

## Development of the freeze–thaw processing technique for disaggregation of indurated mudrocks and enhanced recovery of calcareous microfossils

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**ABSTRACT** – Microfossil extraction from indurated mudrocks is widely acknowledged as challenging, especially for foraminifera. Here we report development of the freeze–thaw extraction method through the addition of rapid heating, detergent and ultrasound stages. We use indurated mudrock samples from the Toarcian (Early Jurassic) of Yorkshire, UK to assess the effectiveness and develop the freeze–thaw method. We compare our results from freeze–thaw with those from standard foraminifera processing techniques, including the use of hydrogen peroxide. Processing by freeze–thaw increased the degree of mudrock disaggregation and resulted in no damage or dissolution of foraminifera. Following the freeze–thaw method with treatment in white spirit and sodium hexametaphosphate aided the separation of foraminifera from the disaggregated clays and was twice as efficient as pressure washing. Samples processed with hydrogen peroxide contained damaged microfossils and an under representation of delicate calcareous foraminifera. Many other studies of indurated mudrocks have used hydrogen peroxide to extract foraminifera, and this might have resulted in apparently barren intervals. The freeze–thaw method outlined here provides a low-cost, low-risk and successful method of disaggregating and extracting calcareous microfossils from indurated mudrocks. We anticipate our method may be relevant for other fossil groups and merits further development.

**KEYWORDS:** *foraminifera, hydrogen peroxide, freeze–thaw, mudrocks, microfossil extraction*

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### INTRODUCTION

Indurated organic-rich mudrocks are regarded as difficult for extracting calcareous microfossils, such as foraminifera and ostracods (Green, 2001). Many authors have described the importance of combining chemicals to extract foraminifera from indurated rocks (Duffield & Warshauer, 1979; Then & Dougherty, 1983; Aldridge, 1990). For instance, Then & Dougherty (1983) reported that indurated organic-rich shales were most successfully broken down by using a combination of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), sodium hypochlorite (NaClO), sodium hydroxide (NaOH) and the detergent Quaternary ‘O’ (C<sub>24</sub>H<sub>47</sub>N<sub>2</sub>O<sub>2</sub>Cl). Current foraminifera extraction techniques typically involve a number of chemicals including organic solvents, such as white spirit (C<sub>6</sub>H<sub>6</sub>), bleach (NaClO), detergents and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Green, 2001). However, some of these techniques can damage the microfossils (Hodgkinson, 1991; Pingitore *et al.*, 1993). The chemicals previously used and their properties are listed below.

**Hydrogen peroxide.** This aids disaggregation of the rock by oxidizing the organic matter. The rock sample is soaked in H<sub>2</sub>O<sub>2</sub> for a variable amount of time; for example, Reolid *et al.* (2012) used 30% solution for 24h and Hylton (2000, unpublished PhD thesis, University of Plymouth) soaked samples of Toarcian age in 10% solution of H<sub>2</sub>O<sub>2</sub> for an unspecified length of time. Aldridge (1990) described the use of a 10–15% solution of H<sub>2</sub>O<sub>2</sub> for ‘black shale’ disaggregation but similarly does not mention the length of time for treatment. H<sub>2</sub>O<sub>2</sub> is designated as a hazardous chemical (Table 1).

**Petroleum ether, paraffin, white spirits and similar organic solvents.** At least some of the organic matter in the samples can be dissolved and hence the rock disaggregated by soaking the sample in an organic solvent such as petroleum or white spirit.

Aldridge (1990) described the use of petroleum ether or similar solvent to extract conodonts and noted that soft or partly indurated mudstones can be disaggregated by soaking them for at least an hour and then covering in hot water, allowing the clay to be sieved off from the fossiliferous fraction. Organic solvents are described by Pingitore *et al.* (1993) as suitable for extracting fossils made of calcium carbonate (CaCO<sub>3</sub>) as part of routine laboratory techniques. Copestake (1978, unpublished PhD thesis, University College of Wales) used petroleum spirit to disaggregate Lower Jurassic rocks. Petroleum ether and white spirit are designated as hazardous (Table 1).

**Detergents.** Detergents can be used to help flocculate the clays; they include Calgon (sodium hexametaphosphate (NaPO<sub>3</sub>)<sub>6</sub>), Decon-90 (KOH) or Quaternary ‘O’ (2-(8-heptadecenyl)-4,5-dihydro-1,3-bis(2-hydroxyethyl)-1H-imidazolium chloride or C<sub>24</sub>H<sub>47</sub>N<sub>2</sub>O<sub>2</sub>Cl). Duffield & Warshauer (1979) extracted ostracods and conodonts from Devonian shales using a combination of bleach and Quaternary ‘O’. Although Quaternary ‘O’ was successful, the treatment could take up to 7 weeks. Detergents are reported to cause no damage to the thicker-walled microfossils when used in low concentrations (Hodgkinson, 1991). There are no hazards identified for sodium hexametaphosphate in the form of flakes or powder by the European Union Directive 67/548/EEC therefore it is not included in Table 1. Decon-90 has several precautions associated with it (Table 1). As Quaternary ‘O’ was manufactured for use in the mining industry it does not have the same well-defined laboratory hazardous phrases associated with it; none the less the known hazards are listed in Table 1 and it is a strong detergent with environmental restrictions.

**Tensides.** Tensides (organic detergents) disaggregate the rock by breaking the surface tension bonds of the organic matter with

**Table 1.** The relative hazards, risks and safety of chemicals commonly used for the breakdown of organic-rich mudrocks for the extraction of microfossils.

Chemical	Associated risk				Safety advice			Additional phrases associated
	Hazard	Eyes	Skin	Respiration	Swallowing	PPE	Fume hood req'd	
Hydrogen peroxide (H <sub>2</sub> O <sub>2</sub> )	Yes	Serious damage	Harmful if contact. Irritant	Harmful by inhalation. Irritant	Harmful if swallowed	Wear eye/face protection	Yes	Eyes: rinse with water and seek medical advice R8. Contact with combustible material may cause fire. R35. Causes severe burns
Petroleum ether/White spirits (C <sub>6</sub> H <sub>6</sub> )	Yes		Repeated exposure may cause skin dryness or cracking	Harmful may cause lung damage. Vapours may cause drowsiness or dizziness	Harmful if swallowed	Wear gloves, eye and face protection	Yes	Swallowed: do not induce vomiting, seek medical advice R10. Flammable, R51/53. Toxic to aquatic organisms. S2. Keep out of the reach of children. S23. Do not breathe in fumes. S24. Avoid skin contact. S29. Do not empty into drains. S56. Dispose of material and container at special waste collection point
Detergents KOH	Yes	Irritant	Irritant	None listed	Harmful if swallowed	Wear gloves, eye and face protection	Yes	Eyes: rinse with water and seek medical advice R35. Causes severe burns
Quaternary 'O'	None listed	None listed	None listed	None listed	None listed	Wear eye/face protection	Yes	To be stored away from strong oxidizing agents
Tenside e.g. Rewoquat	Yes	Irritant	Irritant	None listed	None listed	Wear eye/face protection	Yes	R50. Very toxic to aquatic organisms. R53. May cause long-term adverse effects in the aquatic environment
Sodium salts	Yes	Irritant	Irritant	Irritant	Irritant	Wear eye/face protection	Yes	N/A
Liquid nitrogen	No	N/A	N/A	N/A	N/A	Wear cold insulating gloves/face shield/eye protection		H281. May cause cryogenic burns or injury. P403. Store in a well-ventilated place Get immediate medical attention. Thaw frosted parts with lukewarm water. Do not rub affected area
Sodium tetraphenylborate (NaTPB)	Yes	Irritant	Irritant	Irritant	Harmful if swallowed	Wear eye/face protection	Yes	Eyes: rinse with water and seek medical advice. Skin: rinse with water and seek medical advice. Swallowing: do not induce vomiting. Seek medical attention

