Environ Sci Pollut Res (2017) 24:26185–26193
DOI 10.1007/s11356-017-9304-y

The biological response chain to pollution: a case study from the "Italian Triangle of Death" assessed with the liverwort *Lunularia cruciata*

Adriana Basile[1] · Stefano Loppi[2] · Marina Piscopo[1] · Luca Paoli[2] · Andrea Vannini[2] · Fabrizio Monaci[2] · Sergio Sorbo[3] · Marco Lentini[1] · Sergio Esposito[1]

Received: 3 November 2016 / Accepted: 17 May 2017 / Published online: 31 May 2017

Abstract The liverwort *Lunularia cruciata*, known for being a species tolerant to pollution able to colonize urban areas, was collected in the town of Acerra (South Italy) to investigate the biological effects of air pollution in one of the three vertices of the so-called Italian Triangle of Death. The ultrastructural damages observed by transmission electron microscopy in specimens collected in Acerra were compared with samples collected in the city center of Naples and in a small rural site far from sources of air pollution (Riccia, Molise, Southern Italy). The biological response chain to air pollution was investigated considering vitality, photosynthetic efficiency, heat shock protein 70 (Hsp70) induction and gene expression levels, and chlorophyll degradation and related ultrastructural alterations. Particularly, a significant increment in Hsp70 expression and occurrence, and modifications in the chloroplasts' ultrastructure can be strictly related to the environmental pollution conditions in the three sites. The results could be interpreted in relation to the use of these parameters as biomarkers for environmental pollution.

Keywords Atmospheric pollution · Chlorophyll degradation · Gene expression · Hsp70 · Photosynthetic efficiency · Ultrastructural alterations · Metal bioaccumulation

Highlights
- Severe alterations were observed in chloroplasts of *Lunularia cruciata* exposed to pollution.
- A strong increase in the expression and occurrence of heat shock protein 70 was detected.
- The results suggest *L. cruciata* could be possibly used as a bioindicator organism.
- Hsp70 could be useful biomarkers to measure the effects and the extent of anthropic pollution.

Responsible editor: Philippe Garrigues

Electronic supplementary material The online version of this article (doi:10.1007/s11356-017-9304-y) contains supplementary material, which is available to authorized users.

✉ Sergio Esposito
sergio.esposito@unina.it

[1] Dipartimento di Biologia, Università di Napoli "Federico II"—Complesso Universitario di Monte Sant'Angelo, Via Cinthia 4, I-80126 Naples, Italy

[2] Dipartimento di Scienze della Vita, Università di Siena, Via Pier Andrea Mattioli, 453100 Siena, Italy

[3] Ce.S.M.A, University of Naples "Federico II"—Complesso Universitario Monte Sant'Angelo, Via Cinthia 4, I-80126 Naples, Italy

Introduction

Bryophyta show a high capability to accumulate metals because of the high surface/volume ratio and the presence of a thin cuticle. It is well known that these organisms do not have proper roots: their rhizoids do not primarily contribute to the uptake of substances from the substrate; therefore, most elements absorbed by bryophytes originate from atmospheric deposition, thus, the levels of specific elements in bryophytes reflect the total deposition and can be used to monitor air pollution in space and time.

Many studies used bryophytes to investigate the levels of environmental pollution (Tyler 1990; Harmens et al. 2008), and pollution-tolerant species have been used to investigate the environmental pollution in highly contaminated sites (Basile et al. 2001, 2008, 2012, 2013). Intriguingly, the liverwort *Lunularia cruciata* L. (Family Lunulariaceae; Order Marchantiales) lives anchored to the ground, and it usually uptakes minerals and other elements from soil solutions as well; so, heavy metal (HM) contamination of *L. cruciata* is related

not only to air pollution but rather to overall environmental contamination, depending on different sources.

The town of Acerra, close to Naples (Southern Italy), is one of most polluted areas in Europe; it represents one of the vertices of the so-called Italian Triangle of Death (the other corners are represented by towns of Marigliano and Nola) sadly known for being highly polluted due to illegal management of hazardous waste by criminal organizations. Thus, this situation determined a diffuse and severe contamination by toxic elements and compounds; this has been linked to increasing rates of brain, liver, lung, stomach, and intestine cancer (Senior and Mazza 2004). On the other hand, heavy metals have been widely demonstrated as a main cause of cancer in human cells (Galano et al. 2014). In previous studies, a biomonitoring project on the urban area of Acerra, using moss bags, evaluated the deposition and biological effects of heavy metals; the results indicated that the area is heavily polluted by heavy metals, a large proportion likely present in the atmosphere as particles (Basile et al. 2008). Comparing the heavy metal bioaccumulation capacity of the epiphytic moss *Scorpiurum circinatum* and the epiphytic lichen *Pseudevernia furfuracea*, it emerged that these species are both able to accumulate heavy metals, but *S. circinatum* has to be preferred because of its higher, constant, and linear accumulation rate (Basile et al. 2008, 2009). In previous works, the liverwort *L. cruciata* has been used to evaluate the in vitro effects of heavy metals on a wide range of cellular responses, including changes in gene expression and transcription (Carginale et al. 2004; Basile et al. 2005).

There are changes in cell ultrastructure and induction of heat shock proteins 70 (Hsp70s) in the talloid liverwort *Conocephalum conicum* (Marchantiales) exposed to atmospheric pollution in different urban and rural sites of Italy (Basile et al. 2013). Interestingly, these results were comparable to the effects observed in vitro in samples exposed to different heavy metals. Namely, at urban sites, the cellular ultrastructure was modified, and heavy metals could be accumulated in cell walls; simultaneously, an evident increase in Hsp70s occurrence was found.

