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ACUTE TOXICITY OF HEAVY METALS FOR BENTHIC EPIPHYTIC FORAMINIFERA *PARAROTALIA SPINIGERA* (LE CALVEZ) AND INFLUENCE OF SEAWEED-DERIVED DOC

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Abstract—The acute toxicity of cadmium, copper, and mercury to the benthic epiphytic foraminiferan *Pararotalia spinigera* (Le Calvez) was investigated using seven different vital cytophysiological and cytochemical methods. The ability to enzymatically hydrolyze the fluorogenic substrates fluorescein diacetate or fluorescein dibutyrate was the most sensitive method of LC50 value determination. The LC50 (24-h) values for cadmium, copper, and mercury determined by this assay with fluorescein diacetate was 0.56, 1.4, and 0.07 μM, respectively. The content of seaweed-derived dissolved organic carbon (DOC), measured by absorbance at 436 nm, produced a dramatic increase of LC50 values for the heavy metals in a dose-dependent manner. "Intact" epiphytic foraminifera attached to seaweeds are less sensitive to acute toxicity of cadmium, copper, and mercury than are "detached" foraminifera.

Keywords—Epiphytic foraminifera Heavy metals Acute toxicity LC50 (24-h) DOC

INTRODUCTION

Numerous studies have demonstrated the importance of using various animal species to detect dangerous ecosystem contaminations [1–4]. Species occupying a key position in the life of ecosystems would be useful biomonitors. Thus, the continual global biogeochemical cycles of inorganic and organic compounds are regulated mainly by biological activity of benthic communities, especially bacteria and protozoa [4–7]. Freshwater protozoa play an important role in the utilization of carbon and nitrogen, the food web and the "microbial loop" in both natural and wastewaters [4,8–10]. These protozoa have evolved a variety of mechanisms for protection from toxic chemicals, including heavy metals [11–16]. Therefore, freshwater protozoans have been used for ecotoxicological investigations [4].

Marine protozoa, especially foraminifera, play a significant role in global biogeochemical cycles of inorganic and organic compounds, making them one of the most important animal groups on earth [10,17–19]. Foraminifera have a shell that may remain a long time after their death; shell deposits have been used to determine former population structure, quantity of specimens, and environmental quality [10,17–21]. Benthic foraminifera dwell in the sediment, on submarine outcrops, coral reef, reef rubble, and mollusk shells, or as epiphytes on seaweeds or seagrass [17–19].

Benthic, especially attached epiphytic foraminifera, habituate under very complicated chemical conditions. Dissolved organic carbon (DOC) has been reported in the sediment in concentrations of up to 40 g/L [22]. This DOC consists of humic acids, small alkyl carboxylic acids, and simple aromatic acids as well as amino, nucleic, uronic, and muramic acids [23–25]. Decomposition of seaweeds and seagrass has been shown to produce DOC [26].

The sediment may also contain peptides, proteins including exoenzymes liberated by bacteria, a wide cluster of natural microbial, algal or animal toxins [23,27–30], and various anthropogenic xenobiotics like heavy metals, polychlorinated biphenyls, and polycyclic aromatic hydrocarbons [22,31,32]. The following defense mechanisms against xenobiotics were detected in benthic epiphytic foraminifera: (a) mucopolysaccharide coat that forms additive diffusion barrier and binds some cationic xenobiotics; (b) membrane carrier-mediated transport system for elimination of anionic xenobiotics from the cytoplasm; (c) active intralysosomal accumulation and isolation of some cationic xenobiotics; and (d) haloperoxidases that transform xenobiotics to their haloderivatives [33]. Benthic foraminifera, though rarely used, may be useful for ecotoxicological investigations and monitoring.

As a first step in the research, this study was focused on the following problems: (a) selection and adaptation of some sensitive cytophysiological and cytochemical methods to detect and assess objectively foraminiferal responses; (b) determination of acute toxicity (LC50) of cadmium, copper, and mercury for an epiphytic benthic foraminiferan, *Pararotalia spinigera*; and (c) determination of the interaction of seaweed-derived DOC on the acute toxicity of these metals. The DOC may alter the toxicity of many anthropogenic xenobiotics including heavy metals [22,26,34–37].

The application of test chemicals to indicate cytophysiological or cytochemical responses was complicated by the masking of the cytoplasm by the foraminiferal shell, rendering simple vital microscopy ineffective. Both attached and "detached" foraminifera are sessile, with the pseudopodial

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net often covered by sediment particles, organic detritus, bacteria, and various microalgae. In addition, detachment may cause the pseudopodial net to retract. Staining methods with rose bengal or with Sudan black B [38] are also useless for determination of cell functional state.