Complete details of specific safety information for all chemicals can be found in full in the Material Safety Data Sheet (MSDS) provided with the chemicals. The risk phrases associated with the chemical are the R-phrases assigned to the chemical according to Annex III of the Dangerous Substances Directive 67/548/EEC – the nature of special risks attributed to the substances and preparations.

the clay minerals. Moullade *et al.* (2005) reported processing samples by soaking them in Rewoquat (1-methyl-2-noroleyl-3-oleic acid-aminoethyl-imidazolinium-methosulfate) for several days followed by repeated washes in  $\text{H}_2\text{O}_2$  to remove the tenside. Gräfe (2005) disintegrated marine Jurassic marlstones with Rewoquat but did not specify how the samples were washed afterwards. Gräfe (2005) recorded poor foraminifer preservation from this study, which he attributed to taphonomic processes.

**Sodium compounds.** Sodium compounds disaggregate the rock by mimicking the natural weathering process of shale. The interlayer potassium within the clay mineral structure is replaced with sodium from sodium compounds and causes expansion (Hanken 1979). Sodium tetraphenylborate (NaTPB) and sodium chloride (NaCl) have been described as effective methods to extract intact, clean fossils from indurated mudrocks; however, the cost of NaTPB generally prevents large-scale application (Hanken, 1979). Muller (1990, unpublished PhD thesis, The State University of New Jersey) used Hanken's (1979) method along with dissolution in sodium carbonate to extract benthic foraminifera from Lower Jurassic limestones, marls and shales. NaTPB is assessed as hazardous (Table 1).

**Freeze–thaw.** Freeze–thaw disaggregates the rocks because as the fluids in the pore spaces of the rock cool and solidify they take up greater volume causing the rock to break apart. Non-chemical disaggregation methods involving the repeated freezing and thawing of rocks have been described previously (Hanna & Church, 1928; Sohn, 1961; Sohn *et al.* 1965; Pojeta & Balanc, 1989). Hanna & Church (1928) describe successfully disaggregating Pliocene shales by freezing small, water-saturated, samples for several hours or overnight but they provide no further detail. Green (2001) notes that repeated freeze–thawing will disaggregate most shales and that this method has been successful for disaggregating clay-rich sedimentary deposits for the extraction of microfossils. Hanna & Church (1928) used tap water for the freeze–thawing, whereas Sohn (1961) and Sohn *et al.* (1965) used dissolved sodium salts solution (including sodium acetate 3-hydrate ( $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ )) and Hinchey & Green (1994) used liquid nitrogen. The freeze–thaw technique has no chemical safety risks associated (see Table 1 for the safety risks associated with sodium salt solution and liquid nitrogen).

Here we report development and testing of the freeze–thaw technique for the breakdown of indurated mudrocks and extraction of foraminifera. The freeze–thaw method resulted in both a better breakdown of the indurated mudrocks compared to other standard methods and, when combined with white spirit and sodium hexametaphosphate, produced a more abundant and diverse assemblage of the foraminifera preserved in the sediment with no evidence of damage to the tests. To test the freeze–thaw method, we used organic-rich, indurated mudrocks that were deposited prior to and during the Toarcian (Early Jurassic) Oceanic Anoxic Event (Kemp *et al.* 2005, 2011; Caswell & Coe, 2013). Our results also extend the range of one foraminifer compared to that reported by Hylton (2000, unpublished PhD thesis, University of Plymouth).

## METHOD

The indurated mudrock samples were collected using a cold chisel and hammer from the Toarcian strata exposed at Hawsker

Bottoms [NZ 94795 07849] and Port Mulgrave [NZ 79803 17669] along the North Yorkshire coast near Whitby, UK. The samples were collected with direct reference to the graphic logs constructed by Angela L. Coe and David B. Kemp and presented in Kemp *et al.* (2005, 2011). A ceramic mortar and pestle was used to breakdown a known weight of six samples of indurated Toarcian mudrocks of variable composition (0.3–5 wt%  $\text{CaCO}_3$ ; 1.3–8.4 wt% TOC; 1.78–5.5 wt% S; Table 2) into roughly pea-sized lumps and these were subdivided into aliquots for Methods 1 to 4.

For the freeze–thaw technique (herein referred to as 'Method 4'), an aliquot of known weight of the pea-sized mudrock lumps was saturated in cold water for 24 h. After decanting off the water and placing the sample in a sealed plastic container, the sample was frozen for approximately 3 h. The sample was then removed from the freezer and rapidly heated by covering it in boiling water for several minutes. This rapid heating is different from previous methods involving freeze–thaw, such as Hanna & Church (1928), who describe letting the sample reach room temperature. After decanting off the warm water and sieving on a 63  $\mu\text{m}$  and a 500  $\mu\text{m}$  sieve stacked together under a gentle water jet to extract the fraction between 63  $\mu\text{m}$  and 500  $\mu\text{m}$ , the residue over 500  $\mu\text{m}$  was returned to the freezer and the fraction less than 63  $\mu\text{m}$  was discarded. The 63–500  $\mu\text{m}$  fraction was washed again in the 63  $\mu\text{m}$  sieve under a gentle water jet until the water ran clear. The freeze–thaw steps were repeated on the fraction greater than 500  $\mu\text{m}$  15–20 times until almost all the original sample was disaggregated to less than 500  $\mu\text{m}$ . This is a similar number of freeze–thaw repetitions to that reported by Sohn (1961) and Sohn *et al.* (1965) although they used sodium salts instead of water. Pojeta & Balanc (1989) and Green (2001) reported that the processing could be speeded up in a vacuum and that they used a sodium sulphate solution. In our development of the method for mudrocks, we also added a few drops of Decon-90 to the samples during each of the rapid heating steps to increase deflocculation of the clays. Once 15–20 freeze–thaw cycles had been completed, the whole of the 63–500  $\mu\text{m}$  fraction collected was subjected to ultrasound for 15–30 s, sieved and washed again using a gentle water jet and then dried.

