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A COMPARISON OF SEQUENTIAL EXTRACTION METHODS USED FOR BIOAVAILABILITY ASSESSMENT OF POTENTIAL TOXIC ELEMENT

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ABSTRACT

Understanding potentially toxic element (PTE) bioavailability has eluded the research community for decades. Here we compare three PTE extraction methods that are widely used in foraminiferal pollution studies. Evident discrepancies between methods were observed when diversity indices and relative abundances were influenced differently by the same and, in some cases, by different PTEs depending on the method. This was reflected in the canonical correspondence analysis (CCA), where Cr-Ni (Method B) and Zn-Pb-Cu (Method C) had a respective positive and negative influence over the foraminiferal relative abundances while no positive correlations were observed for Method A. These discrepancies stem from each method extracting different concentration levels of the same PTE from the same sediment samples. We recommend bioavailable PTEs to be extracted from the following fractions: (1) mud-bound from Method A (F1- exchangeable); (2) organic-bound from Method B (F3- oxidizable); and (3) completely avoid using Method C.

INTRODUCTION

In environmental micropaleontology, benthic foraminifera are bioindicators used in marine environmental assessments and monitoring. Their rapid ecological response to changes in abiotic stressors (e.g., salinity, pollutants) is a key trait that has been exploited when conducting anthropogenic pollution studies (e.g., Alve, 1995; Nigam et al., 2006; Frontalini et al., 2016; El-Kahawy et al., 2018; Bergamin et al., 2019; Barik et al., 2022). In addition, the use of benthic foraminifera in describing and assessing spatial/temporal ecological conditions of coastal (e.g., Jorissen et al., 2018; Castelo et al., 2021; Fajemila et al., 2022a; Ghandour et al., 2025) and deep-water settings (e.g., Schwing et al., 2017; O'Malley et al., 2021) have steadily increased in recent years in response to environmental health assessments (Abd Malek & Frontalini, 2024). At present, the methods applied to assess potentially toxic element (PTE; e.g., As, Se) concentrations in sediments have generally been inappropriate because what is bioavailable to the foraminifers has been severely overlooked by most authors.

Discrepancies exist between researcher methods when applying benthic foraminifera as bioindicators of PTE pollution and scaling to the pollutant's bioavailability. No consensus exists to determine the bioavailability for foraminiferal bioaccumulation and, most importantly, which chemical extraction method is most appropriate. Studies have relied on using either very strong acid (Hydrofluoric and Perchloric), relatively weaker acid (three to one ratio of Hydrochloric and Nitric = Aqua regia), or x-ray fluorescence to determine "total" PTE concentrations (e.g., Yanko et al., 1998; Sulkowski & Hirner, 2006; EPA, 2007; Schintu et al., 2016; Oron et al., 2021; El-Kahawy & Mabrouk, 2023; Ganugapenta et al., 2025) from sediments. These approaches report both bioavailable and non-bioavailable PTEs indiscriminately and simultaneously combine them into a single concentration value (Fig. 1; Method C). According to Tessier et al. (1979), using total PTE extraction is not an accurate approach because it does not reflect the physicochemical behavior of PTEs. In addition, they speculated that when using total extraction (p. 844), "…all forms of a given metal have an equal impact on the environment; such an assumption is clearly untenable." We agree with this since PTEs bound to organic matter, for example, should be readily bioavailable to the foraminifers since most are detritivores. Thus, this approach has been shown to not consider the bioavailability of PTEs (Quevauviller et al., 1993; Morillo et al., 2004; Arain et al., 2008; Martínez-Colón et al., 2009, 2017, 2018; Martins et al., 2020) in sediments and to inadvertently overestimate the impact of the pollutants on ecological responses of benthic foraminifera as seen in numerous studies (e.g., Bergin et al., 2006; Ferraro et al., 2006; Frontalini & Coccioni, 2008; Ayadi et al., 2016; Hess et al., 2020; Cong et al., 2022; Schmitz et al., 2024; Ganugapenta et al., 2025). Bioaccumulated PTEs in an organism are not related to the total concentration in the sediment but to what is biologically extracted (Tessier & Campbell, 1987), which is the case with benthic foraminifera.

OBJECTIVES

This paper comprises two sections that address two major objectives. The first objective is to guide the researchers on the general background aspects of bioavailability. In addition, this section provides a brief explanation of PTE chemical fractionation in sediments and three methods of extraction widely used in pollution studies involving benthic foraminifera.

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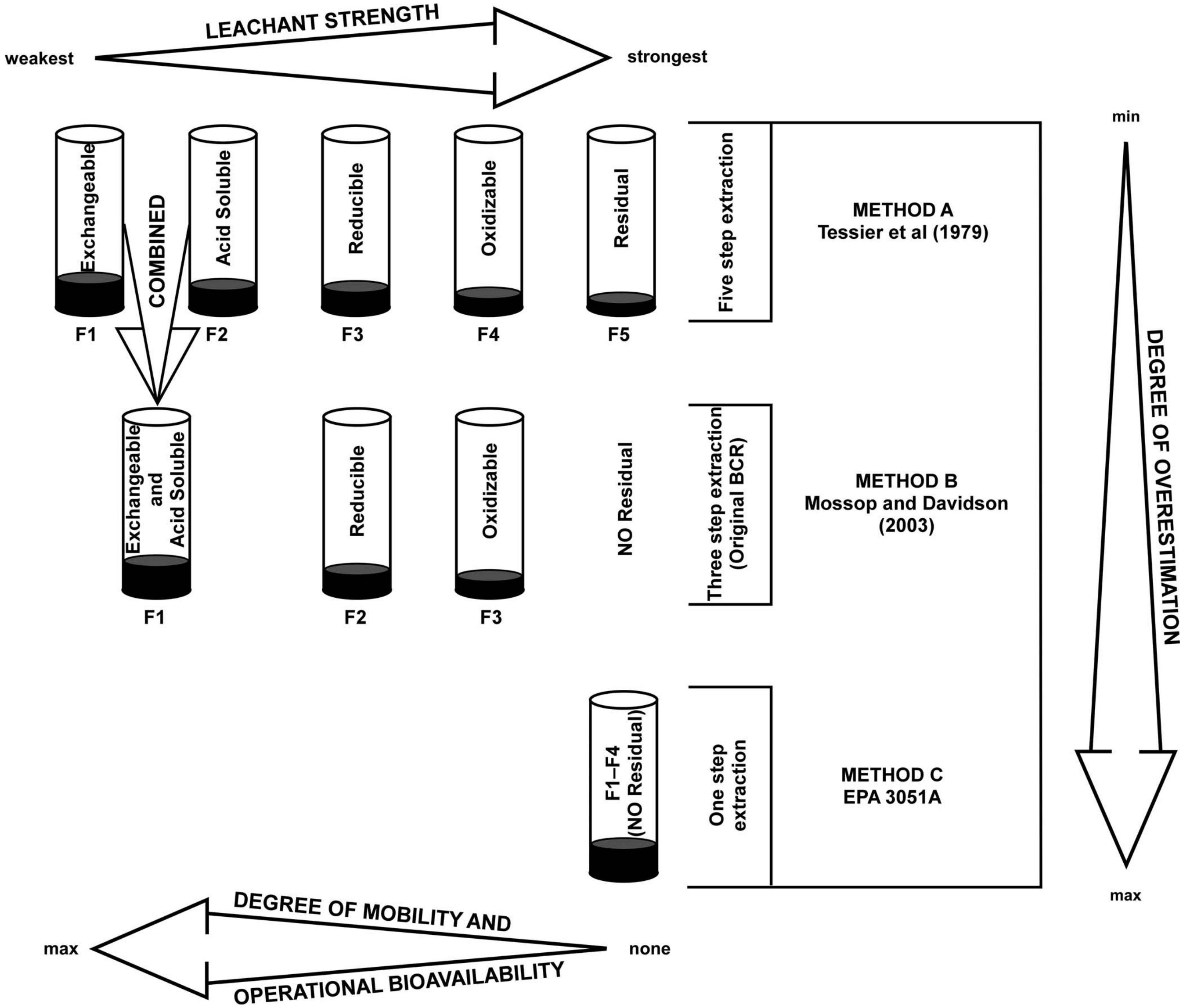

FIGURE 1. Qualitative comparison of all three extraction methods used in this study. Each of the extraction steps is equivalent to a chemical fraction in the sediment. The term fraction is denoted by the letter F.

Given the complexity of PTE fractionation, not addressed here, a more in-depth discussion about the environmental factors that control their fate, transport, and bioavailability was provided by Martínez-Colón et al. (2009).

The main objective of the second section is to use a sediment core, from a known polluted estuary, to examine and analyze the previously described three extraction methods to discuss: (1) how the choice of extraction will lead to different interpretations on PTE bioavailability; (2) how this promotes, from the same data set, different apparent PTE effects on benthic foraminifera; and (3) which PTE chemical fractions should be used as bioavailable to benthic foraminifera and which ones need to be avoided in future pollution studies. The goal of the second section is not to provide an in-depth interpretation of the ecological effects of bioavailable PTEs on benthic foraminifera or to interpret up-core historical changes of pollution, since this is part of a follow-up contribution.

BACKGROUND

OPERATIONAL BIOAVAILABILITY

Numerous protocols were developed that relied on using and applying leachants (e.g., acids, bases, salts; Appendix 1) needed to extract PTEs from different sediment chemical fractions. Unlike the use of strong acids with the capacity to dissolve silicate minerals (e.g., feldspar, pyroxene), sequential extraction methods have been developed to better constrain PTE bioavailability or at least weaker mineral structures recorded in solid phase sediments (Tessier et al., 1979; Davidson et al., 1998; Sulkwoski & Hirner, 2006; Arain et al., 2008; Rosado et al., 2016). Sequential extraction procedures target key sediment chemical components where PTEs are fractionated (Fig. 1). The operational bioavailability or level of "difficulty" in leaching out a PTE is related directly to their substrate affinity (e.g., sediment, particulate organic matter), which is related to a particular set of environmental conditions (e.g., pH, redox, ligands, bonding structures). The stronger the chemical affinity between a PTE and its substrate, the greater the difficulty in being leached out, thus becoming, operationally, less bioavailable (Fig. 1; e.g., Tessier et al., 1979; Ure et al., 1993).

There are numerous PTE extraction methods available in the literature, of which three are widely used in environmental micropaleontology. Two are related to how easily the PTEs are sequentially extracted based on their sediment chemical fractionation and as a function of operational bioavailability (Sulkowski & Hirner, 2006; Bacon & Davidson, 2008; Martins et al., 2013; Martínez-Colón et al., 2017; Barik et al., 2022; Fajemila et al., 2022a,c). The third one is a one-step method that provides a total or pseudo-total extraction depending on the type of leachant used. Since no sequential extraction method is 100% precise, as all sediments have a range of unique properties, the researcher (end-user) needs to understand the differences in the extraction methods (Appendix 1) and how the methodology affects their interpretations when assessing the bioavailability of PTEs to benthic foraminifera (Fig. 1).

