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Roberta D'Onofrio, Valeria Luciani

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Do different extraction techniques impact planktic foraminiferal assemblages? An early Eocene case study

Roberta D'Onofrio*[a] and Valeria Luciani [a]

[a] Dipartimento di Fisica e Scienze della Terra, Università di Ferrara, via G. Saragat 1, 44121, Ferrara, Italy

* Corresponding author.

E-mail addresses: dnfrrt@unife.it

ABSTRACT

Here we report and compare results on planktic foraminiferal assemblages extracted with five disaggregation techniques: acetic acid, H_2O_2 at 10% and 25% concentration, neo-steramina, and liquid N_2. The aim is to estimate whether these laboratory procedures can affect the pristine assemblages or add secondary dissolution effects. We apply these five methods on three samples with different carbonate content from the early Eocene Tethyan Terche section (northeast Italy). For each method we assess: (1) the treatment effectiveness in relation to time required to successfully extract planktic foraminiferal tests and preservation; (2) the degree of dissolution through the analyses of three well-known dissolution proxies including the fragmentation index, the planktic benthic ratio and the weight percent coarse fraction; (3) changes in planktic foraminiferal assemblages through genera and species absolute abundances and the evaluation of multiple species diversity indices.

Our data demonstrate that acetic acid and neo-steramina treatments are the most effective methods as they represent the only ones capable in disaggregating the samples with higher $CaCO_3$ content whereas liquid N_2 revealed to be the best treatment to adopt for samples with low $CaCO_3$ content. The best-preserved foraminiferal specimens derive from the acetic acid and neo-steramina treatments. Nonetheless, the acetic acid along with H_2O_2 treatments, at both concentrations, can impact planktic foraminiferal assemblages affecting the diversity or species abundances. Dissolution of planktic foraminifera shows a complex relationship with the initial carbonate content ($CaCO_3$%) of the samples

and with the different dissolution susceptibility of the species that can differ according to the laboratory procedures.

Key words: Planktic foraminifera; early Eocene; carbonate rocks; disaggregation methods; dissolution susceptibility; quantitative analyses.

1. Introduction

The study of foraminifera requires the observation of their individual tests from washed residues in order to correctly detect the morphological characters that allow species identification. Disaggregation of indurated rocks is therefore necessary to extract them. Multiple techniques for rock disaggregation have been proposed in the past decades (e.g., Saini-Eidukat and Weiblen 1996; Lirer, 2000; Green, 2001 and references therein; Remin et al., 2012; Kennedy and Coe, 2014). Nonetheless, the possible effects of diverse disaggregation methods on the foraminiferal assemblages are much less constrained (e.g., Reolid and Herrero 2004; Kennedy and Coe 2014; Van Bael et al., 2016).

As the requirement of even more detailed data for quantitative analyses has increased in recent years it imposes a detailed re-examination of laboratory techniques to evaluate whether they can modify the pristine microfossil assemblages. Studies based on micropaleontological quantitative data are indeed crucial for paleoenvironmental interpretations thus it is essential to assess whether signals recorded by assemblages are genuine or affected by taphonomic process or laboratory procedures. Particularly relevant are the effects of dissolution on planktic foraminiferal assemblages. Post-mortem dissolution of calcareous microfossils assemblages resulting from taphonomic processes can occur due to acidification within the water column or at the sea floor (primary dissolution). However, certain disaggregation procedures, especially those based on chemical attack, could induce additional dissolution of foraminiferal tests (laboratory or secondary dissolution) (e.g., Hodgkinson, 1991; Pingitore et al., 1993; Van Bael et al., 2016). Regardless of the nature of dissolution, the most notable effect on foraminifera is that their tests can be differentially prone to dissolution depending on genera and species involved

resulting from different coiling arrangements and wall texture (e.g., Berger, 1970; Bé et al., 1975; Thunell and Honjo, 1981; Hancock and Dickens, 2005; Petrizzo et al., 2008; Nguyen et al 2009, 2011; Leon-Rodriguez and Dickens, 2010; Nguyen and Spejier 2014; D'Onofrio et al., 2016). Dissolution can therefore significantly modify the composition of planktic foraminiferal assemblages by enriching them in dissolution-resistant taxa and inducing the loss of the dissolution-prone species.

Several studies have been focalized to identify the dissolution susceptibility of planktic foraminifera, especially for early Paleogene assemblages (e.g., Berger, 1970; Bé et al., 1975; Thunell and Honjo, 1981; Petrizzo et al., 2008; Nguyen et al 2009, 2011; Nguyen and Spejier 2014; D'Onofrio et al., 2016). This is because the early Paleogene interval is characterized by extreme global warming episodes, known as hyperthermals, which were marked by carbonate dissolution in deep-sea settings due to carbonate compensation depth (CCD) rise (e.g., Dickens et al., 1997; Zachos et al., 2005; Kump et al., 2009; Stap et al., 2009; Zeebe et al., 2009; Leon-Rodriguez and Dickens, 2010). The hyperthermals include the most studied Paleocene–Eocene Thermal Maximum (PETM or ETM-1, e.g., Kennett and Stott, 1991; Zachos et al., 2010; McInerney and Wing, 2011) at ~56 Ma, the Eocene Thermal Maximum 2 (ETM-2, also referred to as H-1 or Elmo event, e.g., Cramer et al., 2003; Lourens et al., 2005; Nicolo et al., 2007) at ~54.1 Ma, the Eocene Thermal Maximum 3 (ETM-3, also called K/X event, e.g., Cramer et al., 2003; Nicolo et al., 2007; Agnini et al., 2009) at ~52.8 Ma, and several other additional events spanning the late Paleocene to early middle Eocene (e.g., Cramer et al., 2003; Sexton et al., 2011; Coccioni et al., 2012; Slotnick et al., 2012, 2015; Littler et al., 2014; Kirtland-Turner et al., 2014; Lauretano et al., 2016; Luciani et al., 2016, 2017a, 2017b; Westerhold et al., 2018).

On one hand, it may be relatively simple to recognize markedly altered foraminiferal assemblages because strong dissolution results in extremely impoverished assemblages. On the other hand, it can be much more complicated to recognize minor dissolution effects that are equally important for accurate paleoenvironmental reconstructions. Further issues come with the fact that planktic foraminiferal dissolution susceptibility varies through different time interval following extinction and speciation (e.g., Luciani et al., 2010; D'Onofrio et al., 2016). It is therefore essential to verify the quality of the

micropaleontological data trying to discern between genuine signals and those driven by taphonomic effects or laboratory procedures.

In light of the mentioned evidences, various authors proposed multiple proxies to detect the degree of primary dissolution on foraminiferal assemblages (e.g., Berger, 1970; Hancock and Dickens, 2005; Petrizzo et al., 2008; Nguyen and Speijer, 2014; D'Onofrio et al., 2016). In spite of this, less attention has been dedicated to recognize whether laboratory procedures can induce secondary dissolution.

Following the above critical remarks, the purpose of this study is to examine possible changes within foraminiferal assemblages from three appropriately selected lower Eocene samples of lithified rock with different carbonate content in relation to five disaggregation laboratory techniques: hydrogen peroxide at 10% and 25% concentration, neo-steramina (surface tension-active agent, hereafter surfactant), acetic acid (highly concentrated acetic acid, ~ 80%; CH_3COOH), and liquid N_2. The samples come from the early Eocene Terche section (Venetian Southern Alps, northeast Italy) (Fig. 1). This section was chosen because it is well constrained for its foraminiferal, calcareous nannofossil and stable isotope content (D'Onofrio et al., 2014, 2016). In addition, the Terche section encompasses three early Eocene hyperthermal events, ETM2, H2 and I1, that have induced some taphonomic dissolution on planktic foraminifera (D'Onofrio et al., 2014, 2016). Our analysis allows us to evaluate whether different disaggregation methods can be responsible of additional dissolution for the samples selected here.

We establish for each method and lithology the treatment effectiveness in relation to time required to successfully extract planktic foraminiferal tests and disaggregation degree. Moreover, we illustrate the difference in foraminiferal preservation using a stereomicroscope and a scanning electron microscope (SEM).

We identify the influence of the adopted methods on possible additional dissolution on genera and species abundance. To attain this goal, we here present multiple indices to evaluate species diversity, absolute abundance of genera and species and widely adopted proxies to detect planktic foraminiferal dissolution.

We demonstrate that the best-preserved foraminiferal tests derive from the acetic acid, neo-steramina and liquid N_2 treatments. In general, dissolution effects appear rather moderate but not negligible for some of the methods adopted. Dissolution on planktic foraminifera shows a complex relationship with the initial carbonate content ($CaCO_3$%) of the samples and with the different dissolution susceptibility of the species that can differ according to the laboratory procedures adopted.

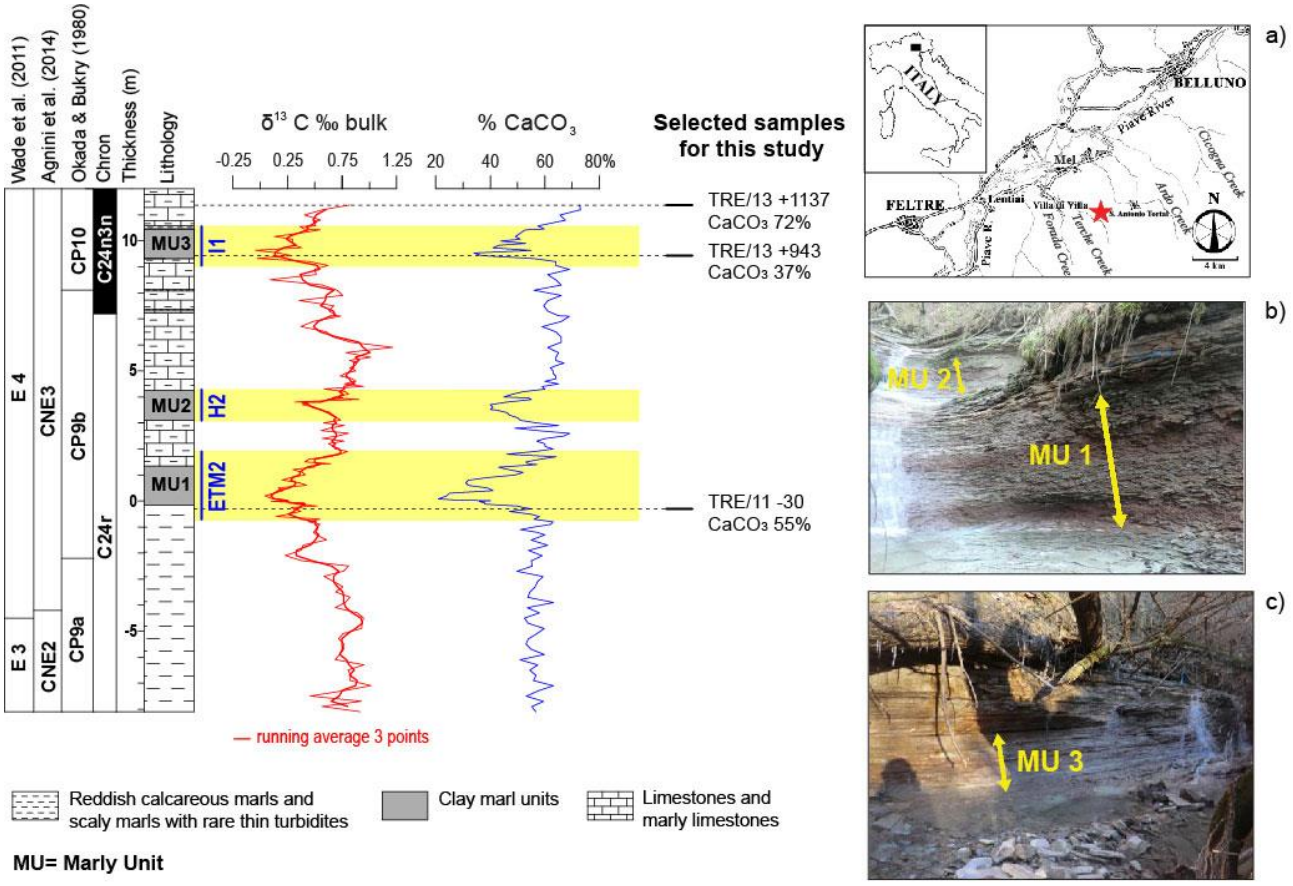


Figure 1. Lithological log of the Terche section (northeastern Italy) plotted against the $\delta^{13}C$ and $CaCO_3$ curves. Black bars indicate the samples here examined. The yellow bands highlight the ETM2, H2, and I1 hyperthermal as defined by the carbon cycle perturbations. The red star on the geographic map (a) shows the location of Terche section in the Piave river valley (Belluno Province). Photographs show the marly-units MU1 and MU2 (b) and MU3 (c) representing the lithological expression of the recorded early Eocene hyperthermals. Modified from D'Onofrio et al. (2016).

2. The Terche section: settings, stratigraphy and lithology

The Terche section (46°2′43.61″N, 12°4′47.78″E) is located in the Venetian Southern Alps (northeastern Italy) and outcrops in correspondence of a Terche Creek right tributary (Fig. 1a). Sedimentary rocks exposed are part of the local Upper Cretaceous–lower Eocene pelagic-hemipelagic

succession deposited in the bathyal setting of the Belluno Basin, one of the main Meso–Cenozoic paleogeographic units of the Southern Alps (e.g., Bosellini, 1989). The plankton benthos ratio and features of benthic foraminiferal assemblages assign to the Terche sediments an average water depth of at least 1000 m (D'Onofrio et al., 2016).

The entire Terche section consists of more than 85 m of pink-reddish to green scaly calcareous marls and marly limestones, locally rhythmically organized, referred to as the Scaglia Rossa Formation (D'Onofrio et al., 2016). The upper part (~27 m thick, Fig. 1) is characterized by the presence of three reddish marly and clayey intervals (marly units, MUs) which thickness varies from 1.1 to 1.5 m. Bio-magnetostratigraphy ascribed the lithological anomalies (MU1, MU2, M3 from the base to the top) and the related negative stable carbon isotope excursions (CIE) to the early Eocene hyperthermals ETM2, H2 and I1 (D'Onofrio et al., 2016). The interval containing the MUs spans the planktic foraminiferal Zones E3 and E4 of the Wade et al. (2011) zonal scheme and the calcareous nannofossil Zones CN2 and CN3 of Agnini et al. (2014) zonation (Fig. 1).

