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LYSOSOMAL MEMBRANE STABILITY IN LABORATORY- AND FIELD-EXPOSED TERRESTRIAL ISOPODS *PORCELLIO SCABER* (ISOPODA, CRUSTACEA)

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Abstract—Two established methods for assessment of the cytotoxicity of contaminants, the lysosomal latency (LL) assay and the neutral red retention (NRR) assay, were successfully applied to in toto digestive gland tubes (hepatopancreas) of the terrestrial isopod *Porcellio scaber* (Isopoda, Crustacea). In vitro exposure of isolated gland tubes to copper was used as a positive control to determine the performance of the two methods. Lysosomal latency and the NRR assay were then used on in vivo (via food) laboratory-exposed animals and on field populations. Arbitrarily selected criteria for determination of the fitness of *P. scaber* were set on the basis of lysosomal membrane stability (LMS) as assessed with in toto digestive gland tubes. Decreased LMS was detected in animals from all polluted sites, but cytotoxicity data were not in agreement with concentrations of pollutants. Lysosomal membrane stability in the digestive gland tubes of animals from an environment in Idrija, Slovenia, that was highly polluted with mercury (260 μg/g dry wt food and 1,600 μg/g dry wt soil) was less affected than LMS in laboratory animals fed with 5 and 50 μg Hg/g dry weight for 3 d. This probably indicates tolerance of *P. scaber* to mercury in the mercury-polluted environment and/or lower bioavailability of environmental mercury. In animals from the vicinity of a thermal power plant with environmental mercury concentrations three to four orders of magnitude lower than those in Idrija, LMS was severely affected. In general, the LL assay was more sensitive than the NRR assay. The LMS assay conducted on digestive gland tubes of terrestrial isopods is highly recommended for integrated biomarker studies.

Keywords—Lysosomal membrane stability Cytotoxicity Lysosomal latency Neutral red retention *Porcellio scaber*

INTRODUCTION

The impact of pollution is an urgent international issue. During the past three decades, damage to the environment has been measured in terms of changes in populations and communities of different organisms. However, whereas changes at the population/community level and in ecosystems are the ultimate concern of these studies, tools for early detection and prediction of the consequences of environmental stress should be developed at lower levels of biological complexity [1].

Cells provide a link between molecular and biochemical events and physiological or whole-organism events [2]. Promising methods that may offer a reliable way of assessing individual health status include assessments of lysosomal membrane stability (LMS) [1,3]. The derivation of lysosomal biomarkers was based on the assumption that because of their key role in cellular functioning, they should be indicative of the functional integrity of cells [1].

Lysosomes are intracellular organelles that contain a battery of approximately 60 hydrolytic enzymes acting optimally at an acidic pH. They are involved in many essential functions, including membrane turnover, nutrition, and cellular defense. They are important organelles for metal and lipophilic contaminant sequestration and detoxification, which can make them prone to oxidative damage that, in turn, can lead to membrane destabilization [2,4]. This is particularly evident in the epithelial cells of the digestive gland, which serves as the major site of intracellular digestion in animals [4].

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Changes in the LMS are the earliest adverse effects following exposure of the organism to elevated concentrations of chemicals, which subsequently lead to effects at higher levels of biological organization [2]. As a result of stress, the permeability of the majority of lysosomal membranes is increased because of structural changes in membranes, such as breaks, gaps [5], and discontinuous overlaps [6].

Techniques to determine lysosomal dysfunction via LMS have been developed primarily in human medicine but have been transferred to environmental research [1]. Reduced stability of the lysosomal membrane as an index of lysosomal function is monitored in the mussel-watch program in Europe and as a biomarker of the effects of pollution in the United States. Usually, cryostat sections of the hepatopancreas or blood cells of marine invertebrates are used [2,7]. For terrestrial organisms, except for earthworms [8] and mollusks [9], very few data exist regarding the subcellular effects of pollutants. Because terrestrial isopods also are the organisms of choice in terrestrial ecotoxicology and ecophysiology [10], LMS would be a welcome additional parameter in laboratory and field toxicity studies. The digestive glands (hepatopancreas) of terrestrial isopods are the main interface between the organism and its environment [11] and, thus, are a suitable organ for studying the effects of environmental pollutants by LMS assay.

The lysosomal latency (LL) assay and the neutral red retention (NRR) assay are most frequently used for measuring LMS by application to cell suspensions or cryostat sections of a tissue. When blood cells are not assessed, sample preparation for LMS is destructive. It would be ideal to assess LMS on isolated, intact tissue.

Lysosomal latency assessment determines alterations in the permeability of the lysosomal membrane to the passage of substrates from the cytosol into the lysosome [12]. This method is the standardized cytochemical procedure regarding cryostat sections for determination of LMS based on evaluation of the activity of the lysosomal enzyme *N*-acetyl-β-hexosaminidase [13]. The method is widely used in biomonitoring programs for assessing the impact of pollutants on marine organisms [14,15].

In the NRR assay, the efflux of the stain (neutral red [NR] dye) from the lysosomes to the cytosol is determined. As a weak base, NR dye permeates the lysosomal membrane in its unprotonated form, where it becomes trapped by protonation because of the low pH in the lysosomes. Release of the NR dye into the cytosol most probably results from impairment of the lysosomal membrane Mg^{2+} adenosine triphosphate–dependent H^+ ion-proton pump, which is responsible for maintenance of the low pH inside the lysosomes [16]. The failure or impairment of this pump leads to increased intralysosomal pH and results in an equilibrium state on both sides of the lysosomal membrane, which allows free passage of the lysosomal contents, including NR dye, to the cytosol [17]. Neutral red staining is a well-established technique for the evaluation of cell toxicity [18]. The ability to retain NR dye is dependent on lysosomal viability or function. The relative release of NR dye therefore often is used as a specific indicator of lysosomal function or activity [19]. The method has been used on earthworm coelomocytes [8] and on hemocytes or disaggregated digestive cells of marine organisms [14,17].