The aim of this work was to compare the effects of environmental pollution, in terms of metal bioaccumulation as well as changes in ultrastructure, vitality, and Hsp70 induction, on *L. cruciata* L. Dumort., collected in a highly contaminated urban area (Acerra), with respect to samples collected in a polluted site located in the city center of Naples and at a relatively unpolluted rural site (Riccia, Molise, South Italy).

Materials and methods

Study sites

Three distinct study sites were chosen; the geographical positions and sites' properties are shown in Supplementary Fig. 1 and Supplementary Table 1.

Site A—Riccia, a small town of ca. 5500 inhabitants, located in Molise region ca. 100 km NE of Napoli, far from local sources of pollution, was selected as a remote rural control site [DMS (degrees, minutes, seconds) 41°29′19.352″ N–14°50′13.596″E; DD (decimal degrees) 41.486463–14.83789].

Site B—Metropolitan area of Naples accounts for about 3.5 million people, and 1 million inhabitants live in the city center. *Lunularia* samples were collected (DMS 40°51′42.908″N–14°15′48.679″E; DD 40.86167–14.26352) at the Botanical Gardens of the University, on the boundary wall along Foria Street, in a central quarter affected by heavy metal pollution, mainly originating from vehicular traffic.

Site C—The town of Acerra (ca. 50,000 inhabitants) is located 15 km north of Napoli (Southern Italy), at an elevation of 25 m asl. The climate is typically Mediterranean, with a mean monthly minimum temperature of 8.5 °C in January and maximum of 24.1 °C in August, and a mean monthly maximum rainfall of 140 mm in December and minimum of 40 mm in August. The Acerra district is characterized by a high traffic load, heavy industry, and intensive agriculture. The whole area is also known for illegal waste dumping (Senior and Mazza 2004). Samples of *L. cruciata* were collected along a road characterized by high vehicular traffic between the city center and the waste thermo-disposal industry (DMS 40°57′48.64″ N–14°22′15.73″E; DD 40.96334–14.370834).

Plant material

The metal-tolerant liverwort *L. cruciata* L. (Dumort.) was collected from moist soil at the sampling sites; the samples were maintained in Petri dishes and processed in the laboratory within 6 h from collection. The protocol proposed for the European moss survey (Harmens et al. 2008) was taken into account and followed with few modifications since this was specifically developed for monitoring spatial and temporal trends in the accumulation of heavy metals in mosses at a wide scale. Specifically, samples were collected using gloves and bags in small open spaces to preclude significant effects of canopy drip.

Samples were collected less than 100 m from roads and/or buildings because the aim of this research was to study the effects of HMs on liverworts in populated areas.

Each sample was a mix of about five sub-samples. Litter and dead parts and litter were removed and only fresh segments were used for the analyses. Soon after collection, samples were refrigerated, frozen as soon as possible, or dried at TR and stored under those conditions until analysis.

Metal bioaccumulation

After collection, 125 mg of *L. cruciata* samples from each site was cleaned from soil particles and other material, dried at 105 °C for 24 h, and homogenized in an agate mortar. Homogenized samples were mineralized in Teflon vessels in

a microwave oven (Milestone MLS 1200 Mega) with 6 mL of 65% HNO_3, 2 mL of 39% H_2O_2, and 0.2 mL of HF. The digested material was diluted in distilled water and analyzed by inductively coupled plasma-mass spectrometry (Perkin Elmer Elan 600) for Al, As, Cd, Cr, Cu, Fe, Mn, Pb, V, and Zn content.

Metal contents were assayed in triplicate and measurements were repeated three times; all concentrations were expressed on a dry-weight basis. Analytical quality was checked by analyzing the Standard Reference Materials ICRM 482 (*P. furfuracea*) and CTA-VTL-2 (tobacco leaves). Precision of analysis was estimated by the coefficient of variation of five replicates and was found to be within 10% for all elements.

The samples of *L. cruciata* collected at the unpolluted Riccia site were used as control instead of M2 and M3 moss reference (Harmens et al. 2008) in order to strictly compare with a standard of liverworts not present in M2 and M3 composite.

Ultrastructural observations

Ultrastructural observations were made by transmission electron microscopy (TEM). Liverwort thin slices underwent fixation with 3% glutaraldehyde in phosphate buffer (0.065 M, pH 7.2–7.4) for 1.5 h at room temperature, post-fixation with 1% osmium for 1.5 h at room temperature, dehydration with alcohol topropylene oxide, and embedding in Spurr's epoxy medium. Ultrathin sections, 50 nm thick, were stained with uranyl acetate and lead citrate. Observations were made with an FEI EM 208S TEM.

Sample vitality

Sample vitality was expressed in terms of photosynthetic efficiency and chlorophyll degradation. Photosynthetic efficiency was checked by the maximum quantum yield of primary photochemistry, as inferred from chlorophyll *a* fluorescence emission: $F_V/F_M = (F_M - F_0)/F_M$, where F_0 and F_M are minimum and maximum chlorophyll *a* fluorescence and $F_V = (F_M - F_0)$ is the variable fluorescence. In addition, the performance index (P_I), a global indicator of the photosynthetic performance, was calculated to express the overall vitality of the samples (Strasser et al. 2000). Measurements were carried out with a Plant Efficiency Analyzer (Handy PEA; Hansatech Ltd., Norfolk, UK) on dark-adapted samples, applying a saturating flash of light of 2400 μmol s^{-1} m^{-2} for 1 s. Chlorophyll degradation was expressed by the ratio between the absorbance at 435 and 415 nm of DMSO extracts (Ronen and Galun 1984). Two extraction cycles of 45 min each at 65 °C were run in 5 mL DMSO; extracts were then combined and centrifuged for 10 min at 4000 rpm. Absorbance was measured using a UV-visible spectrophotometer (Agilent 8453).

