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Freshwater molluscs from volcanic areas as model organisms to assess adaptation to metal chronic pollution

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Abstract

Cellular biomarkers of exposure and biological effects were measured in digestive gland of snails (*Physa acuta*) sampled in sites with and without active volcanism in São Miguel Island (Azores). Metal content in digestive cell lysosomes was determined by image analysis after autometallography (AMG) as volume density of autometallographed black silver deposits (Vv_{BSD}). Lysosomal structural changes (lysosomal volume, surface and numerical densities – Vv_{LYS}, Sv_{LYS} and Nv_{LYS}–, and surface-to-volume ratio – S/V_{LYS}–) were quantified by image analysis, after demonstration of β-glucuronidase activity, on digestive gland cryotome sections. Additional chemical analyses (atomic absorption spectrophotometry) were done in the digestive gland of snails. The highest metal concentrations were found in snails from the active volcanic site, which agreed with high intralysosomal Vv_{BSD}. Digestive cell lysosomes in snails inhabiting sites with active volcanism resembled a typical stress situation (enlarged and less numerous lysosomes). In conclusion, the biomarkers used in this work can be applied to detect changes in metal bioavailability due to chronic exposure to metals (volcanism), in combination with chemical analyses.

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Keywords: Volcanism; Exposure biomarkers; Biomarkers of effect; Snails

1. Introduction

Chemical analyses of tissues of sentinel species have been worldwide used to determine the bioavailable fraction of metals in aquatic environments. However, the accuracy of these analyses has been widely discussed because of the existence of a great number of variables affecting the estimation of metal bioavailability in terms of metal concentrations in soft tissues of molluscs (Fischer, 1986; Soto et al., 1995; Boening, 1999; Marigómez et al., 2002; Rainbow, 2002).

A biomarker approach based on cellular responses to pollutants in sentinel molluscs can be useful in biomonitoring programmes (Soto and Marigómez, 1997a,b). Changes occurring at cell or tissue levels are less affected by abiotic or biotic factors (i.e. salinity, temperature, season, changes in weight,...) and provide realistic and feasible indication of the fraction of bioavailable metals present in the environment and its biological effects (Marigómez et al., 2002).

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The digestive gland of molluscs is known to be the major target site for metal accumulation and detoxification (~~Hemelraad and Herwig, 1988;~~ Soto and Marigómez, ~~1999;~~ Marigómez et al., 2002). A main target cell compartment involved in metal metabolism and sequestration of other xenobiotics is the endolysosomal system of digestive cells (Marigómez et al., 1995; Dimitriadis and Papadaki, 2004).

Two well-established biomarkers were selected for the present study. Intralysosomal accumulation of metals revealed by autometallography (AMG) in digestive cells was used as biomarker of metal exposure. Changes in the structure of digestive cell lysosomes were measured as effect biomarker. AMG has been used in combination with image analysis to localize and quantify metals in cell compartments of invertebrate tissues as volume density of autometallographed black silver deposits (Vv_{BSD}), which has been proposed as a biomarker of metal exposure (Soto et al., 1996a,b, 2002; Soto and Marigómez, 1997a,b; Da Ros et al., 2000; Marigómez et al., 2002; Dimitriadis and Papadaki, 2004). AMG is a sensitive histochemical technique that only requires very few atoms of a given metal in the tissue to catalyse the deposition of metallic silver around them (Danscher, 1984).

Lysosomes are cell organelles with a high content of acid hydrolases devoted to the intracellular digestion of endogenous and exogenous compounds (Cajaraville et al., 1995a,b). Lysosomes have a crucial role in the detoxification of toxic substances, and for that reason changes in lysosomal structure have been used as general marker of pollutant induced stress in a number of field and laboratory studies using molluscs as sentinel organisms (Lowe et al., 1981; Moore, 1988; Cajaraville et al., 1991, 1995a,b; Etxeberria et al., ~~1995;~~ Marigómez et al., 1996; Domouhtsidou and Dimitriadis, 2001, 2004; Marigómez and Baybay-Villacorta, 2003; Koukouzika and Dimitriadis, 2005).