This new freeze–thaw method (Method 4) was tested against three established methods using the same samples (herein referred to as Methods 1–3). Method 1 involved saturating an aliquot of the pea-sized mudrock lumps of known weight in white spirit for 24 h, decanting off the white spirit and then boiling in a 50% solution of sodium hexametaphosphate for 25 minutes. The samples were then soaked in 30%  $\text{H}_2\text{O}_2$  in a water bath for up to an hour. This method is similar to that used by Hylton (2000, unpublished PhD thesis, University of Plymouth) and Reolid *et al.* (2012). Method 2 was the same as Method 1 but the  $\text{H}_2\text{O}_2$  stage was omitted. Method 3 involved soaking an aliquot of the pea-sized mudrock lumps of known weight in Rewoquat (Gräfe, 2005; Moullade *et al.* 2005) for 1 week before repeated washes in  $\text{H}_2\text{O}_2$ .

Method 4 produced a very high degree of disaggregation of the mudrocks into particles <500  $\mu\text{m}$  (see 'Results' and 'Discussion') and some to <63  $\mu\text{m}$ ; however, it left many aggregated clay particles in the 63–500  $\mu\text{m}$  fraction. Two techniques were tested to break down more of the clay aggregates from the 63–500  $\mu\text{m}$  fraction. The first was to combine Method 4 with Method 2. This was done by soaking the whole 63–500  $\mu\text{m}$  fraction recovered from

**Table 2.** Percentage weight of original sample processed for each size fraction for the four methods for the six samples processed.

Sample	Height (m)	Bed no.	Method 1: white spirit and H <sub>2</sub> O <sub>2</sub>					Method 2: white spirit without H <sub>2</sub> O <sub>2</sub>					Method 4: Freeze-thaw					Method 4 and 2				
			Sulphur (wt%)	CaCO <sub>3</sub> (wt%)	TOC (wt%)	Sample weight (g)	>500 µm (%)	63–500 µm (%)	<63 µm (%)	Sample weight (g)	>500 µm (%)	63–500 µm (%)	<63 µm (%)	Sample weight (g)	>500 µm (%)	63–500 µm (%)	<63 µm (%)	Sample weight (g)	>500 µm (%)	63–500 µm (%)	<63 µm (%)	
MA-13	240	34	5.501	2.452	8.414	200	95	2	3	3	100	95	2	3	100	33	52	15	74.78	—	51	49
MD-78	–181	31	4.362	1.571	3.83	200	90	5	5	6	100	90	5	6	100	18	60	22	67.66	—	66	34
MD-146	–351	31	2.841	0.321	1.886	200	64	17	19	7	100	85	8	7	100	1	65	33	56.57	—	80	20
MD-166 / Tse 00.61	–401	30	2.523	2.497	1.356	100	38	28	37	12	100	78	10	12	100	4	67	29	61.25	—	65	35
MD-170	–411	29	1.781	1.964	1.325	100	77	9	15	11	100	83	6	11	100	1	59	40	100	2	42	56
Tse 00.60	–450	29	2.31	1.92	1.79	200	41	27	32	15	90	74	11	15	90	2	72	26	100	4	47	50

The heights of the samples were measured relative to the base of the *Harpoceras exaratum* ammonite Subzone using the graphic log of Kemp *et al.* (2005, 2011). Bed numbers are from Howarth (1992). Sample MA-13 is from the *exaratum* Subzone at Port Mulgrave and the remaining samples are from the *semicellatum* Subzone at Hawksker Bottoms. Wt% TOC, S and CaCO<sub>3</sub> are from Kemp *et al.* (2011). Different sample weights were used initially depending on sample availability. Sample MD-166 is from exactly the same stratigraphic level as sample Tse 00.61. Samples MA-13 and MD-78 are brownish-black poorly laminated organic-rich mudrocks. Samples MD-146, MD-166, MD-170 and Tse 00.60 are dark-grey mudrocks.

Method 4 in white spirit overnight prior to the final drying stage. The sample was then boiled in sodium hexametaphosphate before sieving and drying the 63–500 µm fraction. The second method of breaking down the clay aggregates was to use a high pressure (0.018 ls<sup>-1</sup>) finely dispersed water spray for at least 45 min on the 63–500 µm fraction whilst it was in the 63 µm sieve; this technique was tested on two samples only (Tse 00.60 and MD-170).

The 63–500 µm fraction of the samples processed by all the different methods was picked to 10 g or to 300 specimens (whichever was completed first) using an Olympus SZX12 research stereomicroscope and the fossils were mounted on to slides using water-soluble Gum Tragacanth glue. The preservation of the foraminifera and other calcareous and pyritic microfossils was examined further using the FEI Quanta 200 3D scanning electron microscope (SEM) at the Open University. For the SEM analysis the microfossils were coated in c.15 nm of carbon and recorded using an accelerating voltage of 20 kV and a beam current of 0.6 nA. The software PAST (<http://folk.uio.no/ohammer/past/>) was used to calculate a range of diversity indices.

## RESULTS

The degree of disaggregation of the samples from each of the methods, i.e. the percentage of the sample completely broken up into <500 µm particles, varied considerably from 5% (Method 1, sample MA-13) to 99% (Method 4, sample MD-170) (Table 2, Fig. 1). For all samples, the new freeze-thaw technique (Method 4) produced the highest degree of disaggregation and Method 2 produced the lowest. Method 3 was difficult to use and disaggregation was unsuccessful for sample MD-166, although it demonstrated a 77% disaggregation of sample Tse 00.60 (67% of the sample was the 63–500 µm foraminifera yielding fraction). A combination of Methods 4 and 2, yielded a greater loss of clay particles (<63 µm fraction) from the 63–500 µm fraction than from Method 4 alone (Fig. 2). The average percentage reduction in the weight of the fossiliferous fraction through clay loss from the application of Method 2 after Method 4 was 20% (Fig. 2). In contrast the high-pressure washing of samples Tse 00.60 and MD-170 produced an average percentage reduction in the weight of the 63–500 µm fossiliferous fraction of 10% through clay loss.