Modern cell biology provides numerous cytophysiological and cytochemical methods that can demonstrate the functional and biochemical state of main cellular compartments [8,39–52]. Fluorescent microscopy and special fluorescent probes allow rapid examination of various cell compartments, chemical composition, molecular organization, function, and metabolism with the highest precision and sensitivity [39–44, 50–52].

It is well known that lysosomes, mitochondria, plasma membrane, and some cytoplasmic enzymes play a key role in the development of cellular injury and death [53]. Therefore, the following parameters were selected to detect and analyze foraminiferal response: (a) activity of cytoplasmic enzymes, nonspecific esterases; (b) functional activity and morphology of lysosomes; (c) permeability of plasma membrane; (d) quantity of reactive sulfhydryl groups in the cytoplasm; (e) intensity of inherent ultraviolet (UV) fluorescence of tryptophan-containing proteins; and (f) metabolic state of mitochondria.

MATERIALS AND METHODS

Chemicals

Fluorescein (disodium salt), the fluorescein diacetate, the fluorescein dibutyrate, and the fluorescein mercury acetate were obtained from Serva (Heidelberg, Germany). Acridine orange was obtained from Fluka AG (Basel, Switzerland). The neutral red (BDH Chemicals Ltd., London, UK) was recrystallized twice before using. The cadmium (as $CdCl_2 \cdot 2.5 H_2O$), copper (as $CuSO_4 \cdot 5H_2O$), and mercury (as $HgCl_2$) were obtained from Aldrich Chemical Co. (Milwaukee, WI).

Sampling and aquarium

The major experiments were performed using dominant epiphytic shallow-water species, *P. spinigera*, living on the seaweeds *Sargassum vulgare*, *Cystoseira* sp., and *Jania* sp. In some experiments also *R. macropora* was used. These species are widely distributed along the Israeli coast. Seaweeds with sediment were collected by scubadiving from a depth of 2 to 2.5 m near Mikhmoret, Israel (about 50 km north of Tel Aviv). The material collected was transported within 2 to 3 h in plastic tanks with natural seawater to the laboratory, and placed in an aerated aquarium (60 L of natural seawater). The aquarium was illuminated continuously by a 100-W luminescent lamp. The illuminance at the aquarium bottom was $2.5 \cdot 10^{-3}$ lm/cm^2. The water temperature was 25 to 27°C; pH was 8.1 to 8.4. Part of seaweeds' thalli died and disappeared in this aquarium within 3 weeks, and the water was colored yellow-brown by products of this decomposition including DOC. An absorbance at 436 nm is often used for characterization and quantification of DOC, especially its humic components [23]. We used this method to determine concentration of those compounds in seawater before and after seaweeds' decomposition.

Experimental design

Pieces of seaweed thalli (about 2 to 3 cm long) were removed from the aquarium and placed for further preparation in 100-mm Petri glass dishes. Both "intact" foraminifera (attached to seaweed thalli) and especially "detached" foraminifera (delicately removed from their natural substratum with a fine brush under a dissecting microscope) were utilized in this experimental study. The latter lost or retracted pseudopodia during this manipulation. A vital cytotoxicity assay with neutral red showed clear reversible cellular damage in "detached" foraminifera. However, normal interaction with neutral red was restored in adult specimens within 24 h [33]. Therefore, only adult specimens that were detached 24 h before the beginning of toxicological experiments were used. Fifteen or 20 specimens of "detached" foraminifera were each placed in 50-mm Petri glass dishes with filtered seawater. "Intact" foraminifera were transferred with small pieces (about 5 × 5 mm) of their natural substratum: Ten pieces of seaweeds' thalli with attached foraminifera were transferred to each 50-mm Petri glass dish.

Toxicity assessment and EC50 determination

Stock solution (100 mM) in deionized distilled water was prepared from salts of each test metal and used for the preparation of working solutions in seawater (from 0.01 μM to 1,000 μM). The working solutions were made in seawater without DOC and seawater with different concentrations of DOC. Five or six nominal concentrations of investigated metal ions and 20 specimens of foraminifera for each concentration were used for EC50 (24-h) determination. Five different cytological methods were used to distinguish living from nonliving foraminifera.