Total RNA extraction, cDNA synthesis, and real-time qPCR of Hsp70 expression

Total RNA was isolated from 200 mg of leaves of *L. cruciata* samples with Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA samples were quantified and checked by NanoDrop ND-1000 and by electrophoretic analysis (1% agarose gel—denaturant conditions). Total RNA extracts were purified from genomic DNA with DNase reaction using Ambion DNA-free kit according to the manufacturer's instructions. Complementary deoxyribonucleic acid (cDNA) was synthesized from 1 μg of total RNA using M-MLV reverse transcriptase using Promega ImpPromII kit according to the manufacturer's instructions, using random examers (0.5 μg/μg RNA). Three different and independent cDNA sets were used. A quantity of 0.5 μL of the cDNA was used in the quantitative real-time PCR assay (qRT-PCR), using 0.5 mM of each forward and reverse primers in a final volume of 25 μL, to determine Hsp70 expression. All PCR reactions were performed in duplicate for 40 cycles under the following conditions: denaturation, 95 °C, 30 s; annealing, 60 °C, 1 min; and extension, 72 °C, 1 min. Gene expression was quantified using the SYBR Green PCR Master Mix Kit (Applied Biosystems) and specific primers on the 7500 Real Time PCR System (Applied Biosystems). Six values per sample were used for statistical analysis. PCR reactions were performed using actin gene as internal reference. The following specific primers were used and checked for dimer formation: Hsp70 (At3g12580)—5′-GTCGAAATCATCGCCAACG-3′ and 5′-CGACTTGATTCTTGGCAGCA-3′; actin (At3g18780)—5′-CTCCCGCTATGTATGTCGCC-3′ and 5′-TTGGCACAGTGTGAGACACAC-3′.

Relative gene expressions were calculated with the ΔΔCt method (Livak and Schmittgen 2001).

A standard curve was produced by using four serial dilutions of the mixed qRT-PCR products from all the dose points, and the corresponding efficiencies for each primer pair were calculated according to the equation $E = 10^{-1/\text{slope}}$ (correlation coefficients were >0.98). Hence, the change in expression of Hsp70 transcripts from *L. cruciata* collected in Acerra (site A) and Naples (site B) was compared to those observed in samples collected in Riccia (site C).

Protein extraction, electrophoresis, and Western blot analysis

Samples were quickly frozen in liquid nitrogen in a mortar and powdered with a pestle. Hsp70s were extracted in extraction buffer (30 mM Tris-HCl, pH 7.8, 10% glycerol, 5 mM dithiotreitol, 0.05% Triton X-100); the homogenate was then filtered through four layers of muslin and centrifuged for 20 min at 14,000 rpm and 4 °C in a Beckman JA25 centrifuge equipped with a JA25.50 rotor. The supernatant was designated as the crude extract and used for soluble protein

determination. SDS-PAGE was performed using 10% acrylamide resolving gel with a 4% stacking gel. Before loading, samples were boiled for 10 min in the presence of bromophenol blue to ensure protein denaturation. Proteins were subjected to electrophoresis under a constant voltage of 180 V, 40 mA for 90 min. For Western blot analysis, the separated polypeptides were transferred from gels to a nitrocellulose membrane (Hybond; Amersham Biosciences) soon after the SDS-PAGE run and incubated for 2 h at room temperature with primary antibodies for bovine heart Hsp70 (Sigma). After washing, membranes were incubated with secondary antibodies and developed as described previously (Chiaiese et al. 2011; Esposito et al. 2012). To check the equal loading of the lanes, blottings were probed with anti-tubulin antibodies (SIGMA—not shown). The images shown were representative of at least three different samples.

Statistical analysis

Significance of differences was checked with one-way ANOVA, using the Tukey test ($P < 0.05$) for post hoc comparisons. Prior to analysis, data not matching a normal distribution (Shapiro-Wilk W test at the 95% confidence interval) were log-transformed to correct for skewed distributions.

Results

Bioaccumulation

Concentrations of several metals (Al, Cd, Cr, Pb) showed statistically significant ($P < 0.05$) higher values at Acerra, lower values at Naples, and much lower values at Riccia (Table 1). Concentrations of Fe, Mn, and Zn resulted higher at Acerra and Naples compared with Riccia; As and Cu were higher at Naples, lower at Acerra, and much lower at Riccia; V was the only element not showing any statistically significant difference between sites (Table 1).

Table 1 Mean concentration (±standard deviation; μg/g dw) of heavy metals bioaccumulated in *Lunularia cruciata* samples collected at the three sampling sites

	Riccia	Napoli	Acerra
Al	1123 ± 201c	2754 ± 106b	3511 ± 260a
As	0.51 ± 0.02c	2.3 ± 1.1b	0.74 ± 0.7a
Cd	0.21 ± .0.01c	1.8 ± 0.03b	3.7 ± 0.7a
Cu	4.2 ± 0.4c	22 ± 2.1b	13.5 ± 1.1a
Cr	0.9 ± 0.02c	1.7 ± 0.2b	4.4 ± 0.9a
Fe	624 ± 56b	903 ± 13a	835 ± 43a
Pb	3.2 ± 0.6c	14 ± 0.5b	21 ± 1.3a
Mn	32.6 ± 2.3b	38.0 ± 3.2a	42.0 ± 2.4a
V	1.9 ± 0.2	2.7 ± 0.6	2.5 ± 0.4
Zn	56 ± 3.1b	72 ± 3.7a	74 ± 3.3a

