COPPER UPTAKE BY THE MARINE MUSSEL MYTILUS EDULIS IN THE PRESENCE OF FULVIC ACIDS

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Abstract—Copper uptake and accumulation by the marine mussel Mytilus edulis were studied at different Cu concentrations in chemically defined artificial seawater in the presence and absence of fulvic acids. Both short-term uptake of Cu by excised mussel gills and Cu accumulation in whole mussels after 24 h of exposure decreased in the presence of fulvic acids compared with their absence at similar dissolved Cu concentrations. Calculations of Cu speciation based on previous measurements of labile Cu by anodic stripping voltammetry demonstrated that Cu uptake and accumulation depended on the concentration of labile Cu, in agreement with the free ion activity model. No evidence of a significant uptake of Cu–fulvic acid complexes was observed. Environ. Toxicol. Chem. 2012;31:1807–1813. © 2012 SETAC

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INTRODUCTION

Mussels are the marine species most often used for monitoring marine pollution, because they are sedentary organisms with high filtration rates, easy handling, and widespread distribution [1,2]. Filter feeding bivalves such as the common mussel are exposed to large volumes of water for respiratory and feeding purposes. The gills and digestive system are exposed to the metals present in the environment via these processes. It is therefore important to relate metal accumulation in the mussels with the metal levels and the chemical composition of the medium, both in the dissolved and in the particulate phase [3]. Total metal exposure concentrations are not a good indicator of biological effects, and the chemical speciation in the environment largely determines their availability to aquatic organisms.

Seawater is a buffered medium, with relatively small variations in pH, ionic strength, or ion composition. The inorganic speciation of metals can be determined rather accurately and remains almost constant in marine environments. However, the organic complexation of metals can vary considerably, and its effect on trace metal bioavailability has to be measured or modeled for each specific situation. Dissolved organic carbon concentrations in coastal areas range from approximately 2 to 4 mg C/L and can reach 10 mg C/L [4,5]. Within the pool of dissolved organic matter present in the marine environment, humic substances (as humic and fulvic acids) account for 5 to 25% in surface oceans [6]. In the water column, dissolved fulvic acids (FAs) are much more abundant than dissolved humic acids (HAs), but they can be equally abundant in the suspended particulate matter [7]. Humic substances have a relatively high affinity for metal ions, especially the group of metals that occur naturally as carbonate and hydroxide complexes, such as Cu and Pb [8,9]. They largely control, together with other components of dissolved organic matter, the speciation of these metals in seawater [10].

Several studies from the 1970s until today have proved that the bioavailability of dissolved metals is directly related to the free ion activity under certain conditions (such as constant concentration of competing cations and pH), known as the “free ion activity model (FIAM)” (for reviews see Campbell [11] and Campbell et al. [12]). Although a strong body of evidence supports the FIAM in the presence of inorganic ligands and synthetic chelators, important discrepancies have been observed in the presence of natural organic matter, many times because of the lack of a detailed study of metal speciation (for review see Campbell [11]). More recent studies have started to validate the FIAM for Cu in the presence of natural organic matter, combining direct speciation measurements and bioassays with invertebrate larvae or daphnids [13–16]. However, conflicting evidence still exists concerning the role of dissolved organic matter in the availability of metals to bivalves. Several studies have shown that dissolved organic matter can enhance the uptake of metals by freshwater [17,18] and marine [19] bivalves, possibly as a result of the direct uptake of metal–organic matter complexes or to the effects produced by organic matter on organisms surfaces [20,21]. In the case of Cu, a previous study has shown clear differences in the behavior of gills and whole mussels to Cu exposures in the presence of HAs [22]. Whole-mussel uptake was not reduced in the presence of HAs, whereas Cu uptake by gills was significantly reduced, in agreement with FIAM predictions.

The aim of the present study was to test Cu bioavailability to the common mussel, Mytilus edulis, in the presence of a natural organic matter source more relevant for aquatic systems (FAs) and to address specifically the different behavior found in Cu uptake by gills and whole mussels. For this reason, Cu uptake by isolated gills and Cu uptake by gills exposed for longer periods before excising were compared. By using a combination of chemical speciation measurements and modeling, we verified...
the extent to which Cu uptake was related to the free metal ion activity over a range of complexing conditions.

**MATERIALS AND METHODS**

Mussels were collected from an intertidal site at Westkapelle, The Netherlands. They were transported on ice in a cooling box. Once in the laboratory, they were cleaned from epibionts and transferred to acclimatization tanks filled with artificial seawater of 35‰ and 15±1 °C. The mussels were fed on a commercial yeast formulation used in aquaculture. Two days before the start of the Cu exposure experiments, feeding was stopped and mussels were transferred into clean seawater aquaria to allow depuration.

