

## Article

# An improved cleaning protocol for foraminiferal calcite: HyPerCal - A new practice for micropaleontological and paleoclimatic proxies

Stergios D. Zarkogiannis <sup>1,2\*</sup>, George Kontakiotis <sup>2</sup>, Georgia Gkaniatsa <sup>2</sup>, Venkata S. C. Kuppli <sup>3,4</sup>, Shashidhara Marathe <sup>3</sup>, Kazimir Wanelik <sup>3</sup>, Valia Lianou <sup>3</sup>, Evangelia Besiou <sup>3</sup>, Panayiota Makri <sup>3</sup>, and Assimina Antonarakou <sup>3</sup>

<sup>1</sup> Department of Earth Sciences, University of Oxford, UK;

<sup>2</sup> Faculty of Geology & Geoenvironment, Department of Historical Geology-Paleontology, School of Earth Sciences, National & Kapodistrian University of Athens, Zografou University Campus, Greece; gkontak@geol.uoa.gr; gorgiagkan@gmail.com; wwveua@hotmail.com; vlianou@geol.uoa.gr; pmakri@geol.uoa.gr; aantonar@geol.uoa.gr,

<sup>3</sup> Diamond Light Source, Harwell Science and Innovation Campus, Oxford, UK; shashidhara.marathe@diamond.ac.uk; kaz.wanelik@diamond.ac.uk;

<sup>4</sup> Canadian Light Source, SM beamline, Saskatoon, Canada; charan.kuppili@lightsource.ca

\* Correspondence: stergiosz@geol.uoa.gr

**Abstract:** Paleoclimatic and paleoceanographic studies routinely rely on the usage of foraminiferal calcite through faunal, morphometric and physico-chemical proxies. The application of such proxies presupposes the extraction and cleaning of these biomineralized components from ocean sediments in the most efficient way, a process which is often labor intensive and time consuming. In this respect, in this study we performed a systematic experiment for planktonic foraminiferal specimen cleaning using different chemical treatments and evaluated the resulting data of a Late Quaternary gravity core sample from the Aegean Sea. All cleaning procedures adopted here were made on the basis of their minimum potential bias upon foraminiferal proxies, such as the faunal assemblages, degree of fragmentation, stable isotope composition ( $\delta^{18}\text{O}$  and  $\delta^{13}\text{C}$ ) and/or Mg/Ca ratios that are frequently used as proxies for surface-ocean climate parameters (e.g., sea surface temperature, sea surface salinity). Six different protocols were tested, involving washing, sieving, and chemical treatment of the samples with hydrogen peroxide and/or sodium hexametaphosphate (Calgon®). Single species foraminifera shell weighing was combined with high-resolution Scanning Electron Microscopy (SEM) and synchrotron X-ray Microtomography ( $\mu\text{CT}$ ) of the material processed by each of the cleaning protocols, in order to assess the decontamination degree of specimen's ultrastructure and interior. It appeared that a good compromise between time and cleaning efficiency is the simultaneous treatment of samples with a mixed hydrogen peroxide and Calgon solution, while the most effective way for an almost complete decontaminate of the calcareous components from undesirable sedimentary material is a two-step treatment - initially with hydrogen peroxide and subsequently with Calgon solutions.

**Keywords:** Cleaning protocol; shell weight; climate reconstruction; synchrotron X-ray microtomography ( $\mu\text{CT}$ ); foraminiferal-based proxies

## 1. Introduction

Foraminifera shells are widely used in paleoceanographic and paleoclimatic studies as biostratigraphic or ecological indicators and through physicochemical analyses as proxies of past oceanic conditions [1]. The tests of different foraminifera species can provide environmental information by means of both physical and chemical analyses. Despite the main focus for environmental reconstructions based on stable isotope [2,3] and trace metal geochemistry [4-6] of

foraminifera shells a wealth of information can be attained by their physical analyses that include the study of shell fragmentation [7], abundancies for ecological [8] or biostratigraphic purposes [9] and shell biometry [10-12] including size [13,14] and weight [15,16].