The Tessier et al. (1979) five-step sequential extraction method (Fig. 1; Method A) is used widely in other types of pollution assessment studies (e.g., ecological risk, mining, aquaculture) although it has not found its niche in environmental micropaleontology. In general, the added advantage of employing this type of method is that it provides invaluable information on the provenance (natural vs anthropogenic), fate/transport (mobility), and bioavailability of PTEs (Caplat et al., 2005; Nemati et al., 2011; Li et al., 2022). Regardless of its wide acceptance in the scientific community, variations and modifications have been made to their methodology (e.g., Kim & McBride, 2006; Sulkowski & Hirner, 2006; Bacon & Davidson, 2008; Rosado et al., 2016) based on differences in (1) applicability - PTE leaching from sediments, soils, sewage, sludge; (2) time constraints - length of leaching process; (3) chemical environment - oxidation, diagenesis, carbonate content; and (4) extraction potential - leachant strength.

The Community Bureau of Reference (BCR; Fig. 1; Method B) is a widely used three-step extraction method in pollution studies (Morillo et al., 2004) including in environmental micropaleontology. To simplify the Tessier et al. (1979) protocols, the BCR method was designed to rely on higher acidic conditions, longer extraction times, and leachants without solvent ions as an attempt to minimize re-adsorption and precipitation during the extraction process (Sulkowski & Hirner, 2006). Similar to Method A, modifications have been proposed to improve the accuracy of extraction and reduce time needed. For example, ultrasonication will help in increasing the surface area of the sediments while a microwave-assisted extraction reduces the reaction time, which will maximize PTE leachability from target sediment fractions (Quevauviller et al., 1993; Ure et al., 1993; Arain et al., 2008). One main difference from Method A is that this method relies on only three extraction steps instead of five. The first step in the Method B extraction procedure is to combine the first two Method A fractions (Fig. 1): (F1) exchangeable deemed highly bioavailable to benthic foraminifera (e.g., Martínez-Colón et al., 2009; Fajemila et al., 2022a,c) and (F2) acid-soluble, which Martínez-Colón et al. (2017, 2018) documented as not bioavailable. Researchers need to be concerned that combined extractions steps, including total digestion, of the sediment results in higher PTE concentrations, but much of this fraction is not bioavailable to foraminifera leading to misinterpretations.

The underlying issue for any sequential extraction method is its accuracy in targeting a PTE in a particular chemical fraction. Numerous authors have documented and proved that selecting an appropriate leachant does not guarantee full extraction of the PTE from a given fraction due to cross-contamination (e.g., co-precipitation during an extraction; Martin et al., 1987; Davidson et al., 1998; Mossop & Davidson, 2003; Kim & McBride, 2006; Sulkowski & Hirner, 2006; Bacon & Davidson, 2008; Cuvier et al., 2021). These findings strongly suggest that not only the leachant type but also their concentrations are not 100% target-specific. There is no precise method of extraction given the different types of recovery when adjustments have been made to an extraction method. These are not the only shortcomings of any sequential extraction procedure since improper pH conditions or incorrect leachant concentrations due to user error will affect PTE recovery and concentration.

Aqua regia (three-to-one ratio of Hydrochloric and Nitric acids) is used in one-step extraction methods that produce total PTE concentrations. The short-term extraction time is an added advantage of this method in addition to the values being needed when implementing pollution indices like enrichment factors (EF) and pollution load index (PLI), as well as determining background or baseline values among others. As mentioned earlier, the main disadvantage of this widely used method in environmental micropaleontology is that it does not discriminate between bioavailable and non-bioavailable PTEs. The U.S. Environmental Protection Agency (EPA) protocol 3051A (Fig. 1; Method C) used in this study implements the aqua regia extraction solution.

EXTRACTABILITY VS BIOAVAILABILITY

It is important to remember that both A and B extraction methods were originally designed to assess the impact of sediment-bound PTEs on soils and sediments. Subsequently, these methods were applied to aquatic macrofauna and larger-sized meiofauna (e.g., nematodes) given that they are exposed to PTEs during sediment digestion (passive or active). This exposure does not translate directly to benthic foraminifera given their different mode of life (e.g., feeding, epi/endobenthic); foraminifers at the sediment-water interface will be exposed to PTEs in solution that are truly bioavailable (e.g., Malto et al., 2025). However, it has been found that many PTEs released into porewater are subsequently found associated with refractory organic matter, authigenic carbonates, authigenic clay minerals, and Fe-sulfides that are "permanent" phases of PTE sinks in sediments (e.g., Soliman et al., 2019; Smrzka et al., 2019), hence, immobile, and not bioavailable to benthic foraminifera. As pointed out by Tessier & Campbell (1987, p. 50), "The route of entry of trace metals (water, food, sediments, endocytosis) should be determined for benthic organisms." Thus, sedimentary PTE bioavailability had to be re-evaluated for benthic foraminiferal studies.

Bioavailability is measured by how easily a PTE is leached out from a particular sediment chemical fraction. Method A considered five different fractions (Fig. 1) from which Martínez-Colón et al. (2009) argued that PTEs found adsorbed to sediments and complexed with the organic matter should be considered bioavailable to benthic foraminifera. Although PTEs found co-precipitated with carbonates in the acid-soluble fraction are considered the second most bioavailable (Fig. 1), Martínez-Colón et al. (2017, 2018) demonstrated that they did not correlate with benthic foraminiferal ecological responses but rather with total concentrations.

Here we briefly explain the rationale behind four chemical fractions and its association with PTEs:

(A) Exchangeable fraction (F1) - PTEs readily adsorb to the sediment's surfaces (e.g., clays, minerals). This is related to the ionic charge of the PTE concerning the surface charge of the sediment. Because of this, the PTEs are weakly bonded to the sediments and are thus readily released into the solution due to minute changes in pH or scavenged from the sediment surfaces by changes in salinity (e.g., Cl^- ligands; e.g., Du Laing et al., 2008; Sheng et al., 2023). Based on operational bioavailability, the PTEs in this fraction are very easily leached and thus considered the most available to biota (Tessier et al., 1979; Martínez-Colón et al., 2009; Fig. 1).

(B) Acid-soluble (F2) - PTEs co-precipitated with carbonate minerals. In this type of fractionation, the pollutant is found within the crystalline structure or lattice of the mineral because of cation substitution (Ca^{+2} for Cu^{+2}/Zn^{+2}/Pb^{+2}; e.g., Harstad & Stipp, 2007; Yuan et al., 2016; Smrzka et al., 2019) as this is seen as well during foraminiferal calcification (e.g., van Dijk et al., 2017; Sagar et al., 2021). It is important to realize that this fraction is considered the second most bioavailable (Fig. 1), according to Tessier et al. (1979), but to other fauna that could ingest carbonate minerals. At the expense of not engaging in the intricacies of the marine CO_2-buffer system, a lower pH or a change in alkalinity is needed to dissolve the carbonate minerals as a function of the CO_3^{-2} ion solubility, which will then release any sequestered PTEs. For example, PTEs can remain stable in different carbonate phases [e.g., malachite: $Cu_2(OH)_2CO_3$] and will react differently to dissolution based on solubility and pH, especially in sediments with high amounts of organic matter content (e. g., Martínez-Colón et al., 2009).

(C) Reducible (F3) - PTEs co-precipitated with Fe/Mn minerals. Similar to the acid-soluble fraction, the PTEs are also found within the crystalline structure or lattice of (oxy)hydroxide minerals (e.g., hematite, goethite). The solubility of these minerals is strongly related to the redox environment at the sediment-water interface, thus, during oxic conditions, PTEs are sequestered during mineralization, making them effectively non-bioavailable. Also, it is important to note that Fe oxidation has been documented under anoxic conditions (Dodd et al., 2022). Although this is commonly observed in the subsurface, such conditions are not always observed except in cases where water column stratification occurs and is coupled with long residence times.

(D) Oxidizable (F4) - PTEs are sequestered and complexed by organic ligands associated with organic matter. It is important to remember that the type of organic matter (e.g., labile, refractory) plays a role in terms of food availability. Regarding benthic foraminifera, a debate still stands in terms of organic matter type and preference (e.g., Linke & Lutze, 1993; Alve et al., 2016). Overall, the PTEs found in labile organic matter are more readily bioavailable than those in the refractory form to the benthic foraminifera given the resistance of the latter to weathering (e.g., Baltar et al., 2021; Fajemila et al., 2022b) and subsequent pollutant release. A second ligand (sulfur) promotes sulfide mineral precipitation where PTEs, like with carbonate and (oxy)hydroxide mineralization, are co-precipitated into galena (PbS) or pyrite (FeS_2; e.g., Du Laing et al., 2008) for example. Although living benthic foraminifera have been found to contain framboidal pyrite (most likely by bacterial reductive transformation; Seiglie, 1973; Wang et al., 2010), this type of sequestration only occurs under sulfidic or euxinic conditions at the sediment/water interface, which would already be intolerable to the foraminifers. Under these extreme environmental redox conditions, the PTEs are operationally and chemically not bioavailable to benthic foraminifera given the insolubility of sulfide minerals. This sediment fraction is challenging given that during the extraction method, the PTEs are leached out from both organic and inorganic chemical forms.

MATERIALS AND METHODOLOGY

Study Area and Field Sampling

In September 2022, the Guánica Bay estuary in southern Puerto Rico was included in the National Priority List superfund site by the U.S. Environmental Protection Agency (EPA). For decades this estuary has been receiving copious amounts of pollutants including PTEs, polychlorinated biphenyls, and sewage among others (e.g., Whitall et al., 2013; Kumar et al., 2016). The average water depth is 4 m (Whitall et al., 2013), and the surface sediments are predominantly mud (Bauer et al., 2012; Klaus et al., 2016). A 52-cm push core was collected by SCUBA (Self-Contained Underwater Breathing Apparatus) divers in October 2021 at a depth of 2.5 m in a small cove away from the dredged channel with relatively calm waters, on the northwest section of the bay (Fig. 2). The pollution history of this site ensured that changes in bioavailability vertical gradients (ideal for this study) would be showcased by the extraction method comparisons.