3. Materials and methods

The three selected samples come from –30 cm, +943 cm and +1137 cm levels of the Terche section. Level 0 cm corresponds to the base of MU1 (Figs. 1, 2). The $CaCO_3$ content for these samples is as follows: TRE/13 +943= 37%; TRE/11 –30=55%, TRE/13 +1137= 72% (D'Onofrio et al., 2016). Hereafter samples are labelled as follows: TRE/+943=CM (clayey marl); TRE/11 –30=MA (marl); TRE/13+1137=LM (limestone marl) (Fig. 2).

We summarize below the rationale of our selection of the laboratory techniques to disaggregate rocks and describe the respective procedures adopted in this paper.

3.1. Foraminiferal extraction techniques

The current literature presents a wide range of methods for the foraminiferal extraction from lithified or partially consolidated rocks (e.g., Saini-Eidukat and Weiblen 1996; Lirer, 2000; Green, 2001

and references therein; Remin et al., 2012; Kennedy and Coe 2014). Methods can be based on physical or chemical procedures. We selected for this study five methods that are largely used for rock disaggregation: acetic acid, hydrogen peroxide at 10% and 25% concentration, surfactant neo-steramina and liquid N_2.

We discard rock disaggregation methods such as Calgon ($Na_6P_6O_{18}$), Decon-90 (KOH) or Quaternary 'O' ($C_{24}H_{47}N_2O_2Cl$) and Rewoquat (surfactant) (CH_3OSO_3) because these procedures are known to be aggressive enough to destroy planktic foraminiferal tests unless used in extremely low concentrations (Hodgkinson, 1991). Some authors applied the Rewoquat treatment for extracting foraminifera from pre-Paleogene strongly lithified rocks and reported poorly preserved assemblages (Gräfe, 2005; Moullade et al., 2005). Personal experience on latest Maastrichtian-earliest Danian calcareous marly samples from Scaglia Rossa Formation (Erto section, northern Italy, Luciani, 1997) demonstrates that the Rewoquat treatment destroys all planktic foraminiferal tests whereas the surfactant product called "Desogen" (= neo-steramina) freed rather well preserved foraminifera. Moreover, these chemical products are strong detergent with environmental restrictions according to the European Union Directive 67/548/EEC, especially Decon-90 and Quaternary 'O'.

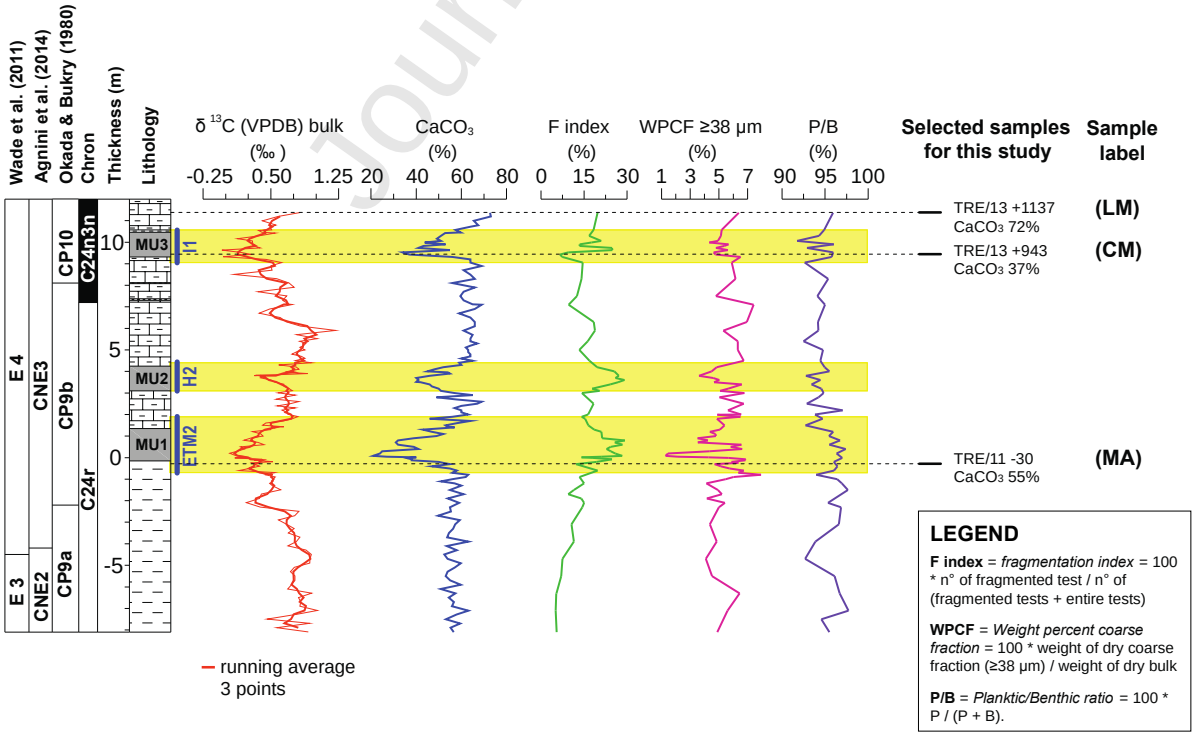


Figure 2. Curves showing the foraminiferal dissolution proxies plotted against the carbon stable-isotope record and the percentage of $CaCO_3$ at the Terche section. Other information is the same as in figure 1. Modified from D'Onofrio et al. (2016).

3.1.1. Laboratory procedures

Some laboratory steps adopted here are common to all the tested methods as described below. Firstly, samples were oven-dried at low temperature (< 50°C) for 24 hours and then broken into small pieces of ~1–2 cm. For each of the samples selected in this study, an almost equal amount of these small fragments (~40 g) were processed with the different methods. After disaggregation, samples were washed through 38 μm, 63 μm and 100 μm stacked sieves. Sieves were immersed in a methylene blue bath after each washing in order to colour planktic foraminifera potentially trapped in the sieve mesh (e.g., Green, 2001). This is an easy method to exclude possible contamination amongst successive samples. Washed sediment residues were then oven-dried at low temperature (< 50°C) and examined under an incident light stereomicroscope for their planktic foraminiferal content and for the dissolution proxies, as described in paragraphs 3.2 and 3.3. We avoid the application of ultrasonic treatment because it would have needed further investigation on its possible effects on planktic foraminiferal assemblages that is beyond the aim of this paper.

3.1.2. Acetic acid

The acetic acid or "cold acetolyse" technique was proposed by Lirer (2000). This method consists of immersion of 50–150 g of dry and crushed sediment (1–2 cm) in acetic acid (highly concentrated, ~ 80%; CH_3COOH) until the rock assumed a characteristic "mousse" appearance. Lirer (2000) suggests a required runtime for soaking (2–10 hours) depending on the lithology. However, our previous experience with the Scaglia Rossa lithology revealed that 4 hours are generally sufficient to reach the characteristic mousse aspect. The "cold acetolyse" method has been, indeed, successfully applied for indurated rocks form several sections in Italy (e.g., Luciani et al., 2007; Fornaciari et al. 2007; Coccioni et al. 2012; Luciani and Giusberti, 2014; D'Onofrio et al. 2016; Luciani et al., 2016) thus representing a

great potential for studies on ancient lithified lithologies otherwise analysable only in thin section.

For this study, we avoid the immersion of washed residues in surfactants as their effects are assessed separately.

3.1.3. Hydrogen peroxide

This category includes those techniques involving the chemical attack of the rock using different concentration of H_2O_2 (e.g., Aldridge, 1990; Reolid et al., 2012). This technique induces rock disaggregation by oxidizing the organic matter. The rock sample is soaked in a solution of water and H_2O_2 at various concentrations and for variable time. Experience suggests that argillaceous and marly samples can be better treated with solution at relatively low hydrogen peroxide concentration whereas lithologies with high $CaCO_3$ content need stronger attack.

In this paper we soaked our samples broken in pieces of 1–2 cm within H_2O_2 at 10% and 25% for the time necessary to reach disaggregation.

3.1.4. Neo-steramina (surfactant)

Neo-steramina is a surface-active agent (also called surfactant, term resulting from contraction of the three words "surface active agents") with the following chemical composition: alkyldimethylbenzylammonium chloride diluted at 10%. This surfactant disaggregates rocks by breaking the surface tension bonds of the organic matter with the clay minerals. The 1–2 cm pieces of rock were here immersed in a neo-steramina bath for the time needed to obtain disaggregation. This procedure is simple and does not require special chemical laboratories. This method was previously cited as "Desogen" or "Neo-Desogen" method (e.g., Luciani, 1997) from the name of the tensioactive chemical product commercially previously distributed by the Ciba-Geigy Company, now out of production. The chemical formula of "Desogen" and "Neo-Desogen" is the same of neo-steramina.

3.1.5 Liquid N_2

The extraction of foraminifera by the liquid N_2 belongs to the physical procedures that include those techniques involving the use of rapid temperature changes which mimic the natural erosional process induced by mechanical weathering. These methods, commonly called freeze-thaw, induce the rock disaggregation by alternating freezing and immersion within hot water of little pieces of rock. The repetition of freeze-thaw cycles allows the progressive growth of ice crystals into the pores and the consequent disaggregation of the rock into finer fraction. The rocks generally disaggregate at their weakest points along cracks within the matrix and/or on the contacts between fossils and matrix.

The freeze-thaw treatment is one of the oldest methods adopted for rock disaggregation being in use for almost a century (e.g., Hanna and Church, 1928; Camp and Hanna, 1937). In recent years some authors proposed to freeze more quickly the rock by using the liquid nitrogen (Hinchey and Green, 1994; Remin et al., 2012). More recently, Kennedy and Coe (2014) assessed the effectiveness of the freeze–thaw method from indurated mudrocks from the Toarcian (Early Jurassic) of Yorkshire, UK, to extracting microbenthic foraminifera. This method is not very successful in disaggregating clay particles in the 63–500 μm fraction such that further treatment with white spirit and sodium hexametaphosphate is required to yield clean foraminifer assemblages. Kennedy and Coe (2014) use hot-cold treatment with water instead of liquid N_2 thus implying longer disaggregation time.

The liquid N_2 method has the advantages that it is cheap, easy to apply, and does not require special chemical laboratories. A well-ventilated room or hood and use of antifreeze gloves to handle N_2 are the only necessities. In addition, the liquid nitrogen, ordinary nitrogen in a liquid state at very low temperature (−196°C), is easily available in most physical and chemical laboratories.

Our analyses consisted of soaking ~1–2 cm pieces of rock in deionized water for about twelve hours and then adding liquid nitrogen that suddenly frozen the samples. After a few seconds, until the liquid N_2 vaporizes, the prompt adding of hot water produced a first disaggregation of sediments. In this step, the rock fragments can be gently crumbled between the fingers. After each cycle, samples were washed through a 38 μm sieve to remove the finest fraction and make the remaining pieces of rock available for a successive treatment with liquid N_2 and hot water. This is an important step because the

foraminifera released from the rock after every cycle are not subjected to repeated freezing and heating in the following cycles thus preventing them from damage. The process was repeated until the samples were disaggregated.

3.2. Qualitative and quantitative foraminiferal analysis

Planktic foraminiferal assemblages from the three disaggregated sample residues were examined at the qualitative and quantitative level.

Qualitative analyses were performed using a stereomicroscope to identify species diversity and to estimate the preservation quality of washed residues. The latter was defined in term of sediment clumps occurrence, tests cleansing or encrustations. The preservation of test-wall characters was qualitatively evaluated at the Scanning Electron Microscope (SEM). Taxonomic criteria adopted in this study follow Olsson et al. (1999) and Pearson et al. (2006).

Quantitative analysis on foraminiferal assemblages is one of the most useful and adopted proxies for paleoceanographic and paleoenvironmental reconstruction. Relative abundance refers to the proportion of a species or genus with respect to a statistical population commonly varying from ~300 to ~500 specimens and it is generally expressed as percentages. This standard procedure based on a fixed number of specimens was introduced within the CLIMAP Projects and developed with the aim to ensure a rapid acquisition and the statistical reproducibility of data (CLIMAP, 1976). By contrast, absolute abundance indicates the number of individuals per unit weight or volume of sediment and alternatively per unit area of sea-floor and it is generally expressed as N/g or N/cm^2 (N=number, e.g., Schott, 1935). The two former are generally used for fossil assemblages whereas the latter for living assemblages.

As in deep-sea sediments planktic foraminifera are extremely abundant (thousands to tens of thousands of specimens larger than 150 μm per gram of calcareous ooze, e.g., Kucera, 1998) they are generally censed with relative counts whereas benthic foraminifera, usually less abundant, are more suitable for absolute counts. The 'relative' quantitative approach is, therefore, mainly adopted because 'absolute' counts are much time consuming. Nevertheless, the relative count bears some caveats as the

relative increase in one genus/species and the decrease in another are auto-correlated as they are both percentages of the entire group (e.g. Murray, 1991). Conversely, absolute abundances are more strictly related to primary productivity when assemblages are not affected by taphonomic problems.

Quantitative analyses were here performed as absolute counts on a fixed amount (10 mg) of washed residues (≥100 μm size fraction) obtained through a precision micro-splitter. We avoid to estimate the absolute abundance of planktic foraminiferal specimens through the density equation of Schott (1935) (N per gram = bulk weight * weight of split-observed residue / total weight of residue). This is because the total weight of washed residues obtained from our analysis varies according to the diverse effectiveness of disaggregation so that the number of specimens per gram is strongly influenced by the values of denominator thus numbers of specimens per gram are not reasonably comparable. We therefore performed counts on fixed amount of washed residues in order to obtain quantitative data directly comparable. We avoid the counts on the smaller fractions (<100 μm) because of the presence of several juveniles.

In addition, we converted our absolute data in relative abundances as expressed in percentages, with the aim to compare our absolute counts with relative counts from D'Onofrio et al. (2016). This comparison was performed by taking in account only those residues treated here with the same procedures adopted by D'Onofrio et al. (2016). Those authors obtained residue from sample CM through the H_2O_2 at 25% treatment and used the acetic acid method for samples MA and LM.

3.2.1 Diversity indices

Absolute counts were also used to calculate a series of diversity indices through the dedicated package in the PAST software (http://folk.uio.no/ohammer/past/) including: Richness, Menhinick, Margalef, Fisher alpha, Shannon, Dominance, Berger-Parker, Simpson, Equitability and Buzas-Gibson' Evenness (for major details see Harper, 1999; Hammer et al., 2001; Hammer and Harper, 2006 and references therein).