Metals can damage lysosomes directly or indirectly. Direct effects are associated with binding of metals to the lysosomal membrane, increased loading of metal-binding proteins within the lysosomes, removal of antioxidant compounds, and inhibition of antioxidant enzymes. The latter two effects promote interactions between oxidants and membranes. Indirect effects are mediated by the formation of oxyradicals, which damage the lysosomal membrane by promoting the peroxidation of membranes and by reducing the antioxidant cellular defense, thus also leading to peroxidation of membranes [20]. Contaminants can impair lysosomal function by disruption of the membrane Mg^{2+} adenosine triphosphatase–dependent proton pump [16], which is responsible for maintaining the acidic internal environment of lysosomes.

Mercury is among the most toxic heavy metals in the environment and transforms into different chemical forms as a result of abiotic factors and biotic activity [21]. It has been demonstrated that Hg^{2+} is able to increase the intralysosomal pH, probably by interacting with the membrane proton pump [22] or by inducing lipid peroxidation [23], leading to a decrease in lysosomal membrane fluidity and an increase in lysosomal membrane fragility [24]. Mercury induces an increase of cytosolic Ca^{2+}, which can modulate lysosomal membrane destabilization via activation of Ca^{2+}-dependent phospholipase A_2 [25]. Also, mercury affects lysosomal membranes by interacting with sulfhydryl groups in the lysosomal membranes [26] and decreasing the activity of the lysosomal enzyme *N*-acetyl-β-hexosaminidase [27]. However, β-glucuronidase, which is present in the soluble phase and is not structurally associated with the membrane [28], was reported not to be affected by mercury [27].

In our previous work, we studied the transformations of mercury in the terrestrial isopod *Porcellio scaber* and its environment, including the assimilation and distribution of inorganic and organic mercury within the animals [29,30]. In the present study, we examined the earliest effects of mercury on this isopod. Thus, the present study has two aims: Application of the modified LL [13] and NRR [7] assays to in toto digestive gland tubes (hepatopancreas) of the terrestrial isopod *P. scaber* (Crustacea), the application of the two methods to laboratory-exposed animals (mercury-fed animals) and to those collected in the field in the vicinity of a mercury mine, a thermal power plant, and an unpolluted location in Slovenia. The results of the two methods are compared. The applicability of LMS assays (LL and NRR assays) as a measure of cytotoxicity in laboratory and field isopod toxicity studies is discussed.

MATERIALS AND METHODS

The present study is divided in four parts. In the first part, application of the two established methods for LMS assessment (LL assay and NRR assay), including some modifications, to in toto digestive gland tubes of *P. scaber* is described. The second part relates to experiments performed on in toto digestive gland tubes of *P. scaber* exposed in vitro to copper (used as a positive test to examine the performance of the two methods for LMS assessment). The positive test is a novel procedure in such kinds of studies, but at the same time, it can be treated as a modification of the methods. In the third part, the methods for LMS assessment are applied to *P. scaber* fed with mercury-treated food in the laboratory, and finally, the methods are used on field animals collected at polluted and unpolluted locations.

Individuals of the terrestrial isopod *P. scaber* (Isopoda, Crustacea) used in laboratory experiments were collected during October 2004, November 2004, and April 2005 at a noncontaminated location in Maribor, Slovenia. Animals were held at a temperature of 20 ± 2°C and a 16:8-h light:dark photoperiod and were fed with hazelnut leaves (*Corylus avellana*). ?3

Before dissection, the weight, sex, molting stage, and presence of the marsupium in laboratory and field animals were noted. During the dissection, the digestive glands were isolated in toto, and their color and shape were described. Also, the fullness and content of the gut were recorded.

Application of two methods for LMS assessment to the digestive gland tubes of P. scaber

LL assay. For LMS assessment, the standardized cytochemical procedure based on evaluation of the activity of the lysosomal enzyme *N*-acetyl-β-hexosaminidase [13] was applied to digestive glands of *P. scaber*. Rather than the cryostat sections recommended by United Nations Environment Program (UNEP/RAMOGE) [13], in toto digestive gland tubes were used. The hepatopancreas consists of two pairs of tubes, which were separated during isolation and used in the following procedure for the LL assay. For each series of gland tubes, five animals needed to be killed.

A series of gland tubes were incubated in a labilizing buffer (0.1 M sodium citrate buffer at pH 4.5) at 37°C for 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, and 180 min to labilize the membrane artificially. The duration of incubation was much longer than described in the UNEP/RAMOGE [13]. After acid labilization, the gland tubes were incubated in 50 ml of 0.1 M sodium citrate buffer containing 3.5 mg of a low-viscosity peptide (for membrane stabilization) and 20 mg of substrate (naphthol AS-BI *N*-acetyl-β-glucosaminide) previously dissolved in 2.5 ml of 2-meth-

oxyethanol for 20 min at 37°C. Then, the gland tubes were rinsed with physiological solution for *P. scaber* [31] and incubated for 10 min at room temperature in 0.1 M sodium phosphate buffer (pH 7.4) containing the diazonium-coupler Fast Violet B Salt (1 mg/ml; Sigma-Aldrich Chemie, Steinheim, Germany). After a 5-min rinse in tap water, the gland tubes were fixed for 10 min at 4°C in calcium formol and mounted in Kaiser glycerine gelatine (Merck KGaA, Darmstadt, Germany).

Compared to the standardized procedure [13], in which only one parameter is assessed, in our procedure two parameters, the maximum amount of stained lysosomes and the staining of the cytoplasm, were assessed microscopically (Axioskop 2 MOT; Carl Zeiss, Goettingen, Germany) in each series of gland tubes by two independent observers. The LMS was assessed as the time of incubation of the gland tube in the labilizing buffer when the maximal amount of stained lysosomes or the staining of the cytoplasm occurs.