Values followed by different letters are statistically different at $P < 0.05$

Ultrastructural observations

Samples from Riccia showed the typical ultrastructure of the Marchantiales (Giordano et al. 1989; Carginale et al. 2004), with a photosynthetic parenchyma with abundant chloroplasts, featured by a well-developed thylakoid and grana system and large starch grains in a moderately electron-dense stroma (Fig. 1a–c). Mitochondria appeared regular, with typical cristae (Fig. 1d, e); double membrane-delimited nuclei with nucleoli (Fig. 1f) were observed and plasmodesmata through cell walls were clearly visible (Fig. 1f). Ultrastructure of photosynthetic parenchyma in samples from Naples showed cells with altered chloroplasts containing starch grains (Fig. 2a–c). The thylakoid system was characterized by a wavy arrangement of membranes (Fig. 2b). Some control-like chloroplasts, less than 10% frequent, were observed, together with some control-like mitochondria (Fig. 2c, d). In the cytoplasm, vesicles (Fig. 2e) and multivesicular bodies were clearly visible (Fig. 2f). Moreover, cells showed severely altered nuclei with condensed material (Fig. 2g). Samples from Acerra showed a strongly altered ultrastructure of photosynthetic parenchyma (Fig. 3a, b). The whole cytoplasm appeared condensed; altered chloroplasts with severely disarranged, wavy thylakoids were present (Fig. 3c–e); in the electron clear stroma, some starch grains were still present (Fig. 3e). Mitochondria showed evident bulges (Fig. 3f).

Vitality

The photosynthetic performance of *L. cruciata* samples showed a statistically significant difference ($P < 0.05$) and increasing values from Acerra to Naples and Riccia (Table 2). This trend was evident both as F_V/F_M and, especially, as P_I. The same situation emerged also for chlorophyll degradation, with increasing values from Acerra to Naples and Riccia (Table 2). Values recorded in samples collected at Riccia are consistent with F_V/F_M, P_I, and OD 453/415 values commonly measured in healthy samples.

Hsp70 expression

Hsp70 expression levels were compared in samples of *L. cruciata* collected in different sites. The results show an up-regulation of this gene in which the expression level, depending on the environment pollution grade, was in the order Acerra (site A) > Naples (site B) > Riccia (site C). Samples collected in Acerra showed the highest Hsp70 expression levels, namely up to 7-fold higher with respect to that of samples collected in Riccia; samples collected in Naples presented

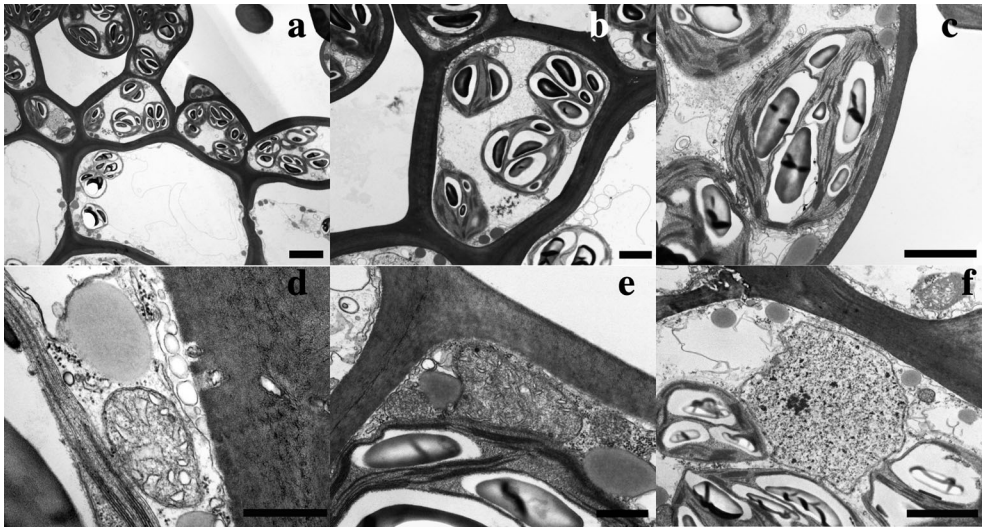

Fig. 1 The table reports TEM micrographs of the photosynthetic parenchyma in *Lunularia cruciata* samples collected from Riccia (site A). **a** The photosynthetic parenchyma at low magnification shows cells with numerous chloroplasts. **b** A single photosynthetic cell. **c** A chloroplast contains a developed thylakoid system, arranged as grana and intergrana, and large starch grains. **d** The micrograph shows, beneath the cell wall, a mitochondrion with cristae next to a cytoplasmic lipid droplet. **e** A mitochondrion with cristae is in tight contact with a chloroplast. **f** A nucleus with nucleolus is visible in the cell. The cell wall is crossed through by a plasmodesma. *Scale bars*: 5 μm (**a**), 2 μm (**b**, **c**, **f**), 500 nm (**d**, **e**)

a 2.3-fold higher Hsp70 expression with respect to samples collected in Riccia (Fig. 4).

Hsp70 occurrence

L. cruciata samples collected at the three different sites showed two different proteins reacting versus Hsp70 antibodies. The heavier exhibited a MW of 76 kDa; the lighter, 70 kDa. In detail, the occurrence of the 76-kDa protein appeared almost unchanged at the three sites, while the signal of the protein weighing 70 kDa considerably increased in the samples collected at Acerra compared with those from Naples and Riccia (Fig. 5).

