GFAAS and ICP-MS Determination of Ag and Cu in the Haemolymph of a Millimetric Marine Crustacean (*Parhyale hawaiiensis*) as a Tool in Ecotoxicology

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INTRODUCTION

The determination of metals in the tissues and fluids of aquatic organisms is an important tool for ecotoxicological studies. The haemolymph is the circulatory fluid of various invertebrates and is functionally comparable to the blood and lymph of vertebrates. It fills the interior of arthropods and molluscs in an open circulatory system (1). When a contaminant is absorbed by an aquatic organism and distributed to the different organs through the haemolymph it is possible to measure the concentrations. This type of study has been performed in relatively large animals such as decapod crustaceans, e.g., the freshwater crayfish *Cambarus diogenes* (weight from 19 to 39 g) (2) and fishes, e.g., the common carp *Cyprinus carpio* (weight of 28.1 ± 12.6 g) (3), but not in smaller aquatic organisms.

Amphipods are invertebrates that live close to the sediment, thus they are an interest group for studies of metal contamination and exposure. The analysis of fluids and organs of this group is still a challenge due to their minimal size. They are appropriate for ecotoxicological studies because they are abundant, widespread, ecologically relevant, and an important link between the producers and the secondary consumers in the food chain (4). The number of studies

ABSTRACT

The determination of metals in aquatic organisms has become an important tool in ecotoxicological studies. In the present study, silver and copper were determined for the first time in the haemolymph of *Parhyale hawaiiensis*, a millimetric marine crustacean. Silver (Ag) was selected because this metal has become of environmental concern due its intense use in nanomaterials. Copper (Cu) was included in this study as a possible biological internal standard. The procedures for haemolymph collection, quantification, and preparation were optimized. The adult organisms were exposed to silver in concentrations from 0 to $100 \mu\text{g L}^{-1}$ in a saline water medium for 96 hours. For quantification and comparison of the method, GFAAS and ICP-MS analysis was used and all operational conditions were optimized. It was found that the Ag concentrations in the haemolymph increased in relation to the increase in Ag concentration in the water, reaching 11 ng mg^{-1} . However, a plateau was observed at the two highest concentrations. More studies are required to verify the usefulness of copper as an internal standard. Both GFAAS and ICP-MS provided satisfactory results in the quantification of Ag and Cu in the haemolymph of these small animals and are an excellent tool for ecotoxicological studies, especially for the evaluation of silver from nanomaterials in the aquatic environment.

with marine amphipods is still scarce, although a large quantity of effluents containing toxic substances is constantly discharged directly and indirectly into the estuarine and marine regions (5, 6). The marine amphipod *Parhyale hawaiiensis* is an epibenthic small organism (less than 2 cm), lives in the rocky shores with tidal fluctuations associated with algae and other organisms. It is a versatile model organism amenable to experimental manipulations (7), easy to cultivate in the laboratory, and tolerates wide variations in salinity and temperature (8).

Several authors have evaluated the metal concentrations in the whole body of amphipods (9–14), but not in haemolymph, probably due to the difficulties to collect it from these tiny animals. For example, an average male of an adult *Parhyale hawaiiensis* reaches no more than 2 cm and weighs less than 0.008 g. The determination of metals in the whole body can be sufficient for some studies, but to prove that a contaminant was in fact absorbed or for understanding its toxicokinetics, internal concentration studies are required (15–17). Evaluation of the haemolymph in amphipods is an interesting tool to help in evaluating metal exposure, such as silver.

Today, the most important and applicable property of silver is its antimicrobial broad spectrum (18, 19) and one of the main reasons for its widespread use in nanomaterials (19–21). As a result of its many

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applications, silver is released into the environment in a variety of compounds and forms (19, 22) ranging from picograms per liter to micrograms per liter (19, 23), and its concentrations are expected to increase (22). Most of the silver toxicity for aquatic life is due to its ionic form (Ag^+) in the water column (23–26), but it is not yet clearly known in marine organisms (25). The increase in environmental Ag concentrations is a fact, but due to the complexity of the chemical behavior of both silver and nanomaterials, especially in marine aquatic systems, it is difficult to know exactly what amount of silver is released and bioavailable to aquatic forms. For this reason, the concentration of metals in the haemolymph can be used as a tool to evaluate the actual exposure levels.

