



The effect of organic ligands exuded by intertidal seaweeds on copper complexation

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ABSTRACT

Copper complexation in marine systems is mainly controlled by organic matter, partially produced by micro- and macroalgae that release exudates with the capacity to bind metals. This feature is important as it influences bioavailability, bioaccumulation, toxicity, and transport of copper through biological membranes. The release of Cu-complexing ligands by seaweeds cultured under copper excess was studied in the laboratory. Five macroalgae belonging to different functional groups were used, including the filamentous *Chaetomorpha firma* (Chlorophyta), the foliose *Ulva lactuca* (Chlorophyta) and *Porphyra columbina* (Rhodophyta), the corticated *Gelidium linguatum* (Rhodophyta), and the leathery *Lessonia nigrescens* (Phaeophyceae). The concentration of ligands and their copper-binding strength ($\log K'$) of exudates released by each species was determined by anodic stripping voltammetry (ASV). The selected algae released exudates in a wide range of concentration (42–117 nM) after 48 h of culture, and addition of 157 nM copper increased the production of ligands up to 8 times. A relationship between structural complexity or thallus thickness and the amount of ligands released was not observed. The binding strength ($\log K'$) varied among species from 7.6 to 8.9, a response that was not modified by exposure to sub-lethal copper excess. The kelp *L. nigrescens* showed a fast response to copper excess, releasing ligands that reduced toxicity of the metal in hours. Results suggest that intertidal and shallow subtidal macroalgae might have been overlooked regarding their role as producers of organic ligands and, therefore, as modulators of metal complexing capacity in coastal waters.

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1. Introduction

There is a general perception that coastal environments are becoming increasingly affected by heavy metals transported in a wide array of industrial, urban and agricultural discharges (Chen et al., 2007). Paradoxically, a number of metals are essential micronutrients for marine organisms even though at elevated concentrations they become toxic (Sunda and Guillard, 1976; Brown and Newman, 2003). Among them, copper is perhaps the best studied and one of the first elements for which the importance of organic complexation was appreciated (Kremling et al., 1981; Wallace, 1982). Thus, it is now widely recognized that organic complexation decreases the toxicity of the metal (Moffett and Brand, 1996; Gledhill et al., 1999) by reducing the levels of free ions. In this context, it has been documented that almost 99% of the total dissolved copper in marine waters is bound to organic ligands (Bruland et al., 1991), whereas the most toxic forms (free ions) are often 4–5 orders of magnitude lower than the total dissolved concentrations of the metal (Croot et al., 2000).

In coastal areas, copper is accumulated by phytoplankton and macroalgae. The kinetics of this process involves two main reactions: adsorption and intracellular uptake (Crist et al., 1994; Vasconcelos and Leal, 2001). Adsorption reflects the capability of metal ions to react with either metabolically active sites that participate in the process of copper uptake by the cells or with non-active sites that contain carboxylic, sulphhydryl and phosphate groups (Levy et al., 2008). Intracellular uptake, on the other hand, leads to metal enrichment within the cells (Xue and Sigg, 1990; Vasconcelos and Leal, 2001). However, adsorption and concentration in the water are not the only aspects involved in the interaction between copper and algal cells. It has been demonstrated that several species of phytoplankton and few macroalgae have the capacity to produce and release organic substances that have the ability to bind copper ions. Copper complexing ligands are released by the marine cyanobacterium *Synechococcus* spp. (Moffett and Brand, 1996), the dinoflagellate *Amphidinium* spp. (Croot et al., 2000), and the marine microalga *Emiliania huxleyi* (Leal et al., 1999). Similarly, there is evidence indicating that macroalgae such as *Ectocarpus* spp. (Sueur et al., 1982), *Fucus vesiculosus* (Gledhill et al., 1999), *Porphyra* spp. and *Ulva* spp. (Vasconcelos and Leal, 2001) also release exudates with the capacity to bind dissolved copper.