Discussion

The results of moss and lichen biomonitoring of heavy metals in the Acerra area clearly indicated severe pollution by heavy metals, a large proportion of which likely present in the

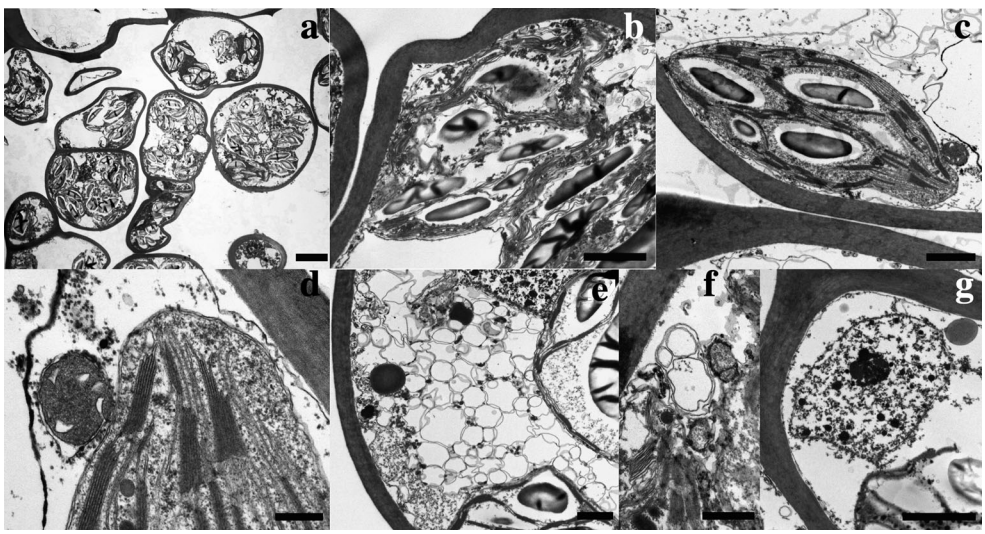

Fig. 2 The table reports TEM micrographs of the photosynthetic parenchyma in *Lunularia cruciata* samples collected from the Botanical Gardens wall facing Foria Street, downtown Naples (site B). **a** Photosynthetic parenchyma at low magnification showing cells with chloroplasts. **b** An altered chloroplast with a disorganized thylakoid system. **c** A control-like chloroplast with starch grains. **d** A mitochondrion with cristae next to a chloroplast. **e** A detail of a vacuolated cytoplasm. **f** A multilamellar body in the cytoplasm. **g** A nucleus with a nucleolus and condensed chromatin. *Scale bars*: 5 μm (**a**), 2 μm (**g**), 1 μm (**b**, **c**, **e**), 500 nm (**f**), 300 nm (**d**)

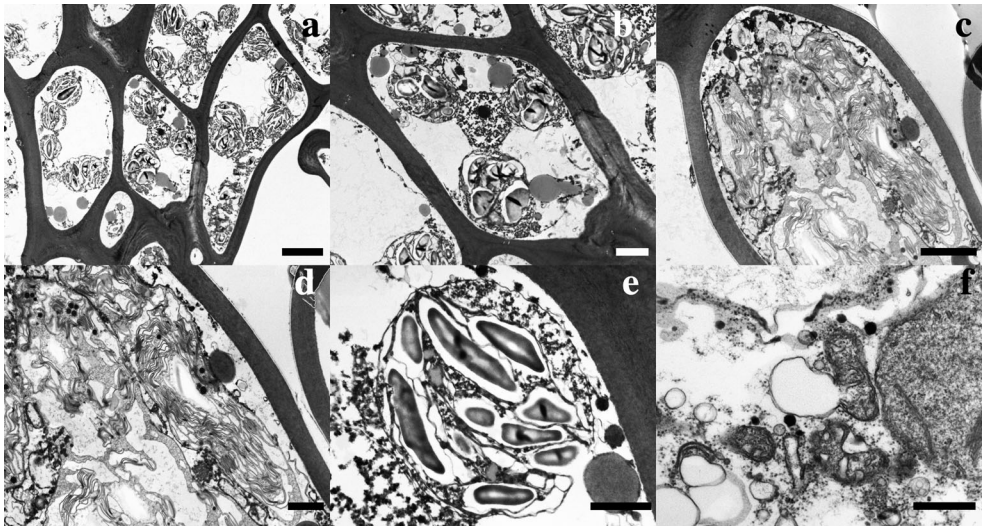

Fig. 3 The table reports TEM micrographs of the photosynthetic parenchyma in *Lunularia cruciata* samples collected from Acerra (site C). **a** Photosynthetic parenchyma at low magnification with cells containing chloroplasts. **b** A single photosynthetic cell with a central nucleus and altered chloroplasts. **c** Cell containing severely altered chloroplasts. **d** Chloroplasts with disarranged thylakoid systems in an electron clear stroma. **e** Altered chloroplast with disarranged thylakoid system and starch grains. **f** At the center of the micrograph, a bulged mitochondrion. *Scale bars*: 5 μm (**a**), 2 μm (**b**, **c**), 1 μm (**d**, **e**), 500 nm (**f**)

atmosphere in particulate form, with several differences between urban, rural, and industrial sites (Basile et al. 2009; Sorbo et al. 2008).

The bioaccumulation results of the present study are consistent with these findings and confirm that the environment of Acerra is heavily polluted by several heavy metals.

Chloroplasts have been shown as a common target of environmental stress (Lage-Pinto et al. 2008): change at the plastid level was reported as the most frequent and visible damage in heavy metal-treated plants, bryophytes, and lichens (Kukkola et al. 2000; Esposito et al. 2007; Zhang et al. 2014; Basile et al. 2012, 2013; Paoli et al. 2014). The appearance of chloroplasts in samples from Naples and Acerra, with the loss of the control-like linear arrangement of the thylakoids, and the condensation of stroma matrix, which appeared electron clear, are evident signs of stress and damage. However, the presence of large starch grains in samples from Naples demonstrated that chloroplasts still preserved part of their metabolic activity. The consequences of such ultrastructural damage to chloroplasts are evident also at the physiological level, with samples showing a reduced photosynthetic performance and a high rate of chlorophyll degradation. Increased vacuolization and cytoplasmic condensation were frequently observed in stressed plant cells and were regarded as primary alterations in plants exposed to heavy metals (Liu and Kottke 2004). Our finding of a large amount of vesicles in the samples from Acerra is in agreement with these studies, demonstrating a connection between the presence of vesicles and heavy metal stress (Nassiri et al. 1996; Liu and Kottke 2004; Carginale et al. 2004). Vesicles could be involved in metal-tolerance phenomena and also in autophagy (Bassham et al. 2006). The need for recycling cytoplasmic material, and damaged organelles, can be related to damages induced by heavy metals in Naples and Acerra (Adamo et al. 2003; Basile et al. 2008).

