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Stability of the copper(I)-cysteine complex assessed using reverse titrations: implications for estuarine and seawater copper biogeochemistry

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Abstract The biogeochemical cycle of copper (Cu) is mediated by its complexation with organic ligands. An emerging strategy for Cu uptake by marine and freshwater phytoplankton involves organic molecules of biological origin, such as cysteine (Cys), indicating an important role for Cys in controlling the redox state and uptake of Cu in surface waters. In this study, Cys-like compounds were detected in the surface layer of the Krka River estuary by cathodic stripping voltammetry (CSV), while high fractions of Cu(I) were simultaneously determined using an adapted solid phase extraction method. The affinity of Cys for associated redox reactions and for Cu(I) stabilisation depends on its complexation affinity towards Cu(I), which remains controversial in the literature, as values of reported stability constants for Cu(I)-Cys are inconsistent. Spectrophotometric and electrochemical approaches were used to determine the conditional stability constant of the Cu(I)-Cys complex (K''_{CuL}), which refers to the equilibrium constant conditional with respect to both Cu and the ligand. Spectrophotometric reverse titration against a known Cu(I) probe, bathocuproine disulfonate (BCS), under seawater conditions (0.55 mol/L NaCl) revealed a previously unrecognised effect of chloride (Cl^-) on the stability of the $[CuBCS_2]^{3-}$ complex due to the possible formation of ternary complexes involving Cu(I), BCS, and Cl^-, which calls for caution when determining the stability of Cu(I) complexes this way. The results of the electrochemical reverse titration of the Cu(I)-Cys complex with BCS in Cl^--containing medium are consistent with the spectrophotometric results. The logarithm of the derived K''_{CuL} for the Cu(I)-Cys complex is 15.35 ± 0.11, corresponding to the conditional stability constants of the strong Cu-binding ligand class (L_1) in seawater, which further supports the importance of Cys for Cu uptake and redox cycling in seawater and estuarine water. By elucidating the stability of the Cu-Cys complex, this study provides insight into how biologically produced and naturally occurring thiol-like ligands influence Cu redox speciation in marine and estuarine waters, which can affect Cu transport and uptake and is therefore essential for accurately representing the Cu biogeochemical cycle.

Keywords Cuprous copper · Cysteine · Reverse titrations · Redox cycle · Speciation

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Introduction

Copper (Cu) is essential micronutrient for marine phytoplankton and is taken up via specific membrane transport systems, i.e. low- and high-affinity transport systems, which are spatially organised according to the oxidation state of Cu and facilitate the transport of Cu(II) or Cu(I) through the cell membrane (Quigg et al. 2006; Guo et al. 2010). The activity of these transport systems depends on several environmental and physiological factors, including iron availability, the affinity of organic matter for Cu and total Cu concentration (e.g. Annett et al. 2008; Barber-Lluch et al. 2023). Only the labile dissolved Cu pool (Cu'), dominated by mostly inorganic ligands and free cupric cation (pCu^{2+}), was previously considered available for phytoplankton uptake (Brand et al. 1986; Moffett and Boiteau 2024). However, several studies have demonstrated that organically complexed Cu can also contribute to Cu bioavailability. Barber-Lluch et al. (2023) found that Cu bound to fulvic acid becomes more bioavailable when total Cu concentrations are low (20 nmol/L, corresponding to 1–10 nmol/L Cu'), resulting in higher cellular Cu accumulation than would be expected from inorganic Cu alone. The authors proposed that this enhancement could result either from the dissociation of organic Cu complexes near the cell surface (diffusion-limited uptake) or from the reduction of complexed Cu(II) to Cu(I), which is then transported by high-affinity Cu(I) transporters (Barber-Lluch et al. 2023). Large-scale ocean biogeochemical models support this view. Simulations using the 3D biogeochemistry model within the NEMO/PISCES global model revealed that the low modelled inorganic Cu concentration cannot meet the cellular Cu demand of phytoplankton, implying that at least a fraction of organically bound Cu must be bioavailable to phytoplankton (Richon and Tagliabue 2019). Nevertheless, studies further show that organically bound Cu(II) complexes can become bioavailable, possibly due to the extracellular reduction of Cu(II) by reductases on the cell surface prior to the uptake of Cu(I) by a high-affinity Cu transporter (Semeniuk et al. 2015). A similar conclusion can be drawn from the study by Walsh et al. (2015), which found that Cu uptake under Cu-limited conditions in the presence of cysteine (Cys) was enhanced by the reductive release of Cu(I) from strong Cu(II) complexes (e.g. EDTA) (Walsh et al. 2015). The authors also found evidence for the direct uptake of the Cu(I)-Cys complex in *Emiliana huxleyi* via a specific Cys transporter (Walsh et al. 2015).