Determination of nonspecific esterases activity

Fluorogenic substrates of enzymes allow the determination of their localization and activity in different compartments of intact living cells [50,51]. Injured or dead cells lose the activity of many cytoplasmic enzymes [53]. Fluorogenic substrates were used as a sensitive marker for assessment of cell viability [50,51]. Fluorescein diacetate (FDA) and fluorescein dibutyrate (FDB) were used for the determination of nonspecific cytoplasmic esterases [33,50]. The fluorescence is due to fluorescein (FLU) which by enzymatic hydrolysis is liberated from the nonfluorescent FDA or FDB through cytoplasmic esterases. The cells were classified as fluorescent (living) and nonfluorescent (dead) under fluorescent microscope [50].

Foraminifera were incubated in seawater with or without metal ions for 24 h and then treated with freshly prepared FDA or FDB solution in seawater (final concentration 1 μM) for 15 min. They were then washed in clean seawater and immediately assayed for fluorescence. Previous experiments gave similar results for FDA and FDB [33].

Examination of functional activity and morphology of lysosomes

The fluorescent probe acridine orange (AO) for a long time was used for the examination of lysosomal functional

activity and morphology in the living cells [43,44]. Proton gradient between cytoplasm and lysosomes produces the force for active intralysosomal accumulation of some cationic compounds including AO and neutral red (NR). Any cell injury decreases this proton gradient and intralysosomal AO or NR accumulation. Dead cells have no intralysosomal accumulation of AO or NR. Nuclei of intact living cells are not well stained by AO or NR. Dead cells demonstrate marked staining of nuclear chromatin and sometimes intensive diffuse staining of cytoplasm [43–45]. Thus, vital staining with AO or NR is often used as a marker of cell viability [43–48]. Extra- and intracellular mucopolysaccharides (glycosaminoglycans) have intensive orange-red fluorescence after vital staining with AO [43,44].

For vital staining with AO, we added AO in seawater to a final concentration of 10 μM. After 24-h incubation with both AO and metal or with AO only (control), we calculated under a luminescent microscope the number of foraminifera with or without small fluorescent red granules (lysosomes) in their cytoplasm (i.e., number of living or dead animals, respectively). We also noted the presence or absence of fluorescence of the nuclei and/or diffuse green fluorescence of the cytoplasm as well as pale orange-red fluorescence of cell coat.

For vital staining with NR, the dye was added in seawater to a final concentration of 0.002% (about 7.0 nM) at the start of the experiments. After 24-h incubation with both NR and metal ions or with NR only (control), the numbers of foraminifera with small red granules (lysosomes) in the cytoplasm (living) and without these granules or with intensive diffuse staining (dead specimens) were calculated. The measurements of living foraminifera were performed in lateral dark-field illumination by vital epifluorescent microscopy, which is necessary to observe delicate small red granules through the shell. In some cases, NR accumulation was measured by microspectrophotometry as described below (see "Technique of microspectrophotometry").

Examination of plasma membrane permeability

It is axiomatic that plasma membrane of intact living cells is practically impermeable to organic anions. Membrane permeability is increased by any cell injury. Dead cells are permeable to anionic compounds. Therefore, examination of cell permeability to some anionic dyes (dye exclusion assay) is often used to distinguish living and dead cells [44,45,49]. For this purpose, we used the fluorescent anionic dye, fluorescein (FLU), in final concentration of 100 μM. After 24-h incubation with both FLU and metal ions or with FLU only (control), we determined FLU penetration in the cytoplasm of foraminifera by fluorescent microscopy and, in some cases, by microfluorometry as described below (see "Technique of microfluorometry").

Biophysical examination of metabolic state of mitochondria

Measurement of blue fluorescence (excitation at 365 nm, emission at 420 to 450 nm) of reduced nicotinamide adenine dinucleotide (NADH) is a classical biophysical method for monitoring the metabolic state of mitochondrial respiratory chain and its response in living cells [8,39,40]. This method was used by us to investigate the metabolic state of mitochondria and its responsibility in living benthic foraminifera [33]. It was shown that intact animals had type IV metabolic state (state of rest). Device and technique of examination are described below (see "Technique of microfluorometry").

Biophysical examination of tryptophan-containing proteins

Monitoring of inherent ultraviolet (UV) fluorescence of tryptophan-containing proteins (excitation at 253 nm, emission at 320 to 380 nm) is a well-known biophysical method for detection of conformational changes of these proteins and their interaction with ligands [33,41]. The binding of metallothioneins with copper ions produced a marked quenching of their UV fluorescence [41]. Therefore, this technique was used for the study of interaction between foraminiferal tryptophan-containing proteins and copper. Device and technique of examination are described below (see "Technique of microfluorometry").