Sampling and Sample Processing

The sediments from the 10-cm diameter core were extruded and sampled at 1-cm between 0–10 cm and 2-cm resolution between 10–46 cm, resulting in 28 samples. The last six cm were extruded at one- (46–47 cm), two- (47–49 cm), and three-cm (49–52 cm) intervals, for three additional samples, given technical difficulties during extrusion. The extruded samples were placed in acid-washed (10% HCl) 8-oz Nalgene©

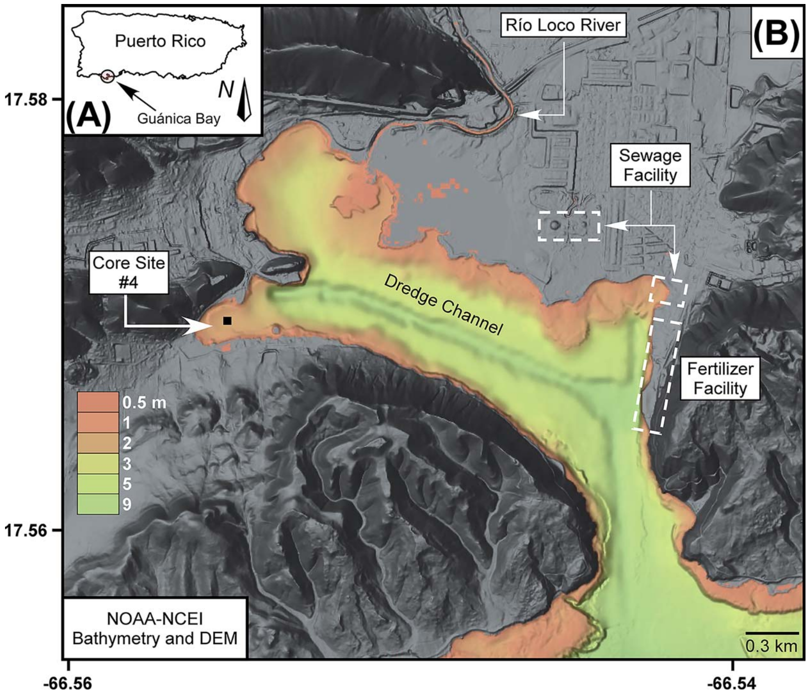

FIGURE 2. Studied area. A) Generalized location of Guánica Bay in Puerto Rico. B) Location of the sediment core.

containers and frozen at 4°C. In the laboratory, the samples were freeze-dried, and subsamples were collected for the following parameters: (1) PTE extraction Method A, (2) PTE extraction Method B, (3) PTE extraction Method C, and (4) analysis of foraminiferal diversity and relative abundances of the dominant taxa.

In summary, 2–20 g of sediment/subsample were initially wet sieved (<63 µm) and further dried at 50°C. For benthic foraminiferal analysis, the goal was to pick 300 individuals (max was 239) from less than 1 g of sediment per subsample (>63 µm). For the PTE concentrations, all subsamples were ground using an agate mortar and pestle. For the sequential extraction methods, PTEs were extracted from subsamples consisting of 0.5 g following Method A (Tessier et al., 1979) and Method B (Mossop & Davidson, 2003; BCR) protocols (Appendix 1; Fig. 1). The original protocols required 1 g of sediment but in this study, half the mass was used (due to limited subsample availability), and all the leachant volumes for the extractions were also halved. For Method C (EPA 3051A; EPA, 2007), a microwave-assisted extraction was done using aqua regia (Appendix 1) and 0.5 g of sediment/subsample. The concentrated acids used in this method are not strong enough to completely dissolve all the residual minerals (e.g., quartz) (Fig. 1). All the leached extracts from the three methods used were diluted in 30% HNO_3. After complete sample digestion, each sample per method was diluted in 2% HNO_3 solution and analyzed using an Agilent 7500cs ICP-MS (Inductively coupled mass spectrometry) at the National High Magnetic Field Laboratory at Florida State University for the following nine PTEs: Cr, V, Co, Ni, Cu, Zn, As, Se, and Pb. Internal standards and spikes were used to ensure instrument stability and to correct data post analysis. Procedural blanks were below detection limits for all reported elements. For this study, we use the term "pseudo-total" for the summation of the extracted PTEs in Method A (ΣF1–F4) and Method B (ΣF1–F3).

DATA ANALYSIS

For this study, we used several ecological parameters. We calculated both 1) Species Richness, where S = the number of species per sample, and the 2) Shannon-Index (Shannon, 1948) using

$$H(S) = -\Sigma p_i x \; ln(p_i)$$

where p_i is the proportion of the i^{th} species in a sample. We also looked at the relative abundance of the most dominant foraminifers: 3) *Ammonia tepida* (Cushman, 1926) Relative Abundance was calculated using

$$ARA = (A/BF_T) * 100$$

where A is the number *A. tepida* and BF_T is the total number of benthic foraminifera in the sample. This was the most dominant species. 4) *Quinqueloculina seminulum* (Linnaeus, 1758) and *Quinqueloculina tenagos* (Parker, 1962) Relative Abundance was calculated using

$$QRA = [(Q_s + Q_t)/BF_T] * 100$$

where Q_s and Q_t are numbers of *Q. seminulum* and *Q. tenagos*, respectively, and BF_T is the total number of benthic foraminifera in the sample. These were relatively the second and third most abundant taxa. It is important to remember that three different PTE extraction methods are being compared in this work. These ecological indices were only used to visualize and conceptualize discrepancies arising from the different "effects" of PTEs.

For the PTEs, a univariate two-sample *t*-test using non-transformed data was done to assess a significant difference between Method A and Method B sequential extractions. Given that the nomenclature and approach for each fraction are different between the methods (Fig. 1; Appendix 1), the following comparisons were done: (a) F1 (exchangeable- Method A) vs F1 (exchangeable and acid-soluble- Method B); (b) F3 (reducible- Method A) vs F2 (reducible- Method B); and (c) F4 (oxidizable- Method A) vs F3 (oxidizable- Method B). In addition, the concentration of PTEs extracted by aqua regia from Method C was compared with the "pseudo-total" values from Methods A ($\Sigma F1 + F2 + F3 + F4$) and B ($\Sigma F1 + F2 + F3$). Lastly, a principal component analysis (PCA) was done using the square-root transform of the PTE data from Method A and Method B. In addition, a canonical correspondence analysis (CCA) was done on the following square-root transformed parameters: (1) Method A PTE fractions (F1, F3, and F4), (2) Method B PTE fractions (F1–F3), (3) Method C PTE aqua regia and "pseudo-total" extractions, (4) S, (5) H(S), (6) ARA, and (7) QRA. The square-root transformations were done using Excel and the two-sample *t*-test, PCA, and CCA were done using the Paleontological Statistics (PAST) software v. 4.15c (Hammer et al., 2001).

RESULTS

Variability of PTEs and Foraminiferal Diversity

PTE Extraction Methods

The nine PTEs of concern (Cr, V, Co, Ni, Cu, Zn, As, Se, Pb) were analyzed following the three different extraction methods (A–C) outlined in Appendix 1. In addition, we want to highlight that for this study we did not include the F2 fraction of Method A (concentration of PTEs associated with carbonates). It has been shown that PTEs in this fraction are not bioavailable to benthic foraminifera (e.g., Martínez-Colón et al., 2009, 2017, 2018; Fajemila et al., 2022a,c).

Method A: F1 (exchangeable) vs Method B: F1 (exchangeable + acid soluble). Figure 3 illustrates the vertical profiles of the sequentially extracted PTEs from the F1 fraction. On the one hand, no observable correlations were established for Ni, Cu, and Zn (Table 1) given they were below the detection limit (BDL) in Method A (Figs. 3D–F). On the other hand, significant statistical differences were observed between methods for the rest of the PTEs. It is important to highlight that V, Co, As, Se, and Pb extracted with Method B had higher concentrations (Figs. 3B–C, G–I). For example, the mean concentration difference for Se was 20x higher in Method B when compared with Method A (Fig. 3H; Table 1) while for V and Pb it was as low as 1.1x (Figs. 3B, I; Table 1). Only Cr had an opposing relationship where the mean concentration from Method A was 1.7x higher than Method B (Fig. 3A; Table 1). Raw data can be found in Appendix 2.

When comparing the methods, the subsamples were clearly distinguished and clustered by method and had different degrees of separation based on the PCA analysis (Fig. 4). For this exchangeable fraction, 92% of the variance is explained by principal component 1 (PC1). Based on their loadings, the most significant PTEs were: Se (0.92) > As (0.35) > Co (0.15) (all positively correlated; Fig. 4A). Chromium has a negative but nonsignificant correlation with PC1. The most significant PTEs correlated within the principal component 2 (PC2, which explained 7% of the variance) were: Zn (0.85) > Cu (0.49) (both positively correlated). Given that PTEs adsorbed to the surface of minerals were extracted with Method A and those adsorbed and co-precipitated with carbonate minerals by Method B (Appendices 1–3; Fig. 1), the subsamples clustered separately along PC1 (Fig. 4A). Method A subsamples tightly clustered along -PC1 and are only influenced (negatively) by Se-As-Co. Concerning Method B, the subsamples were less tightly clustered along the +PC1 as the vast majority (90%) were positively influenced by the same three PTEs (Se-As-Co). The rest of the PTEs do not have a significant influence on the subsample distribution. We want to highlight that two samples (at 1 and 52 cm) were taken out of all the PCA analyses that in most cases both contain extreme PTE concentrations (Figs. 4A–C) which caused overlapping (Appendix 3: A–C). By removing these two samples, unique and distinct clusters are observed with minor to no overlapping (Figs. 4A–C) and with minimal changes to PTE variances and loadings.

Method A: F3 (reducible) vs Method B: F2 (reducible). Figure 5 illustrates the vertical profiles of the sequentially extracted PTEs from the F3 and F2 reducible fractions.

Higher mean concentrations were observed for V, Ni, and Se when extracted with Method A with Se being 300x higher when compared to Method B (Table 1). Conversely, Cu and As showed an opposite profile relationship, and both had higher concentrations when extracted with Method B. For instance, Cu had 71x higher concentrations in Method B than in A. Significant statistical differences around the PTE means were observed except for Cr, Co, Zn, and Pb (Table 1). However, Co-Zn had higher concentrations when extracted with Method A, while for Cr and Pb Method B had the highest with relatively minimal differences. Complete concentration data can be found in Appendix 2.

As expected, the PCA results highlight that the subsamples clustered differently by method (Methods A and B; Fig. 4B). The PC1 explains 57% of the variation between methods, and the most correlated PTEs, based on their significant loadings, were: Cu (0.81) and Pb (0.54) (all positively correlated). The PTEs with the most significant correlation with PC2, and explaining 38% of the variance, were: Zn (0.62) and Pb (0.54) (both positively correlated). Only Cu (-0.45) has a negative correlation along -PC2.

For the reducible fraction, a slight overlap between methods is observed. For Method A the subsamples clustered into two different groups (Fig. 4B): (1) subsamples 49–12 cm show a significant positive influence by Zn and Pb and a significant negative influence by Cu; and (2) subsamples 10–2 cm show a significant negative influence only by Zn and Pb and positive influence by Cu. For Method B a similar grouping is observed, even though the subsamples are more widely distributed along +PC1: subsamples 49–14 cm show a significant positive influence by by Cu–Pb and a negative influence by Ni; and (2) subsamples 12–2 cm are show a significant negative influence by Zn–Pb and a significant positive influence by Cu.