A large body of current research focuses on chemical Cu speciation in seawater, primarily using electrochemical methods such as competitive ligand exchange-adsorptive cathodic stripping voltammetry (CLE-AdCSV) with ligands of known Cu affinity (commonly salicylaldoxime). These studies consistently show that most dissolved Cu is bound to strong organic ligands present in excess relative to total dissolved Cu, as summarised in recent review articles (Ruacho et al. 2022; Moffett and Boiteau 2024). Although the exact composition of Cu binding ligands has yet to be identified, the conditional stability constants of these ligands typically cluster into two operationally defined classes: stronger (L_1, $\log K'_{L1} \approx 10–13$) and weaker ($L_2$, $\log K'_{L2} \approx 13–16$) ligands (Ruacho et al. 2022 and references therein). Dissolved thiols are considered important components of the L_1 in marine and estuarine waters (Whitby et al. 2017, 2018). Most studies assume that Cu bound to these ligands exists primarily as Cu(II), with a few notable exceptions (Leal and van den Berg 1998; Whitby et al. 2017). Whitby et al. (2017) observed a correlation between L_1 ligands and thiol-type compounds of the thiourea class and estimated that up to 90% of Cu in the Duplin River estuary may exist as Cu(I), based on the known affinity of thiols for Cu(I). Similarly, up to 89% of total dissolved Cu in the Krka River estuary was recently found to occur as Cu(I), with the highest proportions coinciding with elevated biological activity (Crmarić et al. 2025). These findings highlight the potential importance of thiol-like compounds in Cu redox chemistry and bioavailability, but surprisingly little work has directly addressed this hypothesis. For example, based on the determined stability constant, some studies suggest that the Cu(I)-Cys complex is too weak to buffer Cu(I) effectively in seawater (Walsh and Ahner 2013; Walsh et al. 2015), contradicting earlier reports of higher complex stability (Stricks and Kolthoff 1951; van den Berg et al. 1988; Çakir et al. 2000).

In this study, we used an electrochemical approach to detect thiol-like compounds in the Krka River estuary, combined with an adapted solid-phase extraction method for Cu redox speciation, to investigate possible interactions between the thiol-like compounds and Cu(II) or Cu(I) species. Furthermore,

we applied spectrophotometric and electrochemical methods to monitor the course of reverse titrations against a known Cu(I) probe, bathocuproine disulfonate disodium salt (BCS), in chloride-containing (Cl^--containing) and chloride-free (Cl^--free) media. This enabled us to determine the conditional stability constants of complexes between Cu(I) and Cys (K''_{CuL}), as the reported stability constants (either overall or conditional) are inconsistent (Stricks and Kolthoff 1951; van den Berg et al. 1988; Çakir et al. 2000; Walsh and Ahner 2013; Walsh et al. 2015).

Materials and methods

Sampling site and sampling procedure

The sampling campaign was carried out on July 15 2024, at 11 a.m. in Krka River estuary on the eastern coast of the Adriatic Sea, Croatia (Fig. 1).

The Krka River is located on the eastern coast of the Adriatic Sea (Croatia) and is a medium-sized river that drains mostly carbonate terrains. The area is covered in limestone, resulting in karstification and the formation of characteristic tufa barriers. The freshwater section is 49 km long, while the salt-wedge type estuary, which begins after the largest and last waterfall (Skradinski buk), is 23 km long. Its geographic features and low tidal influence keep the estuary permanently stratified (Legović 1991). The freshwater-seawater interface (FSI) separates the fresh or brackish upper layer from the bottom seawater layer at a depth between 1.5 and 3.5 m, while its position and thickness depend on river flow, weather conditions and location (Legović 1991; Cindrić et al. 2015; Marcinek et al. 2020). Seawater renewal time ranges from 50 to 250 days and also depends on river flow (Legović 1991). The average annual flow is~50 m^3/s, with a minimum as low as 5 m^3/s and a maximum as high as 450 m^3/s (Bonacci et al. 2017). A sharp halocline at the FSI accumulates sinking particles, therefore, it is enriched with trace elements, organic matter, nutrients and microorganisms of both freshwater and seawater origin (Žutić and Legović 1987; Viličić et al. 1989; Cauwet 1991; Louis et al. 2009). Riverine phytoplankton from Visovac Lake die in the estuary

