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Using proteolytic enzymes to assess metal bioaccessibility in marine sediments

Antonello Bignasca*, Carmela Ianni, Emanuele Magi, Paola Rivaro

Dipartimento di Chimica e Chimica Industriale – Università degli Studi di Genova, Via Dodecaneso 31, 16146 Genova, Italy

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ABSTRACT

In this work we tested two proteolytic enzymes (trypsin and pepsin) to evaluate metal bioaccessibility in marine sediments. Some working conditions were studied, in particular incubation time, enzyme concentration and solution/solid ratio, and metals release mechanisms were investigated using both untreated and denatured enzymes.

The results obtained with trypsin and pepsin were compared with each other and with results from proteinase K extraction, carried out on the same sediment samples, following a procedure reported in the literature. Moreover, a comparison with the first step of BCR sequential procedure was made.

Metal orders of mobility determined with the different enzymes and with acetic acid were compared with the one defined by the enrichment factors in the gut fluids of deposit-feeder organisms.

The overall picture suggests that proteinase K has a better capability of reproducing natural phenomena and is therefore more suitable to determine metal bioaccessibility in marine sediments, when the target of the investigation is deposit-feeder organisms.

1. Introduction

Sediments are generally an important sink for environmental contaminants which can be potentially assimilated by deposit-feeder organisms.

The bioaccessible fraction of a contaminant, defined as the fraction that can be solubilized in the gastro-intestinal environment, can be potentially assimilated by an organism and it is an important vector for its biogeochemical recycling [1].

Measurement of bioaccessibility can be performed by chemical extraction or using biomimetic approaches. The most commonly used chemical extractions employ single reagents [2–4] or sequential selective procedures [5–8]. Biomimetic approaches were first developed using fluids directly excised from the guts of deposit-feeders [9–11], but these methods are time-consuming and often present analytes adsorption instead of release, because gut fluids can be enriched in contaminants [12]. Gastro-intestinal conditions can be simulated using enzymes, such as pepsin, trypsin and proteinase K, involved in the digestive processes of many marine organisms [13,14].

In particular, pepsin is a protease which is secreted from the gastric mucosa in the form of the inactive zymogen, pepsinogen, and is activated by the acidic conditions (optimally, pH 2–4 [15]) generated by the accompanying gastric secretions of HCl [10]. Therefore, this enzyme can be used as a representative of the gastric environment. Trypsin and proteinase K, instead, can be used to simulate intestinal conditions. Trypsin, a representative protease of invertebrates [15], is secreted by pancreatic tissue as the inactive zymogen, trypsinogen, and is activated by enterokinase produced in the intestinal wall, or by active trypsin [10]. Proteinase K is a non-specific, proteolytic enzyme, that is representative of the enzymes encountered in many deposit-feeder organisms. Both enzymes are active under neutral pH conditions [16].

In a previous work [17], the use of proteinase K was already evaluated, focusing on conditions and mechanisms of enzymatic digestion. Moreover, "enzymatic" bioaccessibility was compared with "chemical" bioaccessibility (selective sequential extractions), with the result that, as expected, the latter approach generally leads to an overestimation of the amount of metals actually accessible to benthic organisms.

The aim of this work is to investigate digestive processes, utilizing two other enzymes: trypsin, as an alternative to proteinase K, to study intestinal digestion, and pepsin to simulate the gastric environment. To this end some working conditions (incubation time, enzyme concentration, solution/solid ratio) were tested because it is well-known that all partial extractions are strictly operationally defined. It is therefore very useful to know how results can change depending on different conditions, especially when comparison between different data is necessary.

Moreover, both untreated and denatured enzymes were used to study metal release mechanisms (enzymatic hydrolysis and/or complexation), since unaltered enzymes are able to react with both mechanisms while denatured enzymes can only complex the metals [16,17].

* Corresponding author. Tel.: +39 010 3536179; fax: +39 010 3536190.
E-mail address: bignasca@chimica.unige.it (A. Bignasca).

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Once optimized, these procedures were compared with a chemical extraction (first step of BCR selective extraction [18]) and the previously developed proteinase K method [17].

As in the previous work [17], both bioaccessible metals and proteins were measured to evaluate possible correlations between them. However, data regarding proteins are very variable and are therefore not reported.

2. Methods

2.1. Sampling and sample pre-treatment

Sediments A and B were collected from the Drin Estuary in Albania during an earlier Interreg project. The catchment of the Drin was a highly important metal mining region (chromite, copper ore, and nickeliferous iron ore) in the past, and the estuary is contaminated with trace metals such as Cu, Cr, Pb, Sn and Zn because of the continual leakage of metal-rich waters from old mine adits and exposed spoil tips [6].

Sediment C was collected in the gulf of Venice during the summer of 2003 in the framework of the Corila (COnsorzio RIcerche LAguna) project. More specifically, the sediment was taken from the central part of the lagoon where the risk of pollution from industrial activity is higher. In the vicinity there are several industries and the discharges were uncontrolled in the past [7].

All sediments were collected with a stainless steel box-corer, sub-sampled with a polycarbonate core of about 10 cm from the middle of the box, and immediately frozen at −20 °C. In the laboratory, sediment cores were sliced with a stainless steel hacksaw and the sections were freeze-dried (sediments A and B) or oven-dried at 40 °C (sediment C), homogenized with an automatic agate grinder and stored in a dessiccator at room temperature until analysis.

Since previous studies [6,7] have shown that the sediments are composed of 97–99% fine fraction (<63 μm), they were not sieved.

2.2. Pseudo-total metal content

The aqueous regia soluble metal content was determined weighing 0.15 g of sediment and treating it in microwave teflon vessels with 4 ml of HCl–HNO_3 3:1 (Suprapur grade – Merck). Samples were heated in a microwave oven (CEM MDS 2000) with the following program: power 650 W for 50 min, maximum temperature and pressure 180 °C and 150 psi, respectively. The digests were made up to 10 ml with MQ water, filtered, transferred into polyethylene tubes and stored at 4 °C until analysis.

The accuracy of the procedure was checked with PACS-2 (National Research Council of Canada) certified reference material as reported in Table 1, where reproducibility is also shown as standard deviation for $n = 3$ experiments. Blank analysis was also carried out and showed negligible contamination.

2.3. Selective extraction

Selective extraction was performed according to the procedure proposed by the European Community Bureau of Reference [18]. It consists of three sequential steps, which release respectively acid-labile phase (exchangeable and bound to carbonate metals), reducible phase (metals bounded to Fe and Mn oxides and hydroxides) and oxidizable phase (metals bounded to organic matter and sulphides). In this work we focused on the acid-labile phase, so that only the first step of the procedure was performed. About 1 g of sediment was extracted with 40 ml of 0.11 M CH_3COOH (Suprapur grade – Merck), shaken for 16 h at room temperature and centrifuged for 15 min at 3000 rpm. Supernatants were then filtered, acidified with HNO_3 (Suprapur grade – Merck) and stored in PE vessels at 4 °C until analysis.

The accuracy of the procedure was checked with BCR 701 (Standards, Measurements and Testing Programme of European Community – S.M.&T.) certified reference material as reported in Table 2, where reproducibility is also shown as standard deviation for $n = 3$ experiments. Blank analysis was also carried out and showed negligible contamination.