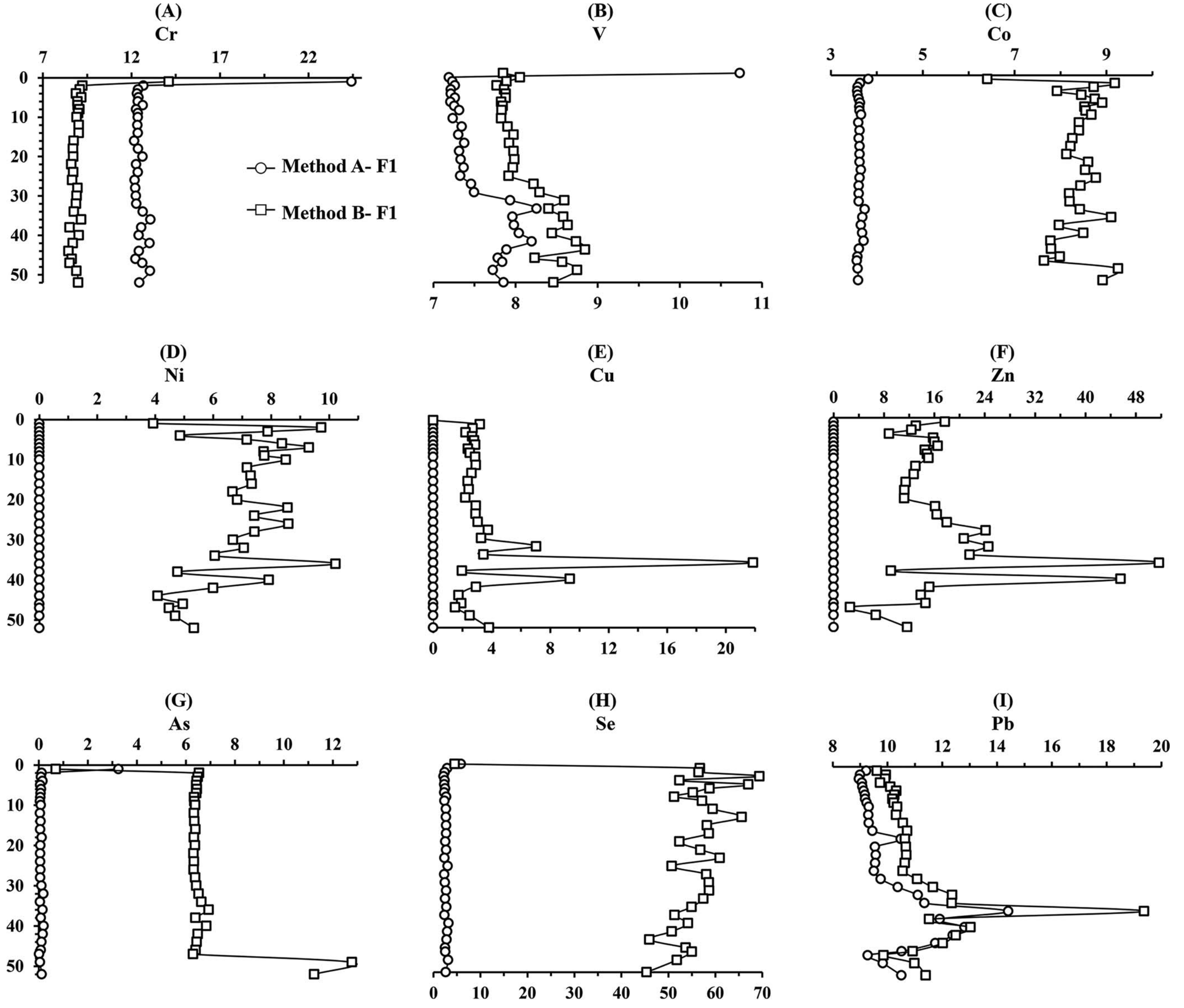

FIGURE 3. Distribution comparison of PTEs extracted from Method A (exchangeable) and Method B (exchangeable + acid soluble). All concentrations are in mg kg^{-1}.

Method A: F4 (oxidizable) vs Method B: F3 (oxidizable). The profiles observed in Figure 6 showed that most PTEs extracted by both methods behave similarly (e.g., Cr, Co, and V) while others (e.g., As and Se) have unrelated trends. Regardless, Ni, Cu, Zn, Se, and Pb had higher concentrations when extracted with Method A, as their means are statistically different (Table 1) with Se having a 33x higher mean concentration than in Method B. Arsenic is the only PTE with a statistically significant mean concentration in which the highest values were recorded from Method B. The only PTEs with no statistical significance around their means were Cr, V, and Co (Table 1). Raw data can be found in Appendices 2 and 3.

The PCA in Figure 4C shows the variance between methods. The PC1 explains 77% of the variation between methods based on the two most correlated PTEs: Cu (0.88) and Pb (0.42) (all positively correlated). The PC2 explains 10% of the variation between methods, and the most correlated PTEs, based on the significant loadings of Ni (0.59) and Cr (0.50) (all positively correlated). Only Zn (-0.33) has a negative but nonsignificant correlation along -PC2. It is important to highlight that the distribution of the subsamples between methods are closer when compared to the previous two fractions (Figs. 5A–B) and are barely overlapping.

Method C: aqua regia and "pseudo-total" extractions. The profiles observed in Figure 7 showed that most PTEs extracted by both methods behave similarly. For methods A and B, the sum of each of their respective fractions was used as a "pseudo-total" concentration. When compared to Method C (aqua regia extraction) it was highly unexpected to find that Cr, V, and Ni were the only PTEs extracted with this method that reached considerably higher concentrations (Figs. 7A–B, D) while Co, As, and Se (Figs. 7C, G, H) and Zn and Pb (Figs. 7F, I) had lower than expected and intermediate values, respectively. Surprisingly, only Cu showed an almost identical profile among the three methods (Fig. 7E). Raw data can be found in Appendix 2.

TABLE 1. Two-sample *t*-test. Gray areas are of no significant correlations. Martínez-Colón Table 1

	Exchangeable Method A (F1)	Exchangeable + Acid soluble Method B (F1)	Two sample *t*-Test p (0.05)	Reducible Method A (F3)	Reducible Method B (F2)	Two sample *t*-Test p (0.05)	Oxidizable Method A (F4)	Oxidizable Method B (F3)	Two sample *t*-Test p (0.05)
	Mean			Mean			Mean		
Cr	12.84	9.03	1.50E-12	20.98	21.50	0.76	55.14	49.04	0.04
V	7.14	7.66	2.80E-04	20.72	15.12	2.30E-03	19.64	20.77	0.26
Co	3.63	8.34	1.37E-49	9.60	9.21	0.62	16.36	16.69	0.63
Ni	0	6.93	n/a	31.37	9.63	2.99E-18	74.55	58.25	9.50E-04
Cu	0	3.57	n/a	1.31	93.03	8.57E-07	189.74	81.32	2.08E-05
Zn	0	16.68	n/a	102.84	79.27	0.06	50.30	36.47	4.30E-04
As	0.19	6.62	3.83E-27	5.34	8.89	3.29E-06	7.16	8.75	3.80E-03
Se	2.72	54.45	3.29E-35	20.32	0.07	3.28E-10	21.60	0.66	4.26E-21
Pb	10.13	11.11	0.02	67.56	92.36	0.17	43.91	12.95	2.54E-05

The PCA in Figure 8 clearly distinguished and clustered the subsamples by method. The PC1 explains 61% of the variation between methods based on the significant positive influence of the two most correlated PTEs: Cu (0.69) and Pb (0.55). The PC2 explains 21% of the variation between methods based on the significant positive influence of Cr (0.45) and Ni (0.37). Only Se (-0.64) had a significant negative loading in the -PC2.

Benthic Foraminifera

A total of 6,717 foraminiferal individuals were counted with *A. tepida* (4,495 individuals), *Q. seminulum* (1,217 individuals), and *Q. tenagos* (350 individuals) as the three most dominant species. Species richness varied from 5–13 and H(S) from 0.39–1.47. The ARA values ranged from 42–93%, while QRA ranged from 1–49%. Raw data for the ecological indices can be found in Appendix 4.

Canonical Correspondence Analysis: Abiotic vs Biotic

Since the main objective of this study is to compare extraction methods, the best approach was to use the PTE concentration data (abiotic) in relation to the ecological parameters (biotic). The following CCA tri-plots with scaling-type 2 illustrate the different environmental effects of the PTEs (by method) on the four main benthic foraminifera ecological parameters (Figs. 9A–F, 10A–C). For example, the proximity of an ecological parameter to the distal end of a PTE vector line indicates that the abiotic parameter is exerting a very strong influence. On the other hand, the closer the parameter is to the proximal end indicates a much lower influence. As expected, and based on the method of extraction, different PTEs exerted a greater, lower, or negligible influence on the benthic foraminifera. In all the methods, ≥98% of the PTE variance is explained by Axis 1, while those found along Axis 2 have very little (≤1.6%) impact on the foraminifers.

Method A (F1, exchangeable) and Method B (F1, exchangeable and acid-soluble) vs foraminiferal ecology. When assessing the effects of the PTEs found in this bioavailable fraction (Fig. 9A), the most significant PTEs associated with Method A were V (0.67) > Pb (0.59) > Se (0.35) (all positively correlated). It is important to remember that Ni, Cu, and Zn were BDL for this Method A (Figs. 3D–F). The QRA seem to be influenced (positively) by V and Se, but weakly by Cr, while H(S) was influenced to a lesser extent by V, Se, Pb, Cr, and Co. PTEs had a very limited positive influence on S. The ARA is found along the -Axis 2 which indicates that this biotic parameter shows a negative correlation with V, Pb, Se, and Cr.

In the case of Method B (Fig. 9B), the whole suite of nine PTEs was present. It is highly important to remember that this method extracted PTEs concurrently from both adsorbed (e.g., mineral surfaces) and acid soluble (carbonate mineral co-precipitation) fractions (Fig. 1). The PTEs that had the highest significance (positively) along +Axis 1 was V (0.62) > Pb (0.49) and to a lesser extent by Cu (0.27). Chromium (-0.41), Co (-0.30) and Ni (-0.39) have significant and negative correlation along -Axis 1. The QRA seems to be influenced (positively) by V and Pb while H(S) is influenced by Cu and Zn. The ARA along -Axis 2 shows that it is influenced (positively) by Cr, Ni, and Co and negatively by V, Pb, and Cu. There is very minimal PTE influence over S.

Method A (F3, reducible) and Method B (F2, reducible) vs foraminiferal ecology. The PTEs most significantly associated with Method A (Fig. 9C) were V (0.89) > Zn (0.86) > Co (0.84) > Pb (0.80) > Cr (0.74) > Ni (0.79) (all positively correlated). The S and H(S) are found in relative proximity to the tri-plot's center axis indicative of no significant influence whereas QRA is positively influenced by the PTEs along +Axis 1. The ARA is found along -Axis 1 which shows that this biotic parameter is negatively correlated with V, Zn, Co, Pb, Cr, and Ni.

In the case of Method B (Fig. 9D), the PTEs that had the highest significance (positively) along +Axis 1 were Zn (0.74) > V (0.73) > Pb (0.61) > Cu (0.59) > Cr (0.55). Arsenic (-0.54) was the only PTE to have a significant and negative correlation along -Axis 1. Of the four ecological parameters in relative proximity to the tri-plot's center axis, QRA is the only one to be influenced (positively) by Zn, V, Pb, Cu, and Cr and negatively by As. The ARA along -Axis 1 shows that it is influenced (positively) by As and negatively by Zn, V, Pb, Cu, and Cr. There is practically no PTE influence over S while very minimal in the case of Cr and Cu over H(S).