Thus, establishing a method for Ag determination in the haemolymph of amphipods is relevant to gaining a better understanding with regard to the toxicity of silver nanomaterials in marine organisms. In addition to the difficulties in the amount of haemolymph available for analysis, trace metal determination in saline water is an additional analytical challenge to overcome, mainly because of the high sodium content (27–30). In order to provide a basis for comparison, the copper (Cu) concentration in the haemolymph was also evaluated in this work as an internal standard, because the hemocyanin, the major constituent (> 60%) of the crustacean's haemolymph, contains copper (1, 31). More than 50% of the total copper in its body is stored in the haemolymph. The concentration remains in a certain range, except when the organism is under stress, e.g., during the moulting process at which time the haemolymph volume decreases (31, 32).

The aim of this work was to develop methods to collect and

prepare haemolymph samples from *Parhyale hawaiiensis* and to determine the Ag and Cu concentrations. Both graphite furnace atomic absorption spectrometry (GFAAS) and inductively coupled plasma mass spectrometry (ICP-MS) were used to verify the applicability of measuring the internal concentrations of these metals after exposure to Ag via water at different concentrations.

EXPERIMENTAL

Instrumentation

A model AAnalyst™ 600 GFAAS instrument (PerkinElmer, Inc., Shelton, CT, USA) and a model 7700x ICP-MS (Agilent Technologies, Hachioji, Japan) were used for the Ag and Cu determinations. Other equipment included a Fisaton 753 magnetic shaker (Fisaton, Brazil); Orion Star A2, 11 pH meter (Thermo Scientific, Singapore); Orion Star A212 conductivity meter (Thermo Scientific, Singapore); YSI 55 oximeter (YSI, USA), Marconi MA403 and Eletrolab EL 202/4 incubator with photoperiod (Piracicaba, Brazil); Zeiss Stemi 2000 C stereo microscope coupled with an AxioCam ERC5 camera (Zeiss, Germany), and Shimadzu AUW220D analytical balance (Cebu, Philippines).

Reagents and Materials

Plastic containers, substrate (crushed coral), aeration pumps, laboratory glassware, pellet food for herbivorous fish (JBL), and marine salt (sea salt from the Red Sea) were used for *Parhyale hawaiiensis* husbandry and the experiments. The reagents used were HNO_3 (Merck; Sigma-Aldrich); AgNO_3 (Sigma-Aldrich); 1000 mg L^{-1} silver and copper mono-elementar standard solution (Quemis), and other reagents generally used in chemical analysis. Glassware, plastic bottles and other materials used during the collection and analysis

of the samples were previously decontaminated with 10% (v/v) HNO_3 for 24 hours. All solutions, dilutions and rinses were performed with ultrapure water (Millipore Corporation, 18 $\text{M}\Omega\cdot\text{cm}$ resistivity).

Identification and Husbandry of the Crustacean

The *Parhyale hawaiiensis* organisms were cultivated in the laboratory of the Ecotoxicology and Environmental Microbiology Department (LEAL - School of Technology/UNICAMP, Limeira, Brazil). Husbandry was performed in plastic containers with reconstituted saline water (salinity of 30) and crushed coral as substrate at $24 \pm 2^\circ\text{C}$ with a photoperiod of 12 hours light and 12 hours darkness, luminosity of 500 to 1000 lux, and constant aeration. The animals were fed with fish food five times per week as described in Artal et al. (33). Saline water media were prepared from the dissolution of marine salt in deionized water. Some physical parameters were controlled to ensure the water quality of the culture, such as at $\text{pH } 8 \pm 2$, dissolved oxygen higher than 3 mg L^{-1} and salinity of 30 ± 2 . This reconstituted saline water was used in the dilution of the chemical substances. All animals used in this work were eight-month old adults. This age was selected to achieve an adequate size of the organism and to allow the collection of enough amount of haemolymph for analysis.

Haemolymph Collection, Quantification, and Sample Preparation

Haemolymph fluid was collected using a thin glass needle that was manually made from a capillary glass with a Bunsen burner. Each organism was washed with deionized water, dried, and placed on a decontaminated glass plate. Using tweezers, the animals were immobilized and placed with the dorsal

segments clearly visible. The needle was inserted into the first or second dorsal segment and the haemolymph was extracted with the capillary needle (Figure 1). The procedure was done carefully to avoid insertion of the needle in the digestive tract. The amount of haemolymph collected was placed into a 2-mL Eppendorf tube containing a diluent solution.