The raw foraminifer abundance and diversity data are shown in Tables 3 and 4 and, Figures 3 and 4. Samples MA-13 and MD-78 are barren of foraminifera (and, therefore, not shown in Table 3). Because of the wide variation in the amount of disaggregation and picking to either 10 g or 300 specimens, we also calculated the amount of foraminifera predicted from each sample if the whole processed sample was picked and 100 g was the initial weight. We calculated this by using the raw foraminifer abundance data in the fossiliferous fraction and multiplying it by the amount of sample available to pick through (i.e. the difference between the size of the picked 63–500 µm fraction and the size of the remaining 63–500 µm residue left to pick). We then normalized to the initial 100 g weight by multiplying those samples with a different start weight by the difference in weight. These results are shown in Table 5 and Figure 5. The range of diversity indices obtained from the software package, PAST, are shown in Figure 4. The diversity indices used were the Simpson, Margalef, Shannon H, Menhinick and Fisher alpha, as well as the dominance and evenness for each sample (see Hammer & Harper (2006) for further details on diversity indices). The raw data for

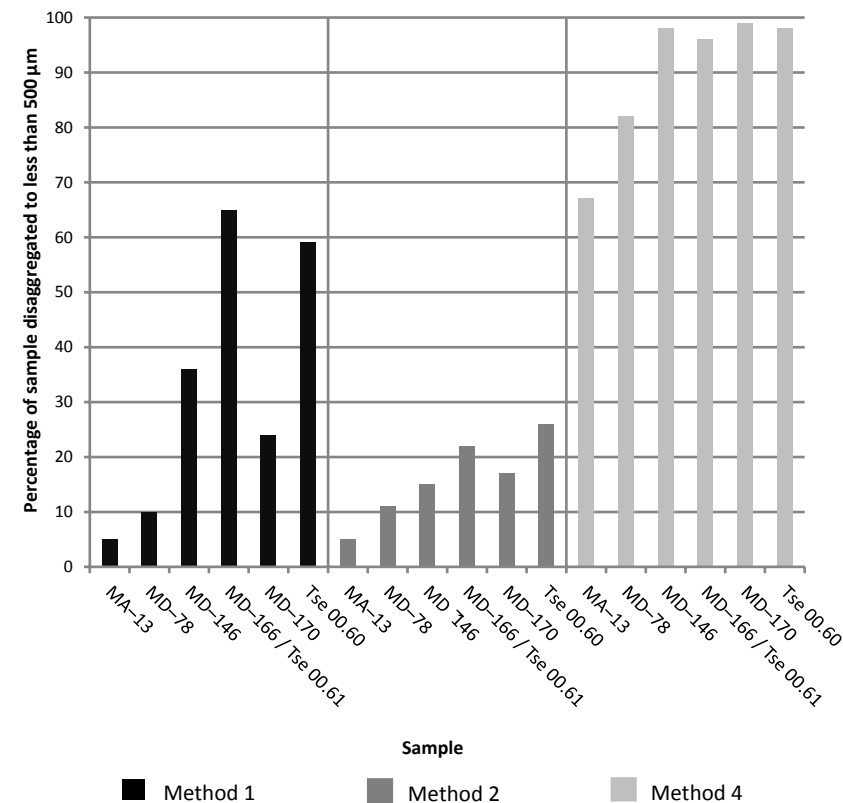


Fig. 1. Histogram showing the percentage disaggregation of samples to less than 500µm from the four different methods.

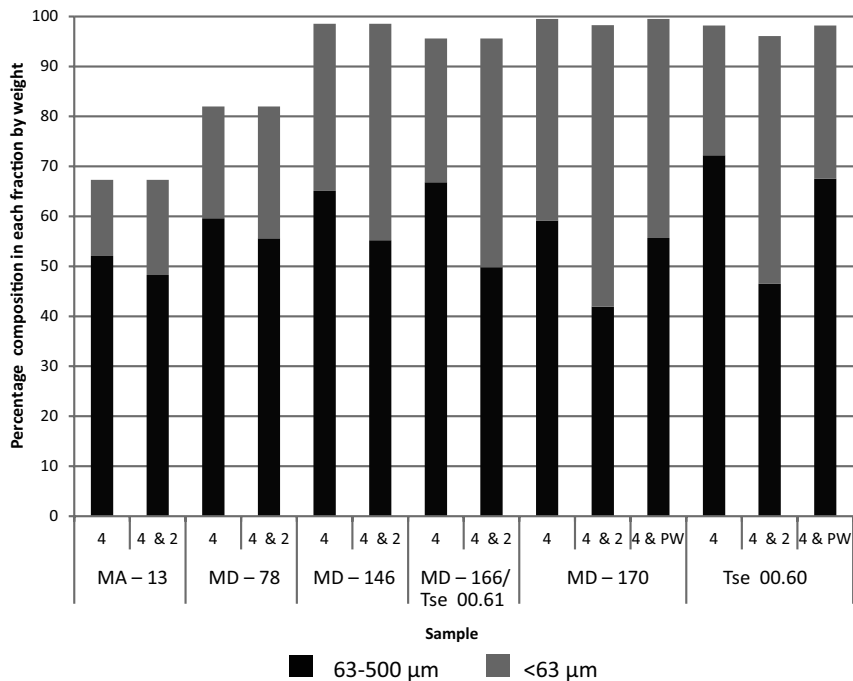


Fig. 2. Histogram showing the percentage composition of the fractions smaller than 500µm and its distribution between the <63µm clay fraction and 63–500µm fraction from which the foraminifera could be extracted. The histogram shows, for all samples, Method 4 and Method 4 combined with Method 2; it also shows for samples MD-170 and Tse 00.60 only, Method 4 combined with high-pressure water spray (PW). The 63–500µm fraction from Method 4 was used to test for further clay removal through the addition of Method 2 for samples MA-13, MD-78, MD-146 and MD-166/Tse 00.61 and through pressure washing for samples MD-170 and Tse 00.60. For samples MD-170 and Tse 00.60 a new aliquot of the samples was used to test Method 4 combined with Method 2.



**Table 3.** Raw foraminifer presence and abundance data.

Sample name	MD-146				MD-166/Tse 00.61				MD-170				Tse 00.60			
Method of processing	1	2	4	4 & 2	1	2	4	4 & 2	1	2	4	4 & 2	1	2	4	4 & 2
<i>Astacolus matutina</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
<i>Astacolus primus</i>	0	2	1	0	0	6	0	11	0	1	0	4	0	3	1	11
<i>A. primus</i> (pyritized)	0	4	0	0	3	5	1	0	2	0	2	5	2	3	1	1
<i>Astacolus primus</i> sp.	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
<i>Brizalina liassica</i>	10	15	6	8	51	115	50	108	2	39	2	28	30	65	23	98
<i>B. liassica</i> (pyritized)	0	2	2	0	0	16	0	5	2	3	0	1	1	9	0	3
<i>Citharina colliezi</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0
<i>Dentalina varians</i>	0	7	3	7	3	3	2	3	13	5	1	0	1	8	2	2
<i>Eoguttulina liassica</i>	0	0	1	0	0	17	1	4	1	4	0	0	0	0	0	8
<i>E. liassica</i> (pyritized)	1	108	41	18	0	30	4	2	72	45	4	3	6	39	0	8
<i>Haplophragmoides</i> cf. <i>kingakensis</i>	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0
<i>Haplophragmoides kingakensis</i>	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>H. kingakensis</i> (pyritized)	3	8	4	0	1	5	0	0	2	1	0	0	2	1	0	0
<i>Marginulina prima</i> ? <i>spinata</i>	0	0	3	0	0	2	0	0	0	3	0	9	0	1	0	1
<i>M. prima</i> ? <i>spinata</i> (pyritized)	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0
<i>Prodentalina terquemi</i>	0	1	0	1	0	1	1	1	1	11	3	15	0	0	1	1
<i>P. terquemi</i> (pyritized)	0	0	0	0	0	1	0	1	0	0	0	2	0	0	0	0
<i>Reinholdella macfadyeni</i>	1	1	1	3	52	82	9	27	10	13	12	69	0	0	0	46
<i>R. macfadyeni</i> (pyritized)	1	1	1	0	20	90	10	19	8	16	4	71	0	4	2	53
<i>Reinholdella pachyderma</i>	1	1	0	0	24	25	0	7	2	22	10	8	0	1	0	18
<i>R. pachyderma</i> (pyritized)	0	1	0	0	24	15	6	1	3	9	8	9	0	1	2	9
<i>Reinholdella planiconvexa</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>R. planiconvexa</i> (pyritized)	0	0	0	0	0	6	0	0	1	0	0	3	0	0	0	0
<i>Reinholdella</i> sp.	0	0	1	0	50	20	4	15	2	2	2	15	2	0	0	2
<i>Reinholdella</i> sp. (pyritized)	0	0	2	0	15	12	6	15	6	6	1	5	0	0	0	2
<i>Spirillina infirma</i> (pyritized)	0	1	0	0	0	1	0	1	0	0	0	0	0	1	0	0
<i>Verneulinoides</i> cf. <i>subvitreus</i> (pyritized)	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Ostracod	0	0	0	0	1	1	0	2	1	0	0	0	0	0	0	3
Unknown pyritized foram.	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0
Microbivalve (pyritized)	5	28	36	26	26	39	20	29	14	25	9	12	29	64	15	24
Microgastropod	1	1	9	10	1	1	2	0	5	2	9	16	13	12	11	3
Microgastropod (pyritized)	13	38	25	29	6	17	7	16	35	19	19	26	53	183	22	18