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Even though the chemical nature of most marine copper complexing ligands is unknown, their concentration in the coastal and oceanic systems can be indirectly determined by complexometric titration with copper ions under specific and controlled conditions. Anodic stripping voltammetry (ASV) is an electrochemical technique widely used to quantify the extent of complexation of copper ions by organic ligands (Bruland et al., 2000; Plavsic et al., 2006). This analytical approach detects free metal ions and labile metal complexes, the two fractions considered to be bioavailable for seaweeds (Deaver and Rodgers, 1996). ASV is also applied to determine how tight copper is bound to an organic ligand (i.e. conditional stability constant K') or the number of available sites in the organic ligand (i.e. water complexing capacity CC) which reflects the ability of a system to complex metals. These features of the algal exudates are important in influencing the bioavailability, bioaccumulation, toxicity, and transport of copper through biological membranes (Moffett and Brand, 1996; Gledhill et al., 1999). It has been hypothesized that phytoplankton blooms determine the complexing capacity of coastal waters by releasing copper-binding ligands (Coale and Bruland, 1988). On the other hand, there is no evidence supporting similar modulating effects by macroalgae. Furthermore, little is known about the influence of copper itself on the production of ligands by seaweeds. In this context, although some studies have reported changes in the concentration of exudates released by phytoplankton in response to increasing copper levels (Moffett and Brand, 1996; Dupont and Ahner, 2005), no information is available for macroalgae, with the exception of that for *Fucus* spp. germlings (Gledhill et al., 1999).

This study assesses the hypotheses that the release of extracellular ligands is a general phenomenon in seaweeds and that copper modulates the quality and quantity of the released exudates. In this context, the possible species-specific nature of the ligands was examined. Species of macroalgae belonging to different functional groups (Steneck and Watling, 1982), including the filamentous *Chaetomorpha firma* (Chlorophyta), the foliose *Ulva lactuca* (Chlorophyta) and *Porphyra columbina* (Rhodophyta), the corticated *Gelidium lingulatum* (Rhodophyta), and the leathery *Lessonia nigrescens* (Phaeophyceae), were exposed to copper excess in the laboratory and the quantity and quality of their exudates were measured.

2. Experimental

2.1. Sample collection

Algae were removed manually during low tide from rocky platforms in central Chile (33°30'S, 71°30'W) using a plastic spatula to prevent mechanical damage. Samples were placed in acid-cleaned plastic bags with seawater from the site to minimize physiological stress, and transported to the laboratory at 4 °C within 2 h of collection. Upon arrival, epiphytes and sediment particles were removed from the surface of the thalli by washing three times in 0.22 µm filtered seawater and gentle ultrasound applications for 5 s. Prior to the experiments, seaweeds were maintained in a culture chamber at 15 °C, under $60 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic active radiation (PAR) on a 12:12 light:dark cycle in filtered seawater (0.22 µm) from the collection site for a maximum of three days.

Seawater was pumped at the sampling site, using a submersible pump and PVC tubes, directly into an acid-washed 50 L polypropylene container. In the laboratory, seawater (33.6 PSU of salinity, pH 8.0) was immediately filtered through 0.45 µm polycarbonate membranes, treated with activated charcoal (<100 µm mesh, Merck) for 48 h to diminish the content of dissolved organic matter, filtered again through 0.22 µm polycarbonate membranes, and enriched with 2 mM nitrate (sodium nitrate, Merck) and

0.1 mM phosphate (sodium dihydrogenophosphate, Merck). Treated seawater was stored in the dark at 4 °C and used for the experiments within a week.

2.2. Experimental set up

Samples of 8–10 g wet weight of each macroalga were placed in 2 L flasks with either 1 L of enriched seawater or with enriched water spiked with 157 nM Cu added as chloride (Titrisol® MERCK). Each experimental condition was done in triplicate and included seawater media and seawater media with copper addition (without algae) as controls for the background levels of ligands. Thalli were exposed to the treatments for 48 h at 15 °C and $60 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR under continuous illumination, long enough for the release of exudates and adequate to avoid degradation of the tissues (Vasconcelos et al., 2002). After incubation, media with algal exudates were obtained by filtering the treatments through acid-washed 0.45 µm cellulose acetate Millipore membrane filters.

2.3. Kinetic of Cu-complexing ligand production by *Lessonia nigrescens*

To determine the relationship between copper complexing ligand production and changes in copper speciation, juveniles of *L. nigrescens* were exposed to enriched seawater spiked with 157 nM Cu for 48 h. Triplicate samples (50 mL) of medium enriched with algal exudates were removed after 2, 12, 24 and 48 h and filtered through acid-washed 0.45 µm cellulose acetate Millipore membrane filters.