Some parameters were evaluated for optimizing the sample preparation by using a minimum amount of sample volume and the lowest diluent interference. The haemolymph from different organisms was combined in a pooled sample consisting of 10, 5, and 4 animals. Ultrapure water and acidified solution with 1.0, 0.5, 0.1, 0.05, 0% HNO_3 (v/v) at the volume of 0.5, 1.0, or 2.0 mL were investigated as diluents. Anti-coagulants (sodium heparin 1:3 and Triton® X-100 0.2% v/v) were also evaluated (34–36). To study the best sample dilution, as well as to evaluate the accuracy of the method to determine Ag by ICP-MS, addition and recovery tests at concentrations of 25, 50, and 100 μg of Ag per liter of haemolymph were performed.

ICP-MS Optimization

The optimization of some instrumental parameters was done before evaluating the analytical calibration curves. These included auxiliary gas flow rate, sample depth reading,

the lenses and the use of helium gas for removing interferences. During the optimizations and analyses, the use of rhodium (^{103}Rh) and indium (^{115}In) as internal standards was also verified. Instrumental optimization was carried out for both the haemolymph and the saline water analyses.

GFAAS Optimization

The measurements for Ag and Cu were made at the 328.1 and 324.8 nm wavelengths, respectively. Sample and chemical modifier volumes [Pd 5 μg / $\text{Mg}(\text{NO}_3)_2$ 3 μg] injected into the graphite tube were 20 μL and 5 μL , respectively. The heating programs for the haemolymph and saline water were also optimized for pyrolysis and analyte atomization. Addition and recovery tests were performed at the three concentration levels of 10, 25, and 50 $\mu\text{g L}^{-1}$ for Ag.

Figures of Merit

The limits of detection (LOD, 3σ criterion) and quantification (LOQ, 10σ criterion) were determined. Due to the lack of a certified reference material (CRM) with a similar constitution of the crustaceans' haemolymph, the accuracy of the method was evaluated using addition and recovery experiments.

Haemolymph Analysis of Exposed Organisms to Ag in Water

The adult organisms (8 months old) were exposed to Ag for 96 hours. The Ag concentrations used were: 0 (control), 5, 10, 25, 50, and 100 $\mu\text{g L}^{-1}$. The animals were individually kept in plastic containers with 100 mL of the exposure solution (AgNO_3 dissolved in reconstituted saline water with a salinity of 30) without feeding and aeration. Twelve replicates were made for each treatment. The haemolymph was collected as previously described. Pooled samples of four organisms (2 male, 2 female) were

made and diluted in 0.05% (v/v) HNO_3 , and Ag and Cu were determined by ICP-MS and GFAAS.

Analyte Determination in Saline Water

A saline water sample was acidified with HNO_3 2% for preservation. A 1:10 dilution of the sample in ultrapure water was done before analysis due to the high concentration of dissolved salts (salinity of 30). For ICP-MS analysis, after the dilution, the sample was directly introduced into the spectrometer using the High Matrix Introduction System (HMI) that supports a high content of dissolved solids (up to 5%). For GFAAS analysis, after the dilution, the sample was directly introduced into the spectrometer.

Statistical Analyses

Statistical analyses were performed by one-way and two-way analysis of variance (ANOVA) with a Tukey test to evaluate the significant differences among the treatments, replicates, and techniques. The Levene's test was performed to the homogeneity of variances. A Student's t -test was performed to compare the recovery test results among the techniques. The p values of <0.05 were considered significant.

RESULTS AND DISCUSSION

Haemolymph Collection, Quantification, and Sample Preparation

The amount of haemolymph collected from the samples was not homogenous ($0.47 \pm 0.39 \mu\text{L}$, $n = 92$) and no correlation was made with regard to age, size, or sex. In different studies, the haemolymph volume of the amphipods was evaluated and varied from 0.2 to 15 μL per animal (33, 37, 38). One of these studies reported more than 80% loss of sample due to difficulties in the collection procedure (39). In addition, it is challenging

Fig. 1. Haemolymph collection procedure. The capillary needle is carefully inserted into the first or second dorsal segment of the *Parhyale hawaiensis*.

to estimate precisely, based on correlations, the collected amount because the volume of the haemolymph can vary from one organism to another. Nevertheless, it is very important to avoid analytical errors when expressing metal concentrations. Therefore, weighing each sample with an appropriate scale was standard procedure for the present work to ensure more reliable results. The weight of the amount of haemolymph was obtained by subtracting the weight of the needle without haemolymph (before) from the weight of the needle with haemolymph (after).

