle often breaks the coccoliths, and thus, the time required for the picking increases. Later, Suchéras-Marx et al. [41] also documented that the isolation of small-sized species using manual picking with a silica spine from dry samples is difficult. All these issues can be overcome using a drop of suspension and a hydraulic micromanipulation system that not only allows the process to be sped up, but also the safe picking of very small and fragile species with no breaking risks and high efficiency still (Table 1).

Our methodology also solves the difficulties related to the initial sample preparation. In fact, when the electrostatic force principle is applied, it is fundamental to prepare a smear slide with a specific concentration of coccoliths to facilitate the picking process, i.e., spaces between each coccolith have to be at least twice the size of the coccolith itself [39]. This kind of preparation is not so easily achieved, especially using the smearing method, and in the case of rare species it may slow down the coccolith selection process. On the contrary, although a preliminary size-sorting can make the procedure easier, by using wet samples and the hydraulic micromanipulator it is also possible to perform precise monospecific picking in highly concentrated sediment samples. This is evident if we compare the data of the picking time in the two samples analyzed here (*H. carteri* and *G. oceanica*, Table 1). Despite the sample in Figure 3a,b being denser and more variegated in the coccolith size range than the sample shown in Figure 3d,e, the picking times are easily comparable, showing the high efficiency of the method even when investigating very different samples (Table 1).

Table 2. Main characteristics of the picking techniques available in the literature compared to this work. N.A.: not available. Hashtag (#) means "numbers".

Picking Method	Picking Tool Material	Picking Tool Ø (µm)	#Picked Coccoliths	Type of Sediment Sample	Picking Principle	References
Micromanipulator	Borosilicate glass	3–20 (inner Ø)	80–100/h	Wet	Controlled suction and release	This work
Manual	Silica	15–20 (outer Ø)	5/h	Dry	Electrostatic forces	[41]
Micromanipulator	Tungsten	N.A.	15/30–60 min	Dry	Electrostatic forces	[39]
Micromanipulator	Tungsten	N.A.	20/45–90 min	Dry	Electrostatic forces	[40]

The methodology presented here has the downside that it requires expensive equipment (micromanipulator and hydraulic control system), despite being commonly used in biology labs, and a specialized technician to achieve its maximum efficiency. Nevertheless, this system allows the isolation of a number of monospecific nannofossils never attained before: up to 1000 coccoliths per day in optimal conditions. This achievement opens up new perspectives in the investigation of fossil coccoliths at species-specific level. For example, it increases the number of single coccoliths that can be analyzed using synchrotron-based instrumentations, strengthening the statistical meaning of the outcomes [50]. Moreover, it may also allow us to apply the ICP-MS to monospecific nannofossil species, previously applied only for culture samples of <20 µm fraction e.g., [21,22,26,27,29]. Improving the

technology and the speed of single coccolith-picking from sediments is important for future research on coccolithophore geochemical analyses, allowing the refining of direct studies of trace elements in monoclonal cultures with the information derived from the geological records. In this way, better calibration of the environmental proxies can be achieved together with a deeper understanding of the role of coccolithophore vital effects during the element incorporation at species-specific level.

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