NRR assay. The NR stock solution was made following the procedure described by Ringwood et al. [7]. Four milligrams of the NR powder (The British Drug Houses, London, UK) were dissolved in 1 ml of dimethyl sulfoxide (Sigma-Aldrich Chemie). The working solution was prepared by diluting 10 μl of the stock solution with 9.9 ml of the physiological solution for *P. scaber*. After dissection, four gland tubes were separated: Three were used for the LL assay, and one was used for the NRR assay.

For each NRR assay, one digestive gland tube was placed on a slide, and 40 μl of the working solution of the NR dye were added to the tissue. The time when the NR dye was added to the gland tubes was noted. Slides were left for 20 min in a light-protected humidity chamber that consisted of a glass Petri dish (r = 9.5 cm) with a lid. To maintain high humidity, the lid was sprayed with distilled water, and the base of the dish was covered with moistened filter paper. After that, the slides were covered with the coverslip and inspected under a microscope (Axioskop 2 MOT; Carl Zeiss) every 30 min. At every inspection, the fraction of the gland tube that became colored pink to red because of the leakage of NR dye from the lysosomes into the cytoplasm was assessed.

Positive test

To test the performance of both methods for assessing LMS in *P. scaber* hepatopancreas, a positive test based on in vitro exposure of digestive gland tubes to copper was introduced. Copper was chosen as a reference chemical because of its known role in the induction of oxidative damage [32]. Immediately after dissection, gland tubes were exposed in vitro to copper by incubation in copper solutions (50 and 100 μg/L) prepared from $Cu(NO_3)_2$ and physiological solution for *P. scaber* for 1 h and then assessed for LMS following the two methods, the LL assay and the NRR assay.

Laboratory mercury-fed animals

The concentrations of Hg^{2+} in the leaves that were offered to the animals in our experiments were selected to be within the range of mercury concentrations measured in the Idrija region of Slovenia, which has been reported to range from 0.1 to 51.8 μg/g dry weight in plants and 1 to 2,500 μg/g dry weight in soil [33]. The hazelnut leaves were prepared following the protocol proposed by Drobne and Hopkin [34]. Solutions with selected concentrations of mercury, to be applied to hazelnut leaves, were prepared by diluting standard $HgCl_2$ solutions with rainwater (to simulate natural conditions).

In the 3-d feeding experiments, three groups of five animals were exposed either to untreated leaves or to leaves treated with Hg^{2+} at 5 or 50 μg/g dry weight. The humidity in the Petri dishes (r = 4.5 cm) was maintained by spraying the lids with distilled water every day and keeping the dishes in plastic bags. Each of the test series was repeated 10 times.

After 3 d of exposure, dissection and the two procedures for LMS assessment were performed. The gland tubes of five animals from one Petri dish were needed for one LL measurement. From each animal, three gland tubes were reserved for LL assay. In addition to the 10 LL assays, the NRR assay was conducted twice. For one NRR measurement, one gland tube of one animal was enough.

Field animals

Field animals were collected from selected parts of Slovenija (Fig. 1). The Maribor site represents a clean, unpolluted reference environment.

Idrija is an environment that is chronically contaminated with mercury (natural background and past mining and smelting activity). Therefore, it is a natural and anthropogenic laboratory suitable for studying the transformation and distribution of mercury in air, water, soil, and biota and the effects of mercury on the organisms and humans living there. In the present study, Idrija was chosen to compare the cytotoxic effects of mercury in chronically exposed animals with the effects to mercury in laboratory-exposed animals.

Velenje, Slovenia, is polluted by emissions from a nearby coal-fired thermal power plant in Šoštanj (~1 km distant). The thermal power plant has been operating since 1956, and it is estimated to emit 0.2 tons of cadmium, 22.1 tons of lead, 4.5 tons of arsenic, 298 tons of zinc [35], and 0.3 tons of mercury [36] per year. After a desulfurization device was built at the thermal power plant, the emissions markedly decreased (see *Discussion*).

Analytical method for total mercury determination

The total mercury content was determined in samples of compost and soil from the garden in Idrija and in leaf litter, decaying wood, and soil from Velenje, where individuals of *P. scaber* were taken for LMS assessment. The soil samples were dried at 30°C and homogenized; other samples were frozen at −22°C, lyophilized, and homogenized. After acid digestion, total mercury concentration was determined by cold-vapor atomic absorption spectrometry [37].

Statistical analysis

For statistical analysis of the data, the Windows statistical software SPSS (Ver 12.01; SPSS, Chicago, IL, USA) was used. The differences in LMS among the unexposed and exposed animals were tested by the nonparametric Kruskal–Wallis test, with the Mann–Whitney post hoc test in which only two samples were compared (level for significance, $p < 0.05$).

RESULTS

Application of two methods for LMS assessment to the digestive gland tubes of P. scaber

LL assay. The method described in UNEP/RAMOGE [13] for cryostat sections was modified for digestive gland tubes and successfully conducted on unexposed *P. scaber*. In Figure 2A, the absence of stained lysosomes is shown. Generally, the

Fig. 1. Sites in Slovenia used for sampling of *Porcellio scaber*.

amount of stained lysosomes in the digestive gland increased with increasing time of incubation in the labilizing buffer, until the majority of lysosomes became destabilized and the amount of stained lysosomes reached the maximum (Fig. 2B). When incubation in the labilizing buffer was continued, the amount of stained lysosomes decreased, and sometimes, they could no longer be observed. Further prolongation of the incubation time in the labilizing buffer causes leakage of the lysosomal content into the cytoplasm. This can be seen as staining of the cytoplasm in the entire gland tube (Fig. 2C), with lysosomes still visible. Both the maximal amount of stained lysosomes and cytoplasm staining were considered in the present study when LMS was assessed.