Determination of reactive sulfhydryl groups

Fluorescent cytochemistry with fluorescein mercuric acetate (FMA) is used for specific detection and measurement of free sulfhydryl groups in different cellular structures. Any agents blocking sulfhydryl groups inhibit this reaction [42]. Therefore, it is useful for revealing of mercury ions bound by sulfhydryl groups in living cytoplasm. After incubation with various nominal concentrations of mercury or without mercury (control), foraminifera were fixed by cold 1% trichloracetic acid in 80% ethanol within 1 h, washed in clean 80% ethanol, stained with FMA solution ($2.5 \cdot 10^{-5}$ M in clean acetone) within 4 h, rinsed in absolute ethanol, rinsed in xylene, and embedded in nonfluorescent immersion oil. These preparations were immediately used for microfluorometry.

Technique of microfluorometry

A special flexible microfluorometric system containing a Nikon measuring head, rectangular measuring diaphragm, and two changeable excitation light sources was constructed by us. A pencil-style spectral calibration mercury–argon lamp and a high-grade fused silica bundle for lateral (dark-field) illumination of living foraminifera were used for microfluorometry of blue or UV fluorescence. A long-wave conversion filter 6042 (Oriel Corporation, Stratford, CT), which absorbs the 253.7-nm line, fluoresces at a maximum of 340 nm, and transmits the 366-nm line, has been used for excitation of blue fluorescence. A combined glass barrier filter was used for blue fluorescence transmitting in the 420- to 450-nm region. Blue fluorescence of 480 specimens of *P. spinigera* was measured.

Ultraviolet light 250 to 280 nm for excitation of UV fluorescence was performed by a chlorobrome gas filter, using a combined barrier filter transmitting in the 320- to 380-nm region. Inherent UV fluorescence of 100 specimens *P. spinigera* was measured.

A stabilized quartz tungsten halogen lamp was used as an excitation light source for microfluorometry in a visible re-

gion. A special combination of dichroic mirror, excitation, and barrier light filters was used. This combination cut off practically all background light and permitted only the light of fluorescence to reach the photomultiplier tube [52].

The size of the measuring diaphragm was 50 × 50 μm in the plane of the specimens. This size was used to eliminate possible influence of background fluorescence in experiments with both "intact" and "detached" foraminifera. The background fluorescence was very low in experiments with "detached" specimens.

Data of microfluorometry are usually expressed in arbitrary units (a.u.), i.e., μA of photocurrent. Microcuvettes with a fluorochrome concentration series were used for standardization and calibration [40,52]. The control measurements for FLU were taken in a microcuvette of 0.1-mm depth, corresponding to the mean thickness of *P. spinigera*. The luminescence intensity of FLU solution depended linearly on the concentration, from 0.005 to 2 mM [52]. Fluorescence intensity of 100-μM FLU solution in the microcuvette was adjusted to 75 ± 5 a.u. It was constant the entire time that measurements were taken.

Technique of microspectrophotometry

Measurements of NR accumulation in foraminiferal cytoplasm were performed by the microspectrophotometric plug method with manual scanning of specimens [52]. Four different locations (cameras) from each foraminiferan and the adjacent vacant area (background) were alternatively combined with a measuring diaphragm in these microspectrophotometric measurements at 450 nm. The value of transmittance coefficient (τ) was determined for each pair of measurements:

$$\tau = \frac{I_s}{I_b} \times 100\%,$$

where I_b is the intensity of the light passing through the background, and I_s is the intensity of the light passing through the specimen.

The transmittance coefficient of the same foraminiferan before NR accumulation was measured in the same manner as described above. This permitted us to make an allowance for the light that was absorbed and dissipated by the structures of the foraminifera themselves. The optical density ($D = \lg 1/\tau$) was calculated from the values. The difference between optical density of specimens before and after accumulation of NR (ΔD) was the quantity proportionate to the amount of dye in the cytoplasm. For each cadmium (Cd) concentration and control, 20 specimens were used, i.e., 800 separate measurements from 100 specimens of *P. spinigera* were performed.

Statistics

In acute toxicological experiments for calculations of EC50 values, the standard probit analysis was usually used [54]. All data obtained by microfluorometry and microspectrophotometry were computed and presented as mean ± 95% confidence limit.