A necessary preliminary step for the use of foraminifera shells in paleoenvironmental studies is the isolation of the test specimens from the bulk ocean sedimentary matrices that consist of several components. A number of methodologies have been employed to transform the bulk sediment samples into useable micropaleontological material [17,18] as a first level treatment. Although the additional cleaning protocols to isolate primary calcite for geochemical analyses are advanced and several cleaning experiments have sought to quantify the effects of each of these methods on measured elements [19-22], there are only a few studies that assess the efficiency of different treatment procedures on the physical properties of the foraminifera shells such as their weight [21,23].

Studies that focus on foraminifera shell weight measurements are particularly vulnerable to the degree of test contamination, due to their foraminous nature, these specimens have the potential to include contaminants (i.e., sedimentary residuals), which can alter or skew the record toward heavier values [24]. Residual clays or nano-ooze in poral spaces and shell surface obstruct the study of test ultrastructure that yield information about the degree of carbonate dissolution [25] or test porosity [26]. Furthermore, such coatings or infillings (in apertures) often precludes automated recognition software, which is based on morphological features of foraminifera shells [27], from classifying their images correctly [28] and greatly complicate specimen segmentation when using high resolution X-ray tomographic techniques [29]. In the present study, by using SEM and X-ray tomography with reagents that are established not to alter the fossil geochemical signal, we propose a methodology that effectively diminishes surface and internal specimen contamination.

## 2. Materials and Methods

For the cleaning test, *Globigerinoides ruber albus* (NCBI:txid2606480) sensu stricto specimens were used, from the 45 cm interval of unconsolidated sediments from the North Aegean Sea core M22-67 (245 m water depth; 38°21.87'N, 25°56.96'E) with a radiocarbon date (AMS  $^{14}\text{C}$ ) of 14.5 ka before present. The core consisted mainly of fine-grained (hemipelagic muds and clays) sediments and represents a sedimentary archive of the last 85 kyr, while the predominant clay minerals in the area are [30] and have been during the study interval [31] illite and smectite. *G. ruber albus* s.s. was chosen for species under investigation because of its high abundance in the sample and its importance in paleoclimatic studies. It is likely that foraminiferal shells from different settings, and possibly different foraminifera species of different size, will respond differently to cleaning.

The sample was oven-dried overnight at 50°C and was weighed unprocessed 4.10 g. Subsequently, it was divided into six aliquots (~0.7 g each) that were transferred into different 50 ml glass beakers and underwent treatment for 20 minutes at room temperature before wet sieving over a 63  $\mu\text{m}$  mesh, using six processing methods: (1) addition of Calgon® (sodium hexametaphosphate,  $(\text{NaPO}_3)_6$ ); (2) 30% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ); (3) 30% hydrogen peroxide and subsequent treatment with Calgon; (4) simultaneous treatment with 30% hydrogen peroxide and Calgon; (5) 49.5% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ); and (6) distilled water without chemical additions (see Table 1 for procedures).

Beaker 1 received treatment with Calgon by filling up the beaker with 5% Calgon solution (50 g  $\text{Na}_6\text{P}_6\text{O}_{18}$  diluted in 950 ml distilled water) as proposed by [23]. Beaker 2 received treatment with 30% hydrogen peroxide by adding 4 ml of the reagent and filling up the beaker to 50 ml with distilled water, producing a 2.5% hydrogen peroxide solution. Beaker 3 received a “two step treatment”. The sample was initially treated with 2.5% hydrogen peroxide solution, like beaker 2, and after washed through a 63- $\mu\text{m}$  sieve the remaining coarse fraction was transferred back to the beaker and treated with 5% Calgon solution, similar to beaker 1 (HyPerCal treatment). Beaker 4 received simultaneous treatment with hydrogen peroxide and Calgon by adding 4 ml of 30% hydrogen peroxide in 46 ml of 5% Calgon solution. Beaker 5 received treatment with 4% hydrogen peroxide solution by diluting 4 ml of 49.5% hydrogen peroxide in 46 ml distilled water, and beaker 6 only received treatment with distilled water. All beakers were periodically sonicated every 2 minutes for 4 seconds, since a 4 sec

sonication step has been found to provide a greater detritus cleaning effect and minimize shell breakage [23].