2.4. Enzymatic hydrolysis (proteinase K and trypsin)

Proteinase K (Sigma), extracted from Tritirachium album, was used following the method developed in a previous work [17]; each experiment had an “enzyme set” and a “denatured enzyme set”, made up, respectively, of samples added with the buffered untreated proteinase K solution and with the buffered denatured proteinase K solution. Proteinase K was denatured by heating the buffered enzyme solution in a microwave oven (CEM MDS 2000), with the following conditions: power 650 W for 5 min, maximum temperature and pressure 150 °C and 150 psi, respectively.

In addition we had a “control set”, made up of samples treated only with the phosphate buffer solution at pH 7.5, prepared with H_3PO_4 85% and NaOH 30% (Suprapur – Merck). This set is useful because the enzyme has to be used in a buffered solution and it is necessary to take into account proteins and metals released by the buffer itself (ionic exchange mechanism).

Sediment samples were weighed in acid-cleaned PE centrifuge tubes, and different volumes of phosphate buffer and enzyme solutions were added, as reported in Table 3. Then the tubes were sealed and placed in a thermostated bath (Shake Bath PBI), constantly shaken at 250 rpm at 25 °C. The samples were then centrifuged for 15 min at 2500 rpm; the samples of the “enzyme set” were centrifuged simultaneously to achieve the same incubation time. The supernatant was then used to determine proteins and trace metals.

Trypsin (Sigma), extracted from Bovine pancreas, was one of the digestive enzymes used in this study. Since this kind of enzyme is similar to proteinase K (it shows a more specific behaviour, but it works in the same pH range and is representative of the same part of the digestive process), some of the same working conditions were used; in particular the temperature was fixed at 25 °C, incubation time was 3 h and phosphate buffer at pH 7.5 was used to solubilize the enzyme. Enzyme concentration (0.1 mg/ml and 0.4 mg/ml) and solution/solid ratio (10:1 and 50:1) were the parameters whose influence was studied.

As already done for proteinase K, each experiment had an “enzyme set” and a “denatured enzyme set”, made up, respectively, of samples added with the buffered untreated trypsin solution and with the buffered denatured trypsin solution. Trypsin was denatured by heating the buffered enzyme solution in a microwave oven, as described above for proteinase K.

Again, we also had a “control set”, made up of samples treated only with the phosphate buffer solution at pH 7.5, useful to evaluate proteins and metals released by the buffer itself.

Sediment samples were weighed in acid-cleaned PE centrifuge tubes, and different volumes of phosphate buffer and enzyme solutions were added as reported in Table 3. Then the tubes were sealed and placed in a thermostated bath (Shake Bath PBI), constantly shaken at 250 rpm at 25 °C: This temperature represents a good compromise between the optimal working temperature of trypsin (about 40 °C) and the temperature of the sea bed (about 13–15 °C). The samples were then centrifuged for 15 min at 2500 rpm, paying attention to process every tube of the “enzyme set” simultaneously to achieve the same incubation time. The supernatant was used to quantify extracted proteins, according to the BCA assay [19], and to determine trace metals.

Table 1
Certified and found metal concentrations (ppm) in CRM PACS-2 marine sediment.

PACS-2	Al	As	Cd	Cr	Cu	Fe	Mn	Ni	Pb	V	Zn
Certified	66,200 ± 3200	26.2 ± 1.5	2.11 ± 0.15	90.7 ± 4.6	310 ± 12	40,900 ± 600	440 ± 19	39.5 ± 2.3	183 ± 8	133 ± 5	364 ± 23
Found	33,698 ± 1168	29.0 ± 0.8	2.26 ± 0.01	65.7 ± 2.9	314 ± 11	40,592 ± 763	336 ± 7	46.5 ± 5.0	178 ± 1	104 ± 4	360 ± 5

Table 2
Certified and found metal concentrations (ppm) in BCR 701 lake sediment.

BCR-701	Al	As	Cd	Cr	Cu	Fe	Mn	Ni	Pb	V	Zn
Certified	–	–	7.34 ± 0.35	2.26 ± 0.16	49.3 ± 1.7	–	–	15.4 ± 0.9	3.18 ± 0.21	–	205 ± 6
Found	137 ± 2	1.95 ± 0.07	5.86 ± 0.06	2.01 ± 0.02	36.5 ± 0.5	73.1 ± 1.0	156 ± 2	16.0 ± 0.2	2.28 ± 0.04	0.399 ± 0.017	173 ± 2

2.5. Enzymatic hydrolysis (pepsin)

Pepsin (Sigma), extracted from Porcine gastric mucosa, was then tested as the digestive enzyme. Pepsin is another proteolytic enzyme, but has a completely different behaviour from trypsin and proteinase K; it is active under acidic conditions (pH between 2 and 4 [15]) and is representative of the gastric part of a digestive process. For its use, some working conditions were checked: incubation time (from 1 to 6 h), enzyme concentration (from 0.1 mg/ml to 2.0 mg/ml) and solution/solid ratio (10:1 and 50:1).

As described above for trypsin, each experiment had an "enzymatic set", a "denatured enzyme set" and a "control set". The only difference is that in this case the solvent was a 0.075 M solution of HCl [20] (Suprapur grade – Merck); therefore the "control set" was made up of samples treated only with the 0.075 M HCl. Since hydrochloric acid was used as a solvent, in these experiments it is even more important to take into account its effect, especially on metal release, because of its low pH (about 1) and because it is part of the digestive process.

Pepsin was thermally denatured by the same procedure previously reported for trypsin.

Samples were processed and analyzed to quantify extracted proteins and trace metals following the same scheme reported above.

2.6. Instrumental analyses

The supernatants from the different experiments were filtered, transferred into PE test tubes, acidified with HNO_3 and stored at 4 °C until analysis.

Elemental concentrations were determined with an inductively coupled plasma atomic emission spectrometer (ICP-OES) Vista Pro (Varian), with internal standard (Lu, λ = 291.139) calibration method. Detection limits regarding enzymatic hydrolyses are reported in Table 4.

3. Results and discussion

3.1. Sediment characteristics

The characterization of Albanian sediments (A and B), performed during the earlier Interreg project, showed that they are rich in organogenic carbonate debris [21], in agreement with general characteristics of Adriatic sediments, which present high amount of carbonate material [22]. Moreover, a high amount of recent organic matter (excretion, dead organisms, etc...) was found in sediments from Drin Estuary [23].

As regards sediment C, we do not have specific information on its geochemistry. Anyway it is possible to consider that, in general, sediments from Venice Lagoon are characterized by a lower percentage of carbonate material compared with Adriatic marine ones, because of terrigenous inputs, which are predominantly siliciclastic [22].

The aqueous regia soluble metal content and the labile fractions for sediments A, B and C, are reported in Table 5. As expected, aqueous regia soluble metal contents are quite high in Albanian sediments (A and B). On the other hand, Venice lagoon sediment (C) presents, in general, much lower amount of aqueous regia soluble metals, with the exception of Cd and Zn.

The high amount of labile Cd in sediment C (more than 80%) can be related to the importance of atmospheric inputs in that area, according to Frignani and Bellucci [22] and to Martin et al. [24]. Martin et al. also suggest the same reasons to justify the high amount of labile Zn and Pb (40% and 5% in our case).

Sediments A and B also show a high percentage of labile Cd (about 20% and 40%, respectively) and this can be explained considering their geochemical characteristics, as described above [21,23]. The high content of carbonate material can also explain the high

Table 4
Detection limits (ppb) for enzymatic hydrolyses.

