Benthic foraminiferal ultrastructural alteration induced by heavy metals

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Abstract

Heavy metals are known to cause deleterious effects on biota because of their toxicity, persistence and bioaccumulation. Here, we briefly document the ultrastructural changes observed in the miliolid foraminifer *Pseudotriloculina rotunda* (d'Orbigny in Schlumberger, 1893) and in the perforate calcareous species *Ammonia parkinsoniana* (d'Orbigny, 1839) induced by exposure to one of three heavy metals (zinc, lead, or mercury). The exposure of these two benthic foraminiferal species to the selected heavy metals appear to promote cytological alterations and organelle degeneration. These alterations include a thickening of the inner organic lining, an increase in number and size of lipid droplets, mitochondrial degeneration, and degradation vacuoles and residual body proliferation. Some of these alterations, including the thickening of the inner organic lining and the proliferation of lipids, might represent defense mechanisms against heavy metal-induced stress.

Key words: protist, pollution, miliolid, ultrastructure, cytoplasm, *Ammonia, Pseudotriloculina*

1. Introduction

Benthic foraminifera are single-celled eukaryotes that are highly abundant in marine environments. Traditionally, benthic foraminifera have been applied to paleoecological, paleoenvironmental and paleoclimatological reconstructions and hydrocarbon exploration.

Their application has been also extended to environmental biomonitoring (for a review, see Alve, 1995; Yanko et al., 1999) and they are now widely used as effective bioindicators in a wide range of marine and transitional marine environments (e.g., Armynot du Châtelet and Debenay, 2010; Frontalini and Coccioni, 2011; Schönfeld et al., 2012; Alve et al., 2016).

Although several ecological studies on benthic foraminifera have been performed over the last 50-60 years, only a few have focused on the specific response to a single pollutant in terms of tolerance, growth, reproduction and/or survival (i.e., Bresler and Yanko, 1995a,b; Morvan et al., 2004; Saraswat et al., 2004; Le Cadre and Debenay, 2006; Nigam et al., 2009; Denoyelle et al., 2012; van Dam et al., 2012a,b; Linshy et al., 2013; Nardelli et al., 2013; Frontalini et al., 2015, 2016) and even more limited is the knowledge of ultrastructural changes induced by exposure to pollutants (Table 1).Fewer ultrastructural studies on the effects of pollution on foraminifera have so far been published as compared to those performed on other marine organisms, ranging from other protists (Ismail et al., 2002; Debelius et al., 2009; Gomiero et al., 2013; Miazek et al., 2015; Sures Kumar et al., 2015) to metazoans (e.g., Storelli and Marcotrigiano 2000; Achard et al. 2004). In fact, most foraminiferal studies have focused more on metals' incorporation into calcite for proxy calibration rather than on cellular responses or alterations resulting from heavy metal exposure (i.e., de Nooijer et al., 2007; Munsel et al., 2010; Nardelli et al., 2016; van Dijk et al., 2017a,b). Under these circumstances, further culture and ultrastructural studies are required to better understand the specific biological response(s) of foraminifera (Nigam et al., 2006), and the potential detoxification mechanisms against heavy metals.

In benthic foraminifera, heavy metals have been suggested to promote 1) a thickening of the inner organic lining (IOL); 2) an increase in the number and size of lipid droplets (LD); 3) mitochondrial degeneration; 4) proliferation of degradation vacuole (reported as autophagosomes), lysosomes, and residual bodies in *Ammonia* species (Morvan et al., 2004; Le Cadre and Debenay, 2006; Frontalini et al., 2015, 2016). Following LeKieffre et al. (this volume), as the separation between degradation vacuoles containing external (food) or internal (organelles) material is rather difficult on transmission electron microscopy (TEM) images, we use the term degradation vacuoles. Alterations on LD (reported as lipidic vesicles) have been attributed to a perturbation in the metabolic regulation of specimens exposed to copper (Cu) (Le Cadre and Debenay, 2006). Similarly, a proliferation of LD, mainly neutral lipids (esterified cholesterol and triglycerides), has been documented in specimens treated with Hg (Frontalini et al., 2016). The IOL has been considered to protect the cell from xenobiotics (Leutenegger,

1977) and its thickening has reinforced the idea of a defense-like mechanism against pollutants (Le Cadre and Debenay, 2006).