After their treatment the sample aliquots were washed through a 63- $\mu\text{m}$  sieve and left overnight in the oven to dry at 50°C. They were subsequently dry-sieved and approximately 15 non-fragmented *G. ruber albus* s.s. specimens from the 300-355  $\mu\text{m}$  sieve fraction were picked from each aliquot for further analyses. In order to minimize the effect of specimen size (i.e. size of apertural openings, chamber size) in the cleaning efficiency the particular size fraction was used because it is also very frequent in paleoceanographic studies. For assessing the effect that each treatment had on the surface ultrastructure of the foraminifera specimens, 5 specimens from each sample were mounted and gold-coated for SEM imaging. The samples were examined with a JEOL JSM-6390 instrument at a  $\times 1100$  magnification, a working distance of 2.1 mm and an accelerating voltage of 20kV in the Department of Geology and Geoscience of the National Kapodistrian University of Athens. In order to evaluate the extent of detritus removal from the interior of the specimens and quantify weight loss from each chemical treatment method, 5 additional specimens from each sample were weighed and subsequently scanned using X-ray computed tomography. The shells were initially weighed as a group of five individuals to obtain a mean and subsequently every two in order to record the variation in each sample. The weight analysis took place using a Sartorius microbalance (1 mg precision) at the Department of Geology and Geoscience of the National Kapodistrian University of Athens.

After weighing, the specimens were tomographically scanned using Synchrotron X-ray radiation at the Diamond Manchester Imaging Branchline (I13-2) at Diamond Light Source. The specimens were transferred into quartz capillaries of 1 mm inner diameter (similar to [29]) that were subsequently attached to magnetic cryo-cap holders and mounted on to a goniometer. The data was acquired with partially coherent, pink X-ray beam which has broader energy spectrum centered around 27 keV. For each of the sessions exposure time of 0.5 seconds were used, at a 0.09 degree rotation step size producing an acquisition of 2000 projections with 2560 $\times$ 2160 pixel resolution using a pco.edge 5.5 camera at a 4 $\times$  magnification, which resulted in an effective pixel size of 0.8125  $\mu\text{m}$ . The reconstruction of the acquisition data and their downsampling to 8bit tomographic images were performed with Savu package [32]. The images were subsequently analyzed in Avizo software, where the shell and sedimentary infill areas were segmented and discriminated as described in section 3.3 of [24].

**Table 1.** Table summarizing the different cleaning methods followed.

Method	Method name	Chemical treatment			Treatment time	Processing	
		Step 1	Step 2	Step 3		Step 4	Step 5
1	“Calgon”	50 ml Calgon 5%	-	-	20 min sonicated every 2 min for 4 sec	Wet sieving over >63 µm mesh	Dried overnight
2	“30% Peroxide”	2.5% hydrogen peroxide (4 ml of H <sub>2</sub> O <sub>2</sub> were added to 46 ml distilled water)	-	-	20 min sonicated every 2 min for 4 sec	Wet sieving over >63 µm mesh	Dried overnight
3	“HyPerCal treatment”	4 ml of 30% H <sub>2</sub> O <sub>2</sub> added to 46 ml distilled water (2.5% hydrogen peroxide)	Wet sieving over >63 µm mesh	4 ml 5% Calgon were added in 46 ml distilled water	20 + 20 min sonicated every 2 min for 4 sec	Wet sieving over >63 µm mesh	Dried overnight
4	“Mixed Calgon and peroxide”	4 ml of 30% H <sub>2</sub> O <sub>2</sub> added in 46 ml of 5% Calgon solution	-	-	20 min sonicated every 2 min for 4 sec	Wet sieving over >63 µm mesh	Dried overnight
5	“49.5% Peroxide”	4 ml of 49.5% H <sub>2</sub> O <sub>2</sub> added to 46 ml distilled water (4% hydrogen peroxide)	-	-	20 min sonicated every 2 min for 4 sec	Wet sieving over >63 µm mesh	Dried overnight
6	“Control”	Treatment only with distilled water	-	-	20 min sonicated every 2 min for 4 sec	Wet sieving over >63 µm mesh	Dried overnight