	Phosphate buffer (proteinase K–trypsin)	HCl 0.075 M (pepsin)
Al	7	5
As	6	4
Cd	0.5	0.2
Cr	1	0.2
Cu	2	1
Fe	10	4
Mn	0.4	0.1
Ni	1	0.5
Pb	2	1
V	2	1
Zn	2	4

Table 3
Sediment amounts and solution volumes for the different sets of experiments.

Samples	Solution/solid ratio	Sediment (g)	Phosphate buffer/HCl (ml)	Enzyme solution (ml)	Denatured enzyme solution (ml)
"Control set"	10:1	1	10	0	0
	50:1	0.5	25	0	0
"Enzymatic set"	10:1	1	0	10	0
	50:1	0.5	0	25	0
"Denatured enzyme set"	10:1	1	0	0	10
	50:1	0.5	0	0	25

Table 5
Total and labile metal concentrations (ppm) in sediments A, B and C.

Samples	Metal content	Al	As	Cd	Cr	Cu	Fe	Mn	Ni	Pb	V	Zn
Sediment A	Total	79,323 ± 3765	20.1 ± 1.2	0.381 ± 0.009	227 ± 5	139 ± 1	58,930 ± 1320	843 ± 15	414 ± 3	59.1 ± 1.7	n.a.	125 ± 1
	Labile	86.3 ± 3.5	1.12 ± 0.09	0.073 ± 0.003	0.471 ± 0.008	9.07 ± 0.03	848 ± 64	260 ± 2	15.0 ± 0.7	0.903 ± 0.020	n.a.	5.84 ± 0.11
Sediment B	Total	85,811 ± 2994	19.6 ± 1.6	0.288 ± 0.007	247 ± 3	147 ± 1	63,541 ± 632	915 ± 12	415 ± 15	80.0 ± 2.7	n.a.	128 ± 1
	Labile	73.5 ± 4.3	1.04 ± 0.13	0.121 ± 0.005	0.498 ± 0.006	13.1 ± 0.3	732 ± 17	294 ± 5	14.8 ± 1.0	0.407 ± 0.044	n.a.	6.98 ± 0.08
Sediment C	Total	43,305 ± 2281	13.3 ± 0.5	1.44 ± 0.04	25.0 ± 0.2	30.2 ± 1.5	21,360 ± 567	463 ± 10	16.0 ± 0.1	25.4 ± 0.8	42.5 ± 1.8	220 ± 8
	Labile	15.1 ± 1.3	0.549 ± 0.113	1.19 ± 0.01	0.139 ± 0.005	1.46 ± 0.03	34.2 ± 0.4	94.3 ± 0.9	0.929 ± 0.037	1.36 ± 0.10	0.521 ± 0.011	90.6 ± 1.7

Mn labile fractions in the three sediments, ranging from about 20% of the aqueous regia soluble content in sediment C to about 30% in sediments A and B.

4. Trypsin

The possible use of trypsin in biomimetic experiments has been evaluated with some preliminary tests; since trypsin is an enzyme with similar characteristics to proteinase K (both enzymes are representative of those present in the neutral-pH part of digestive systems), some of the working conditions were maintained, as reported above.

Both bioaccessible proteins and metals were determined in each experiment, but protein quantification gave very variable results, so only elemental concentration is reported. These problems in protein quantification may be due to the high specificity of trypsin in the hydrolysis of peptidic bound, unlike proteinase K that hydrolyzes every peptidic bound, independently of involved aminoacids [25].

4.1. Influence of enzyme concentration at fixed solution/solid ratio

Fig. 1 shows buffer-corrected concentrations of metals released from sediments A and B as a function of trypsin concentration. The experiments were carried out using both untreated and denatured enzyme to evaluate possible metal mobilization mechanisms [16,17]. Despite the few elements taken into account in these experiments, it is possible to note an increase in Cu, Fe and Mn release when trypsin concentration is taken from 0.1 to 0.4 mg/ml, both with untreated and denatured enzyme. Cd, Pb and Zn were also determined but their concentrations were below detection limit in all experiments.

Results obtained using denatured trypsin are similar to those obtained with the untreated enzyme, especially in sediment A. Nevertheless it is quite interesting to observe the trend of Mn release (and of Cu release in sediment B): the amount of Mn released by denatured trypsin is higher in both sediments and with both concentration conditions; in particular, released Mn increases from a few ppb (untreated trypsin) up to 1 or 2 ppm (denatured trypsin). This shows that thermal denaturation of the enzyme entails structural modifications (such as unfolding processes) that increase its ability to complex metals.

4.2. Influence of solution/solid ratio at fixed enzyme concentration

The influence of solution/solid ratio was tested on sediment C. Two different ratios were tested: a 10:1 ratio, similar to that present in the digestive systems of deposit-feeder organisms [26] and a 50:1 ratio, since it is useful to have a direct comparison with the first step of selective sequential extraction scheme proposed by BCR [18]. It is important to bear in mind that nowadays the BCR scheme is the most used procedure to evaluate metal mobility and bioaccessibility in solid environmental matrices.

In each of these experiments, incubation time was 3 h and trypsin concentration was 0.1 mg/ml. The metal concentrations obtained from all the experiments are reported in Table 6.

It is easy to notice that solution/solid ratio has a strong influence on the amount of released metals by both untreated and denatured trypsin and also by buffer solution. All the determined elements clearly show their dependence on this parameter, even though some metals (Cu, Ni and V) are influenced less evidently compared to other elements. The only exception to this trend is represented by As, whose release seems not to be influenced by solution/solid ratio. This is probably due to the fact that, in these conditions, almost all