Among metals, zinc (Zn) is considered as an essential intracellular element and micronutrient for eukaryotic life because it plays important roles in cellular proliferation, metabolism, reproduction, enzymatic activity and protection against free radicals (e.g., Martín-González et al., 2005; Gallego et al., 2007). However, at high concentrations Zn becomes toxic and produces cellular damage (e.g., Gallego et al., 2007), mainly due to oxidative stress (e.g., de Freitas Prazeres et al., 2011). Lead (Pb) has been regarded among the most damaging elements to organisms as it mimics other biologically essential metals by substituting for Ca, Mg, Fe, Zn, and Na (Lidsky and Schneider, 2003; Flora et al., 2012). Because of its non-biodegradable nature, Pb is known for its prolonged persistence in the environment (Flora et al., 2012). Lead, like Zn, also promotes the generation of reactive oxygen species (ROS) that might result in damage to biomolecules (Flora et al., 2012). Mercury (Hg) and its compounds are extremely toxic and has been considered as one of the most harmful metals for biota (Clarkson and Magos, 2006; Eisler, 2006). Mercury is also reported to cause oxidative stress (McElwee et al., 2013).

The main aim of this contribution for this Benthic Foraminiferal Ultrastructure Atlas special issue is to present the most commonly noted ultrastructural changes observed in two species of benthic foraminifera, *Ammonia parkinsoniana* and *Pseudotriloculina rotunda*, after acute exposure to these two non-essential (Hg and Pb) and one essential (Zn) heavy metals.

2. Materials and methods

2.1 Experimental conditions

Three experiments were conducted with the selected metals (Zn, Pb and Hg).

In unpolluted seawater Zn concentrations are generally < 10 μg/L (Eisler, 1993) but in highly polluted areas concentrations up to 5 mg/L have been reported (Reddy et al., 2005). To test the effect of Zn, a culture experiment exposed the miliolid species *Pseudotriloculina rotunda* (d'Orbigny in Schlumberger, 1893) to 50 mg/L of Zn for 24h. This concentration was chosen on the basis of the results obtained by Nardelli et al. (2013) on the same species. Because a concentration of 100 mg Zn/L was lethal to this species after one week of treatment and <50% of specimens died during a 7-week incubation at 10 mg Zn/L, an intermediate concentration of 50 mg Zn/L was chosen for the acute exposure. A preliminary test was also performed to ensure that all specimens survived 24h of exposure to this concentration of Zn. Specimens were isolated from culture batches (see Nardelli et al., 2013 for more details) and maintained at 18°C;

only those with active reticulopods were used as inoculates and checked for pseudopodial activity before starting the experiment. Zinc solution was prepared, just before starting the experiment, with ultrapure salts ($ZnSO_4 \cdot 7H_2O$, from Sigma) diluted in natural seawater from an established control site (Portonovo, Ancona, Italy) previously filtered at 0.42 μm and stored in the dark, at 4°C. Salinity (37) and pH (8.02) were previously measured. Specimens where picked from culture batches, that were normally kept at 15°C and gradually raised to 18°C in the week preceding the experiment. Fifteen specimens of *P. rotunda* were randomly picked from the pool to be exposed to 50 mg/L and twelve were maintained in control conditions (seawater). Untreated (i.e., control) and treated (i.e., Zn) foraminiferal specimens were incubated at 18°C, for 24h and then separately processed for TEM analyses.

The Pb experiment was based on *Ammonia parkinsoniana* (d'Orbigny, 1839) and has been fully described in Frontalini et al. (2015). In brief, specimens were cultured in sediments exposed to one of three concentrations of Pb (1 ppb, 1 ppm, or 10 ppm) or the control (no lead), up to eight weeks. Similarly, the Hg-based experiment was performed on *A. parkinsoniana*, exposed to one of three concentrations of Hg (1 ppb, 1 ppm, or 100 ppm) or the control up to 12 weeks (Frontalini et al., 2016).