NRR assay. The NRR assay also was used successfully for the in toto digestive gland tube of *P. scaber*. Soon after the start of the incubation in NR dye, the lysosomes in the most distal part of the gland tubes and on sites of the glands injured by the tissue isolation procedure became destabilized first (Fig. 2D and E). Subsequently, lysosomal destabilization was observed to be spread throughout the gland tube. From 110 to 140 min after the start of incubation in NR dye, the majority of lysosomes in the majority of digestive gland tubes of control and exposed animals leached their lysosomal content into the cytoplasm, which could be seen as pink to red staining of the gland tube.

Positive test

LL assay. After in vitro incubation of gland tubes in copper solutions (positive test), a dose-dependent decrease of LMS was detected when the maximal amount of stained lysosomes or cytoplasm staining was assessed (Fig. 3).

NRR assay. At 20 min after incubation in NR dye, the positive test showed differences in LMS between glands of control animals and glands exposed to copper in vitro at 50 and 100 μg/L for 1 h (Fig. 3). However, no differences in LMS were observable between the two copper concentrations to which the gland tubes were exposed in vitro.

Laboratory mercury-fed animals

LL assay. Lysosomal membrane stability in glands of animals exposed to Hg^{2+} (5 and 50 μg/g dry wt) in the food for 3 d was reduced significantly compared to control animals fed with uncontaminated leaves (Fig. 4). No dose-dependent response was observed: The LMS between groups of animals exposed to Hg^{2+} at 5 and 50 μg/g in the food was not significantly different (Mann–Whitney test: Maximal amount of stained lysosomes, $p = 0.372$; cytoplasm staining, $p = 0.110$).

NRR assay. The NRR assay showed similar results to the LL assay: After only 20 min of incubation in NR dye, the lysosomes in digestive glands of control animals were much more stable compared to the glands of animals exposed to mercury in the food for 3 d (Fig. 4). In the fraction of a tube with destabilized lysosomes, no significant difference was observed between animals exposed to mercury at 5 or 50 μg/g dry weight leaf (Mann–Whitney test: $p = 0.161$).

Field animals

LL assay. The results indicated more stable lysosomes in animals from the unpolluted location in Maribor compared to animals from the polluted locations. The most affected lysosomal membranes were observed in animals from Velenje (Fig. 5).

NRR assay. After 20- or 50-min incubation in the NR dye, the NRR assay showed the most stable lysosomes in digestive glands of animals from Maribor compared to animals from Idrija and Velenje (Fig. 5). The differences between the animals from different locations were more significant after 20 min of incubation (Kruskall–Wallis test, Mann–Whitney test: $p < 0.001$) than after 50 min of incubation in the NR dye (Kruskall–Wallis test, Mann–Whitney test: $p < 0.05$). In spite

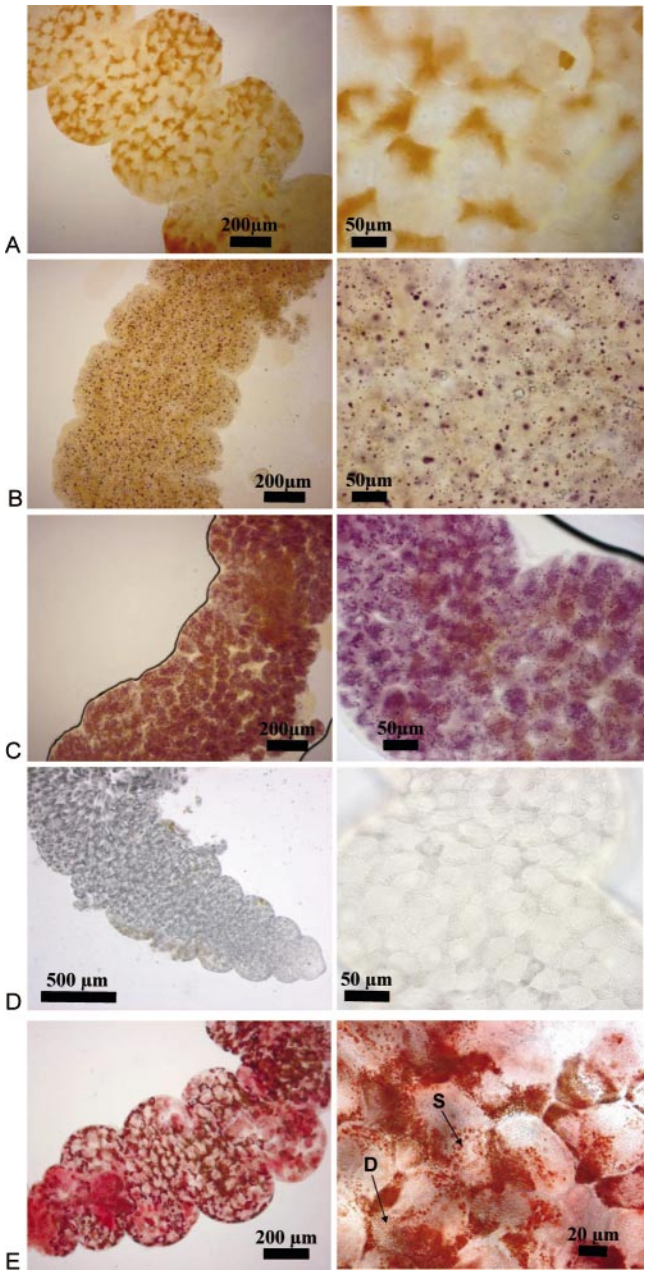

Fig. 2. Lysosomal membrane stability assessment in the digestive gland tubes of *Porcellio scaber*. Lysosomal latency assay: (**A**) absence of stained lysosomes, (**B**) maximal amount of stained lysosomes, and (**C**) cytosol staining. Neutral red retention assay: (**D**) before the incubation of the gland tube in neutral red (NR) dye and (**E**) after the incubation of the gland tube in NR dye. At higher magnification, it is possible to distinguish between the stable lysosomes (S) that contain the NR dye and the destabilized lysosomes (D) where leakage of the lysosomal content to the cytoplasm occur, which is seen as the pink to red dyeing of the cytoplasm. The fraction of the colored gland tube was assessed at every inspection time.

of the trends in Figure 5B showing the most affected lysosomes in the animals from Velenje, the Mann–Whitney test showed no statistically significant differences between the animals from Idrija and Velenje (after 20 min: $p = 0.145$; after 50 min: $p = 0.245$).