Satisfactory results were obtained by ICP-MS when a different number of organisms were used in the haemolymph pooled samples (10, 5, and 4). Therefore, for further experiments, four animals per pooled sample were used to avoid the use of many organisms. The advantage of being an even number enables the use of females and

males in a 1:1 ratio. Haemolymph coagulation was not observed in this work, probably due to the high dilution (approximately 250 times). Better results were obtained in the absence of anticoagulants (sodic heparin or Triton® X-100, see Table I), even though their use in blood samples is recommended (33–35). Thus, anticoagulants, which can also contain impurities, were not used in further experiments.

Based on the capacity of the ICP-MS automatic sampler, 1 mL of diluted sample was used for measurements. Higher concentrations of nitric acid, used as diluent, lead to sample precipitation, probably due to the protein constituents in haemolymph, while dilution in ultrapure water would impair sample preservation. Thus, the lowest acid concentration of 0.05% (v/v) was chosen which was the point at which precipitation was not observed. Best recoveries were

obtained using a haemolymph pool of four organisms diluted in 1 mL of HNO₃ 0.05% (v/v) (Table I).

Optimization of ICP-MS Instrumental Parameters

Two parameter modes were optimized since the ICP-MS instrument was used for both the haemolymph and the saline water analysis. The operating parameters are listed in Table II. The limits of detection and quantification for the haemolymph were, respectively, 0.13 and 0.44 µg L⁻¹ for Ag and 0.55 and 1.8 µg L⁻¹ for Cu. For the saline water method, the LOD and LOQ were, respectively, 1.2 and 4.1 µg L⁻¹ for Ag (see Table II).

Optimization of GFAAS Instrumental Parameters

The optimum temperatures obtained from the results in the pyrolysis and the atomization curves were 800 and 1700 °C for Ag and 1200 and 2000 °C for Cu in the

TABLE I
Silver Recoveries Using 1 mL of Different Diluents and Anticoagulants in a Haemolymph Pool of 4 Non-exposed Organisms (2 male and 2 female) Spiked With Ag and Determination by ICP-MS

Conditions	Ag Added (µg L ⁻¹)	Ag Found (µg L ⁻¹)	Recovery (%)
Ultrapure water	25	25	99
	50	59	119
	100	121	121
HNO ₃ 0.05%	25	29	114
	50	51	101
	100	89	89
HNO ₃ 0.1% ^a	25	29	114
	50	52	103
HNO ₃ 0.1% + 0.2% Triton® X-100 ^a	25	29	118
	50	58	115
HNO ₃ 0.1% + 1/3 heparin ^a	25	5	18
	50	16	32

^a Observed formation of precipitated material.

TABLE II
Optimized ICP-MS Settings for Ag and Cu Determination in Haemolymph and Ag in Saline Water

Common Parameters for Haemolymph and Saline Water Analysis	
Isotopes Selected Analytes	⁶³ Cu, ¹⁰⁷ Ag, and ¹⁰⁹ Ag
On-Line Internal Standard	(¹⁰³ Rh 250 µg L ⁻¹ diluted on-line 20 x)
Plasma Flow Rate	15 L min ⁻¹
Nebulizer Pump	0.10 rps
Spray Chamber	Scott (double pass) at 2 °C
Interface	Platinum Cones
Sampling Cone	1 mm
Skimmer	0.4 mm
Parameters for Haemolymph Analysis	
Radio Frequency Power	1550 W
Sample Depth	4.0 mm
Nebulizer Gas Flow Rate	1.10 L min ⁻¹
ORS ^a on Collision Mode	He at 5.0 mL min ⁻¹
Energy Discrimination	5 V
Parameters for Saline Water Analysis	
Radio Frequency Power	1600 W
Sample Depth	10.0 mm
HMI ^b	0.63 L min ⁻¹

^a ORS3: Octapole collision/reaction system third generation.

^b HMI: High Matrix Introduction System.

TABLE III
Optimized Heating Program for GFAAS Determination of Ag and Cu in Haemolymph and for Ag Determination in Saline Water

Step	Temperature (°C)			Ramp (s)	Hold (s)	Ar Flow Rate (mL min ⁻¹)
	Ag Haemolymph	Cu Haemolymph	Ag Saline Water			
Drying 1	90	90	90	10	20	250
Drying 2	130	130	130	15	30	250
Pyrolysis	800	1200	800	10	20	250
Atomization	1700	2000	1500	0	5	0
Cleaning	2450	2450	2450	1	3	250

haemolymph, and 800 and 1500 °C for Ag in the saline water. Table III lists the optimized heating program for the GFAAS analysis. Good precision and recoveries were obtained for both analytes in the haemolymph and the saline water under GFAAS optimized conditions (Table IV). The LOD and LOQ for the haemolymph were 0.11 and 0.37 µg L⁻¹ for Ag and 0.32 and 1.1 µg L⁻¹ for Cu, respectively. For saline water, the LOD and LOQ were 0.16 and 0.54 µg L⁻¹ for Ag (see Tables III and IV).