2.4. Analytical determinations

2.4.1. Copper speciation and complexing capacity

Copper speciation and complexing capacity were determined in samples enriched with macroalgal exudates resulting from exposure to culture media with and without copper addition. Measurements were done using a µ-Autolab Type II system voltammetric analyzer (Eco Chemie) with a thin mercury film (TMF) deposited in a rotating glassy-carbon disk as working electrode (RGCDE), a double junction Ag/sat AgCl reference electrode with salt bridge (3 M KCl), and a platinum wire counter electrode. Prior to sample analysis, the RGCDE was coated with nafion®, a semipermeable coat that minimizes interferences by inhibiting diffusion of organic complexes to the mercury film (Hurst and Bruland, 2005). Samples (20 mL) were deoxygenated with oxygen-free nitrogen and mounted into the system. The voltammetric scans were performed using the square wave modulation using the following parameters: frequency 20 Hz; step size 2 mV; scan rate 10 nV s^{-1} ; amplitude 50 mV; electrode rotation 5000 rpm; deposition potential -0.65 V (vs. Ag/AgCl); deposition time 30 s; equilibration time 10 s; scan range -0.65 to 0.0 . Samples were titrated with an increasing amount of ionic copper, which was allowed to equilibrate for 10 min with the natural ligands present in the sample. During this period, the working electrode rotated at 5000 rpm to accelerate the mixing process. Measurements of the copper oxidation peak height at each added copper concentration were recorded. A titration plot of peak current vs. total copper concentration (sum of concentrations of copper added + ambient copper) was constructed and the concentrations of ASV-labile copper were calculated for each of the total copper concentrations.

Titration curves were constructed plotting each copper oxidation peak height vs. the total copper concentration added. ASV-labile copper concentration (Cu') was calculated dividing the peak current without copper addition by the slope of the curve. Concentrations of organically complexed copper (CuL) were calculated by difference of concentrations between the total copper and the ASV-labile copper. Titration curves were linearly transformed by

plotting Cu'/CuL vs. Cu' (Ruzic, 1982), and copper complexing organic ligand concentrations (L) and conditional stability constants ($\log K'$) of samples enriched with algal exudates were calculated from the slope and intercept of the linear least squares regression of the linearization plot.

The significance of the differences in copper complexing ligand concentrations produced by algal species exposed to seawater media and to seawater media with copper addition were tested using a one-way (between subjects) ANOVA. A posthoc Tukey's multiple comparison test was applied to identify where significant differences existed.

2.4.2. Determination of total dissolved copper

Total dissolved copper was determined by spectrophotometric measurement of the copper(I) complex of bathocuproine according to Moffett et al. (1985). Spectral absorbance measurements were obtained using a 1 m-long liquid waveguide capillary cell (LWCC, World Precision Instrument), coupled to a miniature CCD-array spectrometer (USB2000, Ocean Optics), and fiber-coupled tungsten halogen lamp (LS-1, Ocean Optics). Absorption spectra were recorded at 484 nm against blank and standard solutions. Analytical quality was tested against certified seawater reference material (CASS-4) from the National Research Council Canada (Ottawa, Canada). Copper content of the certified seawater was not significantly different from the expected values (t test, $p > 0.05$). All analyses and manipulations were conducted at 25 °C in a Class-100 laminar flow bench.

3. Results

All algae used in the study were able to modify the water quality after 48 h of culture, as indicated by a significant increase ($F_{1,9} = 23.4$; $p < 0.05$) in the concentration of organic complexing ligands with capacity to bind copper ions as compared to the control media without algae (22.3 nM; Fig. 1). No relationship between thallus morphology (i.e. filamentous, foliose, corticated or leathery) and the concentration of ligands released was observed. For example, *L. nigrescens*, the species with the thickest thallus (i.e. larger proportion of cell wall) produced as much ligands as those species with delicate thalli (i.e. *P. columbina* and *C. firma*). The maximum concentration of Cu-complexing ligands, 4.9 times higher than the control, was obtained in the culture medium used to incubate the corticated *G. lingulatum*. Other algae produced ligands that reached concentrations ranging from 42.1 nEq Cu L⁻¹ (the filamentous *C. firma*) to 67.5 nEq Cu L⁻¹ (the foliose *U. lactuca*), 1.9 and 2.9 times higher than the control, respectively.