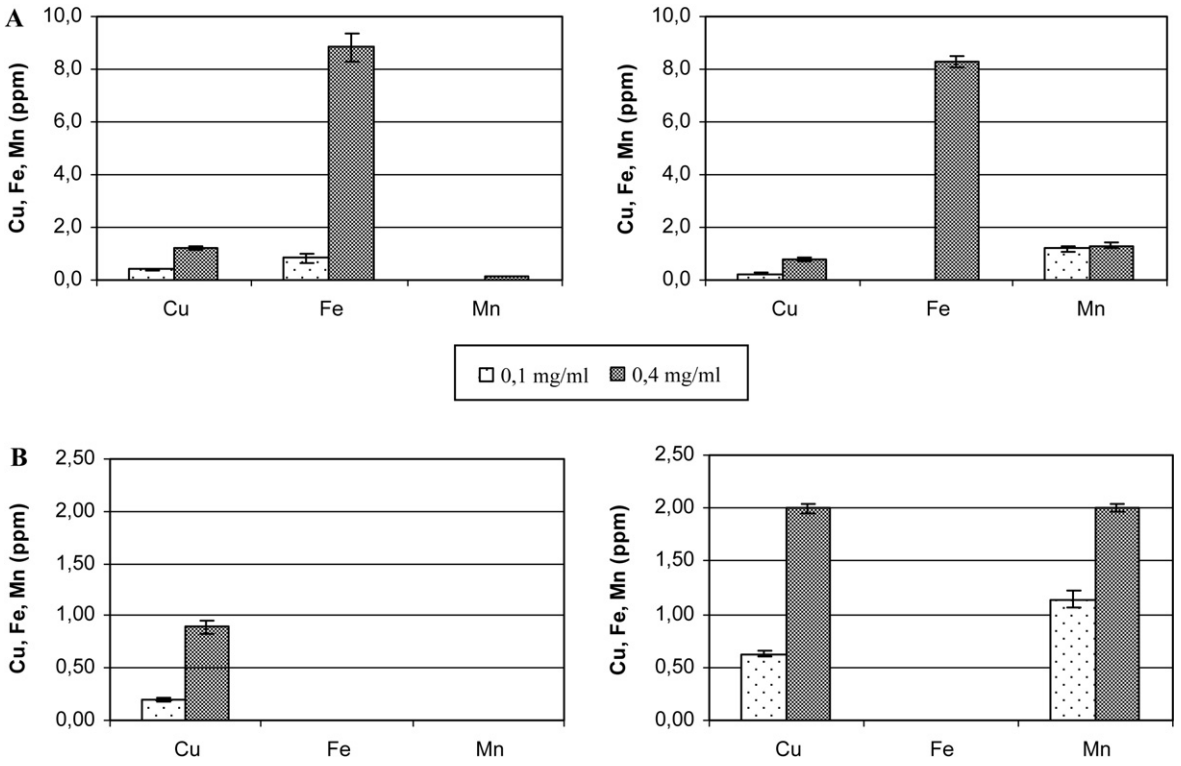

Fig. 1. Buffer-corrected metal amounts released at different untreated (left) or denatured (right) trypsin concentrations from sediments A and B.

bioaccessible As is released in the case of 10:1 ratio and there is no significant increase rising up to 50:1 ratio.

However, despite the exception regarding As, the solution/solid ratio is confirmed to be the most important parameter, as already observed in the previous work on proteinase K [17].

4.3. Comparison between trypsin and proteinase K

Some experiments have been effected with proteinase K on sediments A and B, following the procedure reported in our previous work [17].

Fig. 2 reports the buffer-corrected concentrations of Cu, Fe and Mn (Cd, Pb and Zn were below their detection limits) extracted from sediments A and B using both untreated and denatured trypsin and proteinase K.

With regard to untreated enzymes, released metal concentrations prove to be always higher when proteinase K is used. Opposite behaviour may be observed when denatured enzymes are utilized; in this case the amount of Cu and Mn released by trypsin is higher than that released by proteinase K. It may be supposed that the denaturation process has a stronger influence on the structure of trypsin than proteinase K; a stronger structural modification, with a consequent increase of the complexation ability, could explain this inverted trend. However it is important to consider that the use of denatured enzymes is useful in investigating metal release mechanisms (complexation in particular), but data from untreated enzymes have to be considered in the evaluation of metal bioaccessibility.

In view of the data reported above and results from bioaccessible proteins analyses (protein quantification reliable only using proteinase K) and bearing in mind that these two enzymes are representative of the same part of the digestive process, it is highly recommended that proteinase K is used for this kind of biomimetic experiment.

5. Pepsin

The second proteolytic enzyme used in this study was pepsin. Several working conditions were tested, in particular incubation

Table 6
Metal concentrations (ppm) released from sediment C by 0.1 mg/ml untreated and denatured trypsin and by phosphate buffer at different solution/solid ratios.

	Trypsin		Denatured trypsin		Phosphate buffer	
	10:1	50:1	10:1	50:1	10:1	50:1
Al	5.27 ± 0.24	32.7 ± 0.1	2.11 ± 0.13	23.4 ± 2.9	1.41 ± 0.13	7.09 ± 0.22
As	1.69 ± 0.05	1.53 ± 0.02	1.77 ± 0.05	1.66 ± 0.05	1.76 ± 0.03	1.99 ± 0.03
Cd	0.035 ± 0.002	0.121 ± 0.008	0.035 ± 0.002	0.118 ± 0.006	0.032 ± 0.002	0.127 ± 0.005
Cr	0.070 ± 0.004	0.158 ± 0.003	0.069 ± 0.003	0.147 ± 0.010	0.066 ± 0.003	0.126 ± 0.003
Cu	1.25 ± 0.03	2.03 ± 0.08	1.14 ± 0.01	1.90 ± 0.02	0.929 ± 0.016	1.43 ± 0.02
Fe	3.01 ± 0.32	18.8 ± 0.3	1.66 ± 0.04	15.1 ± 0.7	1.29 ± 0.06	5.78 ± 0.22
Mn	1.02 ± 0.03	4.49 ± 0.06	1.10 ± 0.02	4.55 ± 0.14	1.01 ± 0.02	4.49 ± 0.04
Ni	0.114 ± 0.002	0.156 ± 0.013	0.113 ± 0.005	0.207 ± 0.011	0.102 ± 0.003	0.149 ± 0.004
Pb	<d.l.	0.122 ± 0.048	<d.l.	0.017 ± 0.009	<d.l.	<d.l.
V	1.31 ± 0.03	1.99 ± 0.07	1.30 ± 0.02	1.94 ± 0.05	1.27 ± 0.02	2.15 ± 0.07
Zn	0.891 ± 0.057	6.17 ± 0.72	0.801 ± 0.038	5.67 ± 0.24	0.742 ± 0.032	4.98 ± 0.11

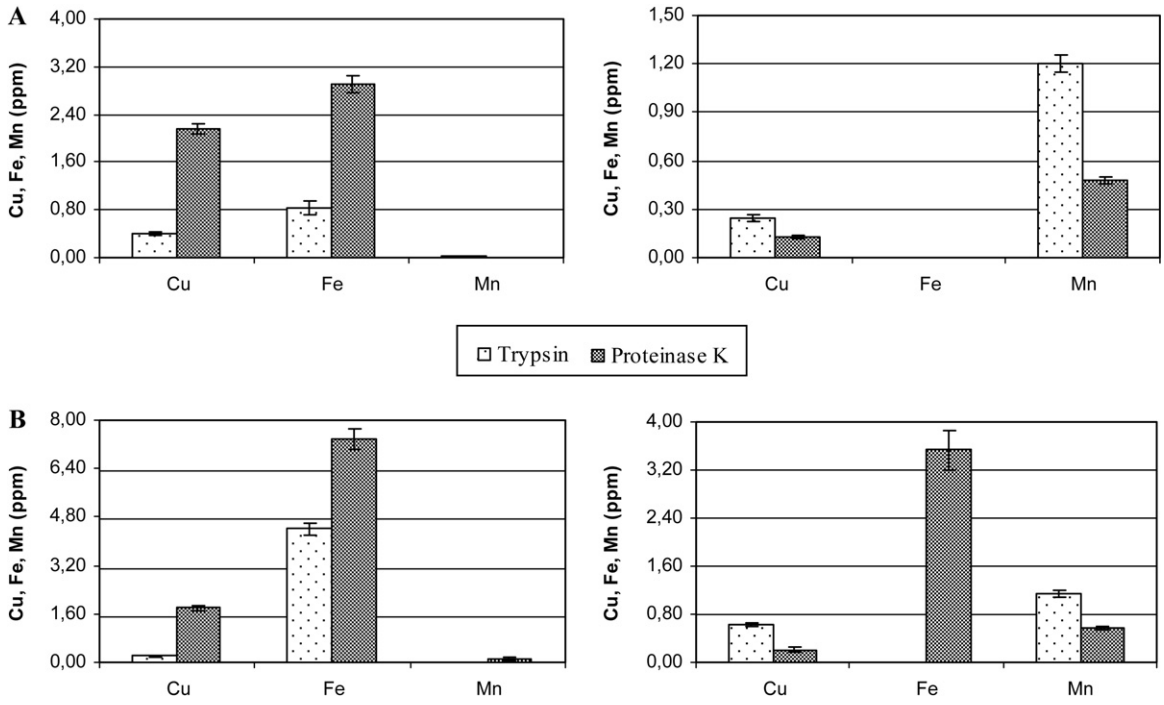

Fig. 2. Buffer-corrected metal concentrations released by untreated (left) or denatured (right) trypsin and proteinase K from sediments A and B. Incubation time: 3 h; enzyme concentration: 0.1 mg/ml; solution/solid ratio: 10:1.

time (from 1 to 6 h), pepsin concentration (from 0.1 mg/ml to 2.0 mg/ml) and solution/solid ratio (10:1 and 50:1).