Method A (F4, oxidizable) and Method B (F3, oxidizable) vs foraminiferal ecology. The most significant PTEs associated with Method A (Fig. 9E) were Cu (0.64) > V (0.58) > Zn (0.54) > Pb (0.39) along +Axis 1 with As (-0.66) > Cr (-0.62) > Ni (-0.61) > Co (-0.45) > Se (-0.37) (all positively correlated) along -Axis 1. The S and H(S) are found in relative proximity to the tri-plot's center axis indicative of no significant influence, and as with the previous comparisons,

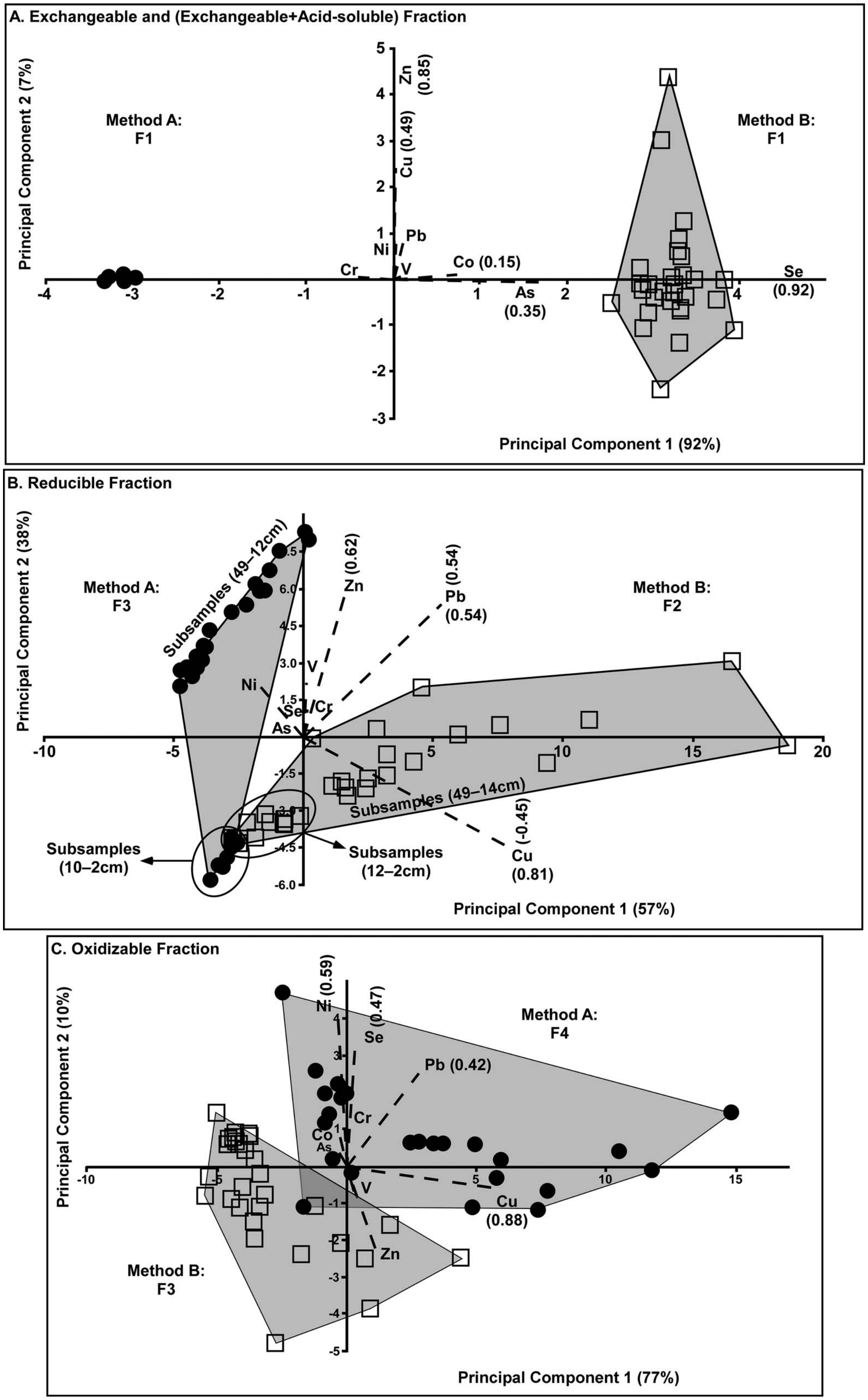

FIGURE 4. PCA of: (A) F1 vs F1, (B) F3 vs F3, and (C) F4 vs F3. Horizontal values in parenthesis represent the loadings of the most important PTEs along PC1. Vertical values in parenthesis represent the loadings of the most important PTEs along PC2. Circles: Method A. Squares: Method B. Gray shade: To "highlight" sample clusters by method.

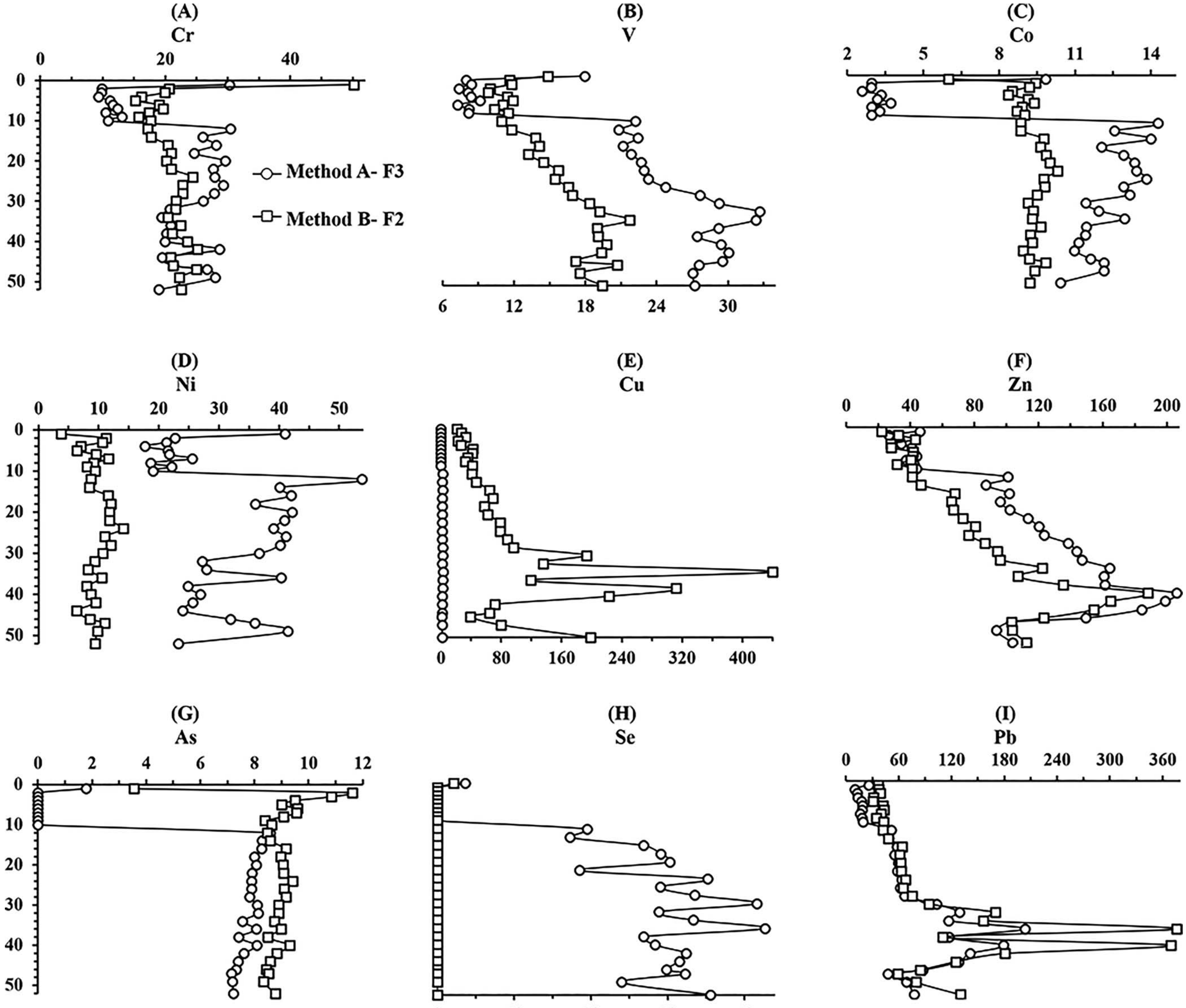

FIGURE 5. Distribution comparison of PTEs extracted from the reducible fraction. All concentrations are in mg kg^{-1}.

QRA seems to be influenced (positively) by Cu, V, and Zn along +Axis 1 and negatively correlated with As, Cr, Ni, and Co along -Axis 1. The ARA is found along -Axis 1 showing a positive correlation with As, Cr, Ni, and Co.

In the case of Method B (Fig. 9F), only two PTEs [Zn (0.59) > Cu (0.49)] had the highest significance (positively) along +Axis 1, whereas Cr = As (-0.73) > Co (-0.72) > Ni (-0.65) > Pb (-0.57) had a negative significance along -Axis 1. Of the four ecological parameters in relative proximity to the tri-plot's center axis, QRA is the only one to be influenced (positively) by Zn and Cu and negatively by Cr, As, Co, Ni, and Pb. The ARA is found along -Axis 1, which showed a different relationship to that of QRA along -Axis 1 (positive: Cr, As, Co, Ni, and Pb) and +Axis 1 (negative: Zn-Cu). The S is not influenced by any of the PTEs while H(S) is minimally influenced by Zn and Cu.

Method C (aqua regia and "pseudo-total") vs foraminiferal ecology. It is important to remember that three approaches were implemented to assess the total PTE concentrations. The first approach was to combine the four fractions Σ(F1 + F2 + F3 + F4) extracted with Method A (Fig. 1) into a "pseudo-total" concentration since we did not extract PTEs from the residual (F5) fraction. The same was done for Method B in which the three fractions Σ(F1 + F2 + F3) were added together (Fig. 1) and lastly, the aqua regia method was implemented for Method C for total concentration.

The most significant "pseudo-total" PTE concentrations associated with Method A were As (0.84)> Se (0.72) > Zn (0.67) > Cu (0.65) > V (0.55) > Pb (0.54) (all positively correlated) along +Axis 1, while Ni (-0.67) > Cr (-0.45) along -Axis 1 were negatively correlated (Fig. 10A). The QRA and H(S) seem to be influenced (positively) by Pb, Zn, As, and Se and negatively by Ni and Cr. The ARA is found along -Axis 1 which shows that this biotic parameter is being negatively influenced by Cu, Pb, As, and Se (+Axis 1) and positively by Ni and Cr (-Axis 1). The S is not influenced by any of the PTEs.