The water used for reagent preparation and rinsing was deionized water purified by ion exchange (resistivity ≥18.2 MΩ cm⁻¹, Milli-Q; Millipore). The Cu exposure experiments were conducted in 35‰ chemically defined artificial seawater prepared from analytical-grade salts and deionized water, following the formulation given by Lorenzo et al. [14]. The seawater was aerated with 0.2 μm-filtered air for 24 h to establish CO₂ equilibrium with the atmosphere and to stabilize the pH (8.05–8.20). Copper concentrations were obtained by adding the necessary volumes from a standard 1 g/L Cu solution (Merck). Fulvic acid stock solutions were prepared by dissolving the acid form of Laurential soil FAs (Fredriks Research Products) in a 10⁻² M NaOH solution and stored at 4 °C in the dark. The carbon content of the FA was 34% of total dry weight [20]. The experimental Cu and FA concentrations were chosen according to the complexation model previously described by Lorenzo et al. [15]. This model was obtained from labile Cu concentration measurements by anodic stripping voltammetry at different Cu and FA levels. In this context, labile Cu is defined as the fraction of the total metal that is measurable by anodic stripping voltammetry, consisting mainly of the free cupric ion plus inorganic Cu complexes.

All experiments were conducted in a 15.0 ± 0.5 °C isothermal room in the dark. The exposure solutions were acclimatized for 24 h to bring them to the experimental temperature and to allow the complexation between Cu and FA to reach equilibrium. Glassware and plastic labware were stored in 5% HNO₃ acid for at least 24 h and rinsed three times with deionized water before use.

**Isolated gill experiments**

Mussels between 50 and 60 mm were selected for these experiments. Mussels were opened with a scalpel and cleaned with artificial seawater, and the gills were cut from the mantle as close as possible to the base. The gills from each individual were placed in a 0.5-L plastic container and removed from the exposure solution after exactly 1 h. The effect of FAs on the uptake of Cu was studied over a 24-h exposure period. Mussels were exposed to different Cu concentrations (0.5, 1.0, 1.5 μM) in combination with different FA levels (0, 2.5, 5, 10 mg/L). Additionally, one blank and one treatment with 0.25 μM Cu were performed. Nine individuals per treatment were collected at the end of the incubation period.

After exposure, the mussels were placed in clean artificial seawater for 10 min and washed with deionized water to remove the exposure solution from the mussel cavity and the weakly adsorbed metal, respectively. Gills and the remaining soft tissues were dissected separately. Both tissues were transferred into preweighed, acid-washed 2-ml and 30-ml polypropylene tubes, respectively, and dried for 72 h at 65 °C. Samples of water were taken concurrently with the animals in order to measure the actual Cu concentrations in the exposure solutions.

**Metal analysis**

Dried samples of mussel tissue were digested with a mixture of ultrapure 69% HNO₃ (5 ml for the whole mussels and 0.25 ml for the gills) and 30% H₂O₂ (0.25 ml for the whole mussels and 0.05 ml for the gills). After these additions, the samples were left overnight at room temperature and then placed in a sealed box and transferred to a microwave oven for complete digestion. Subsequently, they were diluted with deionized water (30 ml for the whole animal and 1.2 ml for the gills). Blank tubes, containing the acids but without the tissues, as well as samples of mussel tissue reference material (CRM 278R, European Community Bureau of Reference Materials) were also prepared and digested together with the mussel tissues.

For seawater analyses, 10 ml was taken before and after the incubations, to check the nominal Cu concentrations. After addition of 150 μl of 69% HNO₃ and 200 μl of 30% H₂O₂, samples were kept at 4 °C until analyzed for Cu and other metals. Samples of each batch of artificial seawater were treated in the same way and measured to check the background Cu concentrations.