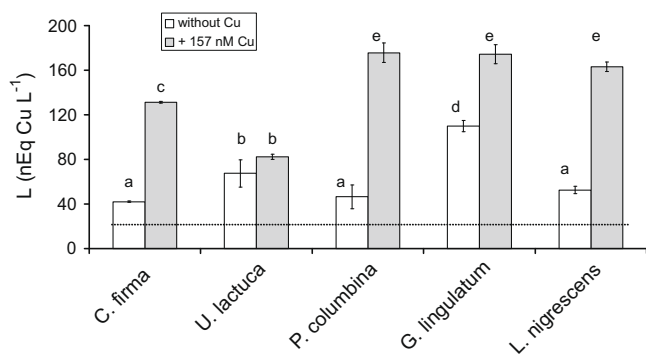


Fig. 1. Concentrations of copper complexing ligands released by seaweeds incubated in enriched seawater without copper addition and medium spiked with 157 nM copper. Continuous line represents the average background concentration (22.3 nEq Cu L⁻¹) of the complex capacity of the media without algae (control) (mean \pm SD, $n = 3$). ^{a, b, c, d, e} Statistical differences (ANOVA, posthoc Tukey $p < 0.05$).

Sub-lethal addition of copper had a clear effect on the production of ligands, and the magnitude of that effect was strongly influenced by the algal species (Fig. 1). For example, the foliose *P. columbina* released almost four times more copper complexing ligands than when incubated in plain medium. Similarly, culture media from *L. nigrescens* and *C. firma* exposed to copper excess showed concentration of copper complexing ligands around three times higher than controls. *G. lingulatum* also increased its release of copper complexing ligands in the presence of spiked copper, although only 1.6 times higher than the values recorded in the enriched seawater without copper. Finally, the release of complexing ligands by *U. lactuca* was not stimulated by copper (Fig. 1). As with the enriched seawater without copper, the magnitude of the responses to medium spiked with copper was not determined by the anatomy of the thallus, which becomes evident when comparing the filamentous *C. firma* with the leathery *L. nigrescens* (ligand concentrations 3–3.1 times higher than when exposed to plain medium) or the foliose *U. lactuca* with the corticated *G. lingulatum* (ligand concentrations 1.2–1.6 times higher than when exposed to plain medium). Binding strength varied according to the species, with values of $\log K'$ ranging from 7.6 in *U. lactuca* to 8.9 in *G. lingulatum* (Table 1). However, for each algal species, $\log K'$ measured in seawater without copper were similar to those obtained from media spiked with copper.

The production of copper complexing ligands by *L. nigrescens* over time and the dynamics of dissolved copper species in a copper-enriched media are shown in Fig. 2. The concentration of ligands (L) increased rapidly after 2 h of exposure to copper-enriched media, from ca. 20 nEq Cu L⁻¹ to 155 nEq Cu L⁻¹. The ligand concentration remained stable afterward. The concentration of copper complexes (CuL) also increased, although more slowly, reaching a maximum after 12 h of exposure. Simultaneously, the level of copper ions decreased from 153 nM to less than 3 nM during the first 12 h, remaining constant afterward.

Table 1

Stability constants of copper complexes ($\log K'$) in culture media enriched with exudates from seaweeds belonging to different functional groups.

Algal functional group	Species	$\log K'$	
		Without Cu	157 nM Cu
Filamentous	<i>Chaetomorpha firma</i>	8.2 \pm 0.1	7.9 \pm 0.3
Foliose	<i>Ulva lactuca</i>	7.6 \pm 0.5	7.7 \pm 0.3
	<i>Porphyra columbina</i>	7.9 \pm 0.3	7.8 \pm 0.2
Corticated	<i>Gelidium lingulatum</i>	8.9 \pm 0.3	8.9 \pm 0.3
Leathery	<i>Lessonia nigrescens</i>	8.7 \pm 0.2	8.6 \pm 0.3