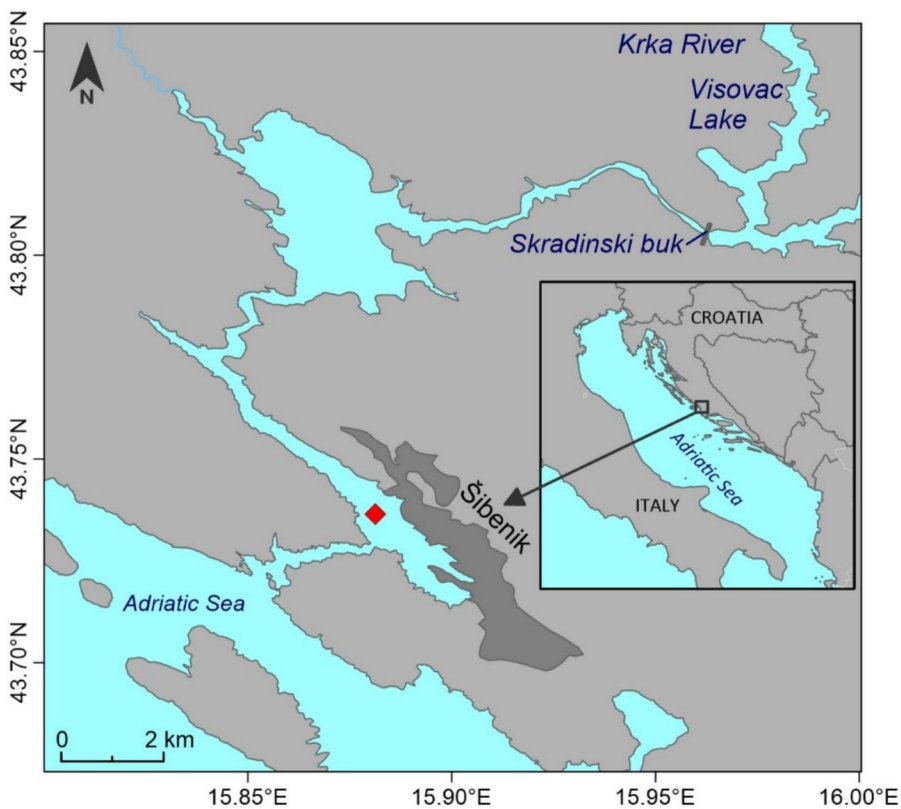

Fig. 1 Map of the Krka River estuary with the sampling site marked (red colored diamond)

due to increased salinity, and the dead phytoplankton sink to the bottom, providing nutrients below the halocline (Legović et al. 1991; Petricioli et al. 1996). The Krka River is an extremely pristine system, and both the river and its estuary are characterised by low concentrations of dissolved organic carbon (DOC) compared to other European rivers and estuaries (Marcinek et al. 2020). It also generally shows low levels of trace metals, although the concentrations of certain metals, particularly Cu, increase manifold during the summer season due to intensive nautical tourism (Cindrić et al. 2015). Longer retention of seawater in summer results in the accumulation of sinking particles, higher bacterial activity, and consequently increased concentrations of nutrients in the estuary.

The samples were collected using a home-built 4.5 L "butterfly"-type water sampler. Vertical profiles of salinity and chlorophyll *a* (Chl *a*) were measured during sample collection by using a EXO2 multiparameter CTD probe (YSI, Xylem, OH, USA) with probe sensors calibrated prior to sampling. Bottles used for sample storage, made of fluorinated ethylene propylene (FEP; Nalgene, NY, USA), were precleaned by soaking in 10% v/v nitric acid for 24 h, followed by several rinses with ultrapure (UP) water. At each depth (n = 7), a total of 2 L of water was collected. Samples were filtered immediately after collection (within 2 h) through 0.45 µm cellulose nitrate membrane filters (Sartorius, Germany) under nitrogen pressure. Square wave (SW) voltammograms of the natural samples were recorded within 20 min of filtration. In parallel, the redox speciation of Cu was determined.