2.2 Sample preparation for TEM analyses

Specimens of *P. rotunda* were prepared for TEM observation following the protocol described by Martín-González et al. (2005), modified after Le Cadre and Debenay (2006). Briefly, after incubation, foraminiferal specimens were pre-fixed in a solution of glutaraldehyde 2.5% (v/v) (Sigma) in sodium cacodylate buffer (100 mM, pH 7.2, TAAB Laboratories Equipment Ltd), for 1 hour. Then specimens were exposed to 0.1M ethylenediaminetetraacetic acid (EDTA) for 36h to remove the calcareous test (following Le Cadre and Debenay 2006). The cells were then rinsed 3 times in 100 mM sodium cacodylate buffer and post-fixed in a 0.5% solution of OsO_4 (v/v; TAAB) in sodium cacodylate buffer (100 mM, pH 7.2) for 45 minutes on ice. Fixed cells were then contrasted in an aqueous solution of 1% (v/v) uranyl acetate (TAAB) for 1 hour, dehydrated in a graded series of acetone baths (25%, 50%, 75%, and 3 times 100% (v/v) in Millipore water), for no less than 20-30 minutes for each step. Foraminifera were then embedded in Embed Low Viscosity Resin, following manufacturer's instructions. These various manipulations were performed in small microcentrifuge tubes (Eppendorf type) using micropipettes. Samples were then processed at the National Center for Microscopy (Complutense University of Madrid). Initially, semi-thin sections (1 μm) of the specimens were stained with 1% (v/v) toluidine blue to check for cell integrity. Ultra-thin (50 nm) sections were collected on 200-mesh copper grids and contrasted with aqueous 8% uranyl acetate (in ethanol 30%) and 0.7% lead citrate solutions, and then examined with a JEM 1010 JEOL Electron Microscope, at 75kV.

Ammonia parkinsoniana preparation for TEM analyses is described in Frontalini et al. (2015, 2016). Briefly, specimens were fixed with 2.5% glutaraldehyde (TAAB, England, UK) in Artificial Sea Water (ASW) for 3 h at 4°C, and decalcified with 0 .1 M EDTA for 36 h. After 5 washings with ASW, specimens were post-fixated with 1% osmium tetroxide (OsO_4; EMS, Hatfield, PA) in ASW for 2 h at room temperature. Specimens were then dehydrated in a graded series of ethanol baths, from 50% to 100%, immersed twice in propylene oxide (10 minutes each; EMS, Hatfield, PA) and embedded in epoxy resin (Durcupan Araldite, SIGMA, UK). Foraminifera were ultimately sectioned using an ultramicrotome (LKB, 2088 Ultrotome®V). Thick sections of 1 µm were stained with 1% toluidine blue in distilled water at 60°C to provide an overview at the light-microscope level. Thin sections (100 nm), collected on 300-mesh nickel grids, were stained with 3% aqueous uranyl acetate and Reynold's lead citrate solutions and finally observed with a Philips CM10 electron microscope at 80 kV.

3. Results

The comparison between control (Fig. 1A) and Zn-treated (Fig. 1B-F) specimens of *P. rotunda* revealed important ultrastructural alterations in zinc-treated individuals but not in control specimens. These alterations included the presence of large numbers of residual bodies, which contained irregular concentric or juxtaposed masses of membranes that can form into a vacuole. Moreover, what were interpreted to be numerous cytoplasmic degradation vacuoles and electron-dense granules were visible in Zn-treated cells (Fig. 1B, C and D). Golgi apparatus (Fig. 1C-D) and mitochondria (Fig. 1B, D and E) were degraded in treated specimens compared to those of the control specimens, where intact organelles (mitochondria and peroxisomes) were commonly observed (Fig. 1A). Some clay particles as interpreted by Goldstein and Corliss (1994) or mineral flake-like crystals were visible both in treated (Fig. 1F) and control specimens and have been interpreted as mica flakes that entered the cell by endocytosis, possibly before the isolation of specimens from natural sediment for culture.