Both bioaccessible proteins and metals were determined in each experiment but, as already observed in the case of trypsin, protein quantification gave very variable results, so only elemental concentration is reported. These problems in protein quantification might be due to the high specificity of pepsin in the hydrolysis of peptidic bound, as already seen in the case of trypsin.

5.1. Influence of incubation time

Fig. 3 shows the time-dependence of metal release from sediment C by pepsin solution (both untreated and denatured) and by 0.075 M HCl (solution/solid ratio 10:1; pepsin concentration 0.4 mg/ml). Results obtained from untreated and denatured pepsin are rather similar and are also similar to those obtained with 0.075 M HCl. This means that most metal release is due to the acidic pH of the solution, but the presence of pepsin has repercussions on the amounts of metal released that are significantly increased because of its proteolytic activity or complexation ability.

The reported trends are quite regular or slightly decreasing when incubation time is raised from 1 h up to 3 or 6 h and this behaviour is closely related to pH variation. In fact in this work we used lower solution/solid ratios (10:1 in these experiments, no more than 50:1 in other experiments, as reported below) compared with other studies [20] and this has a very strong effect on pH. Indeed, using a 10:1 solution/solid ratio, the only addition of the enzyme solution (or of the 0.075 M HCl) to the sample and the subsequent mixing reflects in a quick pH increase, up to 5–6, due to the solubilization of carbonate material by hydrochloric acid. The rise of pH towards neutral values has two direct consequences: the first is the de-activation of pepsin that is active under more acidic conditions (ideal conditions require a pH value between 2 and 4 [15]); the second is the re-adsorption of some elements on the sample.

As reported in the literature, pH does not represent a problem if higher solution/solid ratios are used, but in this case the working conditions do not represent what exactly happens in the digestive systems of deposit-feeder organisms. Therefore, subsequent experiments were carried out choosing an incubation time of 1 h.

5.2. Influence of enzyme concentration at fixed solution/solid ratio

The amount of metals released from sediment C as a function of the pepsin concentration is shown in Fig. 4. Metals concentration are reported without taking into account the contribution of HCl in order to focus only on the enzyme activity.

Cd, Cr, Mn, Ni, Pb and V were often under their detection limits, making it impossible to have significant trends in their releases and so data about these elements are not reported. As already seen for trypsin, all the experiments were carried out using both untreated and denatured enzyme to evaluate possible metal mobilization mechanisms [16,17]. Results obtained using denatured pepsin are quite similar to those obtained with the untreated enzyme, and so the graphs are not reported. The only particularity concerns Cu, as reported in Fig. 5. Trends for untreated and denatured enzyme are similar, but the amounts of Cu released by denatured enzyme, other things being equal, are always higher. This confirms the strong affinity of Cu for proteinaceous material, but also indicates that thermal denaturation of the enzyme entails structural modifications (such as unfolding processes) that increase its complexation ability, as already seen for trypsin.

As regards the other elements, there is a clear influence of pepsin concentration; in fact, by increasing the concentration from 0.1 mg/ml up to 2.0 mg/ml, it is possible to find a constant rise in metals release, particularly noticeable when pepsin concentration is 2.0 mg/ml. However, it is important to consider that although this concentration is useful to study metals release mechanism, it is not realistic and therefore not very appropriate in reproducing natural gastric conditions. Among the considered elements, As shows a different trend: its released amount is practically independent of the enzyme concentration, as already seen for solution/solid ratio

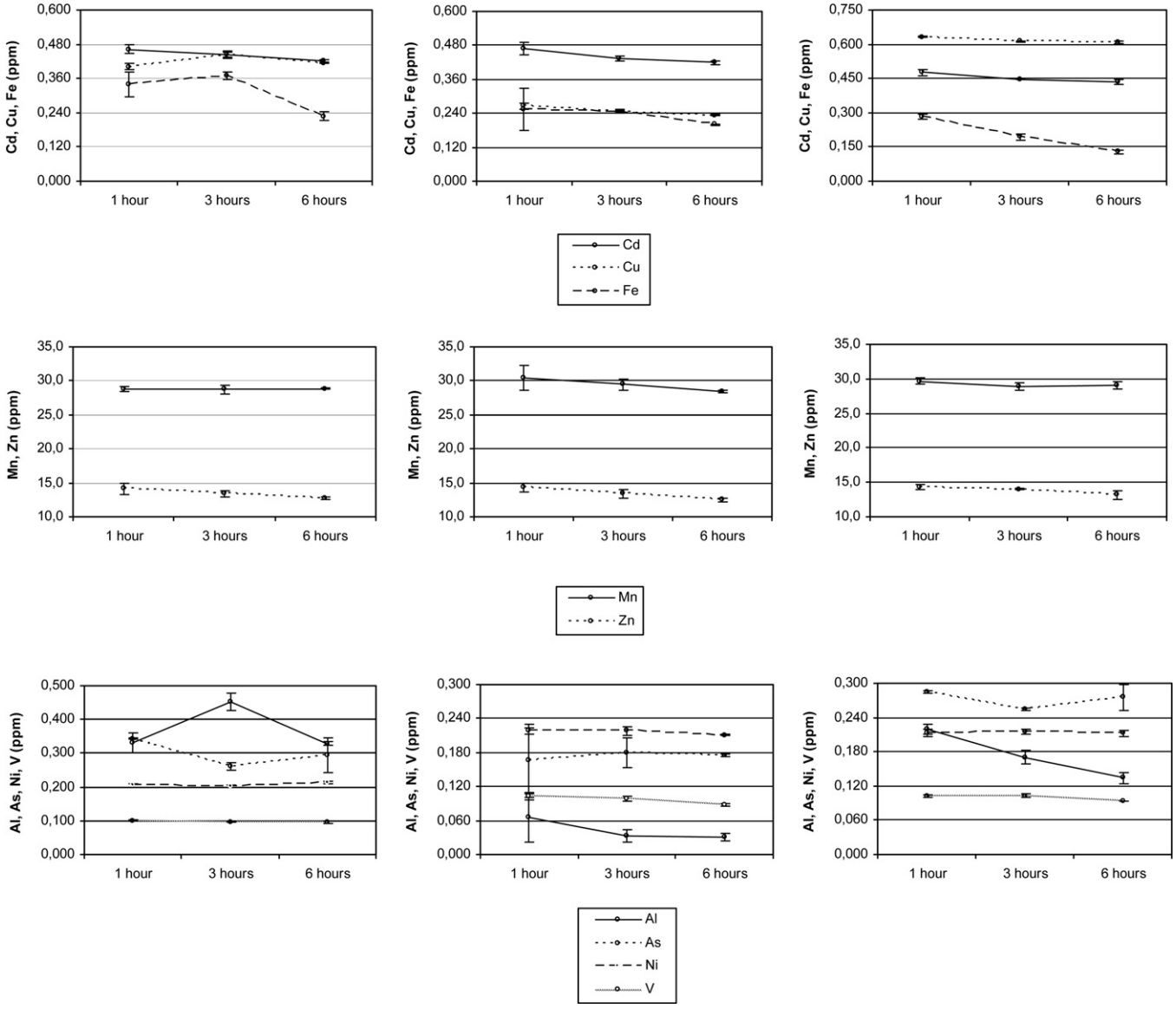

Fig. 3. Time-dependence of metal release by untreated (left) and denatured (right) pepsin and by 0.075 M HCl (central) from sediment C.