RESULTS

DOC concentration in seawater

The seawater was superficially noncolored on the day when the material was brought to the laboratory. The optical density of this water at 436 nm was 0.04 ± 0.017 (mean for 10 separate measurements from two different samplings), and it was designated as seawater without DOC. Aquarium seawater turned brown as a result of the seaweeds' destruction for 2 to 3 weeks. Maximal optical density of this water was 0.74 ± 0.08 (mean for 10 separate measurements from two different samplings). This water was designed as seawater with maximal DOC content.

Determination of EC50 (24-h) for Cd by different cytophysiological assays in "detached" P. spinigera

The EC50 values were determined for action of cadmium in seawater without DOC. Five different cytophysiological assays were used to distinguish living and dead "detached" foraminifera after the action of cadmium (Table 1). Both FDA and FDB had the same EC50 values (0.56 μM). Fluorescent and nonfluorescent specimens were distinguished very easily with both these substrates.

Vital staining with AO demonstrated a more complicated microscopic picture. All control specimens had a weak, diffuse green fluorescence of cytoplasm and orange-red fluorescence of numerous small granules (lysosomes). A mucopolysaccharide coat around foraminiferal cytoplasm was a pale orange or red fluorescence. The small red granules (lysosomes) were absent in the cytoplasm of many foraminifera incubated with cadmium. Green fluorescence of cytoplasm was decreased in some such specimens, whereas pale orange or red fluorescence of the mucopolysaccharide coat was well preserved. The EC50 (24-h) value for cadmium determined by using vital staining with AO was 0.79 μM (Table 1).

Vital staining with NR demonstrated the presence of numerous small red granules (lysosomes) in all specimens of control. These granules were absent in many specimens incubated with cadmium. Some such specimens also demonstrated marked staining of the nucleus and sometimes intensive diffuse staining of cytoplasm. The EC50 value for Cd^{2+} determined with NR was 0.63 μM (Table 1).

Vital staining with FLU showed the absence of its penetration in all control specimens. Many specimens incubated

Table 1. Acute 24-h EC50 values (μM) for "detached" foraminifera *P. spinigera* to cadmium in seawater without DOC

Assay	EC50
FDA	0.56
FDB	0.56
AO	0.79
NR	0.63
FLU	1.26

Data were obtained by vital cytochemical assays with fluorescein diacetate (FDA) or fluorescein dibutyrate (FDB) and cytophysiological assays with acridine orange (AO), neutral red (NR) or fluorescein (FLU).

Table 2. Effect of seaweed-derived DOC on acute 24-h EC50 values (μM) for "detached" and "intact" foraminifera *P. spinigera* exposed to cadmium, copper, and mercury

	"Detached" foraminifera for various % DOC in seawater					"Intact" foraminifera for various % DOC in seawater		
Metal	0%	25%	50%	75%	100%	0%	50%	100%
Cadmium	0.56	1.99	4.47	6.30	8.90	1.33	10.10	16.29
Copper	1.40	–	8.90	–	17.78	3.05	19.49	31.33
Mercury	0.07	–	7.08	–	12.60	0.17	15.08	20.79

DOC concentration expressed as percent to maximal (equal optical density 0.74 at 436 nm).

with cadmium had marked fluorescence of FLU penetration in their cytoplasm. Determination of the EC50 by using FLU gave the highest value in this set of experiments (Table 1).

The influence of DOC on the EC50 values for cadmium, copper, and mercury in "detached" and "intact" P. spinigera

The influence of DOC derived from destructed seaweeds on acute toxicity of cadmium, copper, and mercury was investigated by using an assay with FDA. The results of EC50 (24-h) determinations showed that this DOC dramatically decreased acute toxicity of all tested metals at dose-dependent manner in both "detached" and "intact" foraminifera (Table 2). These results also demonstrated that "intact" foraminifera were less sensitive to toxic action of metals than were "detached" foraminifera. Microscopic investigations showed that in the experiments with "intact" foraminifera the activity of esterases was decreased by heavy metals as it was in other members of the epiphytic community, especially in the ciliates.

Microspectrophotometrical measurements of NR accumulation in "detached" specimens incubated for 24 h in colored seawater (optical density 0.74) with or without cadmium demonstrated a marked decrease of this accumulation produced by 1 to 100 μM cadmium (Fig. 1). The calculation of the EC50 value gave 9.3 μM, which is comparable with the value determined by FDA assay at similar conditions (Table 2).