Comparison of the methods for LMS assessment

To compare the LL assay with the NRR assay, the results for exposed animals are summarized in Figure 6 as a percentage of the median LMS value obtained for digestive glands of unexposed animals.

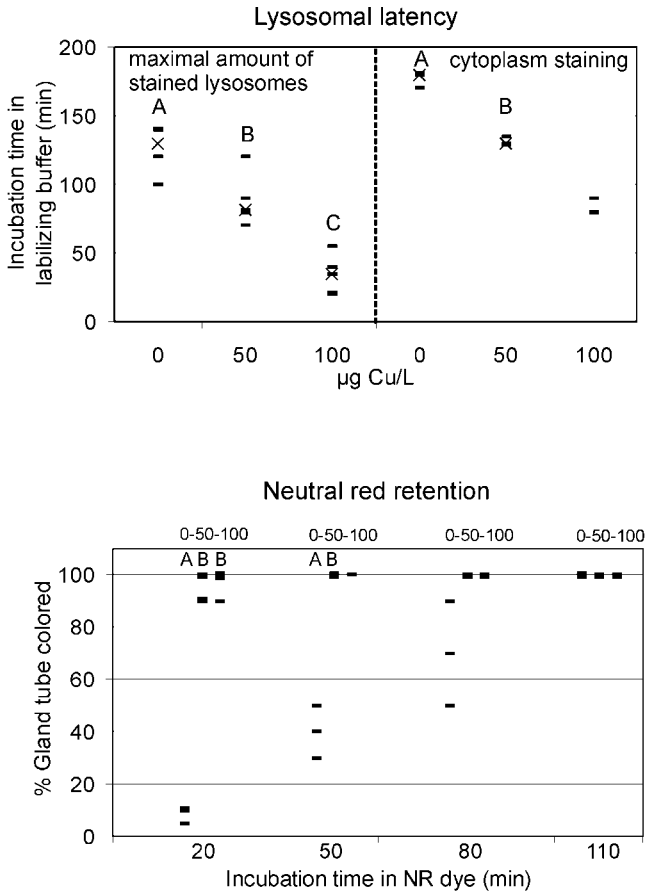

Fig. 3. Positive test: Lysosomal membrane stability (LMS) assessment on digestive gland tubes of *Porcellio scaber*, in vitro incubated in physiological solution (marked as 0) and solutions with 50 and 100 µg Cu/L (marked as 50 and 100, respectively) for 1 h using lysosomal latency (LL) assay and neutral red retention (NRR) assay. Five animals were needed to obtain one result using the LL assay, and one animal was needed to obtain one result using the NRR assay. Median values are marked with a cross. The LMS assessments that differ significantly from each other (Kruskal–Wallis test, Mann–Whitney test, $p < 0.05$) are labeled with different letters.

In general, the LL assay appeared to be more sensitive when the maximal amount of stained lysosomes was assessed. The LL assay (cytoplasm staining) and the NRR assay showed very comparable results when performed on the same animals after 50 min of incubation in NR dye. However, some differences between the LL and NRR assays were obtained in laboratory-exposed animals, for which the LL assay was performed on a much larger population of animals compared to the NRR assay.

Lysosomes were affected least in animals from Idrija if compared to laboratory- and field-exposed animals (LL assay). The most affected lysosomes were observed in the digestive cells of animals collected in Velenje (LL assay, NRR assay after 50 min of incubation in NR).

These data, together with those published in the literature [7], were the basis for setting arbitrary criteria for determining the fitness of the organisms, which were classified as normal, concern, or stress condition (Fig. 6).

Mercury in environmental samples

The concentrations of mercury in the samples from the site at Idrija where animals were taken for LMS assessment were