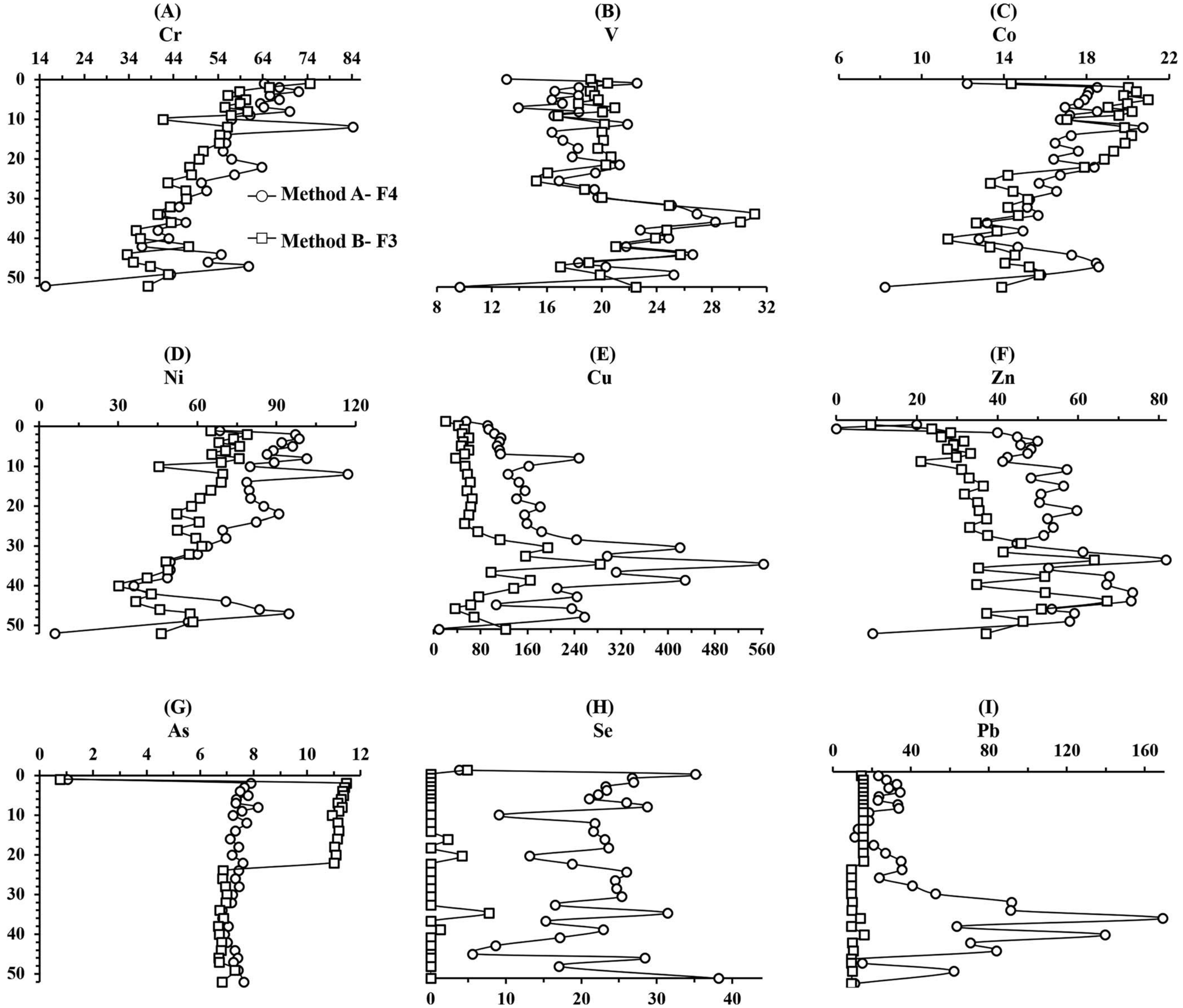

FIGURE 6. Distribution comparison of PTEs extracted from the oxidizable fraction. All concentrations are in mg kg^{-1}.

In the case of Method B, the "pseudo-total" PTE concentrations that had the highest significance (positively) along +Axis 1 were Zn (0.74) > V (0.61) > Pb (0.58) > Cu (0.55) (Fig. 10B). Along -Axis 1, Co (0.72) > As (-0.68) > Cr (-0.66) > Ni (-0.65) had significant and negative correlations. The biotic parameters QRA and H(S) seem to be influenced (positively) by Zn and V (+Axis 1) and negatively by Co, As, Cr, and Ni (-Axis 1). The ARA is found along -Axis 2 which shows that this biotic parameter is being negatively influenced by Zn, V, Pb, and Cu and positively by Ni and Co along +Axis 1. Only S was influenced (positively) by Se.

In the case of Method C, the aqua regia PTE concentrations that had the highest significance (positively) along +Axis 1 were Pb (0.55) > Zn (0.51) > Cu (0.49) (Fig. 10C). Along -Axis 1, Cr = Ni (−0.63) > As (-0.59) > Co (-0.58) had significant and negative correlations. The biotic parameters QRA and H(S) seems to be influenced (positively) by Pb, Zn, and Cu (+Axis 1), while only H(S) is negatively influenced Ni, Cr, As, Co, and Se. The ARA is found along the -Axis 1 which shows that this biotic parameter is being positively influenced by Cr, Ni, As, and Co and negatively by Zn, Pb, Cu, and V. The S is only influenced (negatively) by Se and V.

DISCUSSION

ASSESSMENT OF BIOAVAILABILITY INTERPRETATION AND RECOMMENDATION

Method A (F1, exchangeable) and Method B (F1, exchangeable and acid-soluble)

Method A is more conservative in terms of bioavailability with regards to benthic foraminifera than Method B. Figure 1 illustrates a concern related not only to the level of bioavailability between methods but also to the overestimation produced during the first extraction by Method B (e.g., Rosado et al., 2016). As seen in Figure 3, all the PTE profiles except for Cd had consistent and, in

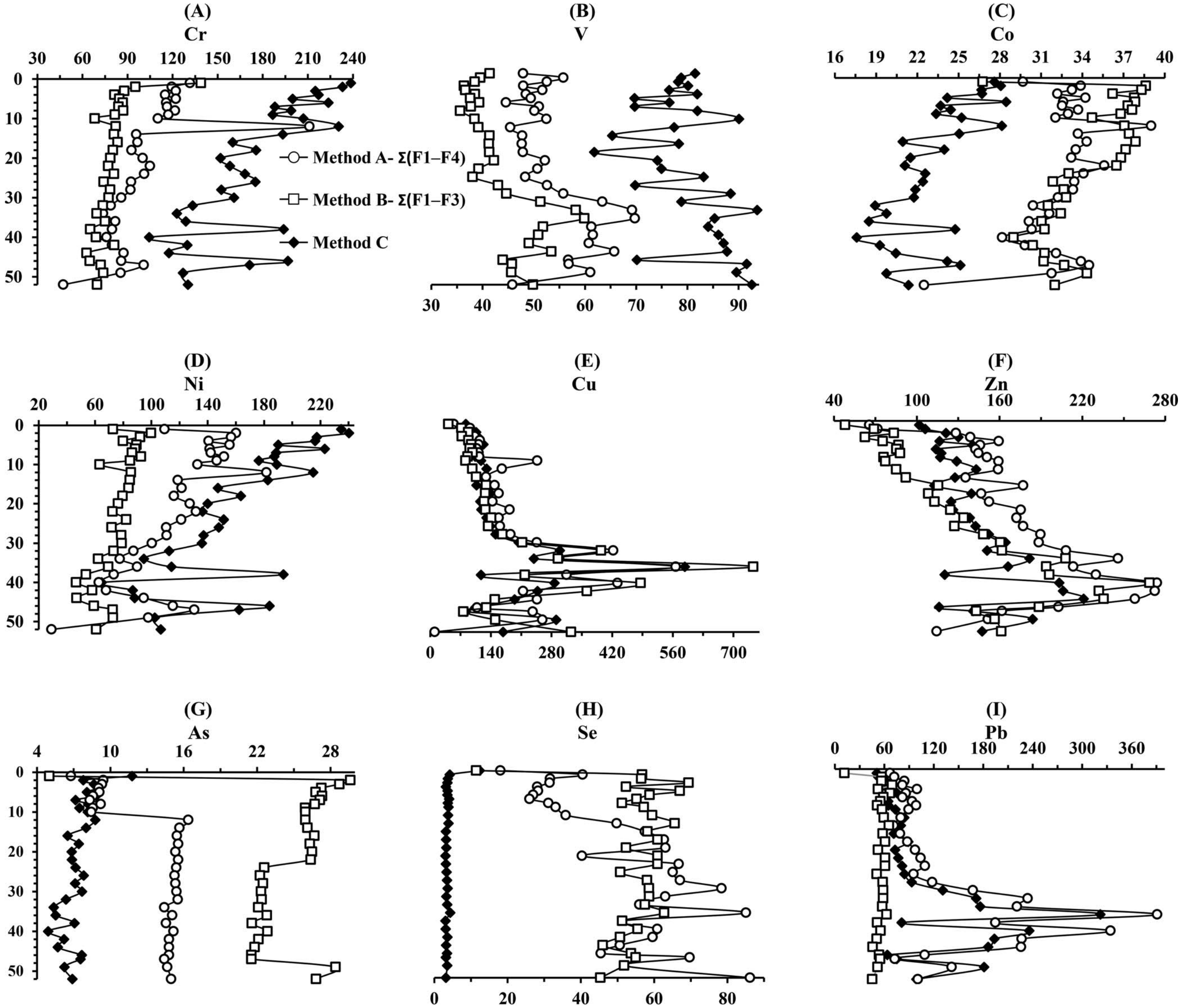

FIGURE 7. Temporal distribution of PTE concentrations per method (aqua regia and "pseudo-total"). All concentrations are in mg kg^{-1}.

some cases, drastically higher concentrations (e.g., Co and Se). This is also reported by other authors (e.g., Ianni et al., 2010; Rosado et al., 2016). This is a result of the level of acidity used in Method B to compensate for the added carbonate fraction during this leaching step (Fig. 1; Appendix 1). This explains why Ni, Cu, and Zn were recorded only in this method, showing that their presence is a result of the combination of the two chemical fractions. This is supported by the presence of Ni, Cu, and Zn in the acid-soluble fraction from Method A (F2, acid-soluble; Appendices 2–3). The PCA in Figure 4A clearly showed dissimilarities between the methods. An interesting fact is that the Method B cluster is being influenced more by the pollutants because two different fractions were leached out in tandem, hence, not only overestimating PTE concentrations, but not being realistic in terms of bioavailability. In contrast, the Method A cluster suggests how precise the extraction process was with very limited PTE influence extracted from only one fraction. This is of concern because the higher PTE concentrations found in Method B will create an overestimation of their effects on benthic foraminifera which will lead to erroneous interpretations. This is supported by the CCA plot (Figs. 9A–B) which clearly showed how the ecological indicators were influenced differently by the same PTE. For example, ARA is only negatively (Pb, Se, Co) impacted by Method A (F1), while influenced positively (Cr, Co) and negatively (Pb, Cu, Zn) in Method B. It is known that benthic foraminifera either passively or accidentally ingest mud-size sediment particles coupled with detritus (e.g., food; Lee et al., 1991; Orsi et al., 2020). During enzymatic digestion, the pH levels within the vacuole will decrease (Martin et al., 2017), and it is expected that this will cause the inadvertent releases of PTEs in their ionic form, which will then become highly bioavailable. This is very plausible given how easily PTEs could be desorbed back into solution by pH changes (e.g., Martínez-Colón et al., 2009). Therefore, we recommend PTEs be extracted from the exchangeable fraction and considered as bioavailable in future studies. We strongly recommend using only