The action of metals on biophysical, cytochemical, and cytophysiological characteristics of "detached" foraminifera in seawater with DOC

Inherent blue fluorescence of foraminiferan, *P. spinigera*, was significantly inhibited by 1 μM of cadmium, copper, and mercury after 24-h incubation; 10 μM of these metals produced a more intensive inhibition. However, a significant inhibition of blue fluorescence by 10 μM of these metals was already detected after 2-h incubation (Fig. 2).

Inherent UV fluorescence of foraminiferan, *P. spinigera*, was significantly decreased by increased concentration of copper; however, 100 and 1,000 μM produced the same effects (Fig. 3).

Fluorescence of sulfhydryl groups in the cytoplasm of *P. spinigera* and *R. macropora* induced by their interaction with

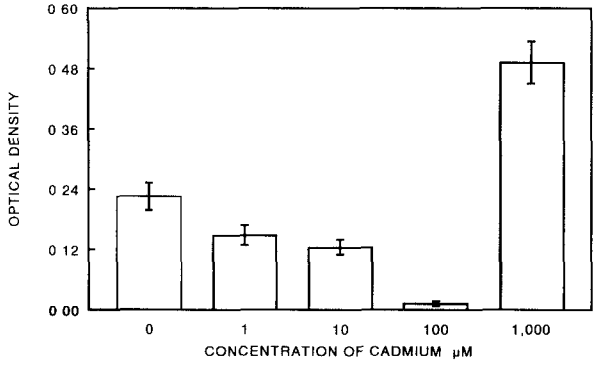

Fig. 1. Action of increased concentrations of Cd on neutral red (NR) accumulation in foraminiferal cytoplasm (expressed as mean optical density of cytoplasm ± 95% confidence limit). Incubation for 24-h in colored (DOC contained) seawater with or without (control) cadmium. Each bar is a result of measurements of 20 specimens of *P. spinigera*.

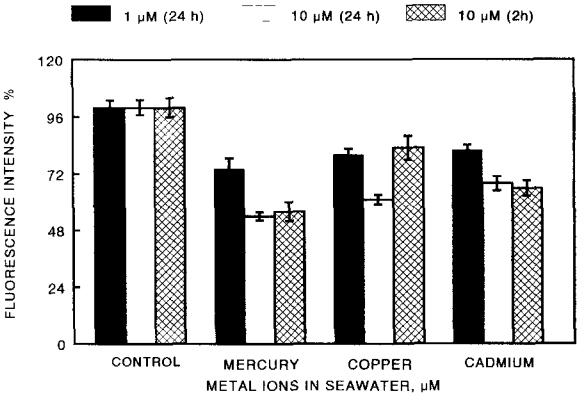

Fig. 2. Action of mercury, copper, and cadmium ions on intensity of blue fluorescence of reduced pyridine nucleotide in foraminiferal cytoplasm (expressed as % to control). Incubation for 24-h in colored (DOC contained) seawater with 1 μM, 10 μM, or without (control) metal ions. Incubation for 2-h in colored seawater with 10 μM or without (control) metal ions. Each bar is a result of measurements of 40 specimens of *P. spinigera*.

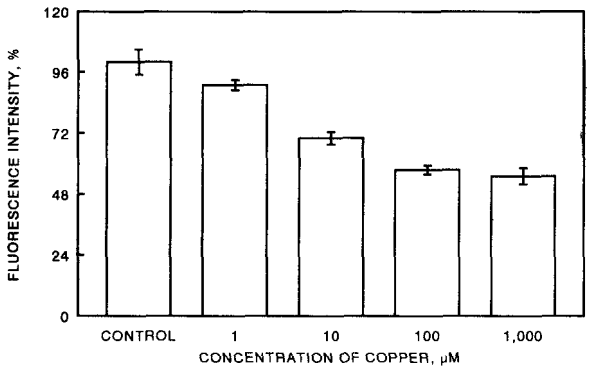

Fig. 3. Action of increased concentrations of copper on intensity of inherent UV fluorescence of tryptophan-containing proteins in foraminiferal cytoplasm (expressed as % to control). Incubation for 24-h in colored (DOC contained) seawater with or without (control) copper. Each bar is a result of measurements of 20 specimens of *P. spinigera*.