Copper concentrations in mussel tissues and seawater were determined by inductively coupled plasma–atomic emission spectroscopy (ICP-AES) using a Varian Liberty Series II ICP-AES. Samples were analyzed against matrix-matched calibration standards, and measurements were performed according to De Wit and Blust [23]. Copper concentration in tissues is expressed on a dry-weight basis (μmol/kg dw). The Cu concentrations obtained from digestion of certified reference material averaged 95.5 ± 12 μg/g (mean value ± standard deviation), a 99% recovery from the certified value (95.5 ± 1.1 μg/g). The coefficient of variation for duplicate sample analyses was approximately 12 and 6.5% for gill and soft mussel tissue analyses, respectively.
Calculation of Cu speciation in the experimental solutions

Labile Cu (Cu') in experimental solutions was calculated according to a complexation model previously described for Cu and FA in seawater [15], using the following equation

\[ \text{Cu}' = -\frac{a + \sqrt{a^2 + 4Cu_T/K_{Cu}'}}{2} \]

where \( a = (-Cu_T + [FA]N + 1/K_{Cu}') \), Cu_T and Cu' are the total and labile concentrations (µM), FA is the fulvic acid concentration (mg/L), \( K_{Cu}' \) is the conditional stability constant of Cu-FA complexes (log \( K_{Cu}' = 5.80 \pm 0.04 \)), and \( N \) is the complexation capacity of the FA (\( N = 613 \pm 38 \mu\text{mol Cu/g FA} \)). This model has been previously shown to describe Cu-FA complexation data in artificial seawater accurately [15].

Data analysis

The normality and the homoscedasticity of the data were tested using the Kolmogorov–Smirnov test and the Levene test, respectively. Differences among groups were detected by analysis of variance and a Dunnett a posteriori test. Regression analysis was performed with GraphPad Prism, and differences between regression parameters in different treatments were tested using the extra sum-of-squares F test [24].

RESULTS

Tissue and seawater analysis

The measured Cu concentrations at the beginning of the exposures were within 5% of the nominal values. Gill incubation experiments did not statistically modify Cu concentrations in exposure solutions, but whole-mussel exposures produced a decrease of 25 \% in exposure solutions, but whole-mussel exposures produced a decrease of 25\% in exposure solutions, but whole-mussel exposures produced a decrease of 25\%. Calculated [Cu']s in the exposure solutions used in the Cu and FA experiments are shown in Table 1.

Isolated gill experiments

To study the effect of FA on Cu uptake by gills, excised gills were exposed for 1 h to various amounts of Cu and FA in a factorial experiment. In addition, treatments with Cu alone were performed at the same time to allow comparison with the expected Cu uptake by the FIAM. Data are represented in Figure 1, in which the Cu concentrations in the excised gills for all the experimental mixtures are plotted versus total Cu concentrations in the water. A decrease in Cu uptake is observed for gills exposed to the same total Cu concentration with increasing amounts of FA. Although this decrease is significant only for the highest FA concentrations tested at 1 µM of total Cu, regression analysis showed that, at 1.5 µM CuT, FA concentration had a significant effect, decreasing Cu uptake by gills with a slope of −7.92 ± 2.7 µmol/kg per mg/L FA (\( p < 0.01 \)). The absence of a significant decrease at 0.5 µM CuT for any of the tested FA concentrations can be explained by considering that the Cu accumulated by excised gills after 1 h of exposure to 0.5 µM Cu in the absence of FA (149 ± 21 µmol/kg) is not significantly higher than that already present in the gills at time zero (119 ± 10 µmol/kg). Therefore, variations in [Cu'] from 0 to 0.5 µM cannot be reflected in significant variations in Cu concentrations in the gills at this short exposure time.

Copper uptake by excised gills in the absence of FA can be described by a linear uptake curve with a slope (uptake rate) of 89.9 ± 2.0 µmol/kg/h. Two alternative regression models (using total Cu or labile Cu as the independent variable) were used to fit the data in the presence of FA, and the resulting uptake rates were compared with that obtained for gills exposed to Cu alone (Table 2 and Fig. 2). If Cu uptake in the presence of FA is described as a simple function of total dissolved Cu, the uptake rate obtained (62.5 ± 3.5 µmol/kg/h) is significantly lower than that obtained in the Cu-alone experiments (89.9 ± 2.0 µmol/kg/h; \( F \) test, \( p < 0.001 \)). This shows that FA protects against Cu accumulation in the gills; this decrease in Cu uptake in the presence of FA likely was related to Cu complexation. This hypothesis was tested by constructing a Cu uptake model, but using labile Cu concentrations instead of total Cu concentrations as the independent variable (Fig. 2b). With this model, the uptake rate constant (109.2 ± 6.8 µmol/kg/h) was not significantly different from that obtained in the Cu-alone experiments (\( F \) test, \( p > 0.05 \)). Therefore, Cu uptake in the presence of FA can be predicted according to [Cu'] in the exposure solution, in agreement with the FIAM.

Whole-mussel experiments

Results of whole-mussel experiments clearly showed that Cu accumulation was much higher in the gills than in the rest of the
tissues for all treatments. As shown in Figure 3, Cu in gills was approximately seven times higher than Cu in the rest of the tissues, and this difference was maintained in all the treatments (different Cu concentrations, absence or presence of FA).