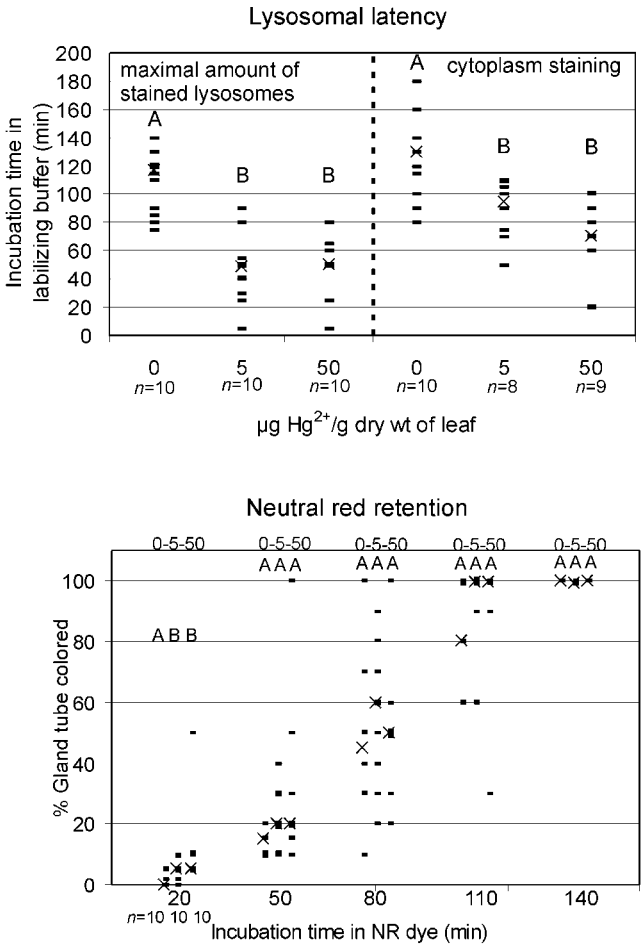

Fig. 4. Lysosomal membrane stability (LMS) assay on the entire digestive gland tube of *Porcellio scaber* exposed in vivo to uncontaminated leaves (marked as 0) and leaves treated with 5 and 50 µg Hg^{2+}/g dry weight (marked as 5 and 50, respectively) for 3 d using lysosomal latency (LL) assay and neutral red retention (NRR) assay. Five animals were needed to obtain one result using the LL assay, and one animal was needed to obtain one result using the NRR assay. Median values are marked with a cross. The LMS assessments that differ significantly from each other (Kruskal–Wallis test, Mann–Whitney test, $p < 0.05$) are labeled with different letters. n = number of results obtained.

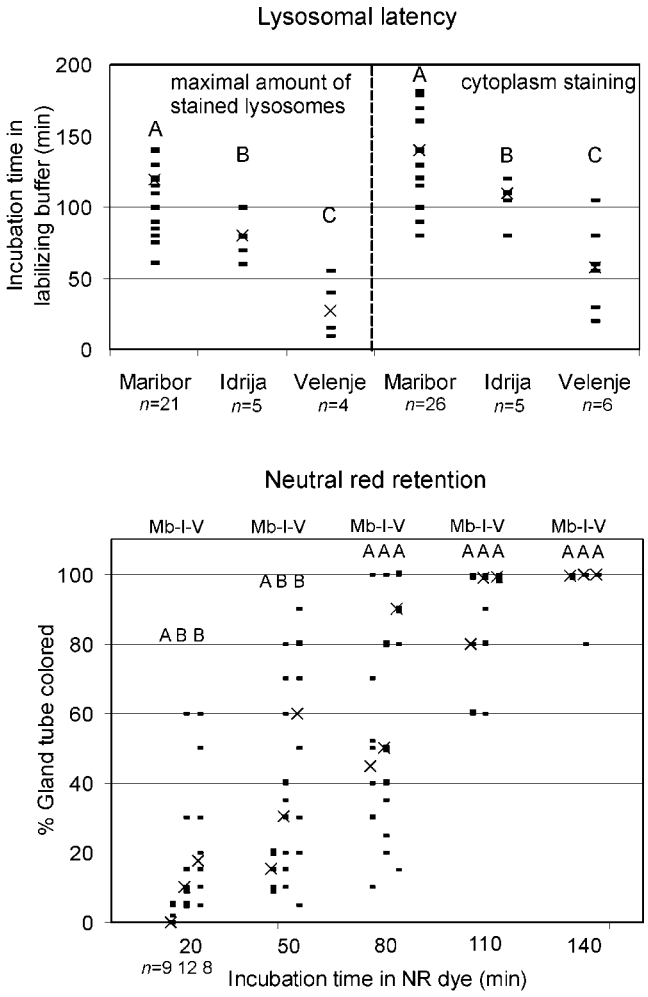

Fig. 5. Lysosomal membrane stability (LMS) assay in whole digestive gland tube of *Porcellio scaber* collected in unpolluted (Maribor [Mb], Slovenia), and polluted (Idrija [I] and Velenje [V], Slovenia) environments in Slovenia using lysosomal latency (LL) assay and neutral red retention (NRR) assay. Five animals were needed to obtain one result using the LL assay, and one animal was needed to obtain one result using the NRR assay. Median values are marked with a cross. The LMS assessments that differ significantly from each other (Kruskal–Wallis test, Mann–Whitney test, $p < 0.05$) are labeled with different letters. n = number of results obtained.

262 ± 8.4 µg/g dry weight in the compost and 1,611 ± 189 µg/g dry weight in the soil. In Velenje, 0.010 ± 0.001 µg/g dry weight was found in the leaf litter, 0.028 ± 0.009 µ/g dry weight in decayed wood, and 1.17 ± 0.17 µg/dry weight in the soil.

DISCUSSION

In the present study, two well-established methods for the assessment of LMS (LL assay and NRR assay) were successfully applied to digestive gland tubes (hepatopancreas) of the terrestrial isopod *P. scaber* from laboratory experiments and from the field.

The modifications introduced into the two methods were as follows: The LMS was inspected on digestive gland tube in toto but not on cryostat sections or cell suspensions. A positive test was introduced to assess the performance of LMS assays. Two parameters related to LMS were assessed, the maximal amount of stained lysosomes and cytoplasm staining, whereas in the standardized procedure, only one parameter is assessed. Criteria to determine the fitness of organisms were arbitrarily set on the basis of LMS.

The advantage of the modified methods for assessment of LMS on the entire digestive gland tube compared to cryostat sections or cell suspension lies in the fact that less damaged cells provide a more relevant response. Moreover, the cells along the entire digestive gland tube do not react at the same time. Therefore, the entire organ/tube should be inspected to assess properly the maximal amount of stained lysosomes or cytoplasm staining.