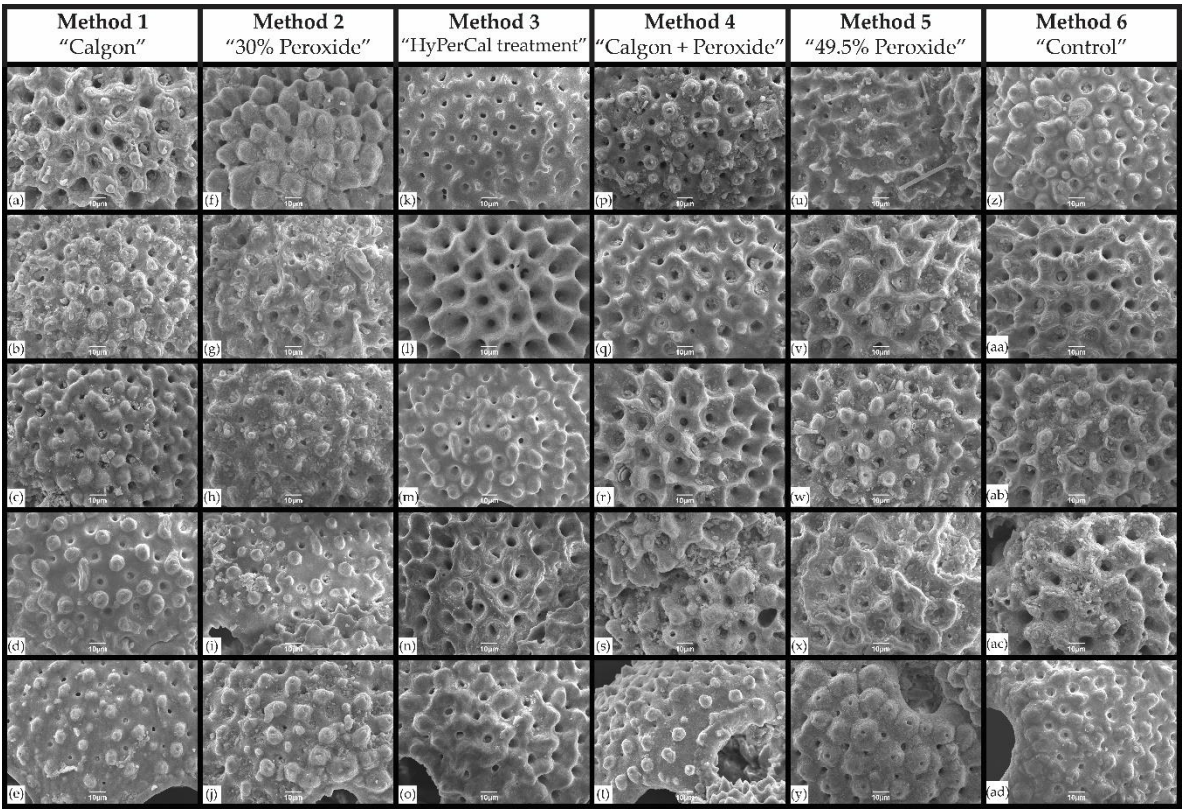


3. Results

There are some general observations of the behavior of the different sample solutions that deserve to be noted here. Due to the cohesion degree of the core sample not all treatment solutions were capable to completely disintegrate the sample’s mass. The most effective reagent to turn the sample solution into homogeneous mud slurry was hydrogen peroxide regardless of its concentration or admixture. The sample aliquots that were treated with water or Calgon solution did not completely disintegrate and small chunks of sediment were left still standing in the beaker that required some extra mechanical effort to better dissolve. Finally, beaker 4 which contained simultaneously both hydrogen peroxide and Calgon exhibited strong foaming during treatment time.

3.1. Scanning Electron Microscopic analysis

The results of the SEM analysis are shown in Figure 1. It can be seen that the surface ultrastructure of the shells that underwent two step treatment – HyPerCal treatment, initially with hydrogen peroxide and subsequently with Calgon, is almost completely free from detrital particles and clay impurities (Figure 1k-o). This treatment method removed detritus from all the different ultrastructural test features such as pores, ridges, interpore area and spine bases, even in the case of dissolved and etched interpore surfaces (Figure 1n). The treatment with hydrogen peroxide of diverse concentrations (Methods 2 and 5) showed that the different ultrastructural features of all the specimens were covered to some degree with detritus. Treatment with Calgon or water had some better cleaning effects especially for some of the specimens (Figure 1a-e and z-ad) and the same is true for Method 4, of simultaneous treatment with hydrogen peroxide and Calgon.