Note: the amount of sample processed and picked varies; see Tables 2 and 4 for further details.

each foraminifer species for each method are shown in Table 3. Species present in consistently high numbers regardless of processing method are *Reinholdella macfadyeni* (Ten Dam, 1952) and *Brizalina liassica* (Terquem, 1858).

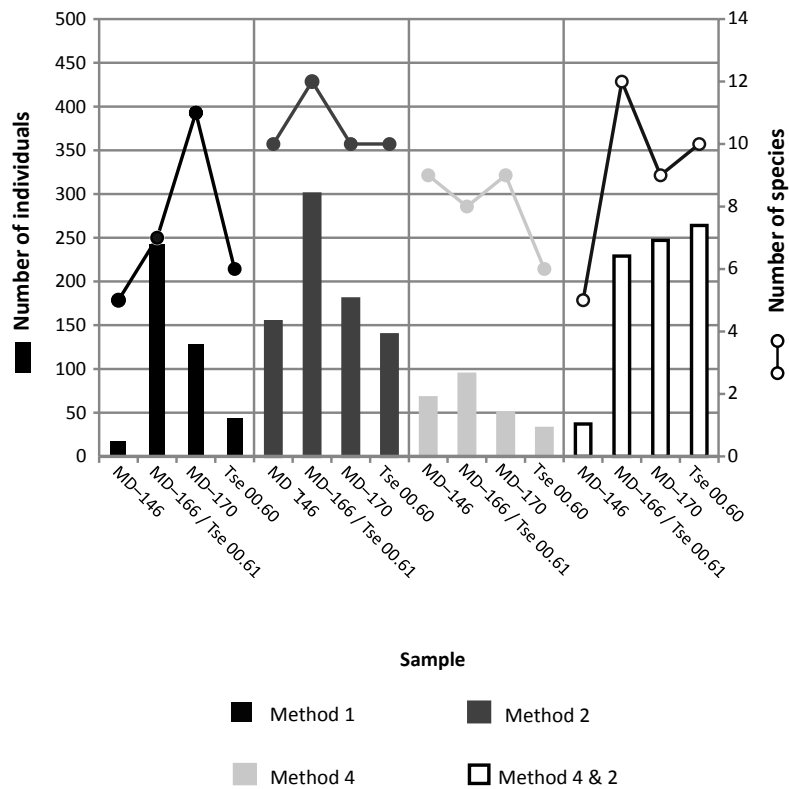
Microfossils from samples processed with Method 1 using H<sub>2</sub>O<sub>2</sub> were degraded in three ways:

- pyritized fossils were damaged (Figs 6a and 7a) compared to those subjected to freeze–thaw (Figs 6b and 7b);
- for the majority of samples there was a decreased abundance, particularly of delicate foraminifera;
- the diversity was lowest, except for sample MD-170 where the diversity was comparable with the other methods. In all samples processed without H<sub>2</sub>O<sub>2</sub> several individuals of the small delicate foraminifer *Eoguttulina liassica* (Strickland 1864) were found in both pyritized (Fig. 7a, b) and non-pyritized forms (Fig. 7c) whereas only one of the four samples processed with Method 1 using H<sub>2</sub>O<sub>2</sub> was found to contain a single specimen of the non-pyritized form of

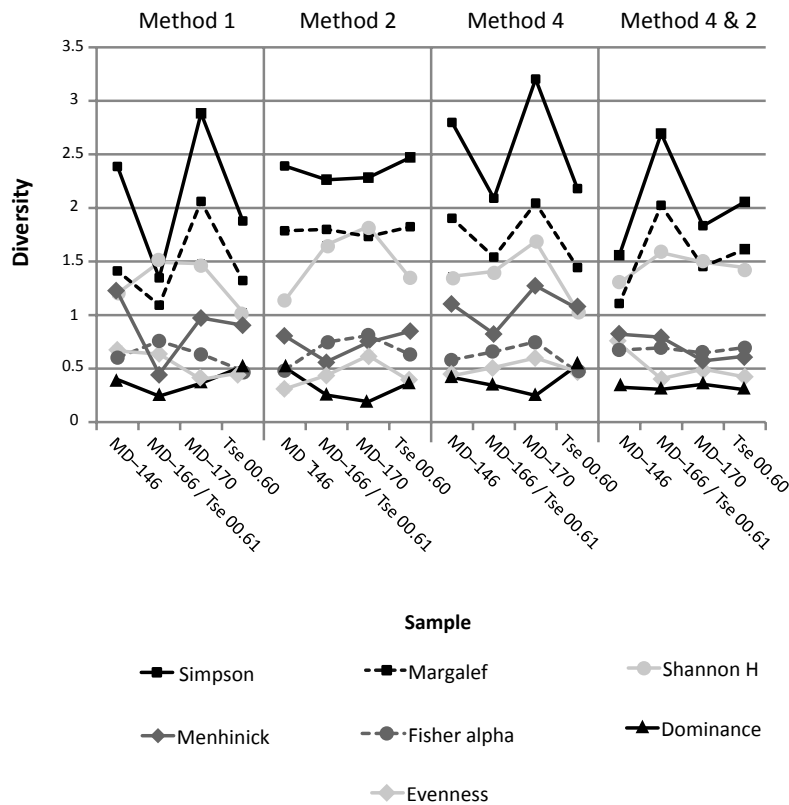
*E. liassica*. The six species that are absent from samples processed with H<sub>2</sub>O<sub>2</sub> but found using other methods are: *Reinholdella* (?) *planiconvexa* (Fuchs, 1989), *Spirillina infirma* (Strickland, 1864), *Verneulinoides* cf. *subvitreus* (Nagy & Johansen, 1991), *Astacolus matutina* (d'Orbigny, 1850), *Citharina colliezi* (Terquem 1866) and *Haplophragmoides* cf. *kingakensis* (Tappan, 1955). Samples processed with H<sub>2</sub>O<sub>2</sub> with only one individual of a species present in 10g or up to 300 counts of the picked residue in addition to *E. liassica* are *Prodentalina terquemi* (d'Orbigny, 1839) and *Marginulina prima* ?*spinata* (Terquem, 1858).