These diversity indices measure different characteristics of the assemblages through different parameters and equations. Specifically, those giving information about the assemblages richness are the following: *Richness index* that simply indicates the number of the species/taxa (S); *Menhinick index* calculated by the equation $S/\sqrt{n}$ also includes the root square of sample or census size (n), i.e., the total number of individuals; *Margalef index* that uses instead the logarithm of the census size $(S-1)/\ln n$; *Fisher alpha index* (α) that defines richness through the formula $S = \alpha * \ln(1 + n/\alpha)$ assuming that abundances are distributed according to a logarithmic model; *Shannon index* (entropy, H) that ranges from 0 (a single taxon) to high values (community well diversified) and considers both the number of taxa and the number of individual through the equation $H = \sum((n_i/n)\ln(n_i/n))$. Other diversity indices highlight the dominance of a certain taxon or taxa over the others in the assemblages such as: *Berger-Parker index* that considers the number of individuals of the dominant taxon (n_i) relatively to the census size (n); *Dominance index* (D) that varies from 0 (equally distributed taxa) to 1 (assemblages dominated by a single taxon) and is defined by the equation $D = \sum(n_i/n)^2$ where n_i is number of individuals of taxon *i*. Conversely, some indices indicate the heterogeneity or the evenness (equitability) of the assemblages, i.e. how the individual are distributed within the different species, these are: *Simpson index* which considers the proportion of the species ($p_i = n_i/n$) and is calculated as $\lambda = \sum(p_i^2)$ or as $\lambda = 1 - D$; *Equitability* (E) that measures the evenness with which individuals are divided among the taxa present and it is defined by the formula $E = H/\ln n$; *Buzas-Gibson's evenness index* defined as $B = e^H/S$.

3.3. Proxies for planktic foraminiferal dissolution

We describe below the three selected proxies here adopted to evaluating planktic foraminiferal dissolution: the fragmentation index (F-index), the plankton benthos ratio (P/B) and the weight percent coarse fraction (WPCF).

3.3.1. Fragmentation index

Planktic foraminifera tend to break into fragments when they begin to dissolve (Berger, 1970, 1973; Bé et al., 1975; Leon-Rodriguez and Dickens, 2010; Nguyen and Speijer, 2014). Consequently, the fragmentation index (F-index), that evaluates the amount of broken tests, is a largely adopted proxy for planktic foraminiferal dissolution. This proxy was evaluated here as number of fragments or partially dissolved planktic foraminiferal tests that showed missing or deteriorated chambers and substantial breakage *versus* entire tests per gram of sediment. We express the values also as a percentage according to Berger (1970) (100 □sum of fragments / (sum of fragments + sum of entire tests)) in order to compare absolute and relative counting. We avoid species level identification of fragmented tests because test recrystallization in many cases does not allow precise identification.

3.3.2. Plankton Benthos ratio

Most planktic foraminifera dissolve preferentially relative to benthic foraminifera. Therefore, the plankton benthos ratio (P/B), often adopted for paleobathymetric estimates (e.g., Murray, 1976; Van der Zwaan et al., 1990), also can be applied as a dissolution index (e.g., Hancock and Dickens, 2005; Nguyen et al., 2009; Nguyen and Speijer, 2014). The P/B index was here calculated on the total number of planktic vs benthic foraminifera from the same aliquot used for the quantitative species count. The obtained data were also compared with relative estimation evaluated as 100 * P / (P + B). We can consider this proxy as reliable to estimate planktic foraminiferal dissolution here because at the Terche section planktic foraminifera dominate throughout (>90%, D'Onofrio et al., 2016) (Fig. 2). Decreases of P/B will therefore indicate loss of planktic foraminifera due to test dissolution instead of paleodepth changes.

3.3.3. Weight Percent Coarse Fraction (WPCF)

Planktic foraminifera, including juvenile specimens, generally exceed 38 μm and thus belong to the coarse sediment grain size. However, dissolution of planktic foraminiferal tests, commonly occur within the lysocline, produce smaller fragments and led to enrich the finer fraction (≤38 μm) (e.g.,

Berger et al., 1982). For this reason, the WPCF it is generally adopted to make inferences about carbonate dissolution besides the primary productivity. This parameter is calculated here as the ratio between the weight of the ≥100 μm dry fraction because it is the fraction here analysed. However, the most commonly used WPCF expressed as the weight of the dry ≥38 μm fraction over the weight of bulk dry sediment (Hancock and Dickens, 2005) is also evaluated for a comparison. We must underline that this parameter can be affected by an increase in calcareous nannofossil and/or planktic foraminiferal production as well as terrigenous input from nearby continental area (Hancock and Dickens, 2005).

4. Results

The effectiveness of the different methodologies here investigated is assessed for each sample as a function of: (1) disaggregation speed; (2) degree to which the sample was disaggregated; (3) quality of planktic foraminiferal preservation as they appear at the stereomicroscope and scanning electronic microscope; (4) variation of dissolution proxies; (5) absolute and relative abundance of planktic foraminiferal genera and species; (6) change in species diversity.

4.1. Disaggregation speed and disaggregation degree

Disaggregation speed and disaggregation degree differ for the different methods adopted, as expected, with a general inverse proportion to the $CaCO_3$ content. We summarize below the main results and show the required runtime and disaggregation steps/response of each sample to the adopted treatment in Table 1.

Table 1. Summary of the main disaggregation steps and runtime observed for the tested methods: required time to reach a mousse aspect (Ma); disaggregation speed (i.e., required runtime for complete disaggregation, CD); required number of freeze-thaw cycle (FTC).

	METHODS											
	ACETIC ACID		H_2O_2 [25%]		H_2O_2 [10%]		NEO-STERAMINA		LIQUID N_2			
SAMPLE	Ma	CD	Ma	CD	Ma	CD	Ma	CD	Ma	CD	FTC	

CM TRE/13 +943 ($CaCO_3$ 37%)	2 h*	3 h	Instant of soaking	1 h	Instant of soaking	1 h	6 h	12 h	Instant of soaking	0.25 h	1
MA TRE/11 -30 ($CaCO_3$ 55%)	3 h	4 h	1h	3 h	1h	3.5 h	12 h	48 h	//	3 h	7
LM TRE/13 +1137 ($CaCO_3$ 72%)	3 h	4 h	//	>72 h	//	>72 h	24 h	48 h	//	8 h	25
			Failed disaggregation		Failed disaggregation				Failed disaggregation		

Sample CM. The more rapid disaggregation (15 minutes) was obtained with the liquid N_2 method that required only one cycle of treatment. Disaggregation with H_2O_2, at both 10% and 25% was also quite rapid (one hour) whereas the acetic acid and neo-steramina treatments needed respectively three and twelve hours. A satisfactory disaggregation was obtained through all the laboratory procedures with the exception of H_2O_2, at both 10% and 25% that left some sediment clumps.

Sample MA. The H_2O_2 at 10% and 25% and liquid N_2 treatments required ca three hours for disaggregating the sample. Seven cycles of freeze-thaw were necessary through the liquid N_2 method. The sample treated with acid acetic was disaggregated after four hours whereas the neo-steramina process released foraminifera after 48 hours. Acetic acid and neo-steramina proved to give a complete disaggregation whereas liquid N_2 and H_2O_2 at both 10% and 25% methods left some clumps of sediment and encrusted foraminifera. Specifically, presence of encrustations on foraminifera extracted with the H_2O_2 treatments allowed only identification at the genera level since obscured some important morphological features (e.g. aperture, sutures, wall-texture etc.) necessary to define the species (e.g. Pearson et al., 2006; Fenton et al., 2018).

Sample LM. Only the acetic acid and the neo-steramina treatments revealed to be effective in disaggregate this sample. Disaggregation occurred in four hours with the acetic acid method whereas the neo-steramina treatment required 48 hours for extracting foraminifera. The latter however left some clumps of sediment. The H_2O_2, at both 10% and 25% even after twelve days of sample soaking showed totally incomplete disaggregation. The liquid N_2 method, instead, was interrupted after 25 freeze-thaw cycles because starting from the 18^{th} cycle proved to be ineffective as no new residue ≥38 and ≥63 μm was released.

4.2. Quality of planktic foraminiferal preservation

The quality of foraminiferal preservation is described below in terms of their appearance at the stereomicroscope (clean tests vs encrusted test wall) (Plate 1) and at the SEM (dissolved *vs* well preserved wall-texture) (Plates 2–5). In general, a good preservation at the stereomicroscope that allows us to recognize the morphological characters was observed in specimens extracted with the neo-steramina, liquid N_2 and with the acetic acid treatments. However, the different wall-textures of early Eocene planktic foraminifera (muricate, cancellate, smooth walled) revealed to show different degree of preservation according to the different laboratory procedures and carbonate content.

Sample CM. Planktic foraminifera appear rather well preserved at the stereomicroscope for all laboratory procedures adopted thus allowing a correct species identification (Plate 1). At the SEM observation, the characteristic 'muricate' *Acarinina*, a term derived from the muricae that form layered pustules on the test wall, appear better preserved with the acetic acid and liquid N_2 whereas the murico-keel of *Morozovella* looks well detectable through the neo-steramina method (Plate 2). Best preserved thin-walled *Chiloguembelina* tests, considered to be as dissolution fragile (Nguyen et al., 2011), were detected in residue obtained with the liquid N_2 method (Plate 3). The honeycomb wall of *Subbotina* is better recognizable with the H_2O_2 at both 10% and 25% and acetic acid treatments (Plate 3).

Sample MA. The neo-steramina, acetic acid and liquid N_2 treatments proved to be the methods revealing the best preserved washed residues (Plate 1). At the SEM observation, acetic acid and liquid N_2 appear again to have better preserved the muricae of *Acarinina* but the murico-keel of *Morozovella* is well detectable with the acetic acid, neo-steramina and liquid N_2 treatments (Plate 2). Best preserved *Chiloguembelina* tests were observed again in residue obtained with the liquid N_2 method. The cancellate wall-texture of *Subbotina* appears better recognizable in washed residue treated with acetic acid procedure (Plate 3).

Sample LM. Acetic acid and neo-steramina provided the sole disaggregated residues (Plate 1). However, some surface encrustations are still present on foraminiferal tests, especially in washed

residue deriving from the neo-steramina method. The SEM images clearly show that best preserved muricae, murico-keel and honeycomb texture was obtained through the acid acetic method (Plate 4, 5).

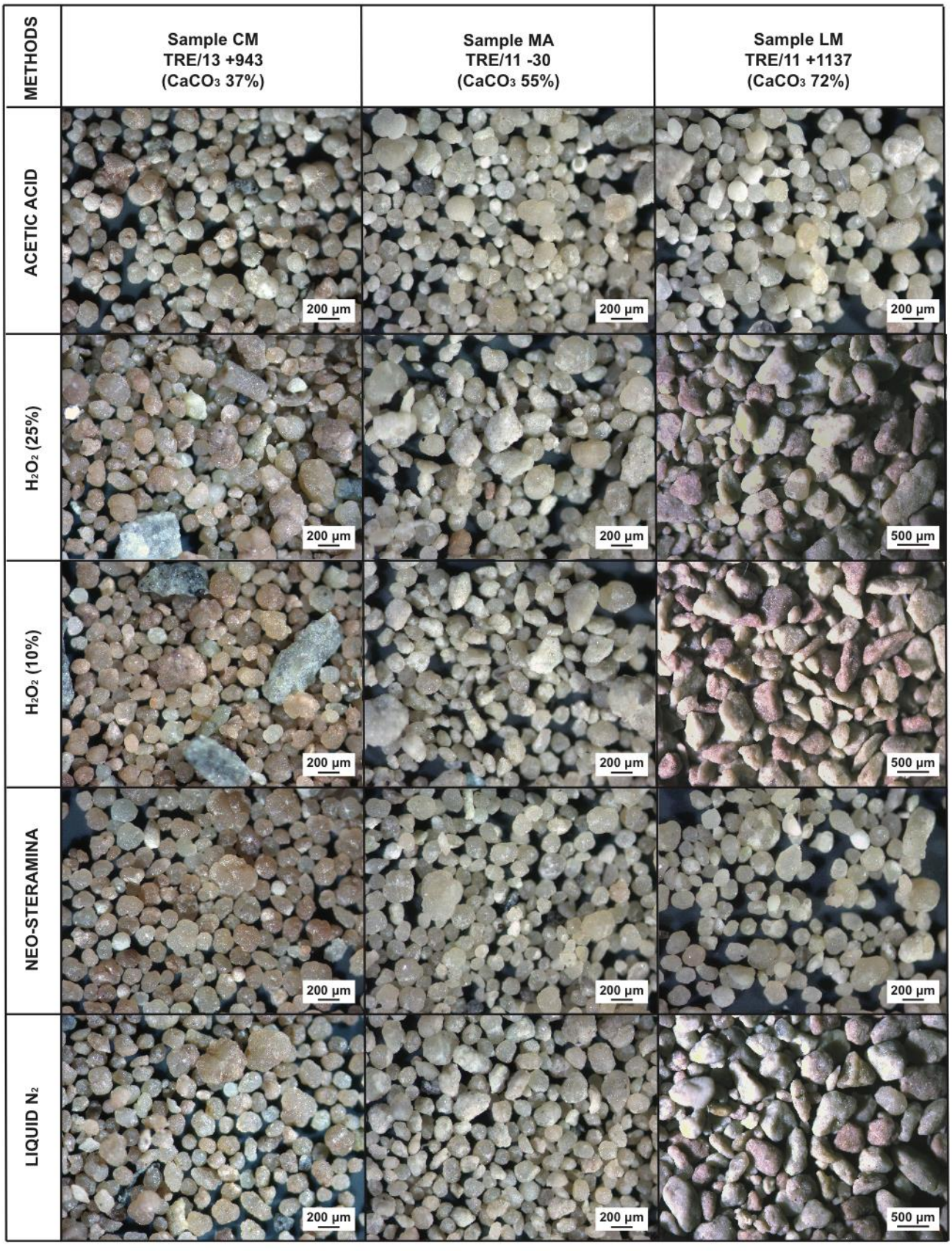


Plate 1. Pictures showing the washed residues obtained for each of the investigated samples through the five extraction techniques selected in this study. Images were taken using a Zeiss stereomicroscope equipped with a camera and the dedicated imaging software ZEN 2 CORE. Note that planktic foraminifera are clean for all the treatments adopted in sample CM although some clumps occur in residues obtained through the H_2O_2 methods. In sample MA encrusted tests and sediment clumps occur in the H_2O_2 (10% and 25%) and liquid N_2 treated residues. Liquid N_2 and H_2O_2 (both 10% and 25%) revealed to be ineffective for disaggregating sample LM.