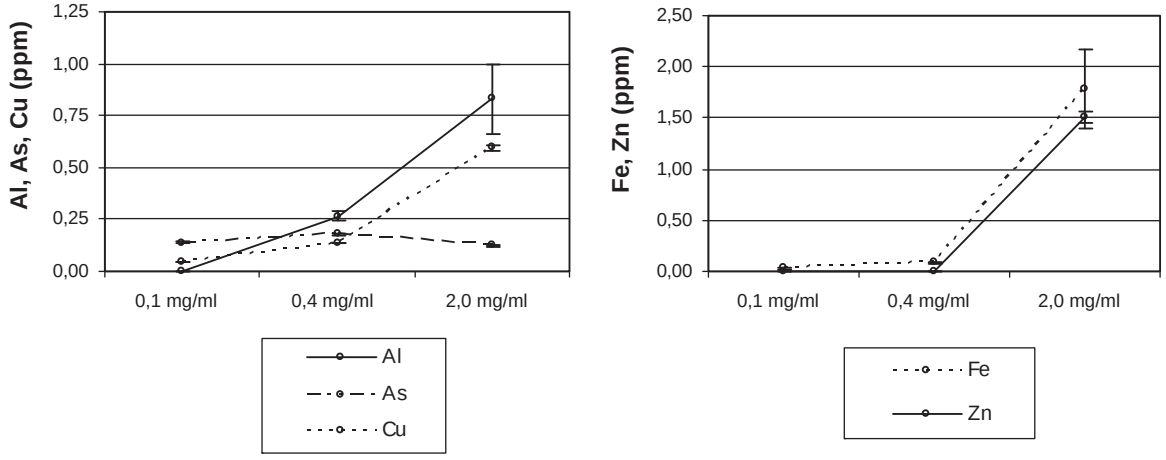

Fig. 4. HCl-corrected metal amounts released by pepsin at different concentrations from sediment C.

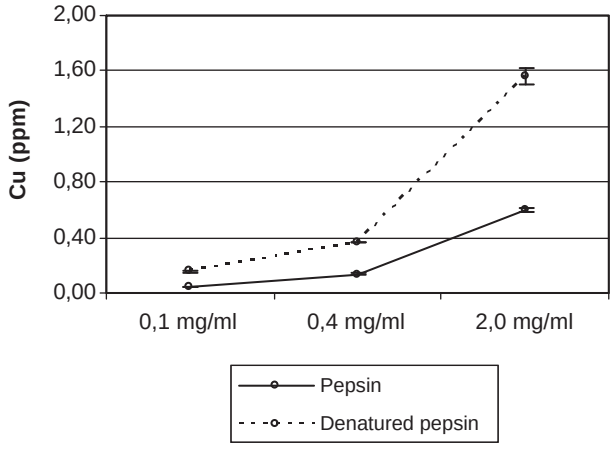

Fig. 5. HCl-corrected Cu amounts released by untreated and denatured pepsin at different concentrations from sediment C.

in the case of trypsin. This is probably due to the fact that the bioaccessible fraction is already completely mobilized (by hydrolytic activity and by complexation) using the lowest pepsin concentration (0.1 mg/ml).

5.3. Influence of solution/solid ratio at fixed enzyme concentration

The last working condition taken into account was solution/solid ratio. Two different ratios were tested, as already done in the experiments with trypsin: a 50:1 ratio and a 10:1 ratio. In each one of these experiments, incubation time was 1 h and pepsin concentration was 0.1 mg/ml.

Table 7 reports all data on metals released from sediment C by pepsin solution (both untreated and denatured) and by 0.075 M HCl, at different solution/solid ratios.

It is noticeable that solution/solid ratio has a strong influence on the amount of released metals; the effect is even more pronounced than in the case of trypsin, especially for some elements like Al and Fe, whose releases rise from a few ppb or less than a ppm up to dozens or hundreds of ppm. Regarding As, it is important to note that, unlike the case of trypsin, there is a clear influence of solution/solid ratio on its release; probably bioaccessible As is released in a lowest amount and not completely in the case of 10:1 ratio, so its release increases proportionally to solution/solid ratio.

Therefore, as already observed for trypsin and in our previous work on proteinase K [17], solution/solid ratio is the most important parameter among the three taken into account. Moreover, in the case of pepsin there is a strong correlation between this parameter and the pH and, consequently, an even stronger correlation between solution/solid ratio and metals release.

Indeed, considering both the effects of incubation time and solution/solid ratio, it is important to underline that the metals release trends that we found are quite different from those indicated by Turner et al. [20]. In this work, the considered metals (Al, Cu, Fe, Mn and Zn) show a biphasic release mechanism, with a first phase of strong mobilization (2–3 h) and a more moderate second phase. In particular, it is reported that Mn, Cu and Zn reach the equilibrium during the second phase while Fe and, in a more restrained way, Al, are continuously released during all the tested incubation time (24 h). From data reported in Fig. 3, on the other hand, it is possible to observe a constant or quite decreasing trend in metals release, but these different behaviours can be easily understood taking into account the solution/solid ratio and the nature of the sample. In the work of Turner et al., in fact, solution/solid ratios from 50:1 to 1000:1 were used. In our work, the ratio used in the experiments regarding incubation time is lower (10:1) and this, together with the characteristics of the sample (high percentage of carbonate material), entails a fast increase of pH. The monitoring of pH in subsequent experiments has shown that it already reaches values between 5 and 6 after only 1 h of incubation, because of the solubilization of carbonate material by HCl. In these conditions, pepsin is de-activated [10] and so it is possible to suppose that the fraction of metals further released when incubation time is increased, is completely mobilized by complexation. Moreover, the strong and fast variation in pH could be another motivation of the re-adsorbtion trend that some metals show when incubation time is increased up to 6 h.

Some experiments were carried out to solve this problem using different buffer solutions (acetic acid/sodium acetate), with pH ranging from 3 to 4, but it was not possible to preserve these conditions for a long time if a 10:1 solution/solid ratio was used. Although better results were achieved using a ratio 50:1, the problem was not solved completely.