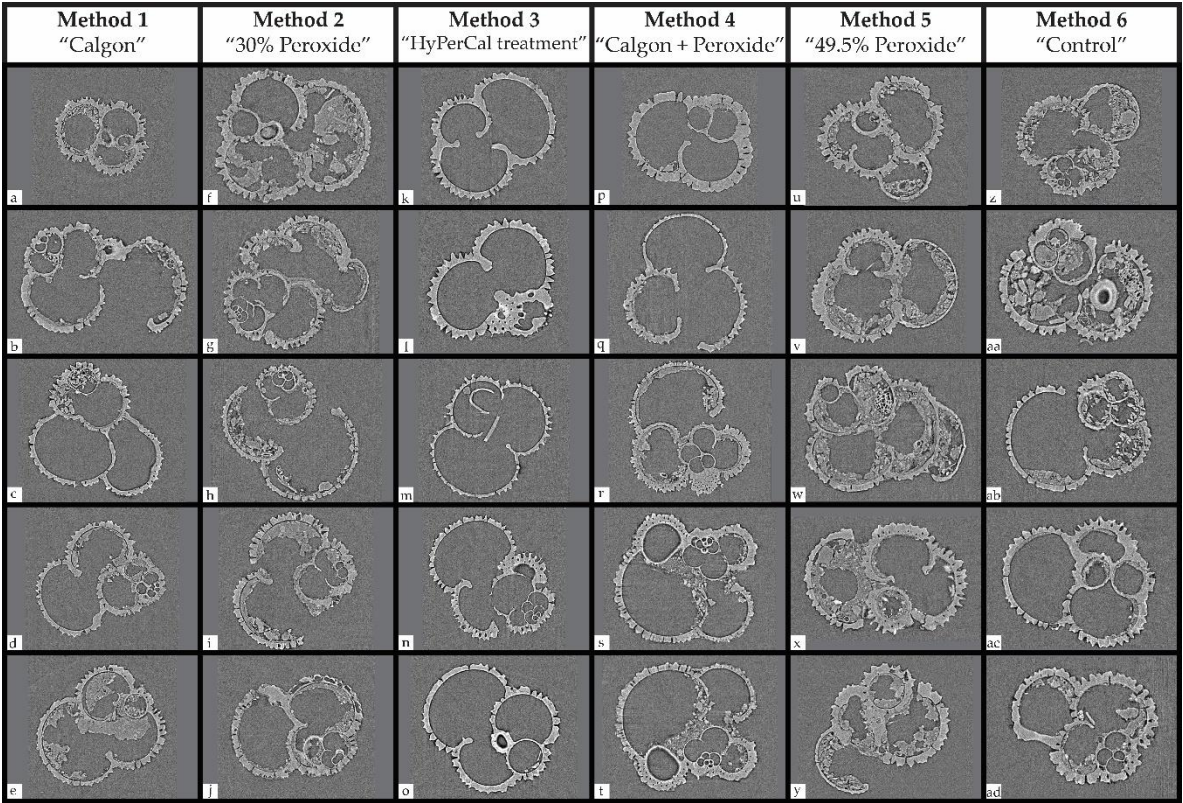


**Figure 1.** Scanning electron microscope images of the ultrastructure of the specimens after their treatment with the different cleaning methods: (a)-(e) images of specimens after treatment with Calgon, (f)-(j) images of specimens after treatment with 30% H<sub>2</sub>O<sub>2</sub> solution, (k)-(o) images after treatment first with 30% H<sub>2</sub>O<sub>2</sub> and subsequently with Calgon solution (HyPerCal), (p)-(t) images after

treatment simultaneously with 30% H<sub>2</sub>O<sub>2</sub> and Calgon solution, (u)-(y) images after treatment with 49.5% H<sub>2</sub>O<sub>2</sub>, and (z)-(ad) images after treatment with only with distilled water.