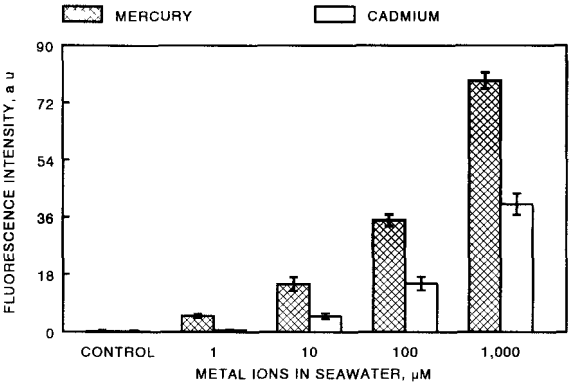

Fig. 5. Action of increased concentrations of mercury or cadmium on permeability of foraminifera to fluorescein (expressed in a.u.). Incubation for 24-h in colored (DOC contained) seawater with mercury or cadmium. Each bar is a result of measurements of 45 specimens of *P. spinigera*.

fluorescein mercuric acetate was significantly inhibited by incubation with mercury for 24 h at a dose-dependent manner (Fig. 4). The calculated EC50 value was 14.1 μM, which was comparable with the EC50 value for mercury determined in water with DOC by using FDA assay (Table 2).

The microfluorometric measurements of FLU penetration into cytoplasm of *P. spinigera* demonstrated that all control specimens were impermeable to FLU. The permeability to FLU was increased at dose-dependent manner by incubation with cadmium and mercury for 24 h (Fig. 5). Calculated EC50 value for the action of mercury was about 100 μM.

DISCUSSION

Results of our toxicological experiments on "detached" foraminifera, *P. spinigera*, in seawater without DOC demonstrated that the EC50 (24-h) values for cadmium determined by using two fluorogenic substrates of nonspecific esterases (FDA or FDB) and cytophysiological assays with AO or NR gave comparable values (Table 1). Our data also showed the lack of enzymatic activity in the cytoplasm and functional activity of lysosomes in many specimens incubated with cadmium. Such specimens had a marked staining of the nuclei by AO and NR. It is well known that such alterations are typical signs of cell death [43-50,53]. Therefore, these EC50 values could be considered the LC50 (24-h) values for cadmium. The most sensitive and useful method for the determination of LC50 values is the enzymatic assay with fluorogenic substrate.

We cannot find in the literature any LC50 values for benthic epiphytic foraminifera. Therefore, our data presented in Table 1 may be compared only with corresponding LC50 values for some freshwater protozoa and cladocerans or marine copepods and amphipods [4,11-16,55,56]. The order of these values for toxic metals is comparable.

The sensitivity of "intact" foraminiferan, *P. spinigera*, to action of cadmium, copper, or mercury determined by assay with FDA was lower than the sensitivity of "detached" specimens (Table 2). This effect may be partly produced by the binding of these metals with the mucopolysaccharide coat that covers all cytoplasmic body of these specimens and attached them to the seaweeds' thalli. It is known that many foraminiferal species produce extracellular acid mucopolysaccharides that can play a significant role in their ecology [57,58]. One of the functions of these mucopolysaccharides may be the antichemical defense of foraminifera. They form a diffusion barrier and have numerous negatively charged groups that can bind cationic compounds, including metal ions. It has been shown that mucous secretions protect mollusks and fish from cationic xenobiotics including cadmium [44,59].

The pieces of seaweeds' thalli may bind heavy metals and eliminate them from the seawater in experiments with "intact" foraminifera. The heavy metal-complexing peptides, phytochelatins, had been isolated from plant cells [60].

Data obtained also demonstrated that DOC derived by

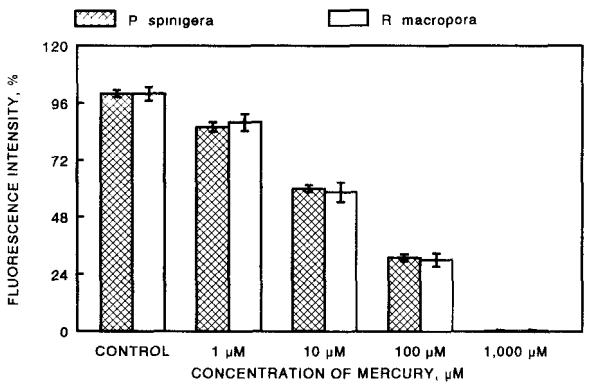

Fig. 4. Action of increased concentrations of mercury on fluorescence of fluorescein mercuric acetate bound by sulfhydryl groups in foraminiferal cytoplasm (expressed as % to control). Incubation for 24-h in colored (DOC contained) seawater with or without (control) mercury. Each bar is a result of measurements of 60 specimens of *P. spinigera* and 20 specimens of *R. macropora*.

seaweed decomposition in the aquarium dramatically decreased at dose-dependent manner the acute toxicity of cadmium, copper, and especially mercury for "detached" and "intact" epiphytic foraminifera (Table 2). These results are not surprising. It is well known that DOC, including humic acids, can bind heavy metals and some organic xenobiotics and modify the toxicity of these chemicals [22,23,35–37]. For example, the humic acids decreased the acute toxicity of cadmium, copper, and some quaternary ammonium compounds for fish and invertebrates. The LC50 values for dioctadecyl dimethyl ammonium chloride increased by approximately 130% for each part-per-million increase in organic carbon [37].