Haemolymph Analysis of Exposed Organisms for Ag in Water

The *Parhyale hawaiiensis* adult organisms were exposed to Ag for 96 hours and both GFAAS and ICP-MS analysis was applied to verify the Ag and Cu concentrations. The sample was collected, weighed, and diluted with 0.05% (v/v) HNO₃ without anticoagulants. Table V lists the results obtained by ICP-MS and GFAAS. Four independent exposure experiments were performed, two for analysis by ICP-MS and two for GFAAS.

The silver concentrations in the haemolymph significantly increased with an increase in Ag in the saline water (Figure 2). The concentrations ranged from 0.7 ng mg⁻¹ in the control sample to 10.8 ng mg⁻¹ in the highest exposure concentration (100 µg L⁻¹) (Table V). Grosell et al. (2) determined the Ag concentration by GFAAS in the haemolymph,

tissues, and other organs of the adult freshwater crayfish *Cambarus diogenes* which was exposed to a 10 µg L⁻¹ Ag nominal concentration for a period of 96 hours. They reported that the Ag concentration in the haemolymph increased from 0.1 ng mg⁻¹ in the control to 0.75 ng mg⁻¹. In the present work, for the same exposure, concentration, and time, a silver concentration of 3.0 ± 0.5 (by ICP-MS) and 7.2 ± 1.9 (by GFAAS) ng mg⁻¹ for the *Parhyale hawaiiensis* was observed (see Table V). Jang et al. (3) exposed the common carp *Cyprinus carpio* to Ag nanoparticles in water containing 0.61 ± 0.05 mg L⁻¹ Ag. The Ag concentrations were determined by inductively coupled plasma optical emission spectrometry (ICP-OES) and were found to be 0.02 ng mg⁻¹ Ag in the blood after 7 days of exposure. Comparing these two studies (2–3), the *Parhyale hawaiiensis* sample efficiently absorbed Ag from the water. However, the Ag accumulation was not linear and reached a plateau in the two highest tested concentrations (50 and 100 µg L⁻¹) for both ICP-MS and GFAAS (Figure 2). This suggests that there is a silver regulation mechanism either by its uptake inhibition or an active excretion in those organisms. Figure 2 shows that there was no significant difference observed between the results obtained from the different replicates and from the different techniques used for Ag determination.

TABLE IV
Ag Recoveries in a Haemolymph Pool of 4 Non-exposed Organisms (2 male and 2 female) Spiked With Ag, Diluted in 1 mL HNO₃ 0.05%, Determination by GFAAS

Found (µg L ⁻¹)	Recovery (%)
10.5 ± 0.6	105 ± 6
27.1 ± 0.4	108 ± 2
49.0 ± 2.6	98 ± 5

Copper concentrations showed no significant difference among all Ag treatments and replicates using both analytical techniques. Thus, the element has proved to be of potential use as an internal standard. The slight variation observed in the Cu concentrations (Figure 3) can be attributed to the differences between the individuals and their moult cycle, resulting in a higher or lower concentration of copper, as described by Depledge and Bjerregaard (31). Because of this variation, more studies are required to confirm the use of Cu as an internal standard for the measurement of metals in haemolymph. For Cu, the measurements performed with ICP-MS provide higher values than with GFAAS (Figure 3). It should be noted that the present experiments are an independent study, using different individuals, as explained above, which may have contained more or less Cu in their haemolymph. Thus, the different values found do not mean that the difference in results is due to the analytical techniques used.