3.2. Synchrotron X-ray absorption and weight analysis

Tomographic slices of the scanned specimens that underwent different treatment are shown in Figure 2 and the results of the tomographic analysis together with the weight measurements are summarized in Table 2. The visual inspection of the tomographs clearly shows that the HyPerCal treatment of Method 3 is the most effective way eliminate contamination from the internal foraminifera shell cavities. As it can be seen in Figure 2(k-o) even the smallest chambers or the secondary apertures and pores are free from detrital material. The two-step treatment with hydrogen peroxide and subsequently with Calgon also show reduced contamination in the smaller chambers but there is still sedimentary material attached to the interior of the larger chamber walls. Calgon alone is less effective in removing contamination, especially in the smaller chambers, while treatment only with water leave the shell infilling in an aggregated form. Treatment with hydrogen peroxide has left the shells with considerable amounts of detritus and the cohesion of this remaining detrital mass seems to increase with hydrogen peroxide concentration.



**Figure 2:** X-ray tomographic images of the interior of the specimens after their treatment with the different cleaning methods: (a)-(e) tomographs of specimens after treatment with Calgon, (f)-(j) tomographs of specimens after treatment with 30% H<sub>2</sub>O<sub>2</sub> solution, (k)-(o) tomographs after treatment first with 30% H<sub>2</sub>O<sub>2</sub> and subsequently with Calgon solution (HyPerCal), (p)-(t) tomographs after treatment simultaneously with 30% H<sub>2</sub>O<sub>2</sub> and Calgon solution, (u)-(y) tomographs after treatment with 49.5% H<sub>2</sub>O<sub>2</sub>, and (z)-(ad) tomographs after treatment with only with distilled water.

Apart from the visual inspection, the X-ray analysis allowed the determination of the total foraminifera cell volume and that of the area in the interior of the shell, which is occupied by sedimentary infill. Thus the degree of contamination of each shell is presented in Table 2 as the percentage of detritus within the cell’s volume. It can be seen that the HyPerCal treatment of the sample with hydrogen peroxide and Calgon in two steps has almost completely removed the