Decomposition of some parts of seaweeds' thalli is a natural permanent process that also has seasonal increases. Such decomposition can produce DOC, including humic acids and other natural organic chelators of metal ions [23–26,60]. Our aquarium experiments cannot reflect all conditions of natural benthic environment; however, it is obvious that the DOC production in brushwood of seaweeds may protect epiphytic attached foraminifera against heavy metals.

Alterations of investigated biophysical and cytochemical parameters produced by incubation with heavy metals demonstrate some mechanisms of their toxic actions on foraminifera. The decrease of inherent UV fluorescence of tryptophan-containing proteins by increased concentrations of copper showed a saturation kinetics. These data testify that not all tryptophan-containing proteins can interact with copper. Only 50% of the tryptophan-containing proteins of foraminiferal cytoplasm had a high affinity to copper. Tryptophan-containing metal-binding proteins were recently discovered in some freshwater ciliates and marine blue crab [15,41]. Copper ions decreased inherent UV fluorescence of apometallothioneins of blue crab [41]. Therefore, some of tryptophan-containing proteins of *P. spinigera* may be metallothionein-like proteins.

The fluorescence of sulfhydryl groups induced by their interaction with fluorescein mercuric acetate was completely inhibited by increased concentrations of mercury in seawater. Our data reflect a competition between mercury penetrated from seawater and mercury of fluorescein mercuric acetate for sulfhydryl groups in foraminiferal cytoplasm. Blocking of proteins' sulfhydryl groups by mercury is the main mechanism of its toxic action [53]. However, some part of these blocked sulfhydryl groups may be connected with protective metal-binding proteins like proteins, which were detected in some freshwater protozoa [14–16].

Our data show that inherent blue fluorescence of "detached" *P. spinigera* was significantly inhibited by incubation for 2 h and 24 h with cadmium, copper, and mercury. This inhibition may reflect oxidation of NAD produced by uncoupling of respiration and oxidative phosphorylation [33,40]. These metals are known uncouplers for all eukaryotic cells [40]. Therefore, they may uncouple respiratory and oxidative phosphorylation also in foraminifera, inhibit energetic metabolism, and produce secondary alterations of cellular function and structure.

Dye exclusion assay with FLU demonstrates that the EC50 values for cadmium in seawater without DOC was more than the EC50 values determined by cytochemical assay with FDA or FDB and cytophysiological assays with AO or NR (Table 1). Also, in seawater with DOC, heavy metals produce relatively low levels of FLU accumulation in the cytoplasm. It was shown previously that benthic epiphytic foraminifera have a carrier-mediated transport system that eliminates anionic xenobiotics from their cytoplasm [33]. A relatively low level of FLU accumulation in the cytoplasm of foraminifera incubated with cadmium or mercury may reflect a remaining activity of this transport system. Similar transport system in proximal tubules of vertebrates was stimulated by low concentrations of cadmium [40]. Loss of effect at 1 μM and low at 10 μM cadmium in seawater may be produced by such stimulation of the transport system in foraminifera.

CONCLUSIONS

Vital cytochemical assay of nonspecific esterase activity with fluorogenic substrates and cytophysiological assay of functional lysosomal activity with AO or NR were used to determine the LC50 (24-h) values for cadmium in benthic epiphytic "detached" foraminiferan, *P. spinigera*. The assay with fluorogenic substrate was the most sensitive and useful method for determination of the LC50 values. "Intact" attached foraminifera had lower sensitivity to cadmium, copper, and mercury than did "detached" specimens. The DOC produced by decomposition of seaweeds in aquarium decreased acute toxicity of cadmium, copper, and mercury for both "intact" and "detached" foraminifera at dose-dependent manner.

Biophysical determination of inherent blue fluorescence of NADH, inherent UV fluorescence of tryptophan-containing proteins, and fluorescent cytochemical determination of sulfhydryl groups allowed the examination of some cellular mechanisms of acute toxicity of heavy metals. These methods may be very useful for further ecotoxicological investigations of benthic foraminifera.

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