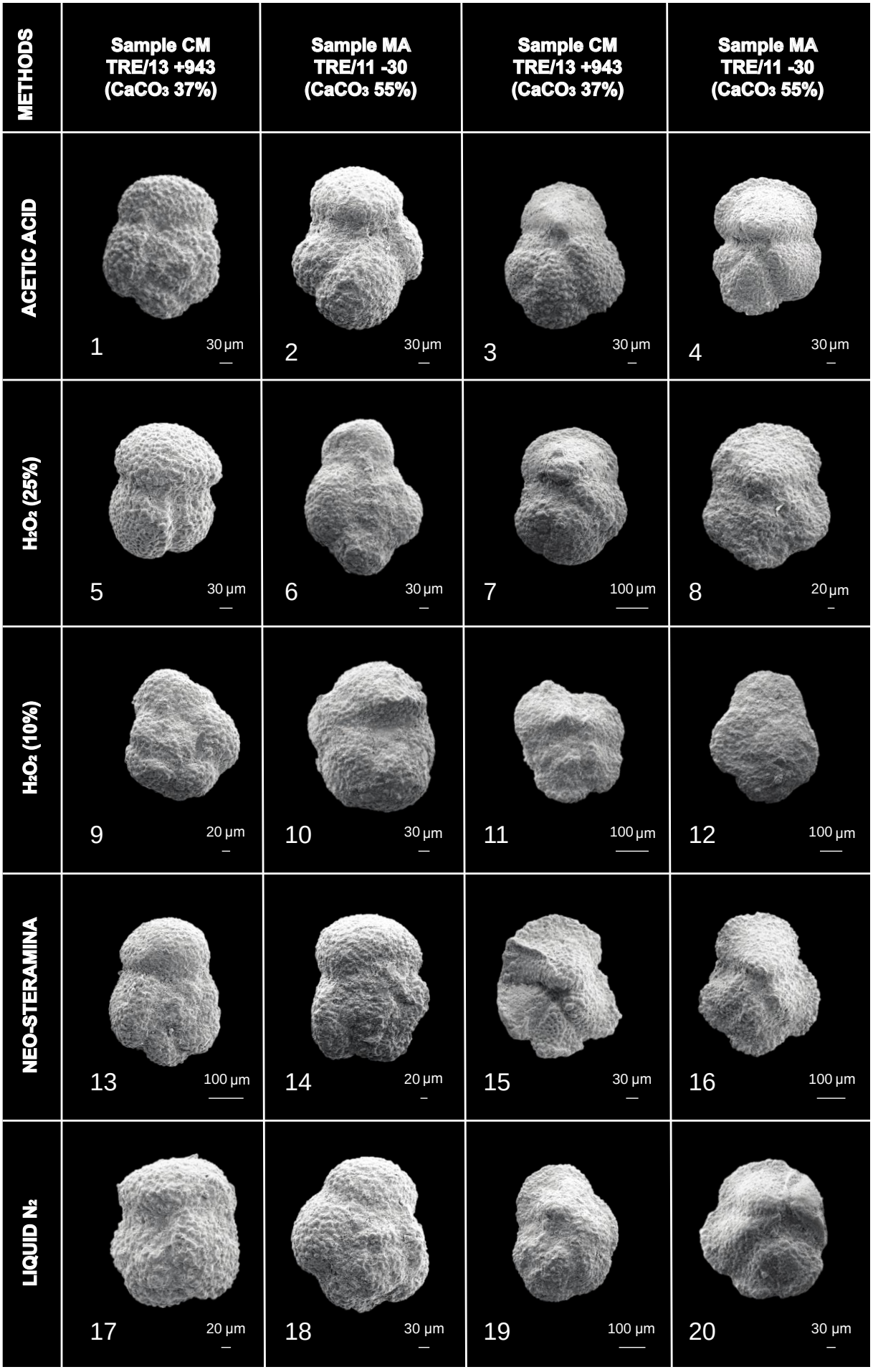

METHODS
Sample CM TRE/13 +943 (CaCO3 37%)
Sample MA TRE/11 -30 (CaCO3 55%)
Sample CM TRE/13 +943 (CaCO3 37%)
Sample MA TRE/11 -30 (CaCO3 55%)
ACETIC ACID
H2O2 (25%)
H2O2 (10%)
NEO-STERAMINA
LIQUID N2
1 30 µm
2 30 µm
3 30 µm
4 30 µm
5 30 µm
6 30 µm
7 100 µm
8 20 µm
9 20 µm
10 30 µm
11 100 µm
12 100 µm
13 100 µm
14 20 µm
15 30 µm
16 100 µm
17 20 µm
18 30 µm
19 100 µm
20 30 µm

Plate 2. SEM images of the muricate walled *Acarinina* and *Morozovella* species picked from washed residues of samples CM and MA obtained with the different extraction techniques here investigated. 1: *Acarinina wilcoxensis*; 2: *A. interposita*; 5: *A. coalingensis*, 6, 9: *A. angulosa*; 10: *A. alticonica*; 13: *A. esnaensis*; 14, 17: *A. quetra*; 18: *A. soldadoensis*. 3, 12: *Morozovella lensiformis*; 4, 7, 8: *M. subbotinae*; 11: *M. gracilis*; 15, 16: *M. marginodentata*; 19: *M. crater*; 20: *M. formosa*.

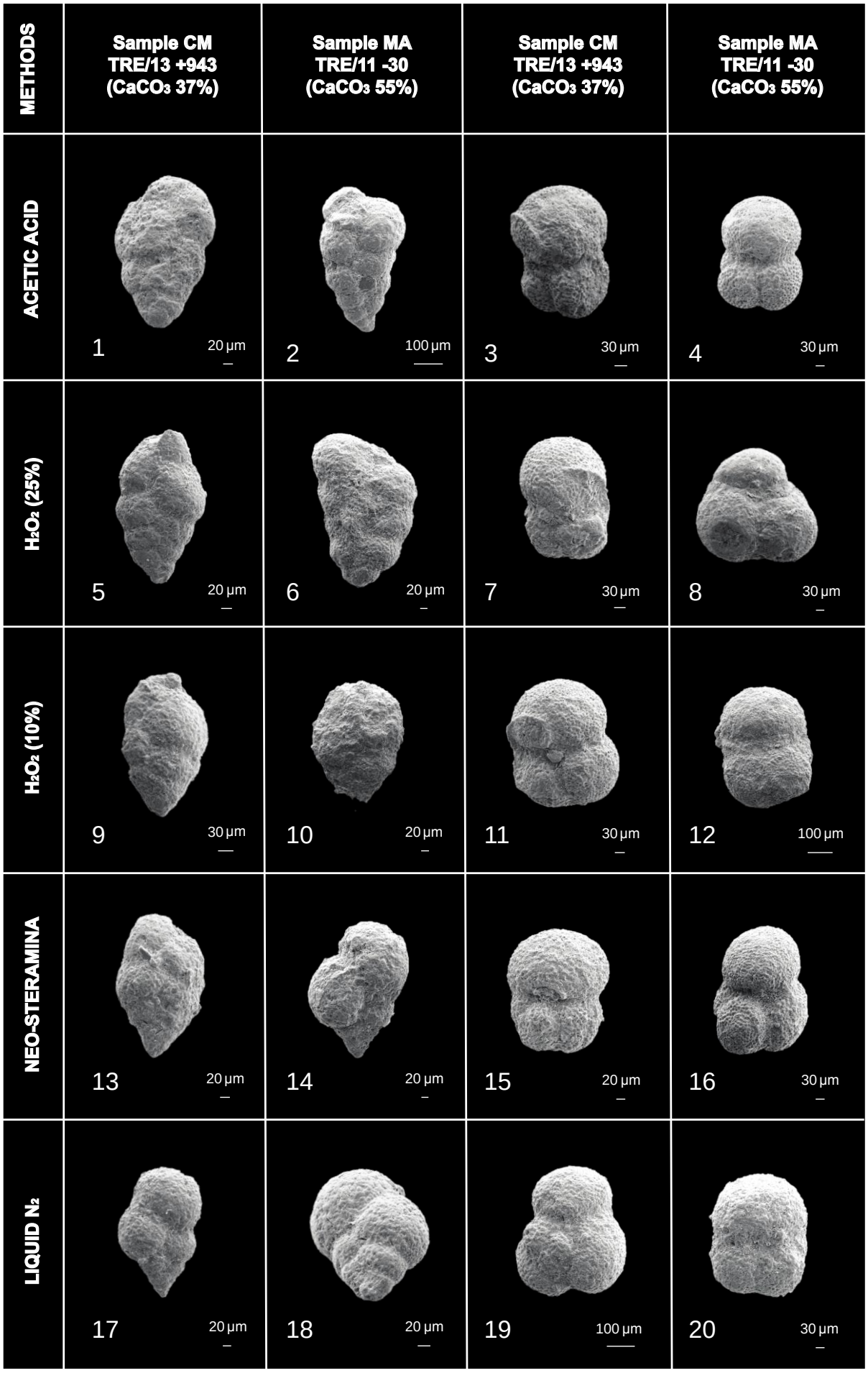

METHODS
Sample CM TRE/13 +943 (CaCO3 37%)
Sample MA TRE/11 -30 (CaCO3 55%)
Sample CM TRE/13 +943 (CaCO3 37%)
Sample MA TRE/11 -30 (CaCO3 55%)
ACETIC ACID
H2O2 (25%)
H2O2 (10%)
NEO-STERAMINA
LIQUID N2
1 20 µm
2 100 µm
3 30 µm
4 30 µm
5 20 µm
6 20 µm
7 30 µm
8 30 µm
9 30 µm
10 20 µm
11 30 µm
12 100 µm
13 20 µm
14 20 µm
15 20 µm
16 30 µm
17 20 µm
18 20 µm
19 100 µm
20 30 µm

Plate 3. SEM images of planktic foraminiferal species picked from washed residues of samples CM and MA obtained with the different extraction techniques here investigated. 1, 2, 6, 13, 18: *Chiloguembelina crinita*; 14, 17: *C. wilcoxensis*; 10: *Chiloguembelina* sp.; 5, 9: *Zeauvigerina sp.*; 3, 4, 7, 11, 12, 15, 20: *Subbotina patagonica*; 8, 16: *S. hornibrooky*; 19: *S. roesnaesensis.* Note that the acid acetic and H_2O_2 methods appear to induce some dissolution in *Chiloguembelina* tests (see in particular images 1, 2, 10, 13).

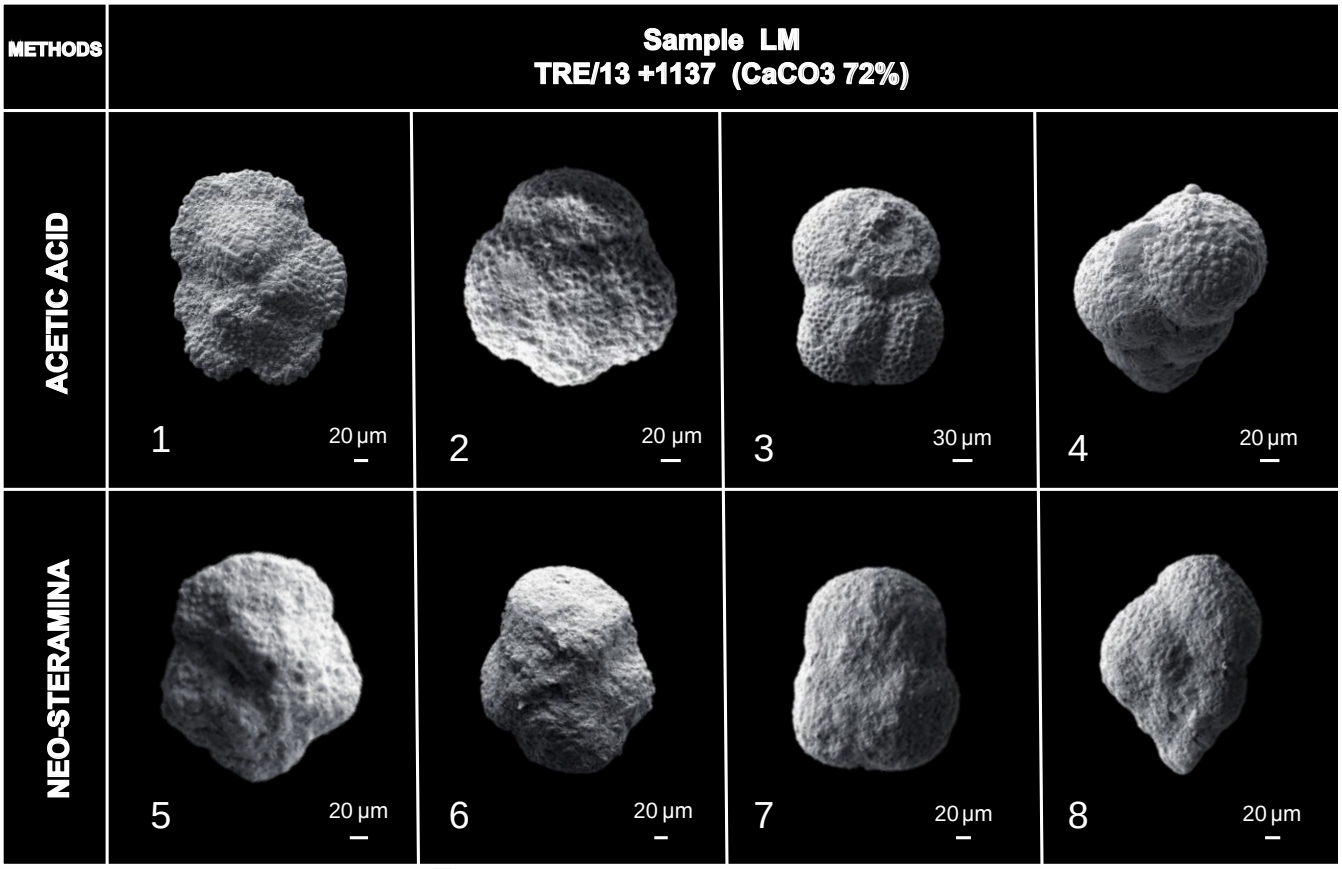


Plate 4. SEM images of the species picked from the two residues obtained for sample LM. 1, 5: *M. marginodentata*; 2: *A. soldadoensis*; 3: *S. patagonica;* 4: *C. wilcoxensis*; 6: *Acarinina* sp.; 7: *Subbotina* sp.; 8: *Chiloguembelina sp.* Note that species treated with the neo-steramina appear badly preserved.