Chemicals and preparation of stock solutions

All solutions were prepared with ultrapure water (18.2 MΩ, Sartorius, Arium® Mini Ultrapure Water System), hereafter referred to as UP water. All chemicals were of analytical grade, unless otherwise stated. For preparation of 1 mol/L borate buffer (pH = 8.3), ortho-boric acid (VWR BDH Prolabo Chemicals, Belgium) and sodium hydroxide (Lachner Chemicals, Czech Republic) were dissolved in UP water. The Cys stock solution was prepared daily by dissolving L-Cys (Thermo Fisher Scientific, Germany) in UP water to a final concentration of 0.01 mol/L. The BCS stock solution was prepared daily by dissolving bathocuproine disulfonic acid disodium salt in UP water to final concentration of 1 mmol/L. The EDTA stock solution was prepared by dissolving ethylenediaminetetraacetic acid disodium salt in UP water to final concentration of 0.1 mol/L. The Cu(II) stock solution was prepared by dissolving copper(II) sulfate in UP water to a concentration of 0.01 mol/L. The Cu(I) stock solutions were prepared under a N_2 atmosphere using two different Cu(I) salts and procedures: tetrakis(acetonitrile)copper(I) hexafluorophosphate (procedure 1) and copper(I) chloride (CuCl) (procedure 2), to assess the possible impact of Cl^- on reverse titrations used for the determining of the Cu(I)-Cys stability constant.

Preparation of Cu(I) stock solution: Procedure 1

A small amount of tetrakis(acetonitrile)copper(I) hexafluorophosphate (Sigma Aldrich, MO, USA) was transferred to an empty Eppendorf tube under anaerobic conditions (in a glovebox). The sealed tube was then removed and weighed outside the glovebox. The mass of Cu(I) salt was calculated by subtracting the mass of the Eppendorf tube containing Cu(I) salt and the empty Eppendorf tube. The Eppendorf tube with the Cu(I) salt, which had been sealed the whole time, was transferred back to the glovebox and 1 mL of deoxygenated acetonitrile, ACN (Sigma Aldrich, MO, USA), was added to obtain a Cu(I) concentration of 3.75 mmol/L. An aliquot of the Cu(I) stock solution (concentration of 3.75 mmol/L) was added to the cuvette containing ACN and fourfold excess of BCS (relative to Cu(I)), and the concentration was determined by UV–Vis spectrophotometry (molar extinction coefficient at 483 nm, ε_{483}, of 13,300 L mol^{-1} cm^{-1}) (Xiao et al. 2004).

Preparation of Cu(I) stock solution: Procedure 2

A modified procedure, originally published by Moffett et al. (1985) was used to prepare the Cu(I) standard solution (Moffett et al. 1985). This involved dissolving CuCl (Thermo Fisher Scientific, Germany) in a solution containing 4 mol/L NaCl (Grammol, UT, USA) and 0.1 mol/L suprapure hydrochloric acid (s.p. HCl; Roth, CA, USA), which had been previously purged with high-purity nitrogen (99.999%; Messer, Germany) to remove oxygen, to obtain a final Cu(I) concentration of 3.49 mmol/L. The consequential

removal of carbon dioxide by purging did not affect the Cu(I) speciation in the solution. The Cu(I) stock solution was freshly prepared for each experiment and used within 30 min. An aliquot of the Cu(I) stock solution (concentration of 3.49 mmol/L) was added to the cuvette containing a fourfold excess of BCS (relative to Cu(I)) dissolved in UP water and the concentration was determined by UV–Vis spectrophotometry ($\varepsilon_{483\ nm} = 13{,}300\ L\ mol^{-1}\ cm^{-1}$) (Xiao et al. 2004).