Control specimens of *A. parkinsoniana* appeared as expected in "normal" foraminiferal cells (see, e.g., LeKieffre et al. this volume), having intact vacuole membranes, mitochondria, peroxisomes, and residual bodies (Fig. 2A). On the contrary, several ultrastructural alterations were observed in Pb-treated specimens, particularly those exposed to the highest concentrations, including cytoplasmic degradation (Fig. 2B). In fact, the membranes delimiting organelles were ruptured, causing the cytosol to appear to fuse between structure. At higher magnification, mitochondria did not show the typical peripheral double membrane integrity, and they appeared swollen with poorly preserved cristae (Fig. 2D) when compared to control specimens (Fig. 2C). Lipid droplets of Pb-treated specimens appeared to have a more irregular outline and to be more electron-dense (Fig. 2F) than LD of control specimens (Fig. 2E). Similarly, Hg-treated specimens showed numerous morphological alterations compared to control specimens. These alterations included cytoplasmic degradation (Fig. 3A), a more electron-dense core in lipids (Fig. 3B), degraded mitochondria (Fig. 3C), and a number of structures interpreted as vacuoles (Fig. 3A).

Discussion

Our results suggest that the ultrastructural alterations induced by exposure to different heavy metals on different benthic foraminiferal species are quite similar, regardless of the involved metal, at least for the three tested divalent ions and the two foraminiferal species. The three tested metals are known to induce the production of reactive oxygen species (ROS) that represent a serious threat to cell fitness during exposure. The ROS are mainly produced in mitochondria (see review by Murphy 2009) and when an excess is produced, mitochondria become dysfunctional or undergo necrosis (which leads to cell- "aging"). The degradation of mitochondria observed in our treated samples is very similar to the ones already reported for several species of ciliates (i.e., Pyne et al., 1983; Martín-González et al., 2005) and could represent an advanced phase of necrosis of these organelles after exposure. This hypothesis also agrees with the results of de Freitas Prazeres et al. (2011) who quantified, for the first time, several biomarkers of oxidative stress (antioxidant capacity, lipid peroxidation, metallothionein-like proteins concentration and total superoxide dismutase activity) in a symbiont-bearing foraminifer, *Amphistegina lessonii*, exposed to Zn. However, it is noted that some foraminiferal species, which thrive within the chemocline of marine sediments where ROS are produced, possess peroxisomes complexed with the endoplasmic reticulum and can cope with ROS (Bernhard and Bowser, 2008).

The presence of higher number of degradation vacuoles (probably autophagosomes) and residual bodies in the cytoplasm of metal-treated specimens could therefore be the consequence of enhanced stressful conditions and represent the tendency of the organism to degrade organelles like mitochondria altered by ROS production. Increased number of degradation vacuoles has been previously reported in other eukaryotic organisms, where they are generally interpreted as cellular stress signals (e.g., Krawczynska et al. 1989; Martín-González et al.

2005). Traditionally, the formation of autophagosomes is considered a specific mechanism induced by starvation or nutritional stress (Klionsky and Ohsumi 1999), for example in yeasts (Abeliovich and Klionsky 2001) and ciliates (Gutiérrez et al. 2001; Gutiérrez and Martín-González 2002). However, it is also known that autophagy plays a major role in the degradation of altered organelles. An increased number of residual bodies has been reported by Le Cadre and Debenay (2006) in two different species of *Ammonia* exposed to Cu. Those authors hypothesized a role of the residual bodies in detoxifying the metals by storing and neutralizing them. Dedicated studies to map metal distributions in foraminiferal cytoplasm are warranted.