As is shown by the agreement obtained between the recovery results and as confirmed by the statistical evaluation, both ICP-MS and GFAAS are suitable to quantify Ag and Cu. The saline water used in the exposure experiments was also analyzed at the end of the exposure period. The Ag concentrations obtained by ICP-MS and GFAAS varied by less than 20% regardless of

TABLE V
Ag and Cu Concentrations in the Haemolymph of Organisms
Exposed to Ag in Water and Determination by ICP-MS and GFAAS (Independent Experiments)

Ag in Water ($\mu\text{g L}^{-1}$)	Ag in Haemolymph (ng mg^{-1})							Cu in Haemolymph (ng mg^{-1})					
	ICP-MS				GFAAS			ICP-MS			GFAAS		
	Experi- ment	Repli- cates	Aver- age	SD	Repli- cates	Aver- age	SD	Repli- cates	Aver- age	SD	Repli- cates	Aver- age	SD
Control	I	0.1	0.7	0.5	0.5	0.7	0.2	70	73	7	40	65	13
		-			0.9			65			74		
		0.3			0.7			71			73		
	II	0.9			0.8			71			79		
		1.3			0.4			85			53		
		1.0			0.8			77			66		
5 $\mu\text{g L}^{-1}$	I	1.0	1.6	0.9	4.9	3.9	0.5	88	88	13	65	61	5
		1.1			3.7			80			67		
		0.6			4.1			81			62		
	II	1.5			3.7			83			58		
		2.2			3.4			114			53		
		3.0			3.5			79			64		
10 $\mu\text{g L}^{-1}$	I	2.9	3.0	0.5	6.0	7.2	1.9	80	91	6	65	83	32
		2.5			5.7			94			58		
		2.3			10.9			93			108		
	II	3.4			7.0			93			72		
		3.3			7.7			96			136		
		3.6			6.1			93			58		
25 $\mu\text{g L}^{-1}$	I	7.6	7.6	0.8	10.7	9.9	2.1	75	75	5	68	64	12
		6.2			11.1			73			70		
		8.1			6.4			81			45		
	II	8.2			8.5			81			56		
		7.2			10.7			71			77		
		8.2			12.1			72			70		
50 $\mu\text{g L}^{-1}$	I	8.8	10.8	3.2	9.1	10.7	1.7	79	82	19	65	69	12
		8.6			9.8			75			63		
		7.6			9.4			58			52		
	II	16.3			11.7			113			76		
		11.4			13.5			76			88		
		12.4			10.8			92			72		
100 $\mu\text{g L}^{-1}$	I	3.3	10.8	4.5	10.4	9.6	4.1	57	82	18	56	57	10
		10.7			4.2			94			41		
		8.5			5.3			64			57		
	II	12.8			11.7			84			68		
		13.1			14.9			85			53		
		16.4			11.0			105			69

Fig. 2. Silver concentrations in the haemolymph of *Parhyale hawaiiensis* exposed to Ag for 96 hours. Determinations by ICP-MS and GFAAS for independent assays.

Fig. 3. Copper concentrations in the haemolymph of *Parhyale hawaiiensis* exposed to Ag for 96 hours. Determinations by ICP-MS and GFAAS for independent assays.

the initial concentration of Ag added to the saline water used in the exposure assays. Two different methods were developed to determine Ag and Cu in the haemolymph using minimal volume (a few microliters) of sample. The choice of method to be used for analysis of the haemolymph will depend on the availability of analytical instruments in the laboratory.

CONCLUSION

Novel methods for the determination of Ag and Cu in microliters of haemolymph of the marine amphipod *Parhyale hawaiiensis* by GFAAS and ICP-MS were successfully developed with good detectability and repeatability. The difficulties inherent in the preparation of samples from small animals for the analysis of Ag and Cu in the haemolymph were overcome. During the exposure experiments, an increase (1 to 11 ng mg⁻¹) in Ag concentration in the haemolymph was observed with an increase in Ag concentration in saline water. Additionally, because changes in any organism may result in a natural response, the Ag accumulation seems to be regulated by the *Parhyale hawaiiensis*, especially

when the concentration reached 50 μg Ag per liter in the saline water. Copper showed to be a promising internal standard because its concentration remained within range in the haemolymph and did not vary with Ag exposure. Due to the use of Ag in nanomaterials and its subsequent release into the aquatic environment, the measurement of metals in the haemolymph of such small organisms can be an important tool to understand and evaluate Ag exposure in laboratory or field studies.

ACKNOWLEDGMENT

The authors thank São Paulo Research Foundation FAPESP 2013/26301-7, National Council of Research CNPq projects 400362/2014-7 and 552120/2011-1, National Institute of Science and Advanced Analytical Technologies (INCTAA), and National Institute of Science, Technology and Information in Functional Complexes Materials (INOMAT) for their financial support. We also thank Agilent Technologies for ICP-MS analysis and Prof. Dr. Marco A. Zezzi Arruda's research group from IQ UNICAMP for the ideas and preliminary experiments.

Conflict of Interest

The authors declare that there is no conflict of interest.

Received July 12, 2017.

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