Analysis of estuarine samples

Determination of Cys-like compounds

The electrochemical determination of Cys-like compounds was carried out on filtered estuarine samples within two hours of collection using cathodic stripping voltammetry (CSV). No further pre-treatment was performed apart from sample filtration. The pH was maintained at the natural values of the estuarine samples (8.10–8.14). The voltammetric measurements were conducted using the Autolab PGSTAT204 instrument (Metrohm Autolab, The Netherlands) which was controlled by the NOVA 2.1 software. An electroanalytical cell with a working volume of 15 mL and three-electrode system were used for the measurements. A hanging mercury drop electrode (HMDE) with a drop surface area of 0.40 mm^2 served as the working electrode, a platinum was used as the counter electrode and an Ag/AgCl (3 mol/L KCl) electrode was used as the reference electrode. SW voltammetry with an amplitude (*a*) of 20 mV and a frequency (*f*) of 25 s^{-1} was used. The accumulation potential was 0.0 V and the accumulation time was 120 s, followed by a potential scan from − 0.2 to − 0.8 V. The presence of Cys-like compounds detected at − 0.48 V was confirmed by standard addition of Cys stock solution to the estuarine sample.

Determination of Cu redox speciation

In this study, we used an adapted solid phase extraction (SPE) method for Cu redox speciation, based on the Buerge-Weirich and Sulzberger (2004) work. Details of the method adaptation are explained in a recent study (Crmarić et al. 2025). Briefly, BCS and EDTA were added to the filtered estuarine sample as Cu(I) and Cu(II) binding ligands to achieve final concentrations of 1 and 5 µmol/L, respectively. A total of 100 mL of samples was prepared. SPE was performed on 50 mL by passing 5 mL aliquots through pre-cleaned Bond Elut NEXUS LRC columns containing 60 mg of polymeric sorbent. After SPE, Cu(I) was retained on the column as the Cu(I)-BCS_2 complex (called the Cu(I) fraction) and eluted with 1 mL of acidified methanol, resulting in a 50-fold pre-concentration relative to the 50 mL of sample passed through the column. In contrast, Cu(II) passed through the column as the Cu(II)-EDTA complex (called the Cu(II) fraction). The remaining 50 mL of the prepared sample, which contains both Cu(I), Cu(II), and their respective ligands, is called the Cu_T fraction and is used to obtain the mass balance (Crmarić et al. 2025).

Determination of total dissolved Cu

Total Cu concentrations in the fractions (Cu(I), Cu(II) and Cu_T) were determined by differential pulse anodic stripping voltammetry (DP-ASV) using the standard addition method (n = 4–6). Prior to the measurements, all the samples were acidified with suprapure nitric acid (s.p. HNO_3; VWR BDH Prolabo Chemicals, Belgium) to pH < 2 and UV digested for 2 days using a home-built system with a 250 W high pressure mercury lamp (Hanau, Germany) to oxidise organic substances. The voltammetric measurements were performed using the Autolab PGSTAT204 instrument (Metrohm Autolab, The Netherlands) with the set up described in the section *Determination of cysteine-like compounds*. The parameters used in DP-ASV were selected according to earlier work in the estuary (Omanović et al. 2006), while their theoretical definitions are described in the literature (e.g. Bard and Faulkner 2000). DP-ASV was conducted under the following experimental conditions: step potential 0.005 V, modulation time 0.05 s, interval time 0.5 s and modulation *a* of 25 mV. The deposition potential was − 0.85 V, while the deposition time depended on Cu concentration in the sample (180 s-600 s). The measurements included an additional desorption step, which involved switching to a negative potential of −1.3 V for 3 s to ensure that no interferences from surface active substances occurred (Louis et al. 2009; Cindrić 2015), followed by 5 s equilibration step and potential scan from − 0.55 V to 0.00 V. The ECD-SOFT (ElectroChemical Data SOFTware) software was used to process the obtained voltammograms

(https://sites.google.com/site/daromasoft/home/ecdsoft), with peak height used as the signal value. Uncertainties (shown as error bars in the plots) for [Cu(I)], [Cu(II)], and [Cu_T] are reported as 95% confidence intervals obtained from the standard addition method used for quantifying unknown concentrations. The uncertainty in the [Cu(I)]/[Cu_T] ratio was calculated by applying the propagation of uncertainty principle.

Measurements of dissolved organic carbon and biological index

High temperature catalytic oxidation was used to measure DOC in estuarine samples with a Shimadzu On-line TOC-L carbon analyser. Excitation-emission matrices for determining the biological activity index (BIX), a fluorescent characteristic of coloured dissolved organic matter (CDOM) used as a proxy for biological activity (Santos et al. 2016), were recorded using a HORIBA Aqualog fluorescence spectrometer (HORIBA Scientific, Japan) in a 1 cm quartz cuvette (Santos et al. 2016). Excitation-emission matrices were processed using TreatEEM software (Omanović et al. 2023).