A thickening of inner organic lining (IOL) or cell membrane was observed in *Ammonia parkinsoniana* exposed to both Hg and Pb (Frontalini et al., 2015). This result is far from rare. In fact, several authors previously reported similar observations in different foraminiferal species, exposed to different metals. For example, Le Cadre and Debenay (2006) who exposed *Ammonia beccarii* (Linnaeus, 1758) and *Ammonia tepida* (Cushman, 1926) to copper (up to 500 μg/L), in culture conditions, reported a thickening of the IOL with a fibrous and stratified appearance particularly at the basal part of the pores. Similarly, a thickening of the IOL with an increase of fibrous material and the proliferation of residual bodies is documented in morphologically deformed *A. tepida* specimens after exposure to oil by Morvan et al. (2004). The IOL, which consists of a complex polysaccharide and glycoproteins bound together in a complex macromolecular structure, represents the matrix between the test and the cytoplasm (Splinder, 1978; Ní Fhlaithearta et al., 2013) that is supposed to have an important role in foraminiferal biomineralization (Langer, 1992; Erez, 2003, Sabbatini et al., 2014). Morvan et al. (2004) suggested that this structure could also have a major role in protecting the cytoplasm, and that a thickening of the IOL may therefore represent a defense mechanism adopted by foraminifera to protect the cell against potential toxicants like heavy metals (i.e., Cu and Pb), and also organic compounds (Sen Gupta et al., 1997; Morvan et al., 2004; Le Cadre and Debenay, 2006; Frontalini et al., 2015). Similar modification of the IOL was also noted by Sen Gupta et al. (1997) for *Cassidulina carinata* Silvestri, 1896 collected in *Beggiatoa* mats around bathyal hydrocarbon seeps in the Gulf of Mexico as well as by Koho et al. (this volume) for *Ammonia* spp. in response to anoxic conditions.

A proliferation of abnormally large LD (reported as lipidic vesicles) and an increase in the number of residual bodies in response to copper exposure were noted by Le Cadre and Debenay (2006). The increase of number and size of LD was suggested to be a perturbation in the regulation metabolism of foraminiferal specimens contaminated by copper (Le Cadre and Debenay, 2006) as also reported in other organisms (Prevot and Soyer-Gobillard, 1986). A proliferation of neutral lipids in the form of LD was documented in specimens of *A. parkinsoniana* exposed to Hg (Frontalini et al., 2016) as well as in other organisms (microalgae, lichens, rat, grey mullet, silver catfish hepatocytes) when exposed to contaminants. Lipid droplets are also hypothesized to sequester toxicants in order to protect cells (Murphy et al., 2008; Rowan-Carroll et al., 2013).

4. Conclusion

Mercury, lead and zinc are important metallic pollutants that, to different degrees, are considered potentially harmful elements for biota. On the basis of our results and literature data, we here present a synopsis of the ultrastructural changes of two benthic foraminiferal species, *A. parkinsoniana* and *P. rotunda* exposed to Hg and Pb, and Zn, respectively. The exposure of the specimens to these heavy metals seems to promote cytological alterations and organelle degeneration. These alterations include a thickening of the inner organic lining, an increase in the number and size of lipid droplets, mitochondrial degeneration, as well as degradation vacuole and residual body proliferations. These alterations suggest cytological oxidative stress induced by the exposure to the tested metals, and some alterations in particular, i.e., thickening of IOL, degradation vacuole, and lipids, are interpreted as potential defense mechanisms against heavy metal-induced stress.

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Table and Figures caption

Table 1. Compilation of experimental results in previous literature about the effects on foraminifera, after exposure to certain pollutants (pollutant, concentration, duration, species, Transmission Electron Microscopy (TEM) and reference).

Figure 1. TEM micrographs of *Pseudotriloculina rotunda.* Low magnification view (A) of a control specimen incubated in natural seawater. Higher magnification views of Zn-treated specimens (B-F). Mitochondria (m), degraded mitochondria (m*), lipid droplet (li), Golgi apparatus (g), degradation vacuole (dv), peroxisome (p), electron-dense granules (e) and clay platelets (c). Scale bars: A-D: 1 µm; E-F: 100 nm.

Figure 2. TEM micrographs of *Ammonia parkinsoniana*. Low magnification views of foraminiferal cytoplasm of control (A) and Pb-treated (B) specimens. Lipid droplet (li), vacuole (v) and residual body (rb). Higher magnification views of intact (C, control) and degraded (D,

Pb-treated) mitochondria, lipid droplets in untreated (E) and Pb-treated (F) specimens. Peroxisome (inset in B). Scale bars: A, B: 1 µm (inset 125 nm); C, D: 50 nm; E, F: 200 nm.

Figure 3. TEM micrographs of *Ammonia parkinsoniana*. Low magnification view of foraminiferal cytoplasm of Hg-treated (A) specimens. Higher magnification views of lipid droplets (B) with electron-dense cores and degraded mitochondria (C). Lipid droplet (li), vacuole (v) and mitochondria (m). Scale bars: A: 1 µm; B: 200 nm; C: 100 nm.