6. Comparison between the use of proteolytic enzymes and the first step of sequential extractions

In addition to experiments already reported, sediment C was also treated with proteinase K (following the scheme indicated in our previous work [17]), to make a comparison between different enzyme behaviours; moreover, the first step of BCR sequential extractions procedure [18] was applied. These experiments allowed the evaluation of metals bioaccessible fractions both by chemical and enzymatic extractions. In particular, to make possible a direct comparison with chemical extractions, enzymatic experiments were carried out using a 50:1 solution/solid ratio; enzyme concentration was in all the cases 0.1 mg/ml, while incubation time was 1 h when pepsin was used and 3 h when proteinase K was used.

Fig. 6 shows net concentrations of metals released from the sediment in these working conditions by pepsin and proteinase K; data are reported as percentage of metals released by enzymatic extractions (not considering the solvents contribution) compared with the amount released by a chemical extraction (CH_3COOH 0.11 M, pH 2.8, under constant agitation for 16 h).

As expected, metals are released differently by each enzyme because of their different characteristics and because of their different hydrolytic and complexation abilities. However, in general, the bioaccessible fractions evaluated by enzymatic extraction are often much lower (<30%) than the ones determined chemically; in the case of this sediment there were two exceptions: Cu and Al which, depending on the enzyme used, show "enzymatic" bioaccessible fractions similar to or even slightly higher than the corresponding "chemical" bioaccessible fractions, probably due to their strong affinity for proteinaceous material. Regarding Cu, this fact confirms previous findings on other studies [9,10,17].

As regards proteinase K, a comparison between "chemical" and "enzymatic" bioaccessibility was also made using data obtained from experiments carried out using a 10:1 solution/solid ratio (closer to the environmental conditions); in this case, all the "enzymatic" bioaccessible fractions were lower than 1% if compared with their corresponding "chemical" ones, with the only exception of Cu (about 70%), due to its affinity for proteinaceous material.

However, it is important to bear in mind that, in addition to complexation and hydrolytic activities, there is a third release mechanism due to the solvent that solubilizes the enzyme. In the case of proteinase K (active under neutral pH conditions), the solvent was phosphate buffer at pH 7.5, which has a ionic exchange effect; however this solvent do not represent the conditions of a digestive system, so this contribution is not considered. In the

Table 7
Metal concentrations (ppm) released from sediment C by 0.1 mg/ml untreated and denatured pepsin and by 0.075 M HCl at different solution/solid ratios.

	Pepsin		Denatured pepsin		0.075 M HCl	
	10:1	50:1	10:1	50:1	10:1	50:1
Al	<d.l.	34.2 ± 1.3	<d.l.	35.1 ± 3.3	0.066 ± 0.044	29.5 ± 0.1
As	0.304 ± 0.013	1.78 ± 0.02	0.266 ± 0.018	1.69 ± 0.31	0.166 ± 0.065	1.67 ± 0.25
Cd	0.466 ± 0.051	1.10 ± 0.01	0.480 ± 0.001	1.11 ± 0.01	0.468 ± 0.023	1.12 ± 0.01
Cr	0.011 ± 0.001	0.189 ± 0.008	0.009 ± 0.001	0.198 ± 0.007	0.012 ± 0.002	0.151 ± 0.019
Cu	0.310 ± 0.007	1.56 ± 0.03	0.422 ± 0.013	1.69 ± 0.01	0.266 ± 0.012	1.36 ± 0.08
Fe	0.286 ± 0.050	231 ± 3	0.213 ± 0.004	228 ± 5	0.253 ± 0.074	217 ± 3
Mn	27.5 ± 0.7	106 ± 2	27.9 ± 0.5	108 ± 1	30.4 ± 1.8	108 ± 1
Ni	0.216 ± 0.006	1.24 ± 0.05	0.211 ± 0.004	1.23 ± 0.02	0.218 ± 0.005	1.24 ± 0.02
Pb	0.002 ± 0.002	0.315 ± 0.011	<d.l.	0.337 ± 0.012	0.011 ± 0.010	0.257 ± 0.013
V	0.115 ± 0.005	0.744 ± 0.017	0.114 ± 0.004	0.771 ± 0.031	0.102 ± 0.005	0.699 ± 0.023
Zn	13.5 ± 3.5	108 ± 1	13.8 ± 0.6	109 ± 2	14.3 ± 0.7	107 ± 1

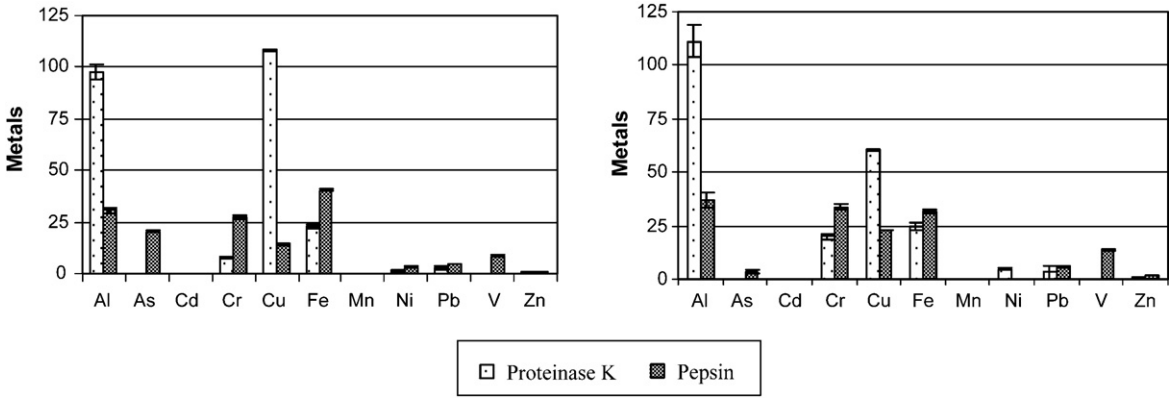

Fig. 6. Net concentrations of metals released from sediment C by untreated (left) and denatured (right) enzymes, expressed as percentage of the amount released by chemical extraction with 0.11 M CH_3COOH.

case of pepsin (active under acidic pH conditions), instead, the solvent (0.075 M HCl) gives a strong contribution to metals release from sediment because of its low pH. Moreover, it is representative of what happens in nature, because hydrochloric acid is actually present in gastric systems, where it also activates pepsin (secreted as pepsinogen, an inactive zymogen [10]). With this premises, when pepsin is the enzyme chosen for enzymatic extractions, it is also important to take into account this third metal release mechanism. The metal concentrations released by pepsin solution (considering also HCl contribute) and acetic acid are reported in Fig. 7.

Bioaccessible fractions evaluated by pepsin are often similar to or higher than the ones determined chemically. This is easily explained considering the effect of pH that is next to 1 in the pepsin solution while in the chemical procedure indicated by BCR is 2.8, and considering the double effect (hydrolysis/complexation) of the enzyme.