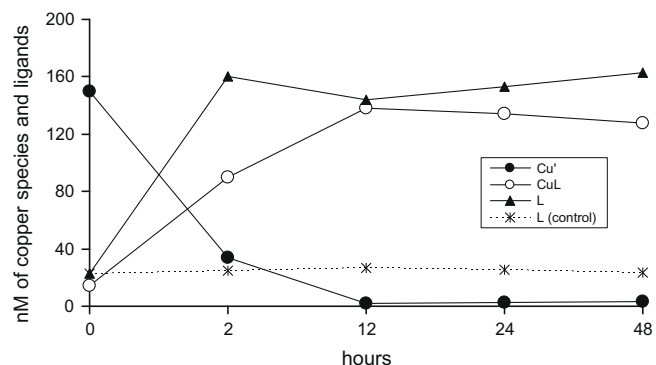


Fig. 2. Dynamics of dissolved copper species and kinetics of ligand production by *Lessonia nigrescens* in copper-enriched media.

4. Discussion

The results demonstrate that (i) all algal species included in the study released ligands, which significantly enhanced the total ligand concentration in the culture medium over the initial level of 22.3 nM and (ii) with the exception of *U. lactuca*, algae responded to spiked copper by enhancing the level of released ligands. Similar results are known for species of *Porphyra* and *Enteromorpha* (Vasconcelos and Leal, 2001) and *F. vesiculosus* (Gledhill et al., 1999). From these studies, it was expected that the release of Cu-complexing ligands by structurally complex species with thicker cell walls, like *L. nigrescens*, would be higher than simpler thalli like those displayed by *P. columbina*, *U. lactuca* or *C. firma*. However, this was clearly not the case, as *G. linguatum* produced almost twice the amount of ligands released by *L. nigrescens*, which in turn produced ligands in concentrations similar to those released by simpler thallus forms. Furthermore, this pattern was not modified by exposing the algae to spiked copper, as revealed by the monostromatic *P. columbina*, which released amounts of ligands similar to *G. linguatum* and higher than *L. nigrescens*. The apparent lack of relationship between structural complexity or thallus thickness and the amount of ligands released remains unanswered and has not been specifically addressed in previous studies. It has to be taken into account that comparisons between the concentrations of ligands released by different seaweed species present severe limitations because (i) only few studies are available and (ii) available information usually comes from studies with different experimental conditions (i.e. incubation times, undefined or different water characteristics, etc.) that may modify the values to be compared (Sueur et al., 1982; Gledhill et al., 1999; Vasconcelos et al., 2002).

The fact that stabilities of Cu-organic complexes ($\log K'$) did not change within species when copper is added suggests that, independent of the concentration of organic material released, the ligands from each seaweed species seem to possess similar chemical characteristics. However, this apparent species-specific stability of the ligands needs further research, as no information on this issue is available.

On the other hand, our results demonstrated that the ligands, in terms of their binding strength, varied among species, a trait that was not modified by exposure to sub-lethal copper excess. This is not entirely surprising considering the high diversity in cell wall composition when comparing green, brown and red macroalgae (McCandless, 1981), or individuals of the same species but of different ploidy (i.e. sporophytes and gametophytes; Craigie, 1990). Thus, the potential release of a diverse array of water soluble anionic polysaccharides, which form the bulk of the seaweeds cell walls, could explain the observed inter-species differences in ligand binding strength.

Seaweeds also exude thiolic compounds as a response to metal excess (Vasconcelos et al., 2002), although no differences were detected in the binding strength of the bulk exudates released by two phylogenetically different species, *Enteromorpha* sp. and *Porphyra* sp. (Vasconcelos et al., 2002). This suggests that the inter-species differences in binding strength recorded in our study cannot be explained by the release of thiolic compounds.