As it was clear that H<sub>2</sub>O<sub>2</sub> was damaging and, in some cases, dissolving, the fossils and the last stage of the Rewoquat (Method 3) involved multiple washings in H<sub>2</sub>O<sub>2</sub>, Method 3 was not tested further.

Table 6 shows the relative costs and processing time for each method. Methods 1 and 2 are the fastest in terms of total time taken for processing, but in terms of 'hands on' time, Methods 2



**Fig. 3.** Histogram showing the abundance of foraminifera and line graph showing the number of species for each of the samples processed by each of the methods.



**Fig. 4.** The diversity of foraminifer assemblages in picked sedimentary rock using the Simpson, Margalef, Shannon H, Menhinick and Fisher alpha indices as well as the dominance and evenness for each sample calculated using PAST software.

**Table 4.** The fossil abundance and diversity in the 63–500µm residue picked to 10g or 300 individuals (whichever was sooner).

Sample	Method 1: white spirit and H <sub>2</sub> O <sub>2</sub>				Method 2: white spirit without H <sub>2</sub> O <sub>2</sub>				Method 4: Freeze-thaw				Method 4 and 2			
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
MA-13	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	1
MD-78	0	0	0	>300	0*	0*	0*	107*	0	0	0	>300	0	0	0	>300
MD-146	17	5	19	81	154*	10*	67*	166*	67	8	70	61	37	5	65	120
MD-166/Tse 00.61	243	7	33	194	>300	12	57	>300	94	8	29	75	229	12	45	230
MD-170	128*	13*	54*	106*	191*	12*	46*	110*	50	9	37	10	247	10	54	37
Tse 00.60	44	6	95	116	139	11	259	>300	32	7	48	52	264	10	45	176

\*Not picked to 10g due to insufficient sample remaining after disaggregation (see Table 2). The columns are as follows: A: abundance of foraminifera in 10g (unless \*); B: diversity of foraminifera; C: abundance of ostracods, microgastropods and microbivalves; D: abundance of prasinophytes (blue-green algae of the group Tasmanitids). Sample MA-13 yielded no foraminifera from any method.

**Table 5.** The abundance and predicted abundance of foraminifera in the residue.

Sample	Method 1		Method 2		Method 4		Method 4 and 2	
	A	PA	A	PA	A	PA	A	PA
MA-13	0	0	0	0	0	0	0	0
MD-78	0	0	0*	0	0	0	0	0
MD-146	17	28	154*	155	67	664	37	166
MD-166/Tse 00.61	243	640	452	630	94	622	229	892
MD-170	128*	131	191*	183	50	296	247	1015
Tse 00.60	44	110	139	147	32	204	264	1349

A: abundance of foraminifera in 10g or the first 300 as in Table 3; PA: the predicted abundance of foraminifera if all 63–500µm fraction initially processed, was picked. Note: the initial sample weight used was normalized to 100g in all cases; \* as Table 4.

and 4 are the shortest and Method 1 is the longest. High-pressure washing adds a minimum of 45 min. At the current (2013) costs for chemicals, Method 4 is the cheapest and Method 1 is the most expensive at approximately double the cost of Method 2 or, Method 4 combined with Method 2.

## DISCUSSION

The effectiveness of the different methodologies was assessed as a function of: (1) the degree to which the sample was disaggregated; (2) the abundance and diversity of extracted foraminifera; and (3) the quality of preservation of the microfossils extracted from the sample.

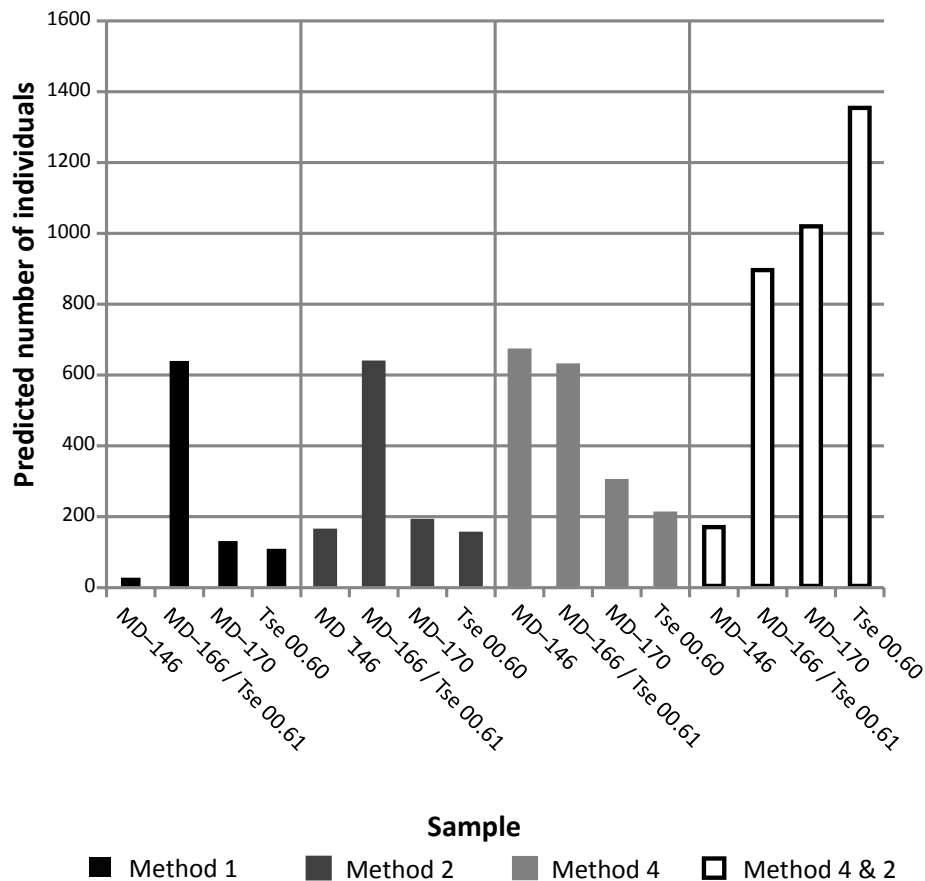
Method 4 was the most effective method for disaggregating the mudrocks, and consistently disaggregated over 65% of the sample (Table 2; Fig. 1). In comparison, Method 2 was the worst and disaggregated less than 25% of the sample to <500µm. However, Method 4 left the microfossils in the 63–500µm fraction very diluted by aggregated clay particles which could not be quickly removed by high-pressure washing. Application of Method 2 after Method 4, on the 63–500µm residue extracted from Method 4, concentrated the microfossils by allowing further disaggregation of the 63–500µm aggregates into clay particles and microfossils from which the clay particles could be removed by gentle washing over a 63µm sieve. The application of Method 2 after Method 4 was twice as effective at breaking down the aggregates and removing the clays than 45 min of high-pressure washing (Fig. 2). Ultrasound preparation of the whole of the 63–500µm fraction at the end of Method 4 also helped