7. Metal mobilization

To demonstrate that an enzymatic extraction leads to more accurate bioaccessibility data compared with a chemical

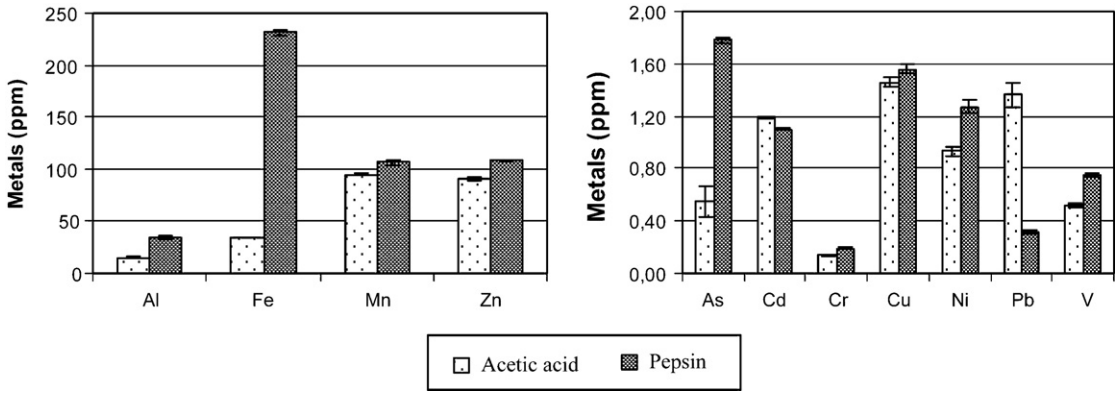

Fig. 7. Metal concentrations released from sediment C by 0,1 mg/ml pepsin solution and by 0.11 M CH_3COOH.

Table 8
Metal fractions mobilised from sediment C by proteinase K (f_k). pepsin (f_p), trypsin (f_t) and acetic acid (f_s).

Sediment C	Al	As	Cd	Cr	Cu	Fe	Mn	Ni	Pb	V	Zn
f_k	0.034	–	0.277	0.008	5.23	0.037	0.015	0.081	0.157	–	0.429
f_p	0.079	13.4	76.0	0.756	5.17	1.08	22.8	7.90	1.24	1.75	49.0
f_t	0.059	–	–	0.128	1.96	0.061	0.001	0.044	0.480	–	0.541
f_s	0.035	4.13	82.6	0.556	4.84	0.160	20.3	5.80	5.35	1.22	41.1

extraction, it is possible to consider the fractions of metals mobilized by the enzymes and by acetic acid (selective sequential extraction) in sediment C. We can define f_k, f_p, f_t and f_s as the metal concentration released from the sediment by proteinase K, pepsin, trypsin and acetic acid, respectively, in a 50:1 solution/solid ratio, in relation to the aqueous regia soluble concentration in the sample. As regards proteinase K and trypsin, buffer-corrected data were considered, whereas as regards pepsin, the contribution of HCl to metals release was not subtracted.

Fractions mobilized by different enzymes and by acetic acid are reported in Table 8.

As regards f_k, the order of mobility was Cu > Zn > Cd > Pb > Ni > Fe > Mn, Cr which is quite similar to other orders reported in the literature [16,17]. The mobility order defined by the use of trypsin was Cu > Zn, Pb > Cr > Fe > Ni > Mn > Cd, proving to be quite different to that defined by proteinase K, even if the two enzymes have similar mechanisms of action (but different specificity).

Orders of mobility obtained with pepsin (Cd > Zn > Mn > Ni > Cu > Pb, Fe > Cr) and with acetic acid (Cd > Zn > Mn > Ni, Pb, Cu > Cr > Fe) are quite similar and show some differences compared with the others. As regards pepsin, this is obvious considering the different mechanisms of action compared with proteinase K and trypsin and confirms that this enzyme is representative of a different part of the digestive process. Moreover, the similarity between orders of mobility defined by pepsin and by acetic acid confirms the strong influence of acidic pH in metals release, whose contribution is higher than the one supplied by the enzyme.

The validity of these data can be evaluated considering factors, reported in literature [27], defining the enrichment of metal in the gut fluid of the lugworm, *Arenicola marina*, relative to continental rock (enrichment factor–EF) and correlating them with the various previously reported f_x.

The best correlation is obtained in the case of proteinase K ($R^2 = 0.6606$), supporting that this enzyme is able to qualitatively mimic the interactions between trace metals and gut fluids of deposit-feeders [16]. Data from trypsin ($R^2 = 0.1887$) lead to a worse correlation and this, in addition to the limitations described above, suggests not to use this kind of enzyme in a biomimetic approach.

Correlation between f_s and EF ($R^2 = 0.4592$) confirms that selective extractions provide less realistic data regarding trace metals mobility, in addition to overestimation, if compared with proteinase K.

The similarity between data from acetic acid and from pepsin ($R^2 = 0.4060$) suggests that even this enzyme should not be used to evaluate metals bioaccessibility in deposit-feeders that live in contact with sediments. Moreover, this enzyme (in addition to practical problems for its use, as described above) is representative of a part of the digestive process that is rarely present in the organisms which are the target of this kind of study.

As regards proteinase K, f_k obtained from experiments carried out with a 10:1 solution/solid ratio (which is a condition closer to the environmental one) were also correlated with EF; despite the low amount of metals released from the sediment, this correlation ($R^2 = 0.5894$) is still better than those obtained with trypsin, pepsin and acetic acid, thus confirming the ability of proteinase K to qualitatively mimic the interactions between trace metals and gut fluids of deposit-feeders [16].

V and As were not considered in the above discussion, the former because its enrichment factor was not available, and the latter because its bioaccessible fractions were not in agreement with enrichment factors in none of the extractions carried out.

8. Conclusions

In this study, different proteolytic enzymes (trypsin and pepsin) were tested for their use as biomimetic reagents to determine metal bioaccessibility in marine sediments. Once the working conditions were studied, their behaviours were compared with each other and with that of proteinase K, already studied in a previous work [17].

As regards trypsin, released metal concentrations are always lower compared with proteinase K. Moreover, bioaccessible proteins quantification showed low reproducibility and non-reliable data. These results may be due to the high specificity of trypsin in the hydrolysis of peptidic bound. Since these two enzymes are representative of the same part of the digestive process, it is better to utilize proteinase K than trypsin.

With regard to pepsin, the release pattern is different from that of proteinase K, because these two enzymes have completely different characteristics and are representative of two distinct phases of digestion process. As for trypsin, bioaccessible protein data were unreliable, probably for the same reason, i.e. the high specificity in peptides hydrolysis. Furthermore, this enzyme requires pH conditions which are difficult to reproduce using a solution/solid ratio similar to the natural one.

The comparison between enzymatic and chemical extractions (first step of BCR selective sequential procedure) showed that, generally, the chemical-defined bioaccessible fraction is overestimated compared to the enzymatic-defined one. This is true if the solvent contribution to metal release is not considered but, in the case of pepsin (active under acidic pH conditions), the solvent (0.075 M HCl) has to be considered, being representative of what happens in nature, where its presence is necessary to activate the enzyme. Therefore, considering the strong contribution of HCl to metals release, the bioaccessible fraction measured by pepsin is often similar to or higher than the one determined with acetic acid.

Moreover, data validity was evaluated considering metal orders of mobility (determined calculating the fractions mobilized by the different enzymes and by acetic acid) and comparing them with that defined by the enrichment factors, reported in the literature for *Arenicola marina* (a lugworm representative of deposit-feeder organisms), finding that proteinase K shows a better capability in reproducing natural phenomena.

With these premises, the use of proteinase K proves to be the most suitable way to determine metal bioaccessibility in marine sediments, when the target of the investigation is deposit-feeder organisms, whose digestion is generally based on neutral pH enzymatic processes.

However, pepsin can be used successfully when the aim of the study is humans [13,28], where gastric digestion plays an important role and where a higher solution/solid ratio is required, making it easier to keep pH in the optimal range.

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