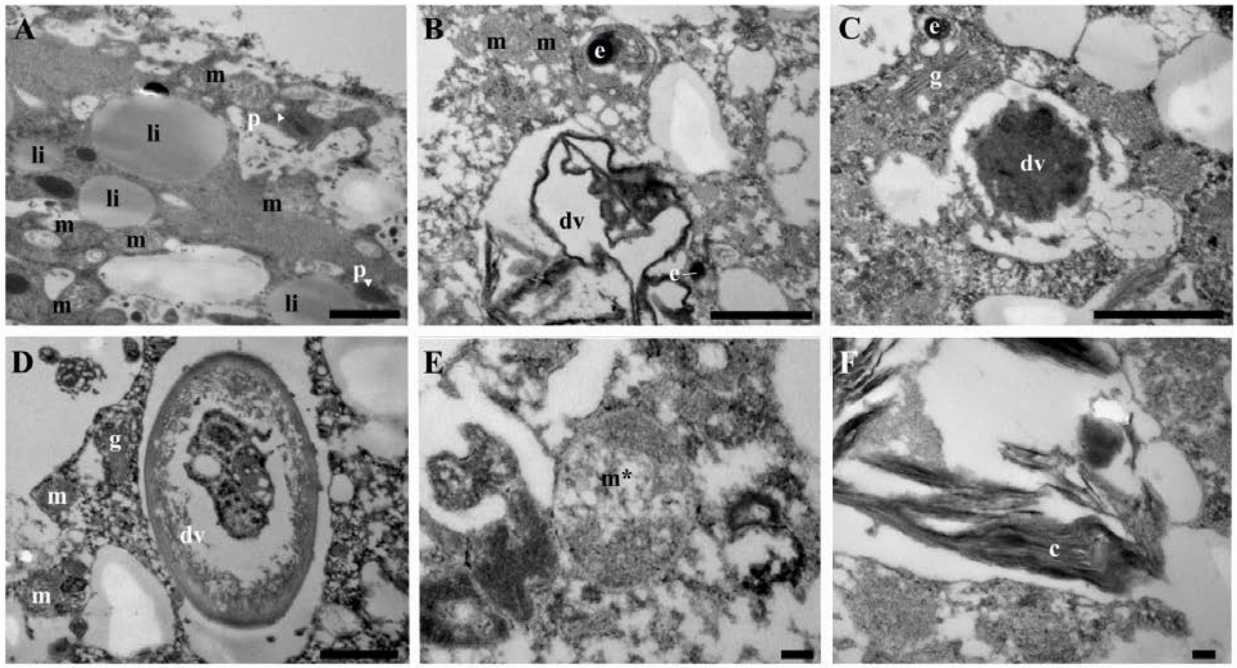
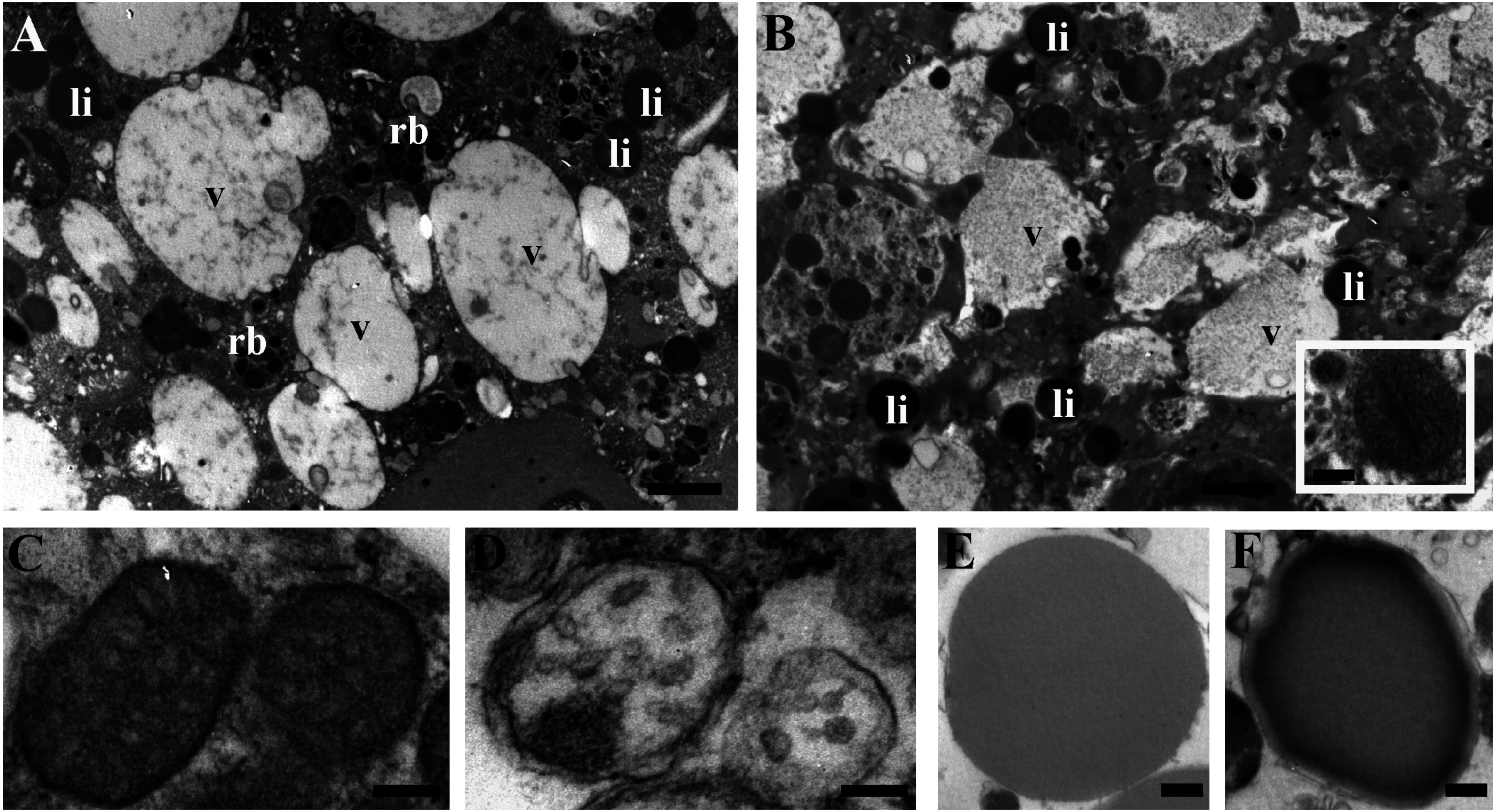