Table 2 Mean values (±standard deviation) of photosynthetic parameters (F_V/F_M and P_I) and chlorophyll degradation ($OD4_{35/415}$) in *Lunularia cruciata* samples collected at the three sampling sites

	Riccia	Napoli	Acerra
F_V/F_M	0.22 ± 0.12c	0.68 ± 0.13b	0.82 ± 0.03a
P_I	0.11 ± 0.02c	0.80 ± 22b	1.51 ± 0.48a
$OD_{435/415}$	1.01 ± .0.02c	1.19 ± 0.01b	1.43 ± 0.01a

Values followed by different letters are statistically different at $P < 0.05$

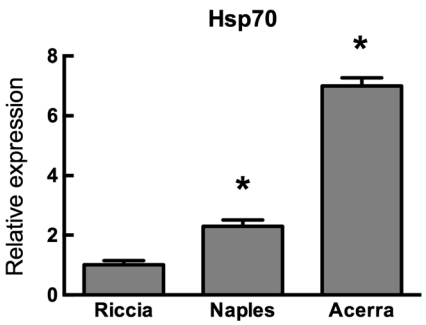

Fig. 4 qRT-PCR expression analysis of Hsp70 gene of *Lunularia cruciata* samples collected in Riccia, Naples, and Acerra. The relative expression of Hsp70 indicated as arbitrary units (a.u.) means the change in expression of the transcripts of Hsp70 genes in comparison to that of the reference housekeeping actin gene in samples from Naples and Acerra with respect to Riccia samples used as control. *Asterisk* indicates a statistically significant difference ($P < 0.05$) compared to control

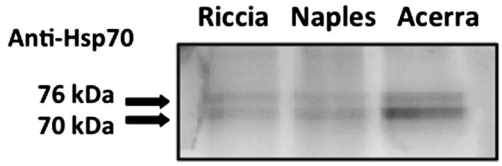

Fig. 5 Western blots of crude extracts of *Lunularia cruciata* samples collected in Riccia rural countryside (site A), on the boundary wall of the Botanical Gardens of Naples (Site B), and in Acerra (site C). Each lane was loaded with 20 μg protein. Antibodies reactivity was checked versus 2 μm pure protein bovine Hsp70 (Sigma—USA) (not shown). Immunodetection was made utilizing antibodies raised against bovine Hsp70 (Sigma). *Arrows* on the left indicate the MW calculated using the relative mobility factor method based on molecular weight markers reference

Air pollution may cause severe cellular oxidative stress (Romieu et al. 2008), and exposure to toxic metals can intensify the production of reactive oxygen species (Gratão et al. 2005). *L. cruciata* samples from the most polluted site C, Acerra, developed multivesicular/multilamellar bodies, an ultrastructure strictly related to autophagy and endocytic phenomena (Thompson and Vierstra 2005; Todeschini et al. 2011). Multivesicular/multilamellar bodies have already been reported in Bryophyta (Basile et al. 2012, 2013; Esposito et al. 2012) exposed to pollutants, and these could originate as accretion of undigested membranes from endocytosis. A dramatic and well-known consequence of oxidative stress is lipid peroxidation, whose products were demonstrated to be very harmful to plant mitochondria (Taylor et al. 2002). Oxidative stress can alter mitochondrial permeability and bring about a collapse of mitochondrial membrane potential, also inducing shape changes of these organelles (Shailasree et al. 2015). Cellular components can swell or shrink when ions move across the membrane, shifting accompanying solvents, as a consequence of either a direct damage to the membrane selective permeability or secondarily to cellular energy depletion (Schwartzman and Cidlowski 1993). All that could explain the finding of mitochondrial ultrastructural changes in our samples, with severe swelling, so that they even bulged to the outer cytoplasm.

Hsp70s are the best-described chaperones involved in protein folding (Hartl 1996), and their central role in stress response has been widely assessed, especially in counteracting the toxic effects of heavy metals on proteins, protecting them from misfolding and proteolysis (Miernyk 1997). Different studies evidenced their role(s) in the response to abiotic stress, such as salinity (Cardi et al. 2015) and drought (Landi et al. 2016). Uptake of heavy metals enhances chaperone synthesis and occurrence, as in *Elodea canadensis* (Esposito et al. 2007), *Lemna minor* (Basile et al. 2015), the moss *Leptodyctium riparium* (Basile et al. 2011; Esposito et al. 2012), and the liverworts *Conocephalum conicum* (Basile et al. 2013) and *Pellia neesiana* (Basile et al. 2016).

Our data clearly demonstrate that pollution increased both HSP expression and synthesis in order to counteract the severe effects caused by the presence of heavy metals in the environment.

Intriguingly, the levels of expression were more than doubled in the city center of Naples, following the heavy traffic of the collection site, but the pollution in the Triangle of Death provoked a further 3-fold increase, inducing a huge 7-fold increase of Hsp70 transcripts. This is accompanied by a corresponding increase in the presence of Hsp70 proteins, mainly of the chloroplastic isoform, in order to preserve the photosynthetic machinery that is the most damaged by pollution, as demonstrated by TEM observations. Changes in ultrastructure, vitality, and Hsp70s clearly indicated that *L. cruciata* samples from Acerra are severely damaged. Although previous studies related ultrastructural damage, physiological responses, and Hsp70 induction to different levels of heavy metal pollution (Basile et al. 2013, 2015; Esposito et al. 2012), in the present study, it is however not possible to infer if such damage has been caused by the uptake of heavy metals or, more likely, by a combination of these pollutants with many others present in the same environment. As an example, high levels of organochlorine pesticides have been measured in soils from Acerra (Qu et al. 2016).