It has been shown that, in aquatic organisms, contaminants cause a significant increase in lysosomal size that eventually can be accompanied by increases in lysosomes number (Lowe et al., 1981; Cajaraville et al., 1991, 1995a,b). Investigations on lysosomal responses in digestive cells of freshwater molluscs are more limited (Giamberini and Pihan, 1997; Giamberini and Cajaraville, 2005; Guerlet et al., 2006). Up to now most of the studies dealt with controlled laboratory exposures to metals or with field studies in areas with strongly marked dissimilar metal availabilities due to industrialisation and release of untreated or partially treated sewage. Few works have been done taking into account natural metal sources with no anthropic origin.

The Azores archipelago is remote from industrial activities despite of the appearance of an expanding tourism. However, the volcanic origin and the unusual geological features of the archipelago may well enhance a continuous availability of trace metals to biota. The archipelago is located in the North Atlantic Ocean at the triple junction of Eurasian, African and North American plates characterised by a complex tectonic settlement, where the seismic and volcanic phenomena are common (Nunes et al., 1993). Therefore, the remaining volcanic activity in certain sites of São Miguel, which is one of the nine islands comprising the Azores archipelago, provides a good "field-laboratory" for investigating the capacity of freshwater snails *Physa acuta* to cope with continuous input of natural metal sources during generations. The main objective of the present investigation is to determine whether freshwater molluscs living in volcanic areas are able to assess adaptation to metal chronic pollution based in the use of selected biomarkers of exposure and effect.

2. Materials and methods

2.1. Experimental design

Different populations of freshwater snails *Physa acuta* were collected from two different sites (Fig. 1) exhibiting dissimilar volcanic profiles at São Miguel: Tanque do Monte (TM) (4.01 m.y.), a site of volcanic origin that has no volcanic activity since 2 million years ago, and Lagoa da Furnas (LF) (0.75 m.y.), a sampling station located inside a crater of a still active volcano showing several active hydrothermal points.

2.2. Histological processing

A portion of the digestive gland of 10 snails per station was fixed in Bouin's fluid at 4 °C for 24 h (Martoja and Martoja-Pierson, 1970), dehydrated in ethanol (70% for 2 h; 96% for 2 plus 2 h; 100% for 2 plus 2 h), cleared in methylbenzoate (overnight), rinsed in benzene (45 plus 45 min) and embedded in paraffin (at 60 °C for 4 h). Histological sections (7 µm) were cut in a Leitz 1512 microtome (Leica Microsystems, Wetzlar, Germany), mounted in albumin coated slides (Menzel-Glaser, Braunscheig, Germany), dried at 40 °C for 24 h, and stored at room temperature until staining.

A second portion of the digestive gland was cryoprotected in phosphate buffer (0.1 M, pH = 7.4) plus sucrose (0.5%), embedded in Cryo-M-Bed, frozen in liquid nitrogen and stored at −40 °C. Frozen samples

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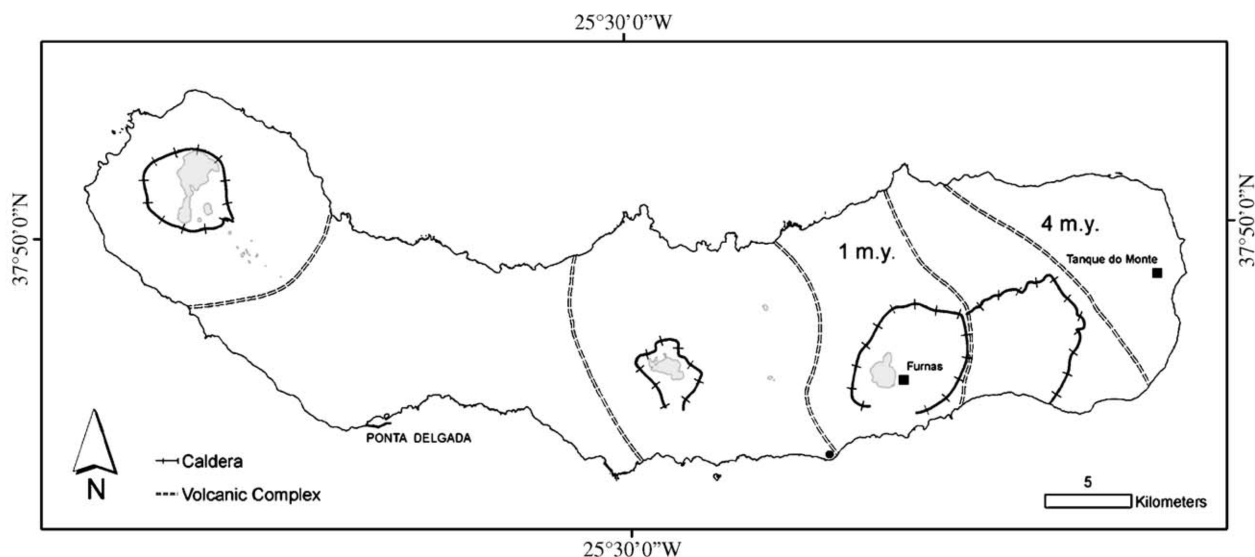