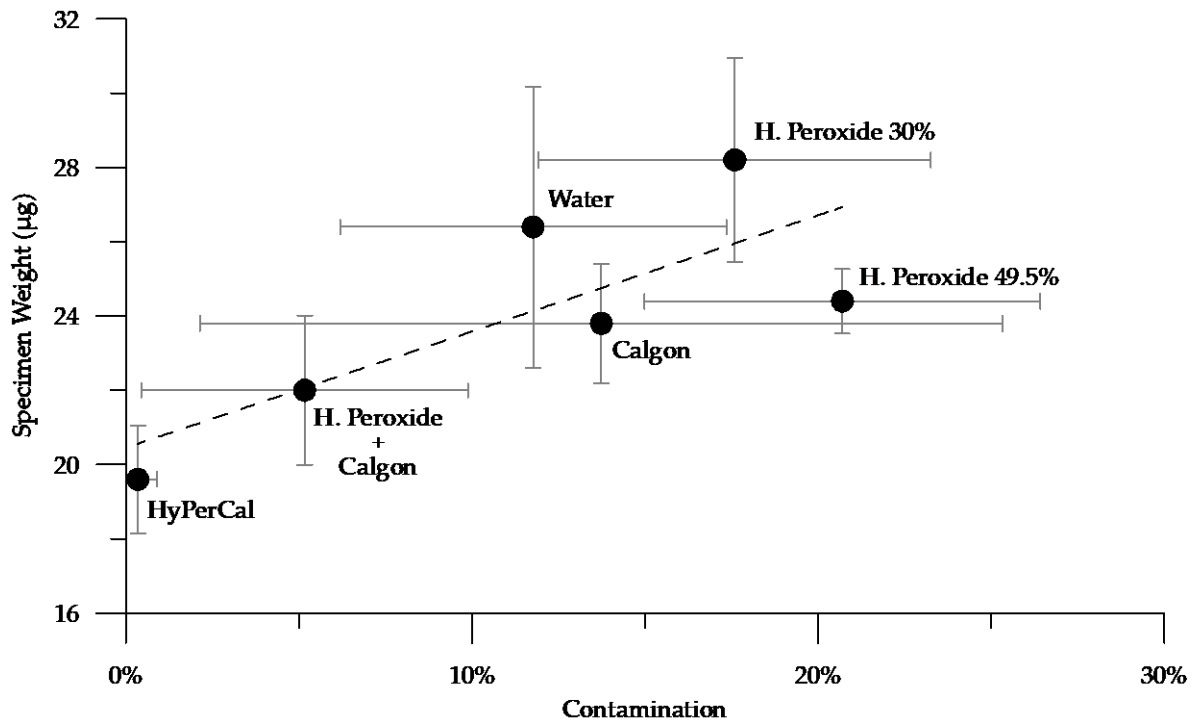


sediment infill (0%, Table 2) from shell's interior, as this is also evident in Figure 2(k-o). The simultaneous treatment with hydrogen peroxide and Calgon within the same solution had adequate results since detrital contamination was reduced to only 5% by volume. Treatment with Calgon or water had a similar effect on detrital removal by reducing contamination to 14% and 12% respectively, while treatment with hydrogen peroxide (of different concentrations) had the minimum efficiency in specimen cleaning.

**Table 2.** Table showing the results of the X-ray tomographic and weight analyses. The degree of contamination is given as a percentage of cell's volume. Furthermore, the difference in the measured weights in regard to the average shell weight of the least contaminated shells.

Method	Method name	Nº of shells	Contamination (%)	Weight (µg)	Weight Diff.
1	"Calgon"	5	14 (±12)	23.8 (±1.6)	21%
2	"30% Peroxide"	5	18 (±6)	28.2 (±2.8)	44%
3	"PerCal treatment"	5	0 (±1)	19.6 (±1.4)	-
4	"Mixed Calgon and peroxide"	5	5 (±5)	22.0 (±2.0)	12%
5	"49.5% Peroxide"	5	21 (±6)	24.4 (±0.9)	25%
6	"Control"	5	12 (±6)	26.4 (±3.8)	35%

Shell weight is found to be a function of to the degree of contamination as shown in Figure 3. It can be seen that samples treated with hydrogen peroxides solution group in the right corner of the graph, while samples treated with aqueous solutions (i.e. water or Calgon) group in its middle. The heaviest shells were the ones that were treated with 30% hydrogen peroxide (Method 2). Their average shell weight was 44% greater than that measured for the least contaminated shells. Although treatment with 49.5% hydrogen peroxide produced consistently lower shell weights its effect on contamination removal was the lowest, suggesting possibly calcite dissolution by the unbuffered solution. Treatment with water produced weights increased by 35% compared to the actual/uncontaminated ones. From the single-constituent solutions Calgon was the one to have the greatest effect on shell weight but also with the greatest variability (12%) in the extent sediment detrital removal. The simultaneous treatment of the sample with Calgon and hydrogen peroxide is found to be an effective method for specimen cleaning since contamination was found consistently reduced to 5%. Finally, the most effective method that almost completely removed contamination (0% ± 1%) was the HyPerCal treatment.



**Figure 3:** Plot of *G. ruber albus* s.s. shell weights after treatment with the different cleaning methods against their contamination as per volume percentage.

#### 4. Discussion

We performed a systematic experiment with chemical treatments commonly utilized to disaggregate marine sediment and which are known to not significantly affect the foraminiferal based proxies: species abundance, shell fragmentation,  $\delta^{18}\text{O}$ ,  $\delta^{13}\text{C}$ , and Mg/Ca. The chemical agents used in solutions were hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in two different concentrations, 5% Calgon (sodium hexametaphosphate,  $\text{Na}_6\text{P}_6\text{O}_{18}$ ), a swap and a combination of them. We find that the most effective way for preparing foraminifera samples for their subsequent micropaleontological or geochemical analyses is the initial cleaning of the sedimentary material with hydrogen peroxide followed by treatment of the sieved sample residual with Calgon solution and we refer to this procedure as HyPerCal. In the present experiment the samples were treated for 20 minutes in every solution and were sonicated every 2 minutes for 4 seconds in order to minimize shell breakage [23] but duration of treatment may vary depending on the cohesion of the sedimentary mass. After their cleaning, single-species specimens from a certain sieve fraction were picked, weighed, and subsequently inspected using SEM and  $\text{S}\mu\text{CT}$ . The analyses showed that the different procedures had a variable effect in contamination removal from the surface and the interior of the examined specimens (Figure 3) and that the HyPerCal treatment left the specimens free of (surface or internal) sedimentary residuals and translucent.