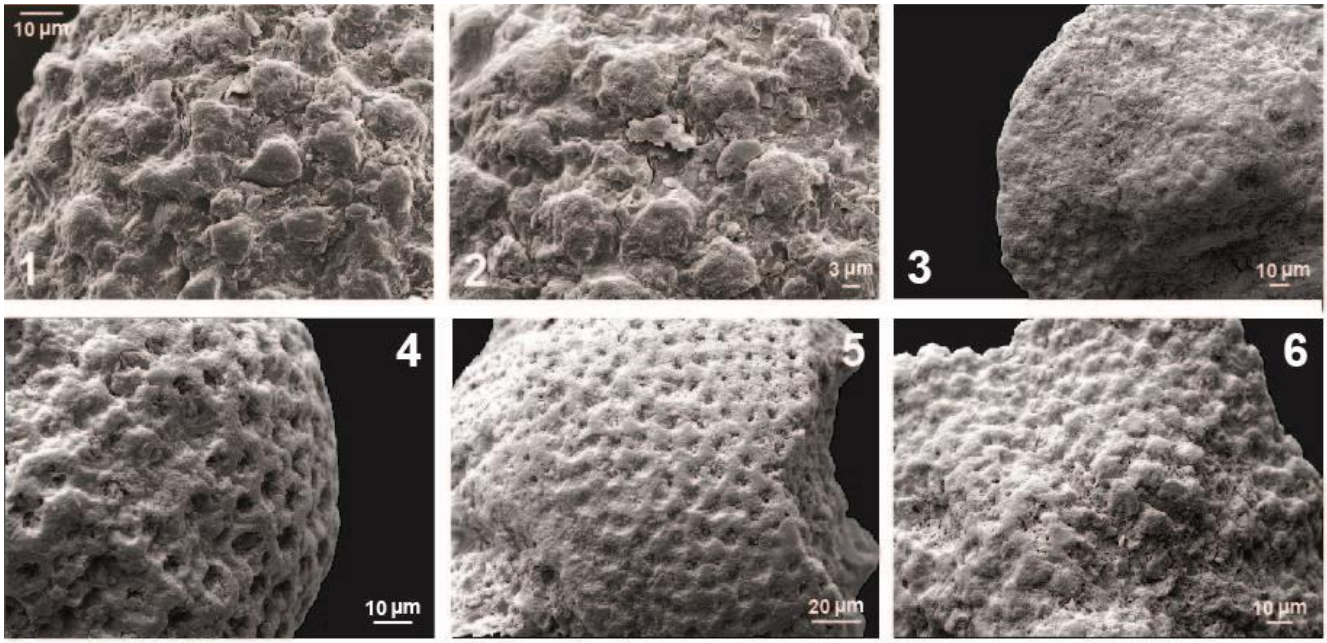


Plate 5. Zoomed SEM images of foraminiferal wall textures as resulted from different treatments. 1 – preserved muricae from spiral side of *Acarinina soldadoensis* (sample CM) treated with the acetic acid method. 2 –very badly preserved muricae from spiral side of *A. esnaensis* (sample MA) treated with the H_2O_2 (25%) method. 3 – badly preserved muricae from umbilical side of *Morozovella marginodentata* (sample LM) treated with the neo-steramina method.4 – well-preserved honeycomb texture from *Subbotina patagonica* (sample LM) treated with the acetic acid method. 5 – badly preserved honeycomb texture from *S. patagonica* (sample LM) treated with the neo-steramina method. 6 – well-preserved muricae from umbilical side of *M. marginodentata* (sample LM) treated with the acetic acid method.

4.3. Dissolution proxies

The main results from analysis of the three dissolution proxies adopted (F-index, P/B and WPCF) are summarized below for each sample and shown in Table S1, figures 3, and 4. In particular, figure 3 shows beside the absolute count also the relative values for a comparison. Variations of the dissolution proxies revealed to be dependent, once again, by both the methods adopted and by the carbonate content. The F-index values display the most evident changes whereas the P/B and WPCF indices show only slight variations for all the procedures and for the three samples investigated.

Sample CM. The greater number of fragments per gram of sediment was recorded in the washed residue treated with H_2O_2 at 25% whereas the lower amount of fragments is present in the residue prepared with neo-steramina. The variation of F-index values as expressed in percentage shows similar

trend with those from absolute count with the exception of the H_2O_2 at 10% which displayed the highest relative value (Fig. 3A).

The lower number of benthic foraminifera was recorded in washed residue treated with liquid N_2 as confirmed by the highest value of the P/B from the same residue (Fig. 3B). Slightly higher values of WPCF were obtained from the H_2O_2 at both 25% and 10% treatments for both >38 μm and >100 μm fractions (Fig. 4, S1).

Sample MA. For this sample F-index shows high values with H_2O_2 at 25% and 10% and liquid N_2 treatments. Similar variations are appreciable when data are plotted as relative abundance of fragment vs entire tests (Fig. 3C). A certain increase of benthic foraminiferal specimens is recorded in the same washed residues whereas acetic acid and neo-steramina residues record higher percentages of the P/B (Fig. 3D).

Greater values for WPCF derive from disaggregation with H_2O_2 at 10%, 25% and liquid N_2 for coarse fraction evaluated for both >38 μm and >100 μm fractions (Fig. 4, S1).

Sample LM. Planktic foraminiferal fragments result relatively high for both neo-steramina and acetic acid treatments. This is also clear from the percentage values (Fig. 3E). The P/B values are somewhat lower with respect to samples with lower carbonate content (Fig. 3F). Slightly higher values of WPCF (>100 μm) were obtained from the neo-steramina method (Fig. 4). Nonetheless, the WPCF proxy based on the >38 μm fraction shows twice the value for neo-steramina method when compared to the acetic acid treatment (8.50% vs 4.82%) (Fig. S1).

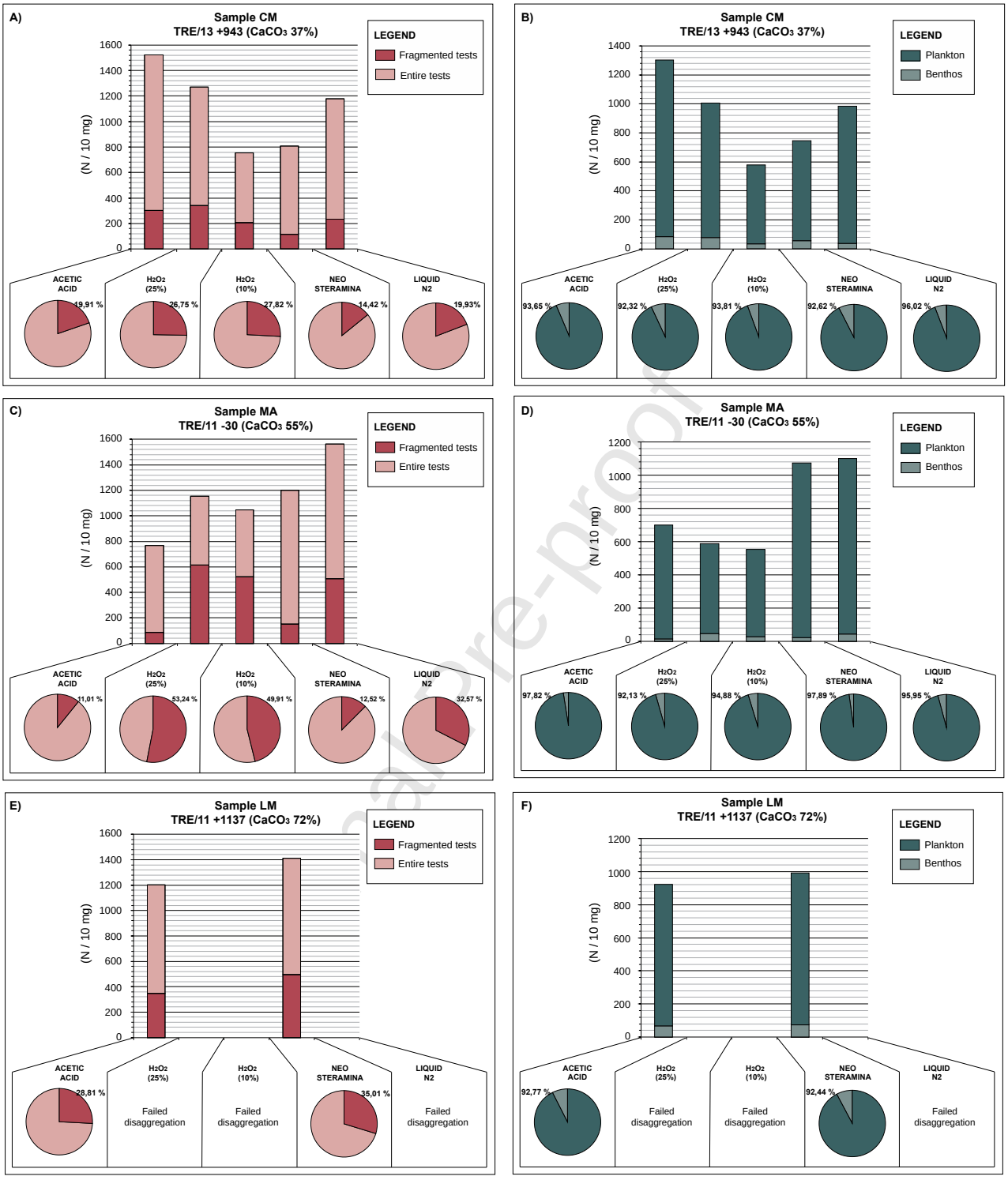
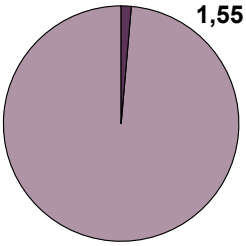
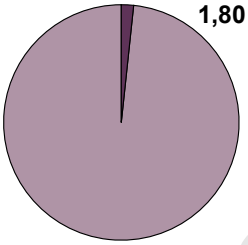
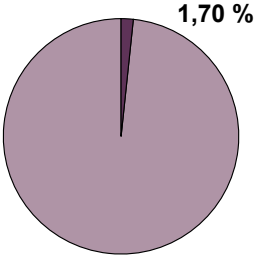
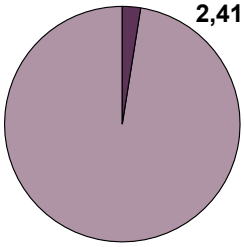
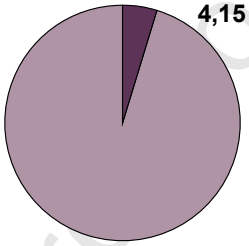
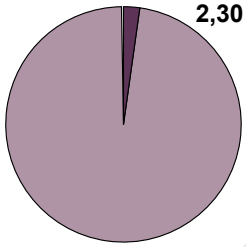
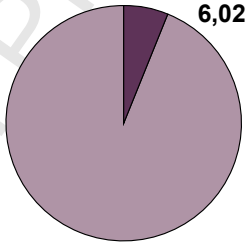
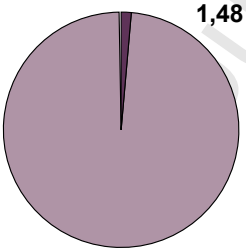
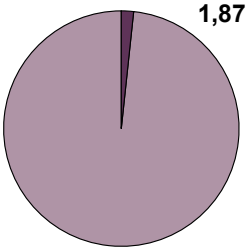
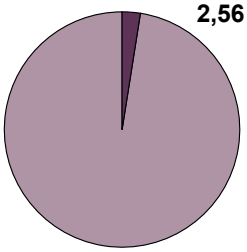
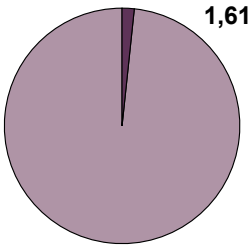
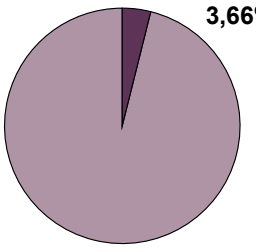


Figure 3. Bar charts showing the planktic foraminiferal dissolution proxies F-index and P/B evaluated as number of fragments vs entire tests and number of planktic vs benthic foraminifera from 10 mg of washed residue. Relative counts are plotted as pie charts for comparison with the absolute values. Note that major values of F-index occur for sample MA treated with the H_2O_2 at both the concentrations whereas lowest values of P/B were recorded in residues obtained from sample LM. See discussion in the text.

METHODS	WPCF >100 µm (%)		
	Sample CM TRE/13 +943 ($CaCO_3$ 37%)	Sample MA TRE/13 -30 ($CaCO_3$ 55%)	Sample LM TRE/13 +1137 ($CaCO_3$ 72%)
ACETIC ACID			
H_2O_2 (25%)			Failed disaggregation
H_2O_2 (10%)			Failed disaggregation
NEO-STERAMINA			
LIQUID N_2			Failed disaggregation

Coarse fraction (>100 µm) Bulk sediment

Figure 4. Pie charts showing the weight percent coarse fraction (WPCF) as calculated for the ≥100 μm fraction for the three samples examined. See discussion in the text.

4.4. Planktic foraminiferal absolute abundances

The absolute abundance of species and genera expressed as number of specimens per 10 mg of washed residue is here presented, for the three analysed samples, in relation with the five laboratory procedures adopted (Fig. 5, Table S2). Several planktic foraminiferal species change their abundance according to the different methods and show different degrees of variations for the diverse carbonate content. The most marked reductions are particularly evident for species with greater abundance. We must specify that for the sample MA treated with H_2O_2 at both concentrations the occurrence of clumps and encrustations prevents the correct taxa identification whereas identification of the biserial species was hampered in some cases by the bad preservation. *Chiloguembelina*, here represented by the species *C. crinita* and *C. wilcoxensis*, was therefore counted at the genus level whereas *Zeauvigerina*, being extremely rare, was excluded from the counts.