Figure 1.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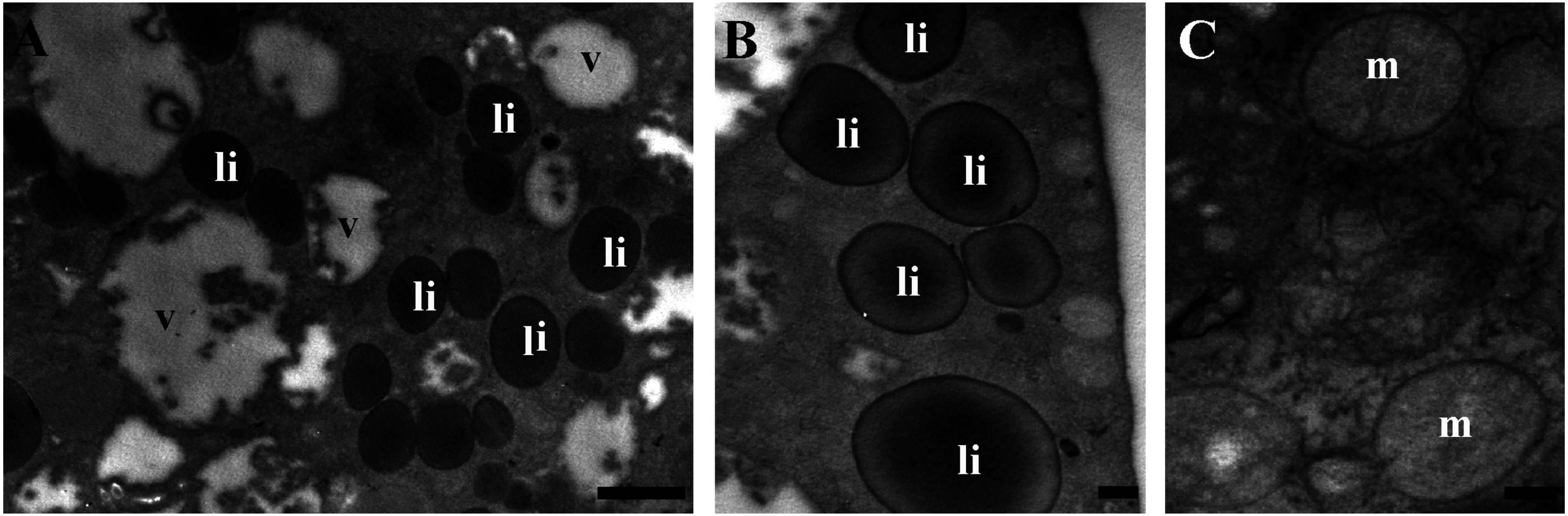
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Table 1.