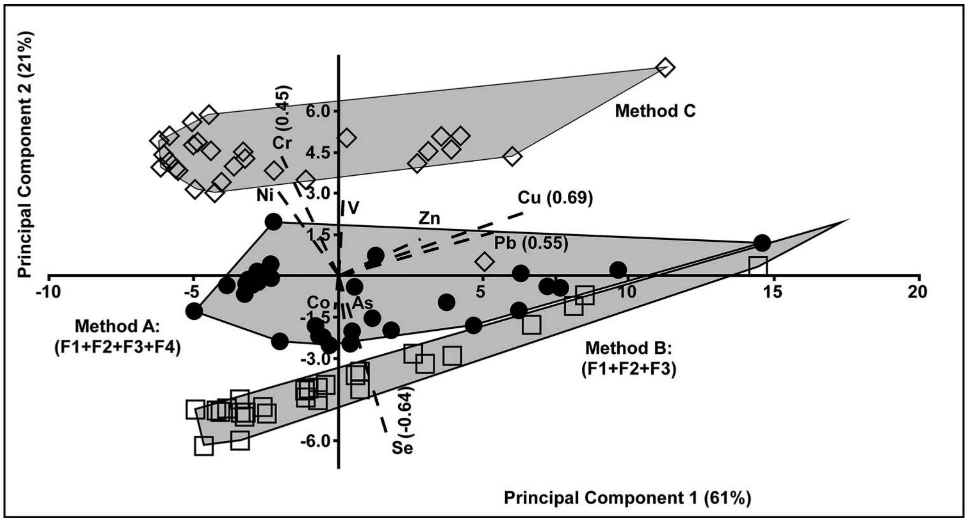

FIGURE 8. PCA of total PTE concentrations. Horizontal values in parenthesis represent the loadings of the most important PTEs along PC1. Vertical values in parenthesis represent the loadings of the most important PTEs along PC2. Circles: Method A. Squares: Method B. Diamonds: Method C. Gray shade: To "highlight" sample clusters by method.

Method A (F1; Fig. 1) from Tessier et al. (1979) because unlike Method B, it does not target the carbonate fraction.

Method A (F3 reducible) and Method B (F2, reducible)

The leachants between these two methods are slightly different in terms of their molar concentrations at a pH of 2 (Appendix 1). This explains why in Figure 5 most PTEs (Cr, V, Co, Ni, Zn, and Se) had higher concentrations when extracted by the more aggressive Method A, since its leachant was diluted in acetic acid (25% v/v) even though Method B had 3x higher molar concentration. It is important to highlight that the overlapping clusters in the bottom-left quadrant are the result of a depositional environmental change between 12–2 cm (Fig. 4B) and not an artifact related to the choice of leachants. This strongly suggests that during changes in redox conditions (reduction > oxidation), the potential extraction of PTEs found associated with Fe/Mn co-precipitates between methods is very similar and more sensitive.

An interesting aspect of comparing these two methods is the PTEs potential effect on the ecological response of benthic foraminifera. As seen in Figures 9C–D, the CCA tri-plot showed that in both cases mostly all the PTEs are influencing benthic foraminifera. The observed correlations, either positive or negative are just statistical artifacts given the choice of leachants used in both extraction methods. The observed differences between methods are based on the degree of influence. For example, V, Zn, Co, Pb, Cr, and Ni have a greater negative influence on ARA with Method A, while V, Zn, Pb, Cu, and Cr have a relatively lesser influence when extracted with Method B. In addition, it is observed that As is positively influencing S and ARA when extracted with Method A and Method B, respectively. As briefly mentioned earlier, the PTEs are found within the crystalline structure of (oxy)hydroxide minerals (e.g., hematite, ferrihydrite, vernadite). Aside from some bacteria relying on oxide minerals for respiration (e.g., Spiro et al., 2010), no reports have been found that documented benthic foraminifera relying on these minerals as a food source or for any other physiological process. Now, no evidence suggests that PTEs found in this fraction are bioavailable especially when both methods rely on stronger leachants (lower operational bioavailability; Fig. 1). Previous studies demonstrate the ability of benthic foraminifera to feed selectively (e.g., Lipps, 1983; Goldstein & Corliss, 1994). However, there is no evidence to suggest that by either active or passive selection of food from sediments, that benthic foraminifera "ingest" and degrade (oxy)hydroxide minerals. Considering these interpretations, we strongly recommend that PTEs in the reducible fraction be avoided in bioavailability studies related to benthic foraminifera.

Method A (F4, oxidizable) and Method B (F3, oxidizable)

According to numerous authors, this organic fraction is considered the least bioavailable aside from the residual or lithic fraction (Fig. 1; e.g., Tessier et al., 1979; Morillo et al., 2004). The rationale for extracting PTEs from this fraction is based on the application of stronger leachants, although Method A has additional chemicals and higher molar concentrations (Appendix 1), which explains the observed higher PTE concentrations for Cr, Ni, Cu, Zn, Se, and Pb (Fig. 6). Unlike the previous two method-fraction comparisons, the PCA showed a relatively higher proximity of the subsample clusters (Fig. 4C). However, most PTEs extracted from Method A are influencing the sample distribution. A very interesting finding is that almost the same types of PTEs (with similar loadings), extracted by both methods, are found to be influencing either positively or negatively ARA, QRA, and H(S) (Figs. 9E–F). This is indicative that this fraction, which is considered the least bioavailable (operationally), should be reassessed when applied to benthic foraminifera given the intrinsic relationship that exists between organic matter, as a PTE vector transport, and foraminifera.

As demonstrated by Jorissen (1999) and others, organic matter plays a pivotal role in benthic foraminifera ecological distributions, including patchiness, dominance of key taxa, and vertical migration (infaunal vs. epifaunal). Other studies

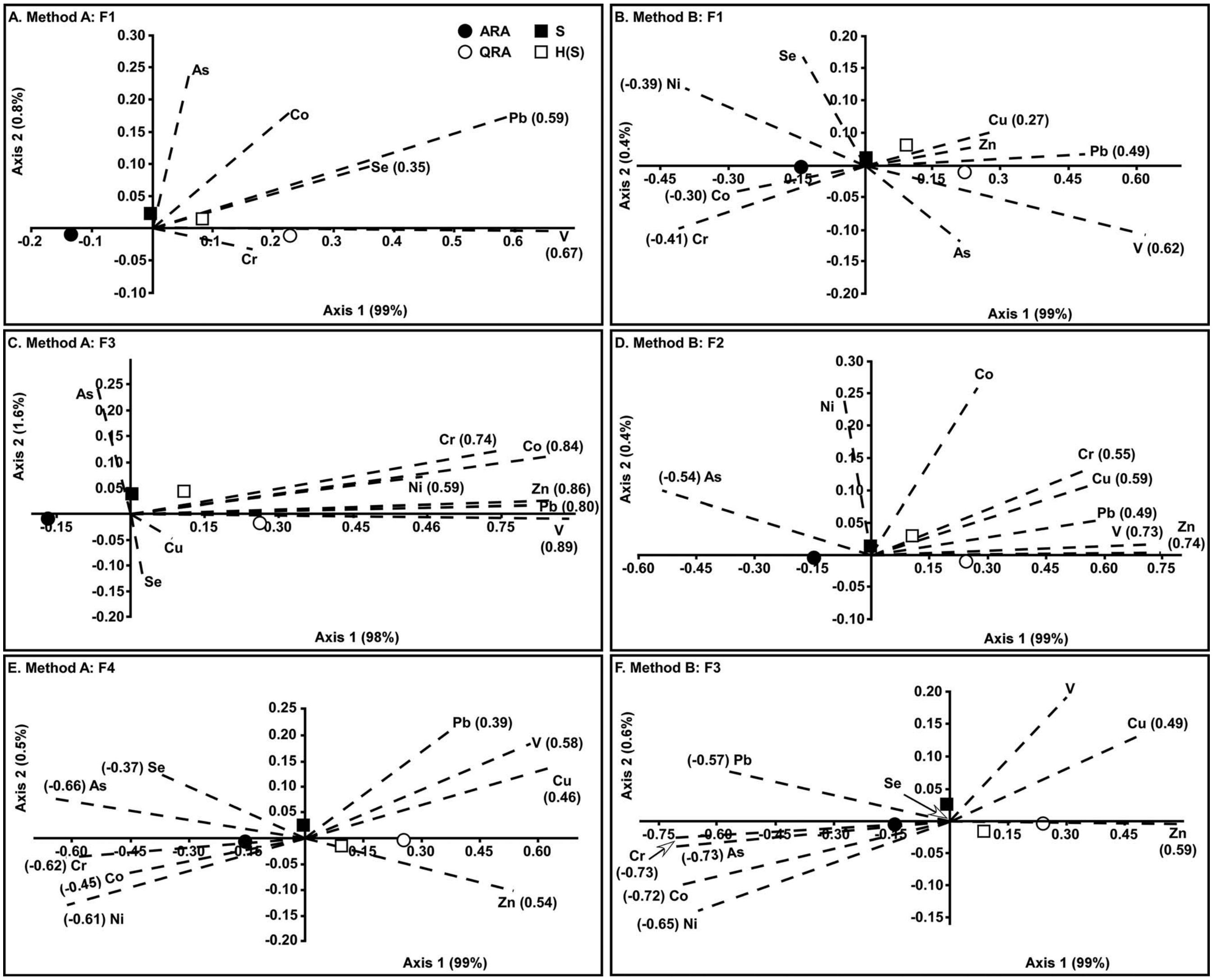

FIGURE 9. Canonical correspondence analysis tri-plots of foraminiferal ecological parameters and PTEs of interest. S: species richness. H(S): Shannon Index. ARA: *Ammonia tepida* relative abundance. QRA: *Quinqueloculina seminulum* and *Quinqueloculina tenagos* combined relative abundances.

have shown the preference for labile (e.g., higher nutritional value) versus refractory organic matter by benthic foraminifera (e.g., Papaspyrou et al., 2013; Cesbron et al., 2016; Cappelli et al., 2019; Fajemila et al., 2022b), while PTEs found in the former are more readily bioavailable to the foraminifers given the resistance of the latter to weathering (e.g., Baltar et al., 2021) and subsequent pollutant release. In addition, studies have demonstrated the minor role of benthic foraminifera in organic matter remineralization (Brückner & Mackensen, 2008; Geslin et al., 2011; Cesbron et al., 2016), especially since PTEs could be released during this process (e.g., Widerlund, 1996; Hullebusch et al., 2003; Li et al., 2020) at the sediment-water interface. As mentioned earlier, numerous benthic foraminifera species are detritivores as well as selective feeders, so this type of food source must be a PTE bioaccumulation transfer mechanism. In addition, when compared to the exchangeable fraction (considered bioavailable to foraminifers), all PTEs except for Se in this oxidizable fraction showed the highest concentrations, so the potential effects of this fraction being bioavailable could be very significant.