Sodium hexametaphosphate is a common dispersing agent in research on marine sediments and is more effective than water in removing clay clumps from shell interiors [33] and foraminifera shell weight loss has been previously reported with [21] and without [23] the use it during cleaning. Our tomographic analysis supports the previous results and confirm that weight differences are the result of sediment contamination removal. The initial treatment with hydrogen peroxide promotes the degradation of organic, which is the major binding agent in benthic sediments [34] and thus minimizing the adhesive forces within the medium. Cohesive forces are at molecular scale the result of the attractive interactions in vacuum between contiguous particles of the same medium, while the adhesive forces are defined as the additional binding forces between particles, due to the presence of a second, interparticle medium [35]. The dispersing action of Calgon, as a second treatment step, helps to neutralize the attraction electrostatic forces between (clay) particles [36] and is thus reducing



particle cohesion. The use of only one of these two reagents alone (Calgon, Hydrogen peroxide) in specimen cleaning did not produce satisfying results both under the SEM and  $\mu$ CT analyses. The use of both reagents in the same solution, compared to HyPerCal, produced fairly satisfactory results by reducing contamination to only 5%. The efficiency, however, of the HyPerCal treatment may stem from the fact that during a two-step treatment the sample processed and sonicated twice as much or from the fact that Calgon is only applied on the coarse fraction of sample, free of a substantial amount of material.

The effectiveness of sediment cleaning procedures is a function sediment matrix mineralogy, grain size and degree of consolidation. The present sediment core material consist fine-grained (hemipelagic) sediment and originate from the South Mediterranean basin, which is also known for the fine particle size of its clay minerals [37]. The chemical treatment tested here has proved appropriate for the fine material that are usually found in sedimentary basins and should remain so for recent sediment, where the depth of burial is not is not considered important to initiate diagenetic alteration of the clay minerals [38]. The efficiency of the HyPerCal procedure in the cleaning of calcitic microfossils makes it complementary for foraminifera shell weight studies since it was shown to bring the measured weight closer to that of an “original” shell. Furthermore, it paves the way for its use in modern analytical techniques that require some degree of automatization, such as image recognition software that are unable to recognize a lot of foraminifera images, whose umbilical aperture is not fully cleaned and is infilled with remaining nannofossil ooze [28]. On the other hand, CT image analyses software cannot easily discriminate between contaminated areas and areas referring to the foraminifera shell unless (subjective) labor intensive segmentation is employed [24].

## 5. Conclusions

In the present study a sediment core sample of late Quaternary age was divided in six aliquots each of which was treated with reagents that do not alter foraminifera calcite geochemistry following different cleaning procedures and the efficiency of each method in specimen cleaning was assessed using SEM and X-ray tomography. The results of the visualization analyses were combined with shell weight measurements and allowed us to conclude that the method that has proven the most effective in removing fine detritus trapped within foraminiferal shells is a two-step treatment of sedimentary material, named here HyPerCal treatment, initially with 2.5% hydrogen peroxide and subsequently with 5% Calgon solutions. The proposed protocol minimizes discrepancies in foraminifera shell weight measurements and greatly facilitates X-ray imaging analyses.

**Supplementary Materials:** The following are available online at <https://figshare.com/>

**Author Contributions:** Conceptualization, S.D.Z.; Original draft preparation, G.K. and S.D.Z.; Laboratory analyses, G.G. and S.D.Z and P.M.; SEM data acquisition, E.B. and V.L.; Synchrotron Data Acquisition, V.S.C.K., S.M., Synchrotron Data Scientific Computing and Analysis, K.W. and S.D.Z.; writing—review and editing, G.K., S.D.Z. and A.A.; supervision, A.A.

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