Fig. 1. Sampling locations in São Miguel Island (Azores). Geological age expressed in Ma (million of years) and ma (thousand of years).

143 were cut in a cryostat (Leica CM3000) at a cabinet 144 temperature of −24 °C. Sections (8 μm) were collected 145 onto glass slides and stored at −40 °C until use.

146 2.3. Autometallographed metal content in lysosomes

147 Intralysosomal metal levels were determined on 148 paraffin sections by autometallography (AMG; Danscher, 149 1984; Soto et al., 1998a,b). Briefly, paraffin sections 150 (7 μm) were dewaxed in xylene, hydrated in ethanol–water 151 mixtures and left in an oven at 37 °C until completely dried 152 (24 h). Tissue sections were covered with a photographic 153 emulsion (Ilford Nuclear Emulsion L4) under safety light 154 conditions. After drying for 45 min in total darkness, 155 sections were rinsed in a developer bath (1:5, b/w Ultrathin 156 Tetenal) for 15 min, rinsed in a stop bath (1% acetic acid) 157 for 1 min, and finally rinsed in a fixative bath (1:10, b/ 158 w Agefix Agfa) for 10 min (Soto et al., 1998a,b). Sections 159 were mounted in Kaiser's glycerol gelatine (Merck). 160 Metals were developed as black silver deposits (BSD) and 161 quantified by means of image analysis (BMS, Sevisan, 162 Bilbo). The volume density of BSD (VD_{BSD}) was 163 calculated by stereology as $VD_{BSD}=V_{BSD}/V_{Ti}$, where 164 V_{BSD} is volume of BSD and V_{Ti} is volume of tissue (Soto et 165 al., 1997b).

166 2.4. Lysosomal structural changes

167 The visualization of lysosomes was based on 168 the histochemical demonstration of β-glucuronidase 169 (β-GUS) activity, according to Cajaraville et al. (1991) 170 and adapted to freshwater organisms as suggested by 171 Giamberini and Cajaraville (2005). Briefly, sections 172 were incubated in freshly prepared β-GUS incubation 173 medium (28 mg naphthol AS-BI-β-D-glucuronide dis- 174 solved in 1.2 ml 50 mM sodium bicarbonate, made up to 175 100 ml with 0.1 M acetate buffer, pH 4.5, containing 176 15% of polyvinyl alcohol) for 20 min at 37 °C in a 177 shaking water bath. After incubation, slides were rinsed 178 in a saline solution (2.5% NaCl) for 2 min at 37 °C in a 179 shaking water bath and then transferred to a postcou- 180 pling medium, 0.1 g Fast Garnet GBC dissolved in 100 181 ml of 0.1 M phosphate buffer (pH 7.4) containing 2.5% 182 NaCl, where they were kept in darkness for 10 min.

t1.1 Table 1
t1.2 Metal concentrations (μg metal/g dry weight) measured by atomic absorption spectrophotometry in the whole soft tissue of snails from two different locations of Sao Miguel island (Azores)

Place	(Cu)	(Fe)	(Zn)	(Cd)	(Cr)	(Pb)	(Ni)
Lagoa da Furnas	82.55 (36.12)	1836.63 (700.98)	1652.12 (641.78)	3.59 (1.53)	3.27 (1.66)	n.d.	36.36 (17.08)
Tanque do Monte	188.21 (80.06)	1008.61 (563.38)	1056.32 (802.36)	16.81 (10.46)	10.77 (5.90)	n.d.	33.78 (14.38)

t1.6 Standard deviation between brackets. $n=5$ replicates of 10 pooled samples each. n.d. below detection limit.