Pollutant	**Concentration**	**Duration**	**Species**	**TEM**	**Reference**
Cd, Cu and Hg	up 1000 µM	24 hours	*Pararotalia spinigera*	No	Bresler and Yanko (1995a)
Mixed compounds	up 1000 µM	up to 4 hours	*Pararotalia spinigera* and *Rosalina macropora*	No	Bresler and Yanko (1995b)
Oil	up to 72 mg/100 mL	up to 12 months	*Ammonia tepida*	Yes	Morvan et al. (2004)
Hg	up to 260 ng/L	100 days	*Rosalina leei*	No	Saraswat et al. (2004)
Cu	up to 500 µg/L	up to 12 months	*Ammonia beccarii* and *Ammonia tepida*	Yes	Le Cadre and Debenay (2006)
Cu	up to 20 µmol/L	up to 2 months	Ammonia tepida and *Heterostegina depressa*	No	de Nooijer et al. (2007)
Hg	up to 300 ng/L	ca. 40 days	*Rosalina leei*	No	Nigam et al. (2009)
Ni, Cu, and Mn	up to 3290 nmol/L	82 days	*Ammonia tepida*	No	Munsel et al. (2010)
Zn	up to 93.4 µg/L	48 hours	*Amphistegina lessonii*	No	de Freitas Prazeres et al. (2011)
Cd	up to 200 mg/L	up to 30 days	*Ammonia tepida*	No	Denoyelle et al. (2012)
Oil	up to 5 g/L	up to 30 days	*Ammonia tepida*	No	Denoyelle et al. (2012)
Drilling muds	up to 100 mg/L	up to 30 days	*Ammonia tepida*	No	Denoyelle et al. (2012)
Diuron (symbiont response)	up to 100 µl/L	up to 96 hours	*Heterostegina depressa, Amphistegina radiata, Alviolinella quoyi, Calcarina mayorii, Operculina ammonoides, Heterostegina depressa, Marginopora vertebralis, Marginopora vertebralis, Sorites orbiculus, Peneropolis planatus, Peneropolis antillarum, Parasorites marginalis* and *Elphidium* sp.	No	Van Dam et al. (2012a)
Diuron (symbiont response)	up to 3 µg/L	up to 96 hours	*Heterostegina depressa, Calcarina mayorii, Alveolinella quoyi, Marginopora vertebralis* and *Peneroplis planatus*	No	Van Dam et al. (2012b)
Cd	up to 14 µg/L	up to 21 days	*Pararotalia nipponica*	No	Linshy et al. (2013)
Zn	up to 100 mg/L	up to 10 weeks	*Pseudotriloculina rotunda*	No	Nardelli et al. (2013)

Pb	up to 10 mg/L	up to 2 months	*Ammonia parkinsoniana*	Yes	Frontalini et al. (2015)
Hg	up to 100 mg/L	up to 3 months	*Ammonia parkinsoniana*	Yes	present paper
Zn	50 mg/L	24 hours	*Pseudotriloculina rotunda*	Yes	present paper
Hg	up to 100 mg/L	up to 3 months	*Ammonia parkinsoniana*	Yes	present paper