Sample CM. Several species show higher abundances when washed residues are prepared with the acid acetic treatment (Fig. 5A). This is particularly evident for *Acarinina esnehensis, A. angulosa, A. wilcoxensis, Morozovella aequa, M. subbotinae, M. gracilis, M. crater, Igorina broedermanni, Subbotina patagonica* and *Globanomalina australiformis*. Conversely, *A. alticonica, A. soldadoensis, A. interposita* and *M. formosa* decrease their absolute abundances with the acetic acid treatment and increase with the H_2O_2 at 25%. *A. coalingensis* and *M. marginodentata* are abundant in residues treated with both the acetic acid and the H_2O_2 at 25% whereas *A. esnaensis* and *Planorotalites pseudoscitula* markedly increase in abundance with the liquid N_2.

The variations of planktic foraminiferal absolute abundance at the genus level mainly reflect the changes recorded by the most abundant species for each genus so that *Acarinina* is more abundant in the washed residue prepared with acetic acid, H_2O_2 at 25% and liquid N_2 treatments (Fig. 5D). Similarly, *Morozovella* and *Subbotina* absolute abundance results greater for the acetic acid treatment. Absolute abundances of *Acarinina* drops with H_2O_2 at 10% and neo-steramina methods. A decrease of specimens

is evident as well for *Morozovella*, *Igorina* and *Subbotina* when washed residue was obtained with H_2O_2 at 10%. *Igorina* and *Planorotalites* are more abundant in residues prepared with the liquid N_2. *Paragloborotalia* is absent with acetic acid and H_2O_2 at 25%. On the contrary, *Globoturborotalita* is present only with the same methods.

Sample MA. From figure 5B appears evident that species show an opposite behaviour with respect to sample CM as many of those decrease in abundance with the acetic acid treatment and increase with liquid N_2 and neo-steramina methods. Specifically, the species *Acarinina soldadoensis, A. coalingensis, Morozovella crater, Subbotina patagonica, S. roesnaesensis,* and *Chiloguembelina* spp. increase with both the liquid N_2 and neo-steramina methods. Conversely, *C. unicavus* and *Globoturborotalita bassriverensis* are only present in sample obtained with the acetic acid method. As compared to sample CM, *A. esnaensis* peaks in abundance in residue prepared with the neo-steramina method instead that with the liquid N_2. A certain increase in sample treated with the neo-steramina was also observed for *M. aequa, M. lensiformis, M. formosa* and *Igorina broedermanni* whereas *A. angulosa, M. subbotinae, G. australiformis* and *Pseudohastigerina wilcoxensis* display higher abundances when residues are prepared with the liquid N_2.

The variations of planktic foraminiferal absolute abundance at the genus level are in line with changes recorded at species level and include data for H_2O_2 treatments (Fig. 5E). With the exceptions of *Catapsydrax* and *Globoturborotalita* that only occur in sample obtained through the acetic acid, all genera show decrease in abundance for these methods with respect to neo-steramina and liquid N_2.

Sample LM. As noted above, species absolute abundances were evaluated only for acetic acid and neo-steramina (Fig. 5C). Most *Acarinina* and *Morozovella* species including *A. soldadoensis, A. esnaensis, A. wilcoxensis, A. coalingensis, M. subbotinae, M. crater* and *M. lensiformis,* as well as *Planorotalites pseudoscitula, Globanomalina planoconica, G. australiformis* and *Pseudohastigerina wilcoxensis*, increase in abundance for the neo-steramina treatment. By contrast, a greater abundance of *Igorina lodoensis, Subbotina patagonica, S. roesnaesensis, S. hornibrooki* and *Chiloguembelina* spp. was reached in residue treated with the acetic acid. *Paragloborotalia griffinoides* occurs only with neo-

steramina method whereas, similarly from sample MA, *Catapsdrax unicavus* and *G. bassriverensis* were only observed in residue obtained with the acetic acid.

At genus level *Acarinina*, *Morozovella*, *Planorotalites*, *Globanomalina*, *Pseudohastigerina* and *Paragloborotalia* display greater abundance with the neo-steramina method whereas *Igorina*, *Subbotina*, *Parasubbotina*, *Catapsydrax* and *Globoturborotalita* with the acetic acid treatment (Fig. 5F).

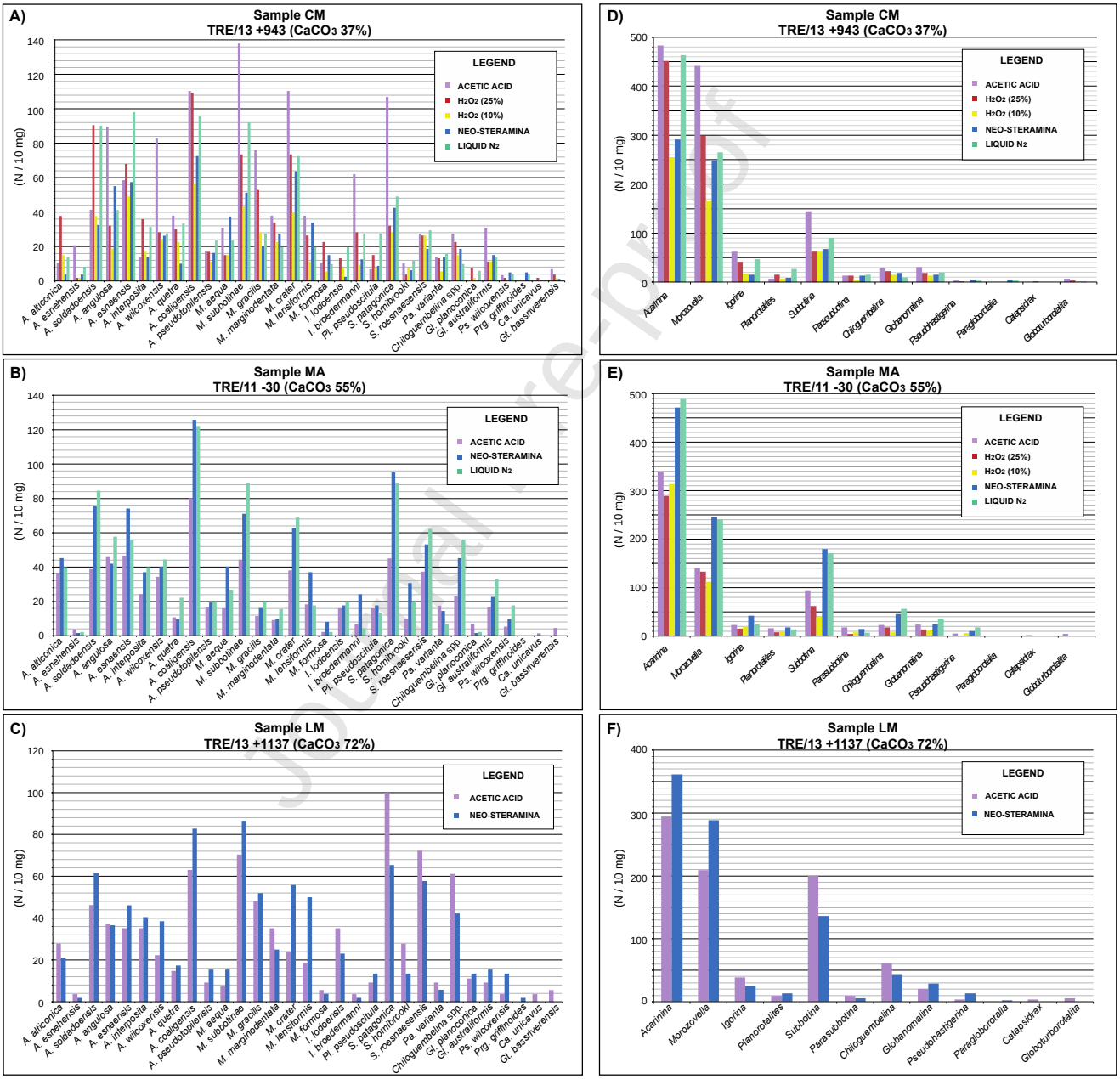


Figure 5. Bar charts showing the number of planktic foraminiferal species (on the left) and genera (on the right) counted as number of specimens per 10 mg of washed residue from the three samples analysed. Note that species and genera abundance changes according to the laboratory method adopted

and the carbonate content. These evidences imply that the laboratory techniques may influence the planktic foraminiferal abundance.

4.5 Planktic foraminiferal diversity indices

Despite the marked variations observed for species and genera absolute abundances, our analysis of diversity indices highlights that the laboratory procedures here compared do not induce significant changes in diversity, dominance, equitability or richness (Table 2 and Figure 6). Moreover, considering the limits of natural variance, there is no straight relationship between changes in diversity indices and the $CaCO_3$ content. The collected data reveal that simple Richness (i.e., number of species) is basically maintained for all the methods thus implying that none of the investigated techniques produced a significant loss of species. However, minor loss of species number was observed in the sample CM as Richness decreases from 31 to 27 with the acid acetic treatment. In the sample MA Richness value is 28 from the washed residue treated with neo-steramina and liquid N_2 and it is 29 in sample LM when prepared with neo-steramina. Other minor variations are observed in residues treated with the acetic acid for the Fisher alpha and Margalef indices that barely decrease in sample CM but increase in both the samples with higher carbonate content. Conversely, the Berger-Parker index for dominance slightly increases in samples CM and LM when prepared with the acetic acid method.

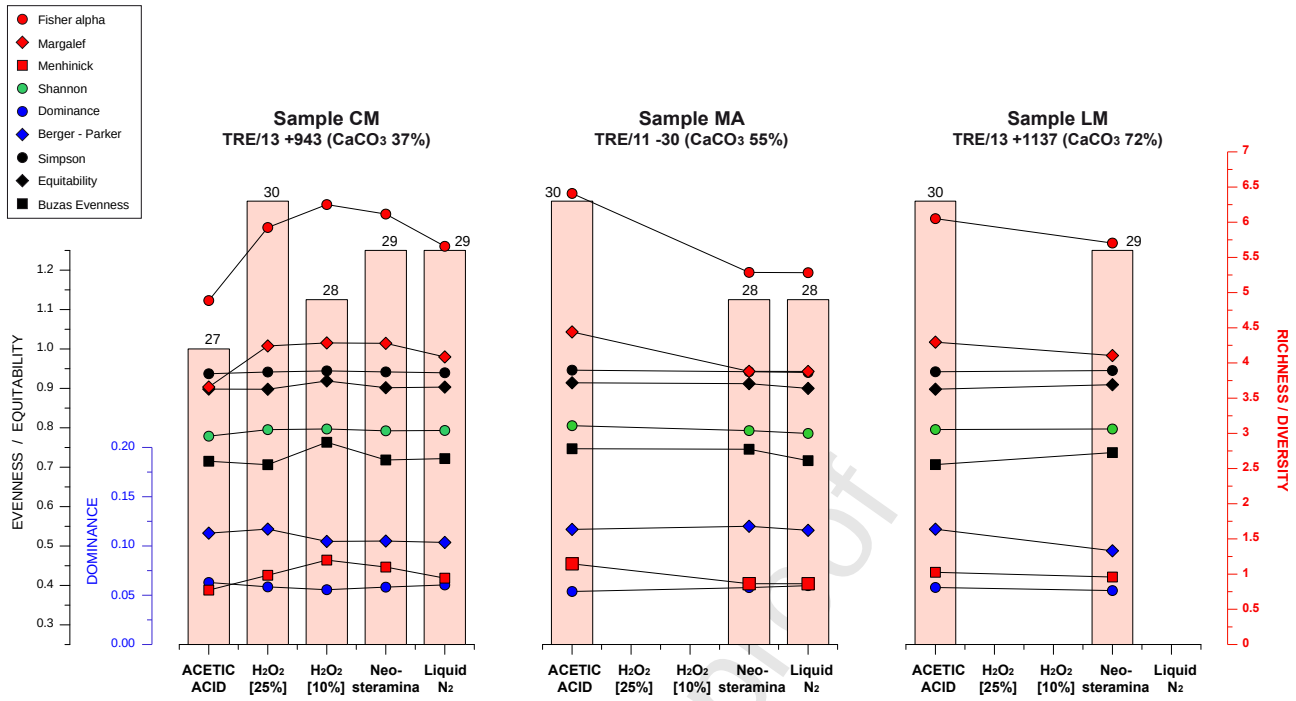


Figure 6. Diagrams showing multiple diversity indices as calculated through PAST software for the three investigated samples. In blue are shown indices indicating species dominance, in black are represented evenness and equitability indices, in red and green are shown richness and other diversity indices whereas simple Richness (i.e., the number of species) is displayed as light red bar charts.

Table 2. Diversity indices as calculated with PAST software

	Sample CM TRE/13 +943 (37% of CaCO3)					**Sample MA TRE/11 - 30 (55% of CaCO3)**			**Sample LM TRE/13 +1137 (72% of CaCO3)**	
	ACETIC ACID	H_2O_2 [25%]	H_2O_2 [10%]	NEO-STERAMINA	LIQUID N_2	ACETIC ACID	NEO-STERAMINA	LIQUID N_2	ACETIC ACID	NEO-STERAMINA
Dominance	0,063	0,058	0,056	0,058	0,060	0,054	0,058	0,060	0,058	0,055
Simpson	0,937	0,942	0,944	0,942	0,940	0,946	0,942	0,940	0,942	0,945
Shannon	2,960	3,053	3,062	3,036	3,041	3,109	3,039	2,999	3,054	3,062
Buzas Evenness	0,715	0,706	0,763	0,718	0,722	0,747	0,746	0,717	0,707	0,737
Menhinick	0,773	0,983	1,199	1,100	0,943	1,146	0,864	0,863	1,026	0,959
Margalef	3,658	4,242	4,285	4,279	4,086	4,441	3,881	3,880	4,296	4,106
Equitability	0,898	0,898	0,919	0,902	0,903	0,914	0,912	0,900	0,898	0,909
Fisher_alpha	4,887	5,925	6,251	6,116	5,658	6,409	5,287	5,283	6,051	5,704
Berger-Parker	0,113	0,117	0,105	0,105	0,104	0,117	0,120	0,116	0,117	0,095
Simple Richness	27	30	28	29	29	30	28	28	30	29

4.6. Comparison between planktic foraminiferal absolute and relative abundance

Our data, based on absolute counts of planktic foraminiferal specimens and dissolution proxies from 10 mg of washed residue, allow us a comparison with the relative counts generated by D'Onofrio et al. (2016) (Fig. 7). We summarize below the main observations.

Comparison of dissolution proxies highlight that major differences between relative and absolute counts mainly concern the F-index which results higher through absolute counts in samples CM and LM of ~20% and 10% respectively.