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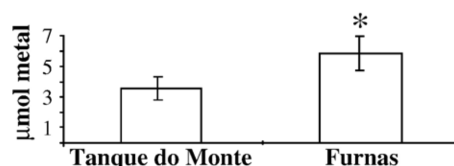

Fig. 2. Total metal load (μmol metal) in snails from Tanque do Monte and Lagoa da Furnas. Asterisk indicates significant differences between pairs of means based on Duncan's tests ($p \leq 0.05$).

183 Afterwards, sections were fixed for 10 min at 4 °C in 184 Baker's formol calcium containing 2.5% NaCl and 185 rinsed briefly in distilled water. Finally, sections were 186 counterstained with an aqueous solution of 0.1% Fast 187 Green for 2 min, rinsed several times in distilled water, 188 mounted in Kaiser's glycerol gelatin and sealed with 189 nail varnish.

190 The structure of lysosomes was determined by image 191 analysis (BMS, Sevisan, Bilbo) according to Cajaraville 192 et al. (1991). Slides were examined using an objective lens 193 of 100× magnification in a Leitz light microscope in order 194 to calculate the following stereological parameters: 195 lysosomal volume density ($Vv_{LYS}=V(L)/V(C)$), surface 196 density ($Sv_{LYS}=S(L)/V(C)$), surface-to-volume ratio ($S/V_{LYS}=S(L)/V(L)$) 197 and numerical density ($Nv_{LYS}=N(L)/V(C)$); 198 where V=volume, S=surface, N=number, L=lyso- 199 somes, and C=digestive cell cytoplasm. Sample size was 200 determined based on previous analyses of mean and 201 standard deviation values of the four parameters which at 202 least resulted to keep constant for a sampling area over 203 16,000 μm^2 (Etxeberria et al., 1994; Marigómez et al., 204 2005). Since the total area of the digestive cells scanned in 205 each measurement was approximately 4000 μm^2, 5 mea- 206 surements were made on one single section (total 207 sampling area per specimen approx. 20,000 μm^2).

2.5. Chemical analysis (atomic absorption spectrometry) 208

209 Another set of freshwater snails was separately pro- 210 cessed for chemical analysis of metals. In order to 211 eliminate the gut contents prior to metal analysis snails 212 were distributed in plastic tanks in a thermostatically 213 controlled (13–15 °C) continuous water-flow system, 214 with active charcoal and glass-wool filtered natural 215 spring water, for 48 h in absence of food. After dis- 216 section, soft tissues were rinsed in distilled water and 217 dried at 120 °C for 48 h until constant weight was 218 reached. Fifty snails per sampling site were grouped in 219 pools of ten snails each giving 5 replicates per sample, 220 digested in concentrated nitric acid, diluted with 0.1 M 221 nitric acid and analysed by atomic absorption spectrom- 222 etry (flame atomic absorption spectrometer Perkin Elmer 223 2280) with simultaneous background correction and a 224 sensitivity of 0.3 mg/l. Merck standard solutions were 225 diluted in 0.1 M nitric acid for calibration. Seven metals 226 were analysed, cadmium (Cd), chromium (Cr), lead (Pb), 227 nickel (Ni), copper (Cu), zinc (Zn) and iron (Fe). The 228 accuracy of the method was verified with the reference 229 material Dorm-2 (dogfish muscle tissue) provided by the 230 National Research Council of Canada, Institute for 231 National Measurement Standards (Ottawa, Canada) with 232 the following results in μg dry weight g^{-1} (n=6 for 233 measured values): Cu 2.59±0.08 measured value versus 234 2.34±0.16 assigned value, Zn 27.47±0.57 measured 235 value versus 25.6±2.3 assigned value, Cd 0.036±0.012 236 measured value versus 0.043±0.008 assigned value, Cr 237 32.4±4.2 measured value versus 34.7±5.55 assigned 238 value, Pb 0.084±0.009 measured value versus 0.065± 239 0.007 assigned value, Ni 22.3±5.2 measured value 240 versus 19.4±3.10 assigned value, Fe 139.52±6.2 mea- 241 sured value versus 142±9.94 assigned value. Metal con- 242 centrations in μg metal/g dry flesh weight were measured 243 and transformed into μmol total metal/g dry flesh.