According to the relationship between Cu accumulation in the mussel and Cu concentrations in the water, the 24-h whole-mussel exposure shows a clear difference with respect to 1-h exposure of excised gills. Regarding excised gills, there was a linear relationship between Cu in gills and Cu in the water, but in this case Cu accumulation appears to follow saturation kinetics, and Cu within the different tissues of the organism reaches a plateau at approximately 0.5 µM Cu in the exposure water (Fig. 4b,d). Copper accumulation was therefore modeled by using a hyperbolic equation, as shown in Table 3. Higher Cu_{max} and lower K_M were obtained for the gills than for the rest of tissues, in agreement with the higher Cu uptake observed in the gills.

**Effect of FA on Cu accumulation in whole mussels**

Copper uptake at 0.5 µM Cu_T was lower in the presence of FA than in its absence, and this decrease was dependent on FA concentration (Fig. 4a,c). This effect was observed both in the gills and in the rest of the tissues of the mussels. At the other Cu_T concentration tested, there is no clear effect of FA on Cu uptake, which seems to be independent of the absence or presence of FA. This is easily understandable if we observe the relationship between Cu accumulation in the mussel with Cu concentration in the water, which loses its linear dependence at concentrations <0.3 µM Cu and reaches saturation at approximately 0.5 µM Cu. Therefore, any changes in Cu' concentration varying from 0.5 µM to 1.5 µM are not reflected in Cu accumulation by the mussels, which is constant at those Cu concentrations.

Experimentally determined Cu_{max} in the absence of FA was used as an input parameter to fit the hyperbolic equation to the data in the presence of FA (Table 3). In this way, the effect of covariation of K_M and Cu_{max} is eliminated [25], and K_M values obtained in the different treatments can be directly compared. Regression analysis showed that there are no significant differences between the K_M obtained for the Cu exposures without FA for gills (0.16 ± 0.06 µM) and for the Cu + FA exposures (0.22 ± 0.02 µM; F test, p > 0.05), showing that Cu' concentration successfully explained Cu accumulation by the gills. A similar analysis led to the same conclusion for the rest of the tissues (Table 3). Total Cu concentrations could not explain the observed Cu accumulation, and the K_M values obtained according to Cu_T were significantly higher than those in the absence of FA (Table 3), showing the protective effect of FA on Cu accumulation by mussels.

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**Table 2. Regression models of Cu uptake for excised mussel gills exposed for 1 h to different Cu concentrations in the absence or presence of fulvic acids (FA)**

<table>
<thead>
<tr>
<th>Regression</th>
<th>Model</th>
<th>k (µmol/kg/h)</th>
<th>p (F test)</th>
<th>r^2 (df)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu only</td>
<td>Cu_{0}gills = Cu_0 + k [Cu]</td>
<td>89.9 ± 2.0</td>
<td>—</td>
<td>0.75 (35)</td>
</tr>
<tr>
<td>Cu and FA</td>
<td>Cu_{0}gills = Cu_0 + k [Cu_T]</td>
<td>62.5 ± 3.5</td>
<td>&lt;0.001</td>
<td>0.45 (81)</td>
</tr>
<tr>
<td>Cu_{0}gills</td>
<td>Cu_0 + k [Cu_T]</td>
<td>109.2 ± 6.8</td>
<td>0.052</td>
<td>0.34 (81)</td>
</tr>
</tbody>
</table>

a Note that in the absence of organic matter (Cu only), [Cu] = [Cu_T] = [Cu']. The Cu_0 value of 119 ± 10 µmol Cu/kg was experimentally determined.

b An extra-sum-of-squares F test was done to compare uptake rates (k) of the different models in the presence of FA with that of the Cu-only model. The null hypothesis is that the parameters are similar.

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**DISCUSSION**

**Copper uptake by mussels and mussel gills**

Copper uptake by the dissolved phase occurs via the gills, which are the main organs exposed to water in the filtering activity of the mussel. This is clearly shown by the high concentrations of Cu in the gills of whole mussels, which are much higher than the concentrations in the rest of the tissues. However, this distribution of Cu within the organism is in clear contrast with that of Pb, a metal that was shown to be very rapidly transferred from the gills to the rest of the mussel tissues, so that the same Pb concentration was found in the different mussel tissues at different exposure times [20].