In vitro exposure of digestive gland tubes of *P. scaber* to copper was used as a positive test to assess the performance of the two LMS methods. Copper was chosen as a reference chemical in the positive test because of its known cytotoxic effects [32]. The results obtained in the positive test with copper confirmed the successful application of both LMS assessments to in toto digestive gland tubes of *P. scaber*. This in vitro positive test is recommended in similar studies to evaluate

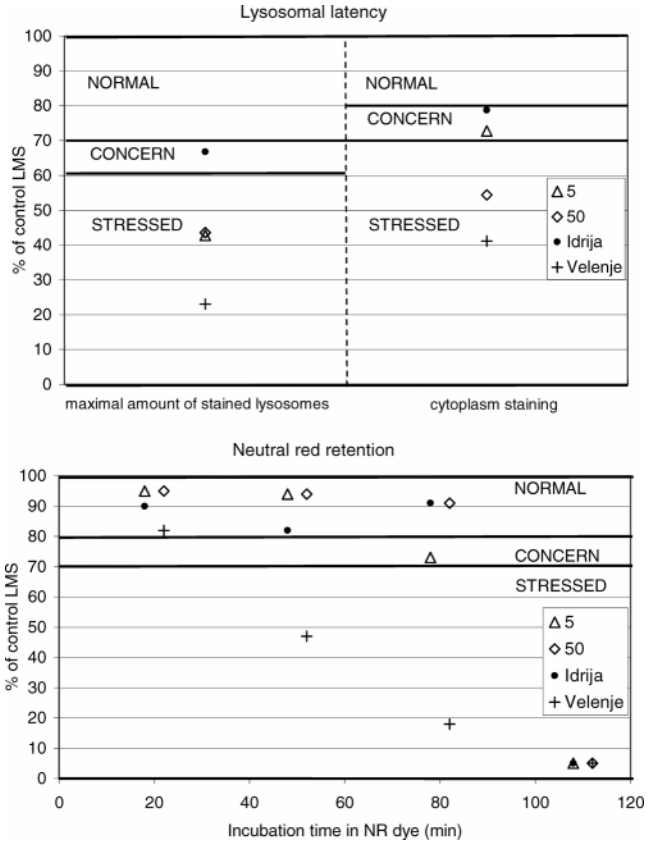

Fig. 6. Lysosomal membrane stability (LMS) in glands of laboratory-exposed (5 and 50 μg Hg^{2+}/g dry weight) and field-exposed (from Idrija and Velenje, Slovenia) animals compared to LMS in glands of unexposed animals and expressed as a percentage of control LMS using lysosomal latency (LL) assay and neutral red retention (NRR) assay. Considering the arbitrarily set criteria described in *Discussion*, the animals are classified as normal, concern, or stressed condition.

the performance of the LMS assay when new tissue or a new experimental setup is used.

To our knowledge, cytoplasm staining has not been assessed previously in other LL assays. In the present study, this parameter showed similar trends to those observed when using the maximal amount of stained lysosomes. The advantage of this parameter is the ease of recording the results in comparison to the maximal amount of stained lysosomes. Furthermore, it sometimes was not possible to detect the maximal amount of stained lysosomes, but cytoplasm staining always was detectable.

Our results were compared to those in the literature [7], and arbitrary criteria for determining the fitness of the animal were set. The median LMS in exposed animals was expressed as a percentage of that in control animals. The criteria were based on both the maximal amount of stained lysosomes and on cytoplasm staining (Fig. 6). The normal condition defines the optimum conditions and range, with up to 20% reduction in LMS compared to control animals when cytoplasm staining (LL and NRR assays) was assessed and 30% LMS reduction when the maximal amount of stained lysosomes was assessed. Further reduction in LMS from 20 to 30% (cytoplasm staining) and from 30 to 40% (maximal amount of stained lysosomes) compared to control animals reflects moderately polluted sites, is named the concern condition, and represents levels outside the normal range limits, indicating that the animals are experiencing some stressful conditions. The stress condition indicates overwhelmed homeostatic and detoxification mechanisms and could lead to serious damage of cells and, subsequently, organism-level effects. They could appear when LMS is reduced by more than 30% (cytoplasm staining) or 40% (maximal amount of stained lysosomes), because a status with more than 40% dysfunctional hepatic cells would lead to significant physiological impairment or death [7]. Lysosomal damage is a prognostic biomarker for pathology and reduced fitness [4]. However, unless complementary biomarker studies at higher levels of biological organization are performed, it is hard to anticipate exactly at which degree of impairment the changes in LMS would lead to effects on higher levels of biological organization [7,38,39].

In general, the cytochemical method for LL assay is not as easy to perform as the NRR assay. Also, approximately five-fold as many animals are needed to obtain the same data as are needed in the NRR assay, but in the LL assay, the stained samples can be stored for future reassessment. However, in both methods, photographs of the preparations also can be obtained. The results of the two LMS methods can be compared directly when the results are expressed as the reduction in LMS in the affected group compared to control. The most comparable results between the LL assay (cytoplasm staining) and the NRR assay appear to be obtained after 50 min of incubation in NR dye. However, the most significant statistical differences between LMS in unexposed and exposed groups were obtained between the LL assay and the NRR assay after 20 min in NR dye. At greater than 50 min of incubation in the NR dye, the differences between the unexposed and exposed groups of animals were no longer significant. Therefore, in the future, examination of lysosomal stability would be reasonable only up to the 60 min of incubation of the digestive gland tubes in NR dye. In addition, a relatively high number of animals are needed to obtain reliable results. We recommend sampling during different seasons over the year (approximately three times per year, with 20–25 animals per location).

Our laboratory experiments showed a very rapid lysosomal response of the hepatopancreatic cells to elevated concentrations of chemicals: After only 1 h of in vitro exposure to copper and after 3 d of in vivo exposure to mercury, injury of the lysosomal compartment was detectable. Similar results were reported by other authors who obtained a lysosomal response after 1 d [17] to 3 d [20] in organisms exposed in vivo and after a few minutes from cells exposed in vitro to metals [22,25]. In particular the lysosomal responses to low concentrations of contaminants appear to be rapid and very sensitive.

The results obtained with the two methods for LMS assessment on in toto digestive gland tubes of metal-fed *P. scaber* were comparable when both methods were performed on the same animals. The LL assay appears to be more sensitive than the NRR assay when the maximal amount of stained lysosomes was compared. A strong positive correlation between the LL assay performed on digestive cells and the NRR assay performed on hemocytes of *Mytilus galloprovincialis* was reported by Koukouzika and Dimitriadis [14]. In contrast, Lowe et al. [17] showed on the same organisms of *Mytilus edulis* that the NRR assay is more sensitive compared to the LL assay when investigating the effect of organic pollutants.