Analysis of model solutions

Spectrophotometric determination of Cu(I)-Cys stability constant

The analytical approach involved the reverse titration of the $[Cu(I)BCS_2]^{3-}$ complex with Cys, as BCS is a commonly used strong Cu(I) chelator that forms an orange-coloured complex with Cu(I) with absorbance maximum at 483 nm (A_{483nm}) and high value of ε_{483}. Therefore, the decrease in A_{483nm} of $[Cu(I)BCS_2]^{3-}$ with increasing Cys addition can be easily monitored by UV–Vis spectrophotometry. Spectrophotometric measurements were obtained by measuring the Cu(I) concentration with a UV–Vis spectrophotometer in a 1 cm quartz cuvette. In procedure 1, a Perkin Elmer Lambda 650 (Perkin Elmer, CT, USA) spectrophotometer was used, and measurements were obtained under anoxic conditions (glovebox). In procedure 2, a Specord 200 Plus (Analytik Jena, Germany) spectrophotometer was used. In some cases, measurements were repeated three times (Table S1 in the Supplementary Information) to determine reproducibility and repeatability. The percentage deviation was less than 1% in all cases analysed.

For the reverse titrations of $[CuBCS_2]^{3-}$ with Cys, both in Cl^--free solutions and in solutions containing 0.55 mol/L NaCl, Cu(I) was added to the solution containing an excess of BCS (approximately twofold excess of BCS over Cu(I)) and 0.01 mol/L borate buffer, while the Cu(I) concentration was 70 µmol/L. The $[CuBCS_2]^{3-}$ complex was titrated with increasing concentrations of Cys, and the decrease in A_{483nm} was followed. During titrations, the Cys concentration was gradually increased from 10 µmol/L to 7200 µmol/L and from 10 µmol/L to 5320 µmol/L in Cl^--free and Cl^--containing solution, respectively.

Electrochemical determination of Cu(I)-Cys stability constant

The stability constant of Cu(I)–Cys was determined by CSV using an Autolab PGSTAT204 instrument, with the instrument settings and measurement parameters as described in the section *Determination of Cys-like compounds*. SW voltammetry with an *a* of 20 mV and a *f* of 25 s^{-1} was used in all voltammograms shown. The other parameters, namely the deposition potential and accumulation time, are shown in the corresponding figures. The electrolyte composition was 0.55 mol/L NaCl and 0.01 mol/L borate buffer and the pH of the electrolyte solution was 8.3.

For the reverse titration of Cu(I)-Cys with BCS, using CuSO as the starting Cu salt, 4 µmol/L Cu(II) was added to a solution containing a twofold excess of Cys over Cu(II), 0.01 mol/L borate buffer and 0.55 mol/L NaCl, which was previously deaerated with high-purity nitrogen. The concentration of BCS was gradually increased from 0.32 µmol/L to 4.56 µmol/L and the decrease in the current of the Cu(I)-Cys corresponding peak, at approximately − 0.66 V, was followed. In the case of CuCl as starting Cu salt, 4 µmol/L Cu(I) was added to the solution containing a twofold excess of Cys over Cu(I), 0.01 mol/L borate buffer and 0.55 mol/L NaCl, which was previously deaerated with high-purity nitrogen. The BCS concentration was gradually increased from 0.80 µmol/L to 30.1 µmol/L and the decrease in the current of the Cu(I)-Cys corresponding peak, at approximately − 0.66 V, was followed.