Conclusions

The data presented here suggest that environmental pollution strongly affects *L. cruciata*, both at ultrastructural and physiological level, inducing a functional adaptation to stress. Therefore, the response chain to stress (and specifically pollution) would comprehend different and complex pathways involving gene expression and biochemical and physiological adaptations in order to counteract the damage observed at the cellular and tissue level. Namely, the heavy metal pollution measured in Acerra, in the "Italian Triangle of Death," caused evident and increased damage with respect not only to Riccia specimens, collected in the unpolluted site, but, intriguingly, even in samples collected in the city center of Naples, an urban area presenting a high anthropic impact due to car traffic.

These results, on one hand, assess the high tolerance of *L. cruciata* to abiotic stress caused by metals; on the other hand, these would suggest that this organism is a useful bioindicator of environmental pollution. Moreover, the utilization of Hsp70 expression and occurrence in Bryophyta can be used as efficient biomarkers of pollution.

Acknowledgements The authors want to thank Dr. Vivana Maresca (Naples) for the statistical analysis of HM accumulation and vitality data. This work was supported by a grant from MIUR, PROGETTO TIDe, Obiettivo realizzativo: 1.9; Bioaccumulo. The authors declare no conflict of interest.

References

Adamo P, Giordano S, Vingiani S, Castaldo Cobianchi R, Violante P (2003) Trace elements accumulation by moss and lichen bags in the city of Naples (Italy). Environ Pollut 122:91–103

Basile A, Cogoni AE, Bassi P, Fabrizi E, Sorbo S, Giordano S, Castaldo Cobianchi R (2001) Accumulation of Pb and Zn in gametophytes and sporophytes of the moss *Funaria hygrometrica* (Funariales). Ann Bot London 87:537–543

Basile A, di Nuzzo RA, Capasso C, Sorbo S, Capasso A, Carginale V (2005) Effect of cadmium on gene expression in the liverwort *Lunularia cruciata*. Gene 356:153–159

Basile A, Sorbo S, Aprile G, Conte B, Castaldo Cobianchi R (2008) Comparison of the heavy metal bioaccumulation capacity of an epiphytic moss and an epiphytic lichen. Environ Pollut 151:401–407

Basile A, Sorbo S, Aprile G, Conte B, Castaldo Cobianchi R, Pisani T, Loppi S (2009) Heavy metal deposition in the Italian "Triangle of death" determined with the moss *Scorpiurium circinatum*. Environ Pollut 157:2255–2260

Basile A, Sorbo S, Conte B, Golia B, Montanari S, Castaldo Cobianchi R, Esposito S (2011) Antioxidant activity in extracts from *Leptodyctium riparium* (Bryophyta), stressed by heavy metals, heat shock, salinity. Plant Biosyst 175:77–80. doi:10.1080/11263504-2010-509935

Basile A, Sorbo S, Pisani T, Paoli L, Munzi S, Loppi S (2012) Bioaccumulation and ultrastructural effects of Cd, Cu, Pb and Zn in the moss *Scorpiurum circinatum* (Brid.) Fleisch. & Loeske. Environ Pollut 166:208–211

Basile A, Sorbo S, Conte B, Cardi M, Esposito S (2013) Ultrastructural changes and heat shock proteins 70 induced by atmospheric pollution are similar to the effects observed under *in vitro* heavy metals stress in *Conocephalum conicum* (Marchantiales—Bryophyta). Environ Pollut 182:209–216. doi:10.1016/j.envpol.2013.07.014

Basile A, Sorbo S, Cardi M, Lentini M, Castiglia D, Cianciullo P, Conte B, Loppi S, Esposito S (2015) Effects of heavy metals on ultrastructure and Hsp70 induction in *Lemna minor* L. exposed to water along the Sarno River, Italy. Ecotox Environ Safe 114:93–101

Basile A, Sorbo S, Lentini M, Conte B, Esposito S (2016) Water pollution causes ultrastructural and functional damages in *Pellia neesiana* (Gottsche) Limpr. J Trace Elem Med Biol. doi:10.1016/j.jtemb.2016.11.014

Bassham DC, Laporte M, Marty F, Moriyasu Y, Ohsumi Y, Olsen LJ, Yoshimoto K (2006) Autophagy in development and stress responses of plants. Autophagy 2:2–11. doi:10.4161/auto.2092

Cardi M, Castiglia D, Ferrara M, Guerriero G, Chiurazzi M, Esposito S (2015) The effects of salt stress cause a diversion of basal metabolism in barley roots: possible different roles for glucose-6-phosphate dehydrogenase isoforms. Plant Physiol Bioch 86:44–54. doi:10.1016/j.plaphy.2014.11.001

Carginale V, Sorbo S, Capasso C, Trinchella F, Cafiero G, Basile A (2004) Accumulation, localisation, and toxic effects of cadmium in the liverwort *Lunularia cruciata*. Protoplasma 223:53–61

Chiaiese P, Minutolo M, Arciello A, Guglielmini F, Piccoli R, Filippone E (2011) Expression of human apolipoprotein A-I in *Nicotiana tabacum*. Biotechnol Lett 33:159. doi:10.1007/s10529-010-0388-4

Esposito S, Castaldo Cobianchi R, Sorbo S, Conte B, Basile A (2007) Ultrastructural alterations and HSP70 induction in *Elodea canadensis* Michx. exposed to heavy metals. Caryologia 60:115–120. doi:10.1080/00087114.2007.10589557

Esposito S, Sorbo S, Conte B, Basile A (2012) Effects of heavy metals on ultrastructure and HSP70s induction in the aquatic moss *Leptodictyum riparium* Hedw. Int J Phytorem 14:443–455. doi:10.1080/15226514.2011.620904