Interestingly, the planktic foraminiferal abundance based on relative percentages does not appear to markedly alter the proportion among the main genera. Nonetheless, results deriving from relative counts by D'Onofrio et al. (2016) show greater abundances from genus *Morozovella* in all the analysed samples with respect to percentages calculated from our absolute count. This is paralleled by lower abundances of *Subbotina* and, at minor extent, of *Chiloguembelina* in sample CM and LM for the former and in all the samples for the latter. Minor components of the assemblages, such as *Planorotalites* and *Pseudohastigerina* are basically recorded only through the absolute counts.

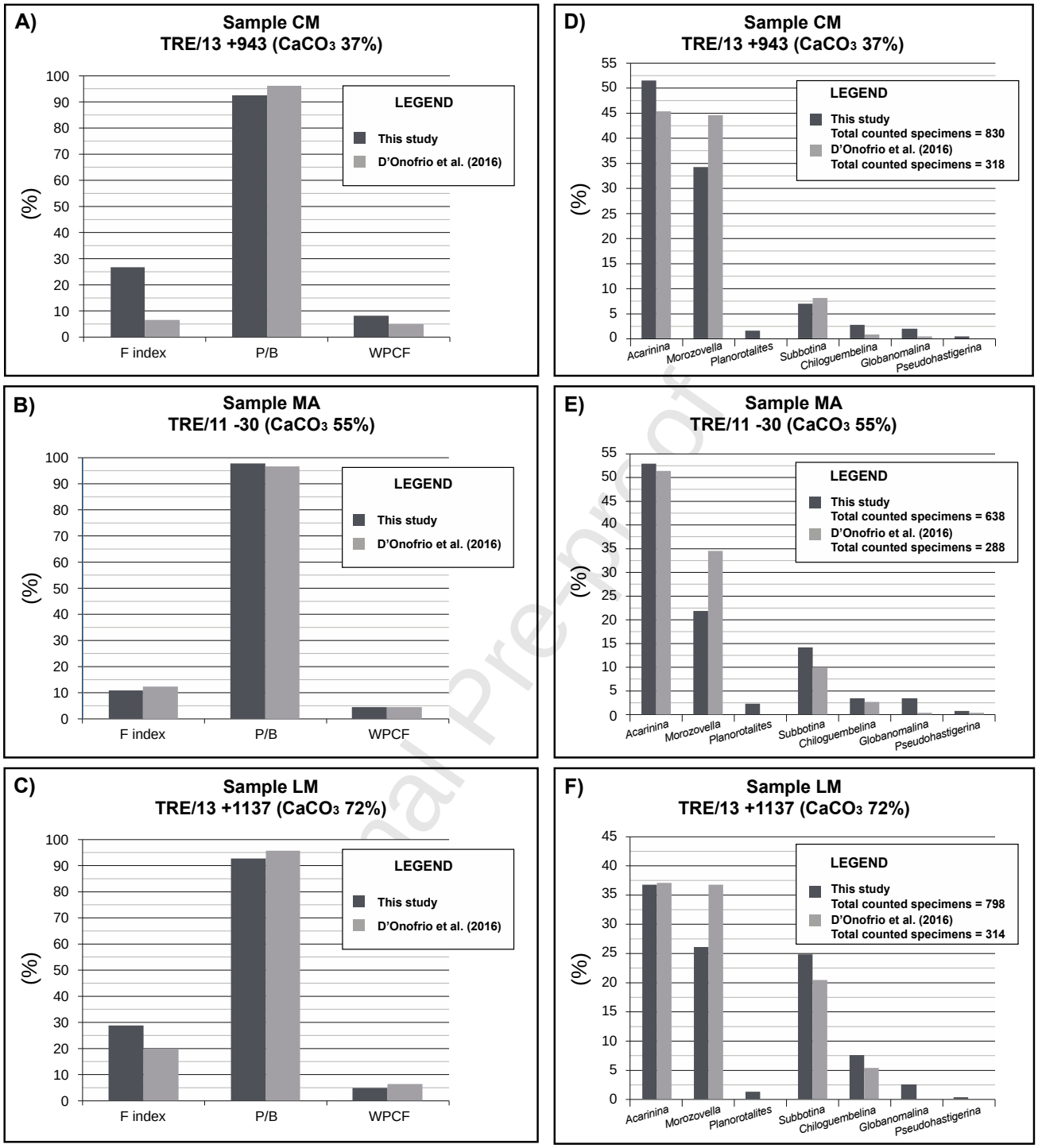


Figure 7. Bar charts showing a comparison between absolute abundance as expressed in percentages (this study) and relative abundance data (from D'Onofrio et al., 2016) on dissolution proxies and genera.

5. Discussion

Our detailed data, obtained from the comparison of five largely utilized laboratory techniques for extracting foraminifera, provide several arguments for discussion.

The first outcome concerns the time required for disaggregating samples that designates liquid N_2 method as the most rapid procedure for sample CM resulting to free clean foraminiferal tests in only 15 minutes. Conversely, whereas the neo-steramina revealed to be the long lasting treatment as it completely disaggregated the sample LM in 48 hours (Table 1).

The most critical information derives from the evaluation of the dissolution proxies and from the absolute abundance of species and genera. Actually, these parameters show variations, sometimes marked, according to different laboratory procedures adopted and carbonate content. These changes imply that some of the methods investigated induce dissolution on planktic foraminiferal tests and allow us to identify the most aggressive techniques and the dissolution susceptibility of the taxa belonging to the early Eocene Zone E4, as discussed below.

5.1. Degree of planktic foraminiferal fragmentation

The F-index is the proxy that shows the most evident changes. These changes classify the H_2O_2, at both 10% and 25%, and acetic acid procedures as the methods inducing additional fragmentation with respect to the other methods, especially for the sample MA. The absolute count approach mirrors that obtained with the relative count shown as bars and pie diagrams respectively in figure 3. These results are in line with the expectation that these methods imply chemical attack of the rock. Conversely, the high F-index value recorded for the liquid N_2 procedure from the sample MA (Fig. 3) is unexpected because this technique does not implicate chemical attack. In this latter case, test fragmentation is probably related to the rock crumbling between the fingers probably due to harder and more brittle feature of this lithology. This effect is, however, not appreciable for the sample with lowest carbonate content (37%). Furthermore, the sample MA was subject to seven cycles of treatment to obtain disaggregation whereas only one was necessary to free foraminifera from sample CM (Table 1). It is thus possible that the repeated cycles may have induced some additional mechanical fragmentation on planktic foraminifera

from the former. Relatively high values of F-index, i.e., 35% that correspond to ~500 planktic foraminiferal fragments over entire test, are recorded also from sample LM obtained with neo-steramina. We hypothesize that the high carbonate content and the hard compactness of the rock produced more fragments with respect to samples with lower carbonate content during the preliminary laboratory phases (crumbling of rocks or washing through metallic sieves, e.g. Plummer, 1945).

The lowest F-index values obtained for all the samples range from ~11% to ~15% in the relative percentages. These values likely indicate a 'background noise' as they are similar to those recorded across the Terche section where no lysocline shallowing is expected, i.e., excluding intervals corresponding to the hyperthermals events (Figs. 2, 7) (D'Onofrio et al., 2016). These 'background' values may derive from early dissolution through the water column (e.g., Ruddy, 1997) and/or from first laboratory phases that are common to all the procedures. On the basis of the consideration above, we can estimate that the H_2O_2 method, both at 10% and 25% concentration induced up to ~40% of additional planktic foraminiferal fragmentation in assemblages deriving from sample MA and up to ~10% in sample CM.

The minor changes of the P/B values (Fig. 3B, D, F) are likely related to the fully bathyal paleodepth (1000–1500 m, D'Onofrio et al., 2016) for the entire Terche section that signifies total dominance of planktic foraminifera throughout (from ~92% to ~98%). However, the minor increase of benthic specimens is recorded when F-index shows higher values, as expected.

The interpretation of the WPCF data is not straightforward. As an example, for sample MA the H_2O_2 and liquid N_2 procedures give higher F-index values, thus suggesting some carbonate dissolution. Consequently, the WPCF (>100 μm) should be expected of lower values instead of greater ones than those deriving from the neo-steramina and acetic acid methods that record lower F-index (Figs. 3, 4). This apparent incongruity can be explained considering that the presence of sediment clumps and encrusted planktic foraminiferal tests, observed in the above mentioned residues, increase the residues weight thus providing higher WPCF values. Furthermore, the WPCF value of the >38 μm is greater probably due to the occurrence of small fragments (Fig. S1).

5.2. Dissolution susceptibility of planktic foraminiferal species to the adopted laboratory methods

We prove here that a number of species significantly change their abundance depending from both the treatment adopted and the carbonate content but showing a complex relationship with these (Fig. 5). The acetic acid method induces, in general, less fragmentation with respect to the H_2O_2 treatments (Fig. 3), but it proved to act selectively on several species (Fig. 5B, C, Table 3). Specifically, all the species with exception of *Acarinina esnehensis, Parasubbotina varianta, Globanomalina planoconica, Catapsydrax unicavus* and *Globoturborotalita bassriverensis*, reduced their absolute abundance with acetic acid from MA with respect to the other methods thus proving to be dissolution prone to acetic acid (Fig 5 B). In sample LM *Acarinina alticonica, Morozovella marginodentata, M. formosa, Igorina lodoensis, I. broedermanni, Subbotina patagonica, S. hornibrooki, S. roesnaesensis* and *Chiloguembelina* spp. revealed to be dissolution resistant as they increase their abundance with acetic acid with respect to neo-steramina treatment instead of decreasing as it occurs in the sample MA (Fig 5C). Only *Globanomalina planoconica*, proved to be dissolution prone to the acid acetic also in sample LM.

Data from sample CM display in several cases marked differences when compared with samples with higher carbonate content as consisting in opposite behaviour. Actually, a number of species appear to be acetic acid resistant differently from samples with higher carbonate content (Fig. 5A). Moreover, in sample CM the species *Acarinina alticonica, A. soldadoensis, A. interposita A. coalingensis, Morozovella marginodentata* and *M. formosa* unexpectedly result dissolution resistant to H_2O_2 at 25%, a method supposed to be rather aggressive. Conversely, several species surprisingly display reduction in abundance for laboratory procedures believed to be less or not aggressive, such as H_2O_2, at 10%, liquid N_2 and neo-steramina. These puzzling evidences deriving from our analyses deserve a reflection. The greater time of soaking needed to disaggregate samples treated with H_2O_2, at 10% (Table 1) with respect to that at 25% may have increased the dissolution effect. Moreover, it must be noted that carbonate content, besides laboratory treatment adopted, markedly influence species abundance variations. We notice that the responses from samples with higher carbonate content, i.e., MA and LM, are rather

'homogeneous' as most of the species suggest acetic acid as the most aggressive method. Response is markedly more 'heterogeneous' in sample with the lowest carbonate content (CM). The latter sample contains a major percentage of clay that is known to better preserve microfossils likely due to the minor permeability to external chemical attacks. It is possible that foraminiferal tests are in this sample less recrystallized thus preserving a major susceptibility to the laboratory procedures. A greater degree of recrystallization could have standardised the response from the lithologies with higher carbonate content. Nevertheless, the general lower planktic foraminiferal abundances for neo-steramina and liquid N_2 treatments in sample CM appears apparently contradictory. The chemical composition of neo-steramina, benzalkonium chloride, should not induce calcite dissolution. To explain the observed incongruity, we have verified whether neo-steramina is able to move Ca^{2+} from $CaCO_3$ thus producing insoluble salt. Specifically, we tested salt precipitation through a solution [2:1] of benzalkonium chloride and Na_2CO_3 and waited 48 hours that was the maximum time necessary to disaggregate samples through neo-steramina (Table 1). Results show that no salt precipitated thus discarding this hypothesis to explain the relatively low number of planktic foraminiferal specimens obtained with this method. The only potential explanation is that, as neo-steramina disaggregate rocks with low carbonate content even better than the other methods, it frees a major number of specimens smaller than 100 μm thus excluding them from analysis.

Even if changes in abundance appears more evident for the more abundant species the low abundance of some species may result up to more than halved depending from the disaggregation method adopted. Minor components of foraminiferal assemblages could be thus overlooked from the widely adopted relative count of ~300 specimens. This result may have important repercussion from biostratigraphic point of view. Notably, the zonal marker *Morozovella formosa* whose first appearance is the bioevent to recognize the base of Zone E4 (Wade et al. 2011) could be even more difficult to detect in washed residues from both the samples CM and MA when prepared with acetic acid or liquid N_2.

The observed different response of species belonging to the same genus may derive from a number of characters such as the weight of the species, the wall thickness, number and size of pores, all

characters that may also influence the degree of recrystallization. Therefore, possible explanations to the record here observed may derive from both diverse original wall microstructures and/or from dissimilar recrystallization processes that may have induced greater brittleness of some species.

We show in Table 3 the dissolution-prone and –resistant species from the early Eocene Zone E4 to H_2O_2 at both concentrations and acetic acid as deriving from our data.

Table 3. Susceptibility of early Eocene planktic foraminiferal species from Zone E4 to the investigated methods inducing dissolution.