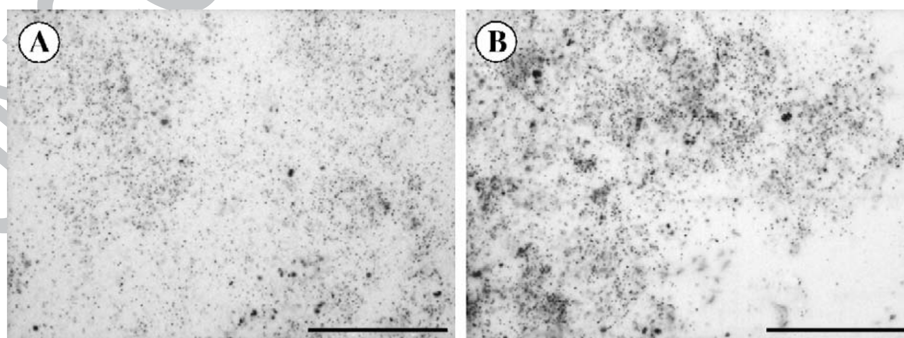

Fig. 3. Autometallographical localization of metals in the digestive cell lysosomes of snails from Tanque do Monte (A) and Lagoa da Furnas (B). Scale bars: 50 μm.

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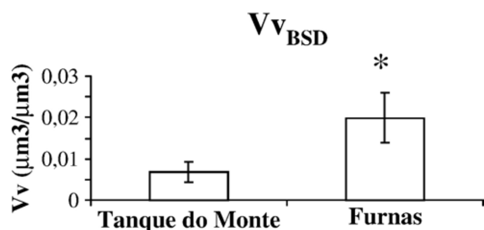

Fig. 4. Volume density of BSD ($\mu m^3/\mu m^3$, Vv_{BSD}) in the digestive cells of snails from Tanque do Monte and Lagoa da Furnas. Statistics as in Fig. 2.

2.6. Statistics

Data were analyzed for homogenity of variances (Levene's test) and normality (Kolmogorov–Smirnov test). Results were reported as mean values ± standard deviations (SD). Data were analyzed by the t test at a significance level of $p<0.05$. Vv_{BSD}, lysosomal Vv_{LYS} and Nv_{LYS} were logarithmically transformed (the variance within individuals depended on the mean) in order to obtain a normal distribution of the data and one-way analysis of variance (ANOVA) was performed. Significant differences between means were established at $p \leq 0.05$ level using the Duncan's test for multiple range comparison between pairs of means. Regression and correlation analyses were made between Vv_{BSD} and total metal load. The statistical analyses were carried out with the aid of the SPSS/PC+(SPSS Inc., Microsoft Co.) statistical package.

3. Results

3.1. Metal content

Metal concentrations recorded in the whole soft body of freshwater snails are shown in Table 1. The dry weights for all the samples were significantly similar ranging from 0.094–0.098. Concentrations (μg metal/g dry tissue weight) of Ni were similar in snails collected in both sampling sites, LF and TM. Cd, Cu and Cr concentrations were higher in snails from TM than in LF although they were statistically similar due to the high variability recorded (ANOVA, $p>0.05$). However, snails from LF exhibited a significantly higher total metal concentration (μmol metal; Fig. 2) mainly due to the high recorded levels of iron (1836 μg Fe/g dry tissue weight) and zinc (1652 μg Zn/g dry tissue weight) that where nearly twofold higher than in snails from TM (Table 1). Pb levels in both populations of snails were below detection limits.

3.2. Autometallographed BSD

After AMG, metal ions were revealed as black silver deposits (BSD) easily distinguished under the light microscope (Fig. 3). BSD were specifically found in digestive cell lysosomes whilst basophilic cells were completely devoid of them in snails collected in both LF and TM (Fig. 3). Digestive cell debris excreted to the lumen of the digestive alveolus (mainly in snails from LF) presented conspicuous BSD in materials of lysosomal origin. The basal lamina of the digestive epithelium of snails from LF exhibited BSD whilst this compartment in snails from TM lacked BSD. Small and tiny BSD were also observed in the stomach wall of snails from both stations.

Vv_{BSD} values recorded in the digestive cells of freshwater snails from LF were significantly higher than in snails from TM ($p<0.05$; Fig. 4).

A significant correlation ($p<0,05$) was found between Vv_{BSD} and total metal loads taking into account all samples available from both TM and LF (r^2: 0.7031, $n=18$).