The results obtained on animals from Maribor (reference location) reflect the natural variability in LMS in a field population of an unpolluted environment. The animals from Idrija are chronically exposed to mercury because of the elevated natural background level and approximately 500 years of mer-

cury mining and smelting. Animals were collected in a place where mercury was found at approximately 260 μg/g dry weight compost and approximately 1,600 μg/g dry weight soil. Compared to animals from the unpolluted environment, the lysosomal stability in hepatopancreatic cells of animals collected in Idrija was affected. Following our arbitrary criteria described above, the animals from Idrija can be ranged either in the concern condition or somewhere between concern and normal conditions according to the LL assay. The NRR assay placed the same animals from Idrija in the normal condition (Fig. 6). Comparison of the results obtained by the two methods for LMS assessment indicates a lower sensitivity of the NRR compared to the LL assay.

In addition, LMS in animals exposed in the laboratory to mercury at 5 μg/g dry weight leaf corresponded to stress (maximal amount of stained lysosomes) and concern (cytoplasm staining) conditions of the organisms, whereas LMS values of animals exposed to mercury at 50 μg/g dry weight of food indicated the stress condition in both cases (Fig. 6). Comparison of results obtained in the LL assay in all laboratory animals exposed to mercury at 5 and 50 μg/g dry weight food showed a decreased lysosomal stability compared to those from Idrija, which are chronically exposed to much higher environmental mercury concentrations. This suggests that the animals from Idrija might have developed tolerance to mercury contamination during historic mercury exposure. The tolerance could be associated with selection of food with lower metal concentration [40] or food colonized with bacteria having mercury resistance (see [21] and references within). Another explanation suggests that autophagy, which is highly related evolutionarily with lysosomal function, could play a protective role in the survival of animals chronically exposed to stress and pollution [41]. Also, it should be pointed out that the mercury applied on the leaves in the laboratory in the form of $HgCl_2$ is readily available to the animals, which is not the case for environmental mercury. Total mercury concentrations in environmental samples measured using standard analytical procedures do not provide information about its actual availability to organisms. Sequential extraction of forest and meadow soil samples from Idrija indicated that up to a maximum of 1% of the total mercury measured was available [42]. However, to our knowledge, there are no data regarding the bioavailability of mercury from leaf litter in the Idrija region, and this also should be investigated in the future.

Velenje is another polluted region. It is located in the Šalek Valley, which is affected by a variety of pollutants (SO_2, NO_x, and metals) originating from emissions of the Šoštanj coal-fired thermal power plant. The average concentrations of cadmium, lead, mercury, and zinc measured in the soil from the Šalek Valley by Kugonič et al. [43] were as follows: Cadmium, 0.76 μg/g; lead, 45.6 μg/g; zinc, 181 μg/g; and mercury, 0.18 μg/g. At the location where *P. scaber* used for LMS assessment were collected, total mercury concentrations in the soil were approximately 1.17 μg/g. These values exceed the average world background soil values (cadmium, 0.3 μg/g; lead, 17.0 μg/g; zinc, 70 μg/g; mercury, 0.05 μg/g) [44] but not the warning values defined by Slovenian legislation (cadmium, 2 μg/g; lead, 100 μg/g; mercury, 1.2 μg/g; zinc, 300 μg/g) [45] (http://www.gov.si/mop/zakonodaja/zakoni/okolje/register/tla/uredba_imisijske_vrednosti_nevarnih_snovi_tla.pdf). However, the total mercury concentrations in the leaf litter and decayed wood were 0.01 and 0.028 μg/g dry weight, respectively. The LMS of animals collected here was most affected when the LL or NRR (after 50 min of incubation in NR) assay results were compared to LMS values for animals collected in Idrija and Maribor. The results indicate the stress condition of animals (Fig. 6). Although low concentrations of single pollutants were reported [43], their possible synergistic action might be the reason for severely affected lysosomes in the hepatopancreas of *P. scaber*. A similar observation was reported by Hankard et al. [46] from a field study using earthworms and by Viarengo et al. [22] in laboratory-exposed mussel hemocytes. However, the observed response also could result from some unmeasured contaminant present at high levels in the environment.

In polluted terrestrial environments, terrestrial isopods likely are exposed to high concentrations of pollutants, which might affect their activity in decomposing organic material and nutrient cycling. As shown in the present study, measured concentrations of pollutants may not truly reflect the risk posed to isopods. Therefore, it is of crucial importance to have appropriate methods for assessing their biological effects. The LMS assay is recommended for use in integrated biomarker studies with terrestrial isopods.

CONCLUSION

Modifications of methods for measuring LMS (LL assay and NRR assay) in isopod digestive glands were introduced: LMS was inspected on the entire digestive glands; a positive test was introduced to assess the performance of LMS assays; two parameters related to LL assay, the maximal amount of stained lysosomes and cytoplasm staining, were assessed; and criteria to determine the fitness of organisms were arbitrarily set on the basis of LMS and ranked the isopods as in normal condition, concern condition, and stress condition. The toxicity data obtained with the two LMS assays were not in agreement with concentrations of pollutants analyzed in the environment. In a highly mercury-polluted environment in Idrija (260 μg/g dry wt food and 1,600 μg/g dry wt soil), the isopods were ranged as in a normal to concern condition. In laboratory experiments, when the animals were exposed to concentrations of mercury in the food that were lower by one and two orders of magnitude (5 and 50 μg/g dry wt) for 3 d, the isopods were ranged in a concern to stress condition. This probably indicates tolerance to mercury in chronically mercury-exposed field animals and/or lower bioavailability of environmental mercury. In an environment polluted by thermal power plant emissions with environmental mercury concentrations three or four orders of magnitude lower than in Idrija, the isopods were ranged in stress condition, probably because of the synergistic effect of contaminants.

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