Results and discussion

Cys-like compounds and Cu(I) in estuarine waters

High fractions of Cu(I) in the total dissolved Cu pool have previously been reported in the estuarine waters of the Scheldt River (Buerge-Weirich and Sulzberger 2004), as well as in recent work in the Krka River-the study site for this research (sampling campaign conducted in July 2023) (Crmarić et al. 2025). In addition, Whitby et al. (2017) reported that up to 90% of dissolved Cu in a salt marsh estuary may be present as thiol-bound Cu(I). The authors determined log K'_{L2} and log K'_{L1} values for two detected ligand classes ranging from 12.10 to 15.20, with L_2 consisting primarily of humic compounds and L_1 of thiourea-like thiols (Whitby et al. 2017). The values reported by Whitby et al. (2017) are consistent with those in recent work in the Krka River estuary, where log K'_{L2} and log K'_{L1} ranged from 12.21 to 15.50, and the Cu(I) fraction in dissolved Cu was up to 89% (Crmarić et al. 2025). Nonetheless, the maximum fraction of Cu(I) in total dissolved Cu corresponded to the highest biological production, leading to the hypothesis that this was due to Cu(I) complexation with ligands of biological origin (Crmarić et al. 2025). This prompted us to investigate the presence of ligands of biological origin using CSV at the same site (Krka estuary) in July 2024 and to compare the results with DOC and Chl *a*, an indicator of phytoplankton biomass. In parallel, the redox speciation of Cu was determined using the adapted solid phase extraction method with BCS and EDTA as Cu(I) and Cu(II)-binding ligands, respectively.

Cys-like compounds in the Krka River estuary

CSV analysis of estuarine samples in SW mode revealed a peak at approximately − 0.55 V in all samples (seven depths at the same site), while an additional peak at approximately − 0.47 V appeared in the subsurface samples (Fig. 2, depths 0.2 m and 1.5 m) where the maximum Chl *a* was measured (Fig. 3b). The origin of the SW voltammetry peak at around − 0.47 V was confirmed by the standard addition method, as the peak increased after the addition of Cys to the sample, indicating its biological origin and thiol nature (Fig. S1 in the Supplementary Information). The correlation between thiol concentrations

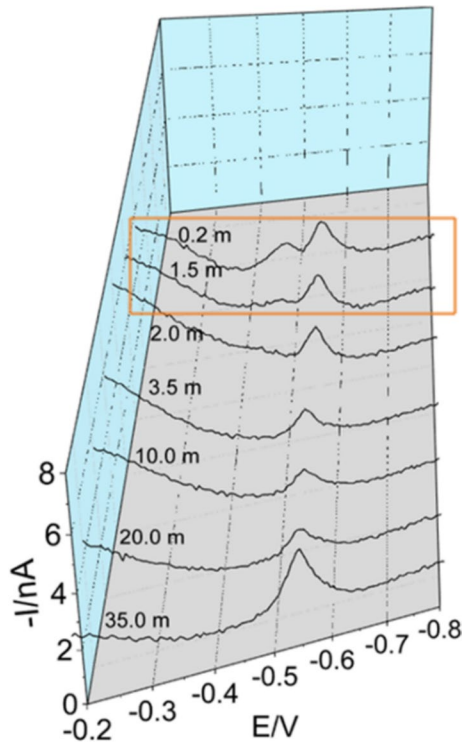

Fig. 2 Square wave (SW) cathodic stripping voltammograms of Krka River estuary samples (July 15 2024, sampling site marked in Fig. 1) after 120 s accumulation at 0.0 V vs. Ag/AgCl. The sampling depths (n = 7) in meters are shown on the left side of the voltammograms. Additional voltammetric parameters (amplitude (*a*), frequency (*f*)) can be found in the section *Determination of Cys-like compounds*. The depths at which the Cys-like compounds were detected (0.2 m and 1.5 m) are indicated (orange colored open square)

and Chl *a* has already been established in the literature (Matrai and Vetter 1988; Al-Farawati and van den Berg 2001). Al-Farawati and van den Berg (2001) reported that fluctuations in thiol concentration paralleled those of Chl and that this correlation, together with the low concentration in areas of greatest estuarine input (low-salinity waters), led to the conclusion that marine phytoplankton is an important source of thiols in the area studied. Similar observations were made for glutathione and Chl in the work of Matrai and Vetter (1988). Analogous results regarding the origin of the observed peaks have been reported in the literature (e.g. Superville et al. 2013; Laglera et al. 2014). Specifically, cathodic stripping peaks characteristic of reduced sulphur species (thiourea, thioacetamide, glutathione, elemental sulphur) in the potential range between − 0.5 V and − 0.6 V (E vs.

Ag/AgCl) have previously been detected in estuarine and seawater samples after accumulation at potentials between − 0.2 V and 0.0 V (Superville et al. 2013; Laglera et al. 2014). Quantification of the individual species' contributions can be achieved by using different deposition potentials in combination with Cu additions