Harmens H, Norris D, the participants of the Moss Survey (2008) Spatial and temporal trends in heavy metal accumulation in mosses in Europe (1990–2005). Centre for ecology and hydrology, environment centre wales, Bangor, UK. http://nora.nerc.ac.uk/id/eprint/3928

Hartl FU (1996) Molecular chaperones in cellular protein folding. Nature 381:571–580. doi:10.1038/381571a0

Galano E, Arciello A, Piccoli R, Monti DM, Amoresano A (2014) A proteomic approach to investigate the effects of cadmium and lead on human primary renal cells. Metallomics 6:587. doi:10.1039/c3mt00344b

Giordano S, Castaldo-Cobianchi R, Basile A, Spagnuolo V (1989) The structure and the role of hyaline parenchyma in the liverwort *Lunularia cruciata* (L.) Dum. G Bot Ital 123:169–176

Gratão PL, Polle A, Lea PJ, Azevedo RA (2005) Making the life of heavy metal-stressed plants a little easier. Funct Plant Biol 32:481–494

Kukkola E, Rautio P, Huttunen S (2000) Stress indications in copper- and nickel exposed Scots pine seedlings. Environ Exp Bot 43:197–210. doi:10.1016/s0098-8472(99)00057-x

Lage-Pinto F, Oliveira JG, Da Cunha M, Souza CMM, Rezende CE, Azevedo RA, Vitória AP (2008) Chlorophyll a fluorescence and ultrastructural changes in chloroplast of water hyacinth as indicators of environmental stress. Environ Exp Bot 64:307–313

Landi S, Nurcato R, De Lillo A, Lentini M, Grillo S, Esposito S (2016) Glucose-6 phosphate dehydrogenase plays a central role in the response of tomato (*Solanum lycopersicum*) plants to short and long-term drought. Plant Physiol Bioch 105:79–89. doi:10.1016/j.plaphy.2016.04.013

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)). Methods 25:402–408

Liu D, Kottke I (2004) Subcellular localization of copper in the root cells of *Allium sativum* by electron energy spectroscopy (EELS). Bioresour Technol 94:153–158

Miernyk JA (1997) The 70 kDa stress-related proteins as molecular chaperones. Tr Plant Sci 2:180–187. doi:10.1016/j.tplants.2004.03.006

Nassiri Y, Ginsburger-Vogel T, Mansot JL, Wéry J (1996) Effects of heavy metals on *Tetraselmis suecica*: ultrastructural and energy-dispersive x-ray spectroscopy studies. Biol Cell 86:151–160

Paoli L, Fiorini E, Munzi S, Sorbo S, Basile A, Loppi S (2014) Uptake and acute toxicity of cerium in the lichen *Xanthoria parietina*. Ecotoxicol Environ Saf 104:379–385. doi:10.1016/j.ecoenv.2014.02.028

Qu C, Albanese S, Chen W, Lima A, Doherty A, Piccolo A, Arienzo M, Qi S, De Vivo B (2016) The status of organochlorine pesticide contamination in the soils of the Campania plain, Southern Italy, and correlations with soil properties and cancer risk. Environ Pollut 216:500–511

Ronen R, Galun M (1984) Pigment extraction from lichens with dimethylsulfoxide (DMSO) and estimation of chlorophyll degradation. Environ Exp Bot 24:239–245

Romieu I, Castro-Giner F, Kunzli N, Sunyer J (2008) Air pollution, oxidative stress and dietary supplementation: a review. Eur Respir J 31:179–197. doi:10.1183/09031936.00128106

Schwartzman RA, Cidlowski JA (1993) Apoptosis: the biochemistry and molecular biology programmed cell death. Endocr Rev 14:133–151

Senior K, Mazza A (2004) Italian "Triangle of death" linked to waste crisis. Lancet Oncol 5:525–527

Shailasree S, Venkataramana M, Niranjana SR, Prakash HS (2015) Cytotoxic effect of p-coumaric acid on neuroblastoma, N2a cell via generation of reactive oxygen species leading to dysfunction of mitochondria inducing apoptosis and autophagy. Mol Neurobiol 51:119–130

Sorbo S, Aprile G, Strumia S, Castaldo Cobianchi R, Leone A, Basile A (2008) Trace element accumulation in *Pseudevernia furfuracea* (L.) Zopf exposed in Italy's so called Triangle of Death. Sci Tot Environ 407:647–654

Strasser RJ, Srivastava A, Tsimilli-Michael M (2000) The fluorescence transient as a tool to characterise and screen photosynthetic samples. In: Yunus M, Pathre U, Mohanty P (eds) Probing photosynthesis: mechanisms, regulation and adaptation. Taylor & Francis, London, pp 445–483

Taylor NL, Day DA, Millar AH (2002) Environmental stress causes oxidative damage to plant mitochondria leading to inhibition of glycine decarboxylase. J Biol Chem 277:42663–42668

Thompson AR, Vierstra RD (2005) Autophagic recycling: lessons from yeast help define the process in plants. Curr Op Plant Biol 8:165–173. doi:10.1016/j.pbi.2005.01.013

Todeschini V, Lingua G, D'Agostino G, Carniato F, Roccotiello E, Berta G (2011) Effects of high zinc concentration on poplar leaves: a morphological and biochemical study. Environ Exp Bot 71:50–56

Tyler G (1990) Bryophytes and heavy metals: a literature review. Bot J Linn Soc 104:231–253

Zhang X, Gao B, Xia H (2014) Effect of cadmium on growth, photosynthesis, mineral nutrition and metal accumulation of bana grass and vetiver grass. Ecotoxicol Environ Saf 106:102–108. doi:10.1016/j.ecoenv.2014.04.025