Operating under the realization that the oxidizable fraction is the most bioavailable to benthic foraminifera, it is important to briefly discuss the composition of the sediment detritus. Schmidt et al. (2022) described detritus feeding (i.e., mud-size sediment, dead particulate organic matter, and other microscopic organisms) as a trophic transfer strategy. Furthermore, it has been shown that within the degradation or digestive vacuoles (Goldstein & Corliss, 1994; LeKieffre et al., 2018; Frail-Gauthier et al., 2019; Tsuchiya et al., 2020; Schmidt et al., 2022) of benthic foraminifera, clay as well as organic detritus are present in relatively high quantities. According to Pascal et al. (2008) and references therein, some benthic foraminifera rely on bacteria (e.g., biofilms), which are part of the detritus and these microbes are known to engage in PTE bio-transformations (e.g., mineralization, accumulation, sorption; e.g., Lloyd, 2003; Choudhary & Sar, 2011; Newsome et al., 2014). Thus, mud-sized sediments and organic matter are vector transports of bioavailable PTEs. This is of importance because the pH levels within the digestive vacuoles in other phagotrophic protists have been reported to be low (3–5) and in some

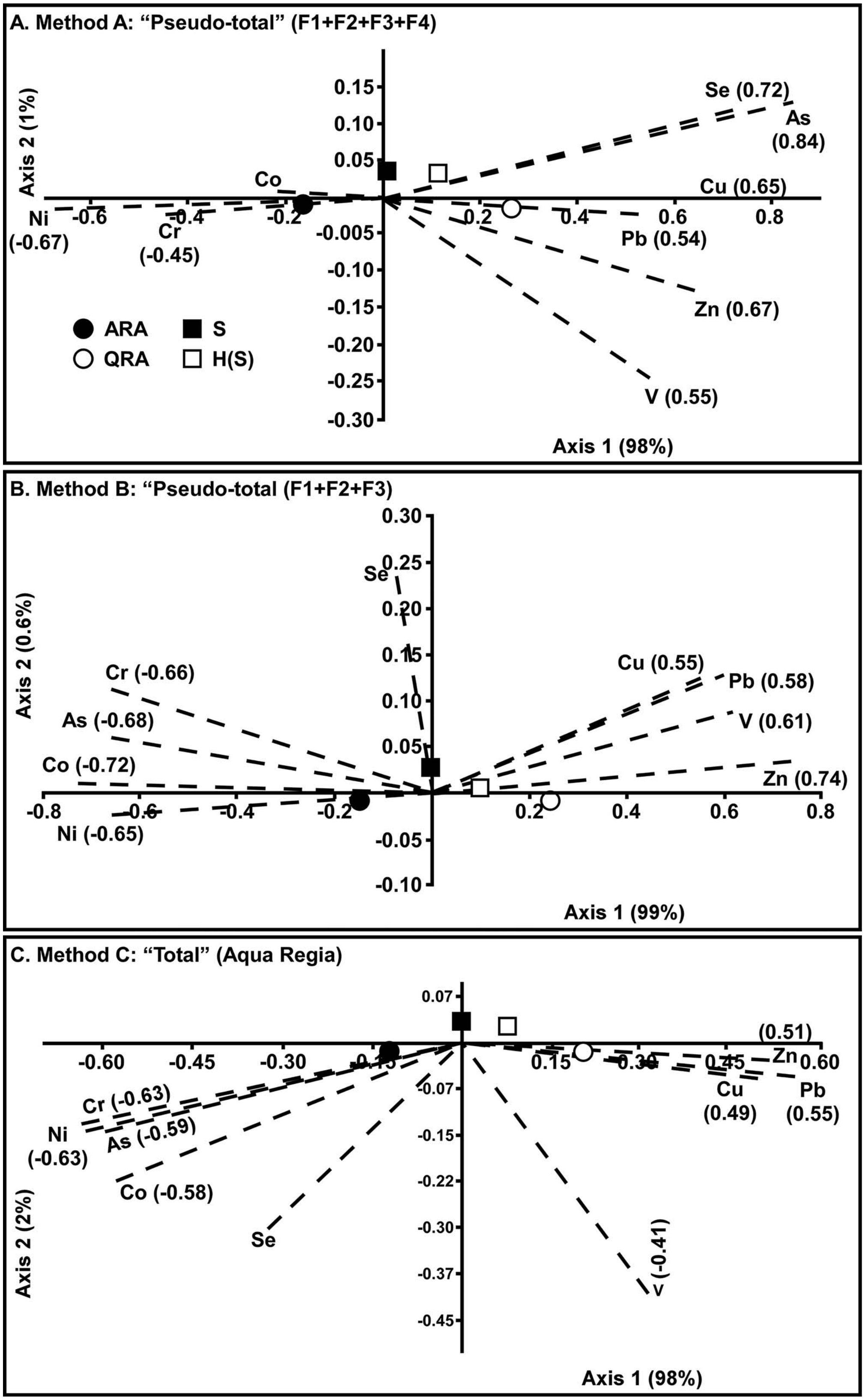

FIGURE 10. Canonical correspondence analysis tri-plots of foraminiferal ecological parameters and PTEs of interest related to total concentrations. S: species richness. H(S): Shannon Index. ARA: *Ammonia tepida* relative abundance. QRA: *Quinqueloculina seminulum* and *Quinqueloculina tenagos* combined relative abundances.

cases down to 1.4 (Fok et al., 1982; González et al., 1993). Enzymatic digestion has been reported to accelerate during higher acidic conditions (González et al., 1993), and acidic vacuoles have been reported in benthic foraminifera (Anderson & Bé, 1976; Frontalini et al., 2019), which could possibly release PTEs back into ionic forms within the vacuoles. This is supported by Orsi et al. (2020) who reported food digestion within acidic vacuoles of benthic foraminifera due to H^+ ATPases, an enzyme that others have reported to be involved in PTE transport in Archaea (*Archaeoglobus fulgidus* Stetter, 1988; Argüello et al., 2007), fungi (*Saccharomyces cerevisiae* Hansen, 1883) (Techo et al., 2020), and diatoms (*Phaeodactylum tricornutum* Bohlin, 1897; Brownlee et al., 2023).

This sediment fraction is challenging given that during the extraction method, the PTEs are leached out from both organic (labile and refractory) and inorganic chemical forms. For the latter, sulfur ligands promote co-precipitation of PTEs into biogenic pyrite (FeS_2; Du Laing et al., 2009). For example, Ni-Cu-Zn had lower concentrations in Method B (Fig. 6). This is interpreted as this method being less aggressive in extracting PTEs from sulfides; hence, they were extracted more from the organic matter. Based on our interpretations of the overall data, we recommend PTEs be extracted from the oxidizable fraction and considered as bioavailable in pollution studies involving benthic foraminifera. We also strongly recommend that only Method B (F3; Fig. 1) from Mossop & Davidson (2003) be used as the extraction method in future studies.

CONCLUDING REMARKS

We agree that the lack of an appropriate definition for PTE bioavailability has eluded the foraminiferal community. For researchers to take advantage of this contribution, they need to remember that the fate of PTEs in sediment dictates its bioavailability. Thus, the assumption that all extracted PTEs, regardless of the method employed, are bioavailable is incorrect. Stemming from this are the misinterpretations of PTE effects on the ecological responses of benthic foraminifera. We are not dwelling on establishing a new or modified definition, but instead wanted to shed light and make recommendations in terms of what is bioavailable to benthic foraminifera.

Researchers employing PTEs in pollution studies need to be aware of the following recommendations related to bioavailability when using benthic foraminifera as bioindicators:

(1) The exchangeable fraction shows the PTEs adsorbed to sediment particles that are considered labile and readily bioavailable to benthic foraminifera during digestive vacuolization. Minor changes in pH or salinity at the sediment/water interface will desorb and remobilize the PTEs, but they will be translocated into other sediment fraction (s). We strongly recommend PTEs extracted only by Method A (F1; Tessier et al., 1979) be used in bioavailability studies using benthic foraminifera.

(2) The PTEs in the acid-soluble and reducible fractions are bound to the crystalline structure of carbonate minerals and (oxy)/hydroxide minerals, respectively. If water parameters like pH and dissolved oxygen change within the water column or at the sediment-water interface due to natural cycling (e.g., tides) or by anthropogenic means (e.g., dredging), the PTEs' chemical mobility will be altered. It is not correct to assume that these two fractions are bioavailable in the sediments simply because the PTEs will be released back into solution. For example, PTEs remobilized into solution can be subsequently re-sequestered or adsorbed, or complexed into a higher or lower bioavailable fraction(s). This chemical behavior has been observed as well during the actual leaching of PTEs during sequential extractions (e.g., Martin et al., 1987; Tessier & Campbell, 1987). Following Martínez-Colón et al. (2009, 2017, 2018), we recommend avoiding PTEs extracted from these two fractions in bioavailability studies using benthic foraminifera.

(3) The PTEs complexed with organic matter are considered labile (dependent on organic matter quality) and readily bioavailable to benthic foraminifera during digestive vacuolization. This oxidizable fraction also represents that the PTEs be released from the organic matter if oxidation increases at the sediment-water interface. Like the previous fractions, the solubilized PTEs will be re-sequestered into other fractions. As noted for example by Skarbal et al. (2000) and Smrzka et al. (2019), during organic matter remineralization or by reductive liberation of oxide-bound Cu, the released PTE is found in a solution complexed to dissolved organic matter. We strongly recommend PTEs extracted by Method B (F3; Mossop & Davidson, 2003) to be used in bioavailability studies using benthic foraminifera.

(4) We do not recommend the use of PTEs from total extraction procedures (e.g., Method C- aqua regia; summation of extracted fractions from Method A and B); it should be completely avoided in pollution studies when only making interpretations about PTE bioavailability on benthic foraminifera. Researchers need to remember that both bioavailable and non-bioavailable PTEs are extracted simultaneously. The same recommendation is given when calculating "pseudo-total" concentrations since all fractions are added together.

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APPENDIX CAPTIONS

APPENDIX 1. Chemicals used in each of the extraction methods. *Synonym for Hydroxilammonium chloride. **No specifics were given about the peroxide pH. We used Rauret et al. (2000) to determine the peroxide pH.

APPENDIX 2. Data of extracted PTEs by fractions: Method A (F1, F2, F3, and F4), Method B (F1, F2, and F3), and Method C (extraction with aqua regia). "Pseudo-total" concentrations were determined by adding the sequentially extracted PTEs by fraction: Method A (F1 + F2 + F3 + F4) and Method B (F1 + F2 + F3).

APPENDIX 3. PCA including the core top (1 cm) and core bottom (52 cm) samples. A: Method A- F1 vs Method B-F1. E: Method A- F3 vs Method B-F2. C: Method A- F4 vs Method B-F3.

APPENDIX 4. Temporal distribution of ecological indices and relative abundances (%) of benthic foraminifera. S = Species richness, H(S) = Shannon Index, ARA = relative abundance of *Ammonia tepida*, QRA = relative abundance of *Quinqueloculina seminulum* and *Quinqueloculina tenagos* combined.