to deflocculate the clays. Hodgkinson (1991) reported that ultrasound preparation damaged agglutinated foraminifera, but no evidence of damage was found on the few specimens of agglutinated foraminifera extracted in the samples tested here.

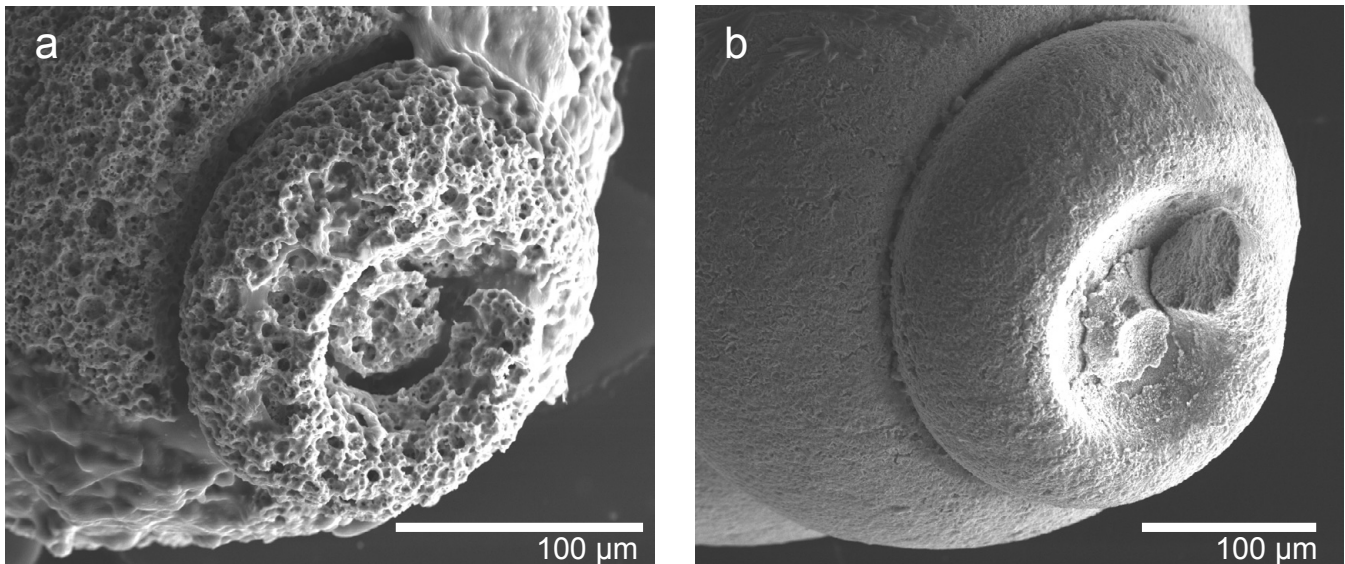
Method 2, and Method 4 combined with 2, yielded within uncertainty the highest raw abundances of all foraminifer species (Table 4; Fig. 5). Method 4 alone did not yield such high raw abundances as Method 4 combined with 2 because the first 10g picked using Method 4 was more diluted by 63–500µm clay aggregates due to the success rate of the disaggregation to particles of <500µm. It follows that Method 4 combined with Method 2 yielded the highest predicted foraminifer counts (Fig. 5) because more of the original sample was disaggregated by the freeze-thaw (compared to Method 2 alone) and the use of both methods concentrated the foraminifera in the raw counts. The success of the freeze-thaw method in both disaggregating more of the sample and not damaging the fossils clearly increases the potential number of foraminifera that are available for picking. However, the efficient breakdown of the rock makes picking more time consuming because aggregated clay particles remain in the 63–500µm fraction even after Method 4 and Method 2 are combined.

The different diversity indices from PAST are indistinguishable between all the methods within the limits of natural variance and there is no clear relationship with either wt% TOC, or wt% CaCO<sub>3</sub> or wt% S for the few samples tested (Fig. 4). However, the raw abundance and diversity counts for the foraminifer assemblages (Table 3) show that Method 1 has a lower abundance of





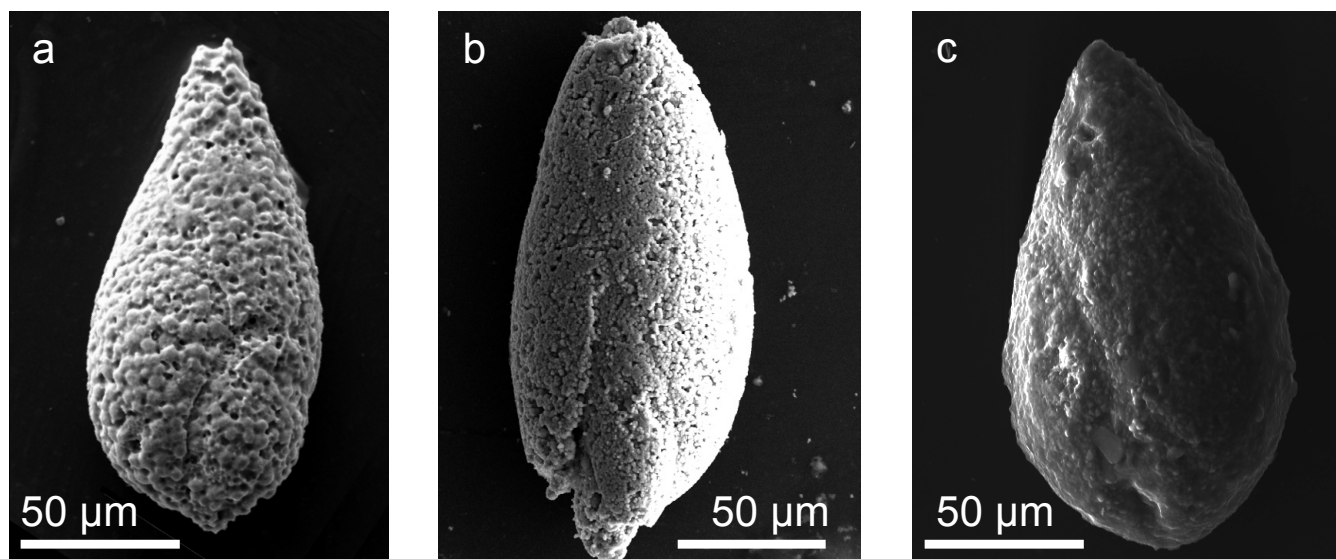
**Fig. 5.** The predicted number of foraminifera in the 63–500µm fraction assuming the initial processed weight was 100 g and that the entire sample which was successfully disaggregated was picked for foraminifera.