	Dissolution-resistant taxa			**Dissolution-prone taxa**			
METHODS	**Sample CM TRE/13 +943 ($CaCO_3$ 37%)**	**Sample MA TRE/11 -30 ($CaCO_3$ 55%)**	**Sample LM TRE/11 +1137 ($CaCO_3$ 72%)**	**Sample CM TRE/13 +943 ($CaCO_3$ 37%)**	**Sample MA TRE/11 -30 ($CaCO_3$ 55%)**	**Sample LM TRE/11 +1137 ($CaCO_3$ 72%)**	**METHODS**
ACETIC ACID	*A. esnehensis* *A. angulosa* *A. wilcoxensis* *A. quetra* *A. coalingensis* *M. aequa* *M. subbotinae* *M. gracilis* *M. marginodentata* *M. crater* *I. broedermanni* ***S. patagonica*** *G. australiformis* ***G. bassriverensis***	*A. esnehensis* *P. varianta* *G. planoconica* ***C. unicavus*** ***G. bassriverensis***	*A. alticonica* *I. lodoensis* ***S. patagonica*** *S. hornibrooki* *S. roesnaesensis* *Chiloguembelina* ***C. unicavus*** ***G. bassriverensis***	*A. interposita* *P. pseudoscitula* *G. planoconica* ***P. griffinoides*** *C. unicavus*	***A. soldadoensis*** *A. esnaensis* *A. interposita* *A. quetra* *A. coalingensis* *M. aequa* *M. subbotinae* ***M. crater*** ***M. lensiformis*** *M. formosa* *S. patagonica* *S. hornibrooki* *S. roesnaesensis* *Chiloguembelina* *G. australiformis* ***P. wilcoxensis*** ***P. griffinoides***	***A. soldadoensis*** *A. wilcoxensis* *A. coalingensis* ***M. crater*** ***M. lensiformis*** ***P. wilcoxensis*** ***P. griffinoides***	**ACETIC ACID**
H_2O_2 (25%)	*A. alticonica* *A. soldadoensis* *A. interposita* *A. coalingensis* *M. marginodentata* *M. formosa* *I. lodoensis*			*A. esnehensis* *M. aequa* *G. australiformis* *P. griffinoides*			**H_2O_2 (25%)**
H_2O_2 (10%)				*A. angulosa* *A. esnaensis* *A. wilcoxensis* *A. coalingensis* *M. aequa* *M. subbotinae* *M. crater* *M. lensiformis* *M. formosa* *I. broedermanni* *S. patagonica* *P. varianta* *G. australiformis* *P. wilcoxensis* *P. griffinoides* *C. unicavus*			**H_2O_2 (10%)**

5.3. Insights from diversity indices changes

Analysis of the diversity indices calculated in this study emphasizes that the minor but noteworthy changes observed in richness and dominance mainly concern residues obtained through the acetic acid method (Fig. 6, Table 2). The variations of richness indices recorded through the acetic acid appear somehow related to the carbonate content as they increase in samples with the higher $CaCO_3$ content (MA and LM) and decrease in samples with the lowest carbonate content (CM). A different trend results from the dominance indices that barely increase for the acetic acid in all the analysed samples.

Changes in simple richness can be explained with presence/absence of minor components of the assemblages such as *Globanomalina planoconica* and *Paragloborotalia griffinoides* that proved to be prone to dissolution induced by the acetic acid treatment. However, variations of the other richness indices, that decrease in sample CM (Fig. 7), are instead mainly controlled by the census size rather than the carbonate content. In fact, Margalef, Menhinick and Fisher alpha indices are all calculated as a function of the census size (see paragraph 3.2.1; Margalef, 1958; Menhinick, 1964) so that their values decrease with the increasing of the census size. In sample CM where these indices show lower values the census size is indeed greater for the acetic acid with respect to the others methods.

By contrast, the dominance index of Berger-Parker can be more strictly related to the abundance of dominant species when referred to similar census size (Magurran, 1988). This could explain the relatively higher value recorded by this index in sample LM treated with acetic acid where *Subbotina patagonica* (100 individuals) is more abundant with respect to dominant species *A. coalingensis* (83 individuals) in sample treated with the neo-steramina procedure.

5.4 Record of planktic foraminiferal genera

Our results on genera abundance (Fig. 5D-F) emphasize that dissolution susceptibility to acetic acid varies depending from the carbonate content revealing a more complex and partly different scenario than previously thought. In previous papers, mainly focalised on the PETM interval, *Acarinina* proved to be more resistant to dissolution than *Morozovella* and the latter more resistant than *Subbotina* and the

small muricate *Igorina* (Petrizzo et al., 2008; Nguyen et al., 2009, 2011). Specifically, Nguyen et al. (2011) performed their test on Ocean Drilling Program sediments by exposing planktic foraminifera to buffered acetic acid solution with a pH of 6.6 in two hour increments until the last specimen was fully dissolved or disintegrated. However, our results are not directly comparable with those from Nguyen et al. (2011) as our analyses were performed from on-land sediments that contain recrystallized and infilled planktic foraminiferal tests.

On the basis of our data, we observe that *Acarinina* and *Morozovella* prove to be dissolution resistant to H_2O_2 at 25% and acid acetic treatments in the sample with lowest carbonate content (CM) but they appear dissolution prone to the acetic acid method when the initial composition of $CaCO_3$ is higher than 37%. The H_2O_2 method at both the concentrations induced in residues from sample MA decline in *Acarinina, Morozovella* and *Subbotina* abundances, similarly to those observed with the acetic acid treatment. The genus *Subbotina,* that should be the most dissolution prone taxon according to the aforementioned authors, show instead greater resistance in the washed residues CM and LM prepared with the acetic acid treatments. Only in sample MA *Subbotina* is dissolution prone to the acetic acid treatment. The record from *Parasubbotina,* not analysed in previous studies, is similar to the results from *Subbotina*.

Igorina appear resistant to the acid acetic method in samples CM and LM whereas in sample MA its abundance shows minor decrease.

The small-sized chiloguembelinids are considered as dissolution-prone taxa. According to Nguyen et al. (2009, 2011) this is not only related to their thin wall but also to the less robust biserial coiling chamber arrangement that characterises this group with respect to the throcospiral growth. However, our results unexpectedly demonstrate that chiloguembelinids revealed to be relatively resistant to acetic acid method in samples CM and LM.

Among the minor component of planktic foraminferal assemblages, the genus *Pseudohastigerina* decreases its absolute abundance in samples MA and LM as treated with acetic acid thus revealing to be dissolution prone to this method. Moreover, *Paragloborotalia* proves to be extremely dissolution prone

to the acetic acid because it is totally lacking from all the washed residues. Conversely, *Catapsydrax* and *Globoturborotalita* appear resistant to the acetic acid procedure.

5.5. Differences between absolute and relative analyses

The comparison between data derived from our absolute counts and those from relative counts of D'Onofrio et al. (2016) highlights, as expected, that the quantitative absolute approach gives a more extensive scenario on both assemblage composition and test fragmentation (Fig. 7).

The F-index is, indeed, markedly underestimated, up to ~20%, in samples CM and LM through the relative approach. The difference in absolute and relative based percentages of the F-index is likely controlled by the census size. This is because in these samples the size of the population is significantly greater (of ~500 specimens) through the absolute approach (Fig. 7).

The planktic foraminiferal assemblages from the relative counts of D'Onofrio et al. (2016) appear less diversified as minor components of the assemblages, such as *Planorotalites*, *Globanomalina* and *Pseudohastigerina*, appear virtually absent in sample LM. The abundances of these genera, especially *Planorotalites*, appear highly underestimated also in the other samples analysed by D'Onofrio et al. (2016). As suggested by Pflaumann et al. (1996) the census size should be increased when using the relative approach, in order to emphasize or detect significant variations of rare species. Similarly to the F-index, this is evidently related to the different census size that involves at least ~300 specimens more through the absolute count. The overestimation of *Morozovella* abundances observed within the relative counts of D'Onofrio et al. (2016) derives from auto-correlation of the genera. As an example, in sample LM the increase in *Morozovella* percentages is balanced by the decrease in percentages of *Subbotina* and *Chiloguembelina*.

6. Summary and conclusions

We have evaluated here changes within foraminiferal assemblages deriving from three early Eocene lithified samples with different carbonate contents from the Tethyan Terche section (northeast

Italy) when disaggregated through five widely adopted laboratory techniques: hydrogen peroxide at 10% and 25% concentration, surfactant neo-steramina, acetic acid, liquid N_2. Our results concern the treatment effectiveness for each lithology in relation to time required to successfully extract planktic foraminiferal tests and to the degree of foraminiferal preservation. In addition, we outline the impact of laboratory-induced dissolution on tests fragmentation and variations in species and genera absolute abundance. We summarise the main conclusions as follows:

(1) Disaggregation is easier for samples with low carbonate content that freed better preserved assemblages. Liquid N_2 is the fastest methods to disaggregate the sample CM and one of the best methods to disaggregate the sample MA. Acetic acid and neo-steramina revealed to be the only treatments effective to disaggregate the sample LM. Interestingly at SEM observation, acarininids and morozovellids muricae appear better preserved with acetic acid and liquid N_2 treatments.

(2) The F-index values change at different extent depending on the laboratory treatment adopted and on the carbonate content. Specifically, the surfactant neo-steramina and Liquid N_2 are the methods inducing minor test fragmentation. Conversely, fragmentation index increases in washed residues treated with the acetic acid and H_2O_2, both at 10% and 25% concentrations with the latter being markedly more aggressive for sample MA. We estimate that the H_2O_2 method, both at 10% and 25% concentration, and acetic acid treatments can induce up to ~20% of additional planktic foraminiferal fragmentation in assemblages deriving from the sample MA and up to ~10% in the sample CM.

The WPCF appear to be less reliable proxy to detect laboratory-induced dissolution from on land sections with respect to the fragmentation index. This is because the WPCF is also influenced by the presence of clumps of sediment, beside the number of fragments that can increase the values resulting in apparent less dissolved assemblages.

(3) Species diversity, including thin-walled taxa, is basically preserved for all the methods adopted. However, several species significantly change their absolute abundance according to the different methods and carbonate content. According to our results, methods best preserving the major number of specimens per species in samples with low carbonate content are acetic acid, H_2O_2 at 25% and liquid N_2.

However, the lower number of specimens derived from the neo-steramina treatment is influenced by the fact that it frees a major number of specimens smaller than 100 μm therefore not available in our count. Marly rocks can release a greater number of specimens per species through the neo-steramina and liquid N_2 treatments. Finally, for rocks with high carbonate content, the neo-steramina method generally freed the major number of specimens per species for most planktic foraminifera.

(4) Species dissolution susceptibility varies according to laboratory procedures and $CaCO_3$ content. Interestingly, some species are more susceptible to the H_2O_2 treatments whereas other species decrease their abundance when washed residue is treated with the acetic acid method. In general, the acetic acid method appears to influence at greater extent the relative abundance of dissolution-prone species. Our results allow us to individuating the dissolution susceptibility of taxa belonging to the early Eocene Zone E4. The observed different response of species belonging to the same genus may result from diverse wall microstructures and/or from dissimilar recrystallization processes that may have induced greater breakability and dissolution susceptibility in some species.

(5) Dissolution susceptibility at the genus level reveals a more complex and partly different scenario than previously thought that ranked *Acarinina* more dissolution resistant than *Morozovella* and the latter more resistant than *Subbotina* and *Igorina* with particular reference to the PETM interval (Petrizzo et al., 2008; Nguyen et al., 2009, 2011). According to our results, the most abundant genera *Acarinina*, *Morozovella* and *Subbotina* all revealed to be dissolution prone to the acetic acid method when the initial composition is of $CaCO_3$ at 55%. The genus *Subbotina* demonstrates to be dissolution resistant in the washed residues prepared with the acetic acid treatments with $CaCO_3$ at 37% and 72%. The record form *Parasubbotina*, not analysed in previous studies, is similar to the results from *Subbotina*. The genus *Igorina* appear resistant to the acid acetic method in samples CM and LM. The small-sized chiloguembelinids that are considered as dissolution-prone taxa (Nguyen et al., 2009, 2011) actually increase their abundance with acetic acid method in sample LM and also, though at a minor extent, in sample CM. We explain these differences considering that recrystallization of tests in our on-land samples may have reinforced test wall thus making them more resistant to acetic acid attack. The

genus *Pseudohastigerina* revealed to be dissolution-prone to the acetic acid method in samples MA and LM.

(6) Our data, based on absolute count of planktic foraminiferal specimens allowed a comparison with the relative abundances from D'Onofrio et al. (2016). Interestingly, the counts produced through the relative approach do not appear to markedly alter the proportion among the main genera. Nevertheless, an overestimation on *Morozovella* is paralleled by underestimation of *Subbotina* and *Chiloguembelina* with the relative counts as a result of auto-correlation of the different genera abundances. Moreover, minor components of the assemblages as well as the F-index are largely underestimated with relative counts thus implying that paleonvironmental reconstructions could be less exhaustive. To reduce this problem we suggest to increase the census size when using the relative approach.

In conclusion, we demonstrate that acetic acid and H_2O_2 treatment, both at 10% and 25% concentration can impact species abundance and planktic foraminiferal test fragmentation. Following these evidences, a good practice to assess the reliability of detailed quantitative analyses should be producing preliminary tests to evaluate the best disaggregation methods according to the lithology in use. We clearly demonstrate that each treatment here tested can affect the assemblages with different degree that can dissimilarly involve species diversity, test fragmentation, species and genera abundance. Therefore, the combination of different methods as applied to a single sample that is a practice widely adopted, adds the distortive effects of the laboratory procedures thus increasing bias to the assemblage analysis.

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Taxonomic Appendix

Acarinina alticonica Fleisher, 1974

Acarinina angulosa (Bolli, 1957a)

Acarinina coalingensis (Cushman and Hanna, 1927)

Acarinina esnaensis (Leroy, 1953)

Acarinina esnehensis (Nakkady, 1950)

Acarinina interposita Subbotina, 1953

Acarinina pseudotopilensis Subbotina, 1953

Acarinina quetra (Bolli, 1957)

Acarinina soldadoensis (Brönniman, 1952)

Acarinina wilcoxensis (Cushman and Ponton, 1932)

Catapsydrax unicavus Bolli, Loeblich, and Tappan, 1957

Chiloguembelinia crinita (Glaessner, 1937)

Globanomalina planoconica (Subbotina, 1953)

Globanomalina australiformis (Jenkins, 1965)

Globoturborotalita bassriverensis Olsson and Hemleben, 2006

Igorina broedermanni (Cushman and Bermúdez, 1949)

Igorina lodoensis (Mallory, 1959)

Jenkinsina coloumbiana (Howe, 1939)

Morozovella aequa (Cushman and Renz, 1942)

Morozovella crater (Hornibrook, 1958)

Morozovella formosa (Bolli, 1957b)

Morozovella gracilis (Bolli, 1957b)

Morozovella lensiformis (Subbotina, 1953)

Morozovella marginodentata (Subbotina, 1953)

Morozovella subbotinae (Morozova, 1939)

Parasubbotina varianta (Subbotina, 1953)

Planorotalites pseudoscitula (Glaessner, 1937)

Pseudohastigerina wilcoxensis (Cushman and Ponton, 1932)

Paragloborotalia griffinoides Olsson and Pearson, 2006

Subbotina hornibrooki (Brönnimann, 1952)

Subbotina patagonica (Todd and Kniker, 1952)

Subbotina roesnaesensis Olsson and Berggren, 2006

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

HIGHLIGHTS

- Five laboratory methods to isolate early Eocene planktic foraminifera are compared
- Laboratory methods are: neo-steramina, H_2O_2 at 25 and 10%, acetic acid, liquid N_2
- Investigated methods can differently affect planktic foraminiferal assemblages
- Best-preserved tests derive from acetic acid, neo-steramina and liquid N_2 treatments
- Foraminiferal dissolution susceptibility is related to methods and $CaCO_3$ content