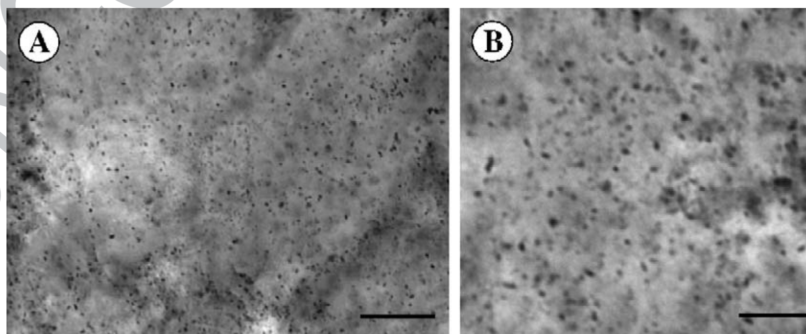

Fig. 5. Lysosomes in cyostat sections of the digestive gland of snails after demonstration of β-glucuronidase activity. (A) Tanque do Monte (B) Lagoa da Furnas. Scale bars: 20 μm.

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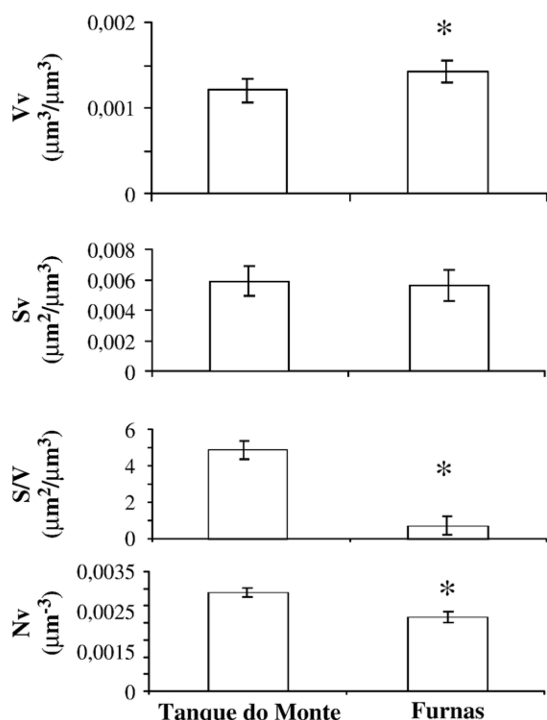

Fig. 6. Results of the stereological analysis of digestive cell lysosomes of snails collected in Tanque do Monte (C) and Lagoa da Furnas. Vv, volume density; Sv, surface density; S/V, surface to volume ratio; Nv, numerical density. Statistics as in Fig. 2.

3.3. Lysosomal structural changes

Lysosomes were visualized as red–purple precipitates in the digestive cells of snails after the histochemical demonstration of β-glucuronidase (β-GUS) activity (Fig. 5). Increased environmental metal concentrations in snails from LF provoked enlargement of the lysosomal system of the digestive cells in comparison with the small lysosomes visualized in the digestive cells of snails from TM (Fig. 6). This enlargement resulted from increased Vv_{LYS} and increased lysosomal size (reduced S/V_{LYS}). The lysosomes in the digestive cells of snails from TM were much more numerous (increased Nv_{LYS}) than in snails from LF (Fig. 6).

4. Discussion

Chemical analyses of molluscan tissues have been widely suggested to be accurate tools to determine metal levels in different environments (Soto et al., 1995; Rainbow et al., 2002). In the present work, soft tissue concentrations of Fe and Zn measured in freshwater snails from the two locations selected (LF and TM) were very high. However, snails from LF, which is a volcanically active area, exhibited higher total metal concentrations than those from TM. Accordingly, LF was formerly known for its high iron bioavailability because of the presence of very active hydrothermal sources. Some previous works have reported the existence of high levels of certain metals (Zn, Cu and Cd) in relation with thermal hot springs in barnacles (Weeks et al., 1995) and with volcanism in littoral, marine, terrestrial, and euterrestrial crustaceans (Moore et al., 1995).