**Fig. 6.** (a) SEM image of a pyritized microgastropod processed with  $H_2O_2$  showing pitting and dissolution damage. (b) SEM image of pyritized microgastropod processed by freeze-thaw.

the ‘rarer species’ and that some species are absent altogether and we interpret this to be the result of complete oxidation and dissolution by  $H_2O_2$ . In contrast, Method 4 combined with 2 generally has a higher abundance of all species.

Our SEM work shows that  $H_2O_2$  resulted in significant damage to pyritized microfossils and our abundance and diversity data show that  $CaCO_3$  microfossils were significantly reduced in number or absent when extracted using  $H_2O_2$ . That  $H_2O_2$  could



**Fig. 7.** SEM images of the foraminifer *Eoguttulina liassica* (Strickland, 1864): (a) pyritized, and processed with  $\text{H}_2\text{O}_2$ ; (b) pyritized, and processed without  $\text{H}_2\text{O}_2$ ; (c) non-pyritized, and processed without  $\text{H}_2\text{O}_2$ .

**Table 6.** Relative cost and processing time for each method tested in this paper.

	Method 1	Method 2	Method 4	Method 4 and 2	High-pressure washing
Total time taken per sample	27h (24h in white spirit, 25 min in sodium hexametaphosphate, 1 h in $\text{H}_2\text{O}_2$ )	26h	2 weeks (24h saturation, 45–60h freezing time)	2 weeks (same as Method 4, but additional 24h in white spirit, 25 min in sodium hexametaphosphate)	The time taken per sample for method undertaken first, 45 min
Hands-on time per sample	1 h 30 min	30 min	30 min	1 h	45 min
Cost per 100 g sample	Twice the cost of Method 2	Approximately £3 at 2013 prices	Negligible costs	As Method 2	The cost per sample for the method undertaken first

Method 1 used white spirit, sodium hexametaphosphate and hydrogen peroxide, Method 2 used white spirit and sodium hexametaphosphate, Method 4 used freeze–thaw. See text for further details.

cause damage to  $\text{CaCO}_3$  was also noted by Hodgkinson (1991) and Pingitore *et al.* (1993). Reolid *et al.* (2012, p.91) described ornamented foraminifera tests that they had extracted as ‘pitted, smooth or polished surfaces on the test periphery’ and damage to final chambers of the test. We note that this pitting is similar to that which we observed on SEM images of the pyritized microfossils processed using  $\text{H}_2\text{O}_2$  (Figs 6a and 7a) and suggest that the damage Reolid *et al.* (2012) observed may have been caused by the  $\text{H}_2\text{O}_2$ . We also suggest that the poor foraminifera preservation reported by Gräfe (2005) and attributed to taphonomic process could be instead due to  $\text{H}_2\text{O}_2$  treatment if this was used in their processing. The lower abundance of  $\text{CaCO}_3$  forms observed in the samples we processed using  $\text{H}_2\text{O}_2$  is interpreted to result from dissolution of delicate foraminifera, such as *E. liassica* and *Haplophragmoides* cf. *kingakensis*. Under the SEM, some of the  $\text{CaCO}_3$  forms of *R. macfadyeni* and *Brizalina liassica* exhibited evidence of dissolution which we interpret to be due to the use of  $\text{H}_2\text{O}_2$ .

Although Hodgkinson (1991) suggested sodium hexametaphosphate in high concentrations could cause damage to foraminifer

tests, there was no visible damage to those processed with methods using sodium hexametaphosphate when compared with Method 4.

Our preliminary foraminifer data from the Toarcian reported here extends the range of *H. kingakensis* by approximately 10 m in Yorkshire compared to that reported by Hylton (2000, unpublished PhD thesis, University of Plymouth). Testing whether the Toarcian OAE is truly barren of foraminifera as reported by Hylton (2000, unpublished PhD thesis, University of Plymouth) awaits further study, but these early results suggest that studies where microfossils have been extracted using  $\text{H}_2\text{O}_2$  may have inadvertently dissolved delicate species.

## CONCLUSIONS

Our new freeze–thaw technique (Method 4) was the most successful technique for disaggregating mudrocks. Our results suggest that  $\text{H}_2\text{O}_2$  is an unsuitable chemical for extracting delicate  $\text{CaCO}_3$  and pyritized microfossils because of the damage and dissolution it causes. Sodium hexametaphosphate, white spirit and Decon-90 caused no visible damage to the foraminifera. The only disadvantage

of Method 4 as a single processing technique was that it left clay aggregates in the foraminifer-bearing fraction that had a dilution effect on the abundance of foraminifera, which makes microfossil picking more time consuming.

The technique of repetitive freeze–thaw combined with ultrasound treatment and using Decon-90 disaggregated at least 50% of the original sample. The application of white spirit and sodium hexametaphosphate (Method 2) after Method 4 resulted in increased separation of the clay from the microfossil fraction and left the most diverse and abundant foraminifer assemblage with no evidence of chemical damage. Following Method 4 with Method 2 is quicker and more effective than using high-pressure washing to remove clays.

Our results demonstrate the importance of investigating the processing technique to maximize the preservation of microfossils and extract a true representation of the preserved foraminifer assemblage. We anticipate that the freeze–thaw method could be the basis of further development of a foraminifer extraction process across a wide range of sedimentary deposits.

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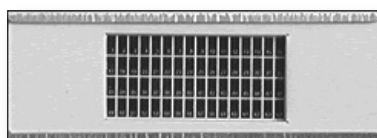
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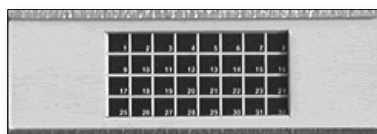
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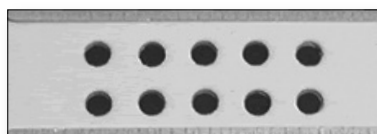
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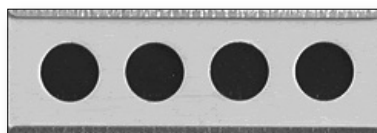
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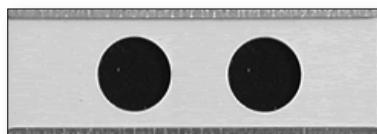
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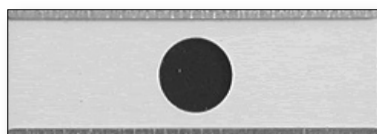
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