Excised mussel gills have been previously employed, resulting in an interesting preparation for studying the uptake of dissolved metals by bivalves [21,22]. The gills remain completely active several hours after dissection, and they show ciliary movement for more than 1 d after being excised. Metal uptake rates of excised gills have been shown to be equivalent to those of whole-mussel gills for Cu [26] and Pb [20].

**Effect of FA on Cu uptake by mussels and their gills**

The present results clearly show that FAs decrease Cu uptake by mussels and that this decrease is in agreement with Cu complexation by FA as previously determined using anodic
stripping voltammetry [15]. No evidence of a significant uptake of Cu–FA complexes was observed. This was confirmed by using both excised-gill and whole-animal exposures and is in agreement with previous experiments showing that another type of organic matter (HAs) also protected against Cu accumulation by mussel gills [22]. These results are in agreement with the FIAM and demonstrate that dissolved organic matter decreases dissolved Cu bioavailability in the marine environment also for bivalves.

Mussels are filter-feeding organisms that retain particles during their filtration process, and both dissolved and particulate metals contribute to their metal body burden. In the case of HA, higher Cu uptake than predicted by FIAM was observed when whole mussels, instead of isolated gills, were exposed, which was interpreted as being due to the ingestion of Cu–HA particles [22]. In the case of FA, the digestive route does not seem to have a significant influence, given that the results followed the FIAM also for whole-mussel exposures. Humic acids are less soluble than FAs in seawater, which can be easily observed by the formation of precipitates in solutions with high HA concentrations in artificial seawater. The higher presence of colloidal or particulate forms of HA compared with FA could explain why this kind of organic matter contributed to Cu accumulation via the digestive route. It should be noted that although the mussels were starved for 2 d before the exposure experiments, production of feces was observed in the tanks in which mussels were exposed to FA, as observed by Lorenzo et al. [22] in the presence of HA, whereas feces were absent in the tanks in the absence of organic matter. This indicates that FAs also form colloids that mussels can ingest, but their contribution to Cu accumulation seems to be considerably lower compared with the decrease of dissolved Cu uptake by the gills caused by Cu–FA complexation.

These results confirm the applicability of the FIAM for dissolved Cu in seawater in the presence of dissolved organic matter. Studies concerning the effects of dissolved organic matter on Cu bioavailability in seawater are normally based on toxicity tests with larval stages of marine invertebrates [14, 15, 27, 28], and all them have shown results in agreement with the free ion model. Similar tests in natural waters successfully relate Cu toxicity to concentrations of dissolved organic carbon [4, 5]. In addition, some studies with marine microalgae

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**Fig. 3.** Comparison of Cu concentration in the gills and in the rest of mussel tissues after 24 h of exposure of whole mussels to different concentrations of Cu in the absence (solid circles) and presence (open circles) of fulvic acids.

**Fig. 4.** Copper accumulation in the gills and the rest of the tissues of *Mytilus edulis* exposed during 24 h to Cu in the absence or presence of fulvic acids (FA) in artificial seawater. In a and c, the results are grouped according to [Cu] in exposure solution; in b and d, they are represented versus calculated [Cu'] in exposure solutions. Significant differences with respect to the uptake in the absence of FA (first bar of each group in a and c) are marked as ‘*’ *p < 0.05, ‘**’ *p < 0.01, and ‘***’ *p < 0.001. Uptake curves in the absence (solid line) or presence (dashed line) of FA are also represented in b and d. Regression parameters and significance are shown in Table 3. Means ± SD (n = 9) are represented.
have shown that Cu uptake and toxicity for these organisms also respond to labile Cu concentrations in the presence of organic matter [29,30]. In the case of mussels, the present results confirm that dissolved Cu uptake by the gills also depends on the labile Cu concentrations in the presence of dissolved organic matter.

Copper concentrations in the marine environment are generally in the nanomolar range, but in polluted estuaries and bays values as high as 30 μg/L (0.5 μM) have been reported [31]. At the low Cu concentrations usually found in unpolluted marine waters, more than 90% of dissolved Cu is usually found to be complexed with natural organic ligands [32], which results in very low Cu concentrations. Although these very low Cu concentrations would result in very low availability of Cu to be taken up by the mussel gills, the steady-state Cu concentration in the mussels living in a water body will be influenced by other important mechanisms, such as the ability of the mussels to regulate internal Cu concentrations to a certain extent and the above-mentioned uptake via the particulate phase. However, knowledge of the important effect of Cu speciation on Cu uptake via the gills can be used in mechanistic models, such as pharmacokinetic and biodynamic models [3,33], to predict metal bioaccumulation based on water chemistry.

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Cu uptake by blue mussels in the presence of fulvic acids