Chemical speciation studies strongly suggest that more than 99% of copper in seawater can be complexed by single or multiple ligands with conditional stability constants ($\log K'$) ranging from 6.6 to 15.6 (Bruland et al., 1991). Furthermore, two classes of ligands have been proposed; one corresponds to weak ligands ($\log K' = 7-11$) normally restricted to the mixing layers of the ocean, and the other include strong ligands ($\log K' = 12-15$) found throughout the ocean column. Several organisms have been identified as producers of extracellular copper complexing ligands with conditional stability constants very similar to the strong-class ligands (Coale and Bruland, 1988), including cyanobacteria (Moffett

and Brand, 1996), dinoflagellates (Croft et al., 2000), heterotrophic bacteria (Gordon et al., 2000), and coccolithophores (Leal et al., 1999). In seaweeds the information seems much more limited and without clear patterns. For example, our results showed that conditional stability constants varied from 7.6 to 8.9 falling within the weak-class range, similarly to those reported for ligands from *Ectocarpus* sp. and *Ulva rigida* (Sueur et al., 1982; Scoullios et al., 2004). In contrast, it was reported that *Porphyra*, *Enteromorpha* and *Fucus* produced strong-class ligands ($\log K' = 11.2-12.5$) (Gledhill et al., 1999; Vasconcelos and Leal, 2001; Vasconcelos et al., 2002), ca. 3–4 orders of magnitude stronger than those determined in this study. These apparent differences, however, should be interpreted carefully, as some studies suggest that methodology may have an influence on the reported values of stability constant (Voelker and Kogut, 2001). In general, different analytical approaches, including fixed-potential amperometry (Waite and Morel, 1983), ASV (Plavsic et al., 2006), and CSV (Donat and van den Berg, 1992; Vasconcelos et al., 2002) have yielded different copper complexing strengths for the same source of natural seawater. In this context, Bruland et al. (2000) suggested that differences observed when estimating copper complexing ligand concentrations and their conditional stability constants with different methodological approaches are likely due to the existence of a mixture of different natural Cu-chelators that form complexes displaying a range of stability constants. Differences in the conditional stability constants have not only been found in ligands from micro and macroalgae, but also in samples of natural seawater (Gledhill et al., 1997 and references therein). As discussed by Mackey and Zirino (1994) and Bruland et al. (2000), the apparently high stability constants of organic Cu-complexing ligands measured in seawater may be an artifact resulting from a slow reaction kinetics with the surface of the electrodes. In summary, while estimates of complexing strength by metal titration can be a powerful tool for understanding ligand behavior in seawater, broad comparisons between laboratories should be conducted with caution.

It is well known that the major contribution to net primary production in coastal waters comes from benthic seaweeds that contribute significant quantities of dissolved and particulate organic matter (Lobban and Harrison, 1994). However, the specific biological origin of organic ligands in near-shore ecosystems, particularly in coastal areas without the influence of rivers and their supply of allochthonous material, is poorly known. In this context, our findings, together with those from the Vasconcelos's group (Vasconcelos et al., 2002; Vasconcelos and Leal, 2008), suggest that intertidal and shallow subtidal macroalgae might have been overlooked regarding their role as producers of organic ligands and, therefore, as modulators of the metal complexing capacity of coastal waters. This could be particularly relevant in coastal zones heavily impacted by copper pollution, where concentrations of organic ligands are usually saturated by the excess of copper and, as a consequence, free copper ions and toxicity are enhanced. A specific example occurs in the south eastern pacific coast in Northern Chile where an area impacted by mine tailings has remained chronically enriched with copper (current levels: 100–200 nM dissolved copper; Andrade et al., 2006). The exceedingly low diversity and abundance of seaweeds at that site is accompanied by an excess of labile copper and high toxicity to most seaweeds (Correa et al., 1999; Medina et al., 2005; Contreras et al., 2009). The brown kelp *L. nigrescens* is a key primary producer along the Chilean coast and modulates the structure of the entire lower intertidal zone (Correa et al., 1999). Its macroscopic diploid phase alternates with a microscopic gametophytic phase, which does not tolerate the levels of copper in the area (Contreras et al., 2009). This intolerance to copper excess has been used to explain the absence of this species from the mine-impacted zone during more than 60 years (Medina et al., 2005). On the other hand, the macroscopic phase used in our

experiments demonstrated to have the capacity to rapidly respond to copper excess by producing organic ligands that increase the complexing capacity of the water, attenuating the level of labile copper within hours. Based on the above, we suggest that an assisted re-introduction of *L. nigrescens* in a copper-impacted coastal area as that described above could significantly reduce the level of labile copper, allowing the re-establishment of other copper-sensitive seaweeds and invertebrates.

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