Nowadays, apart from using chemical analyses in sentinel organisms to detect metal levels in the environment, the use of cell and tissue biomarkers is gaining attention to assess the level of exposure and the biological effects produced by changes in metal bioavailability (Cajaraville et al., 2000). One of these exposure biomarkers used in this work is based on the use of a histochemical technique, autometallography, which detects metals (loosely bound to proteins) present in tissue sections as black silver deposits (BSD) (Danscher, 1984; Soto et al., 1998b, 2002). These BSD can be quantified with the aid of automated image analysis to assess the bioavailable fraction of metals in the environment (Hemelraad and Herwig 1988, Herwig et al., 1989; Soto et al., 1996a,b, 1998a,b; Soto and Marigómez, 1997a,b). Autometallography revealed different pictures of metal bioavailability between LF and TM. Interestingly, conspicuous BSD were observed (but not quantified) in the basal lamina of digestive tubules of snails from LF while this compartment was devoid of BSD in animals from TM. This cell compartment has been previously reported as a preference site for Zn accumulation in seawater snails *Littorina littorea* (Soto et al., 1998). Likewise, Zn concentrations were very high in snails from Lagoa da Furnas, and therefore, it can be concluded that one of the main cellular sites for zinc accumulation can be the basal lamina of the digestive tubules.

Several investigations have outlined the relevant role of the digestive cells of the digestive epithelium of molluscs, and more precisely their lysosomal system, in metal handling, accumulation and detoxification (see review by Marigómez et al., 2002). Accordingly, the quantification of BSD (Vv_{BSD}) in the digestive cell lysosomes of the digestive gland of snails revealed that snails from LF exhibited higher metal bioavailability than snails from TM. These values were strongly correlated with the total metal loads quantified in the whole soft body of snails. It can be concluded that Vv_{BSD}, quantified in the digestive cell lysosomes of snails accurately reflects increased metal bioavailabilities in freshwater related to active volcanism.

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Changes in lysosomal structure have been used as a general marker of pollutant impact in a number of field and laboratory studies using molluscs as sentinel organisms (Lowe et al., 1981; Moore, 1988; Cajaraville et al., 1991, 1995a; Etxeberria et al., ~~1995;~~ Marigómez et al., 1996; Domouhtsidou and Dimitriadis, 2001; Marigómez and Baybay-Villacorta, 2003). Combined enzyme histochemistry and image analysis have been used to determine changes in lysosomes (Cajaraville et al., 1995b). In the present work, the stereological analyses revealed significant differences in the structure of the digestive lysosomal system from both snail populations. Snails from LF exhibited (together with a higher metal load and higher Vv_{BSD}) a higher volume density of larger and less numerous lysosomes (enlarged lysosomes) than in TM. It has been previously reported that the presence of contaminants may cause a significant enlargement of lysosomes that eventually can be accompanied by a reduction in lysosome numbers (Lowe et al., 1981; Cajaraville et al., 1991, 1995a,b; Bilbao et al., ~~2005).~~ Therefore, changes in these parameters clearly reflect the influence of active volcanism in Furnas.

5. Conclusions

Changes in metal bioavailability due to active volcanism can be assessed by quantifying the Vv_{BSD} in tissue sections of the digestive gland of freshwater snails. Moreover, the stereological parameters indicative of the structure of the lysosomal system in the digestive gland of freshwater snails reflect that snails inhabiting places with active volcanism suffer a typical stress situation possessing enlarged and less numerous lysosomes (Cajaraville et al., 1991, 1995a,b; Marigómez and Baybay-Villacorta, 2003). The ability of molluscs to survive in polluted sites might be related to their ability to detoxify pollutants or to avoid excessive exposure (i.e. sequestering metals within lysosomes). There is clear evidence of severe adaptive changes in the distribution and relative occurrence of cell types in the digestive gland of molluscs after long-term/chronic exposure to pollutants (Rasmussen et al., 1983; Widdows et al., 1984; Cajaraville et al., 1990a,b; Marigómez et al., 1990, 1996, ~~1998; Zaldibar,~~ et al., 2002). The fact that the population of snails is present in these places by generations, may suggest a kind of plastic adaptation. The mechanism of this possible genetic adaptation in the case of the digestive gland of snails deserves future investigations to determine if animals from a chronically metal-exposed area (active volcanism) are significantly different from animals from clean places in metal metabolism and if there are differences in cell composition.

6. Uncited references

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~~Köhler et al., 2002~~
~~Moore, 1976~~
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~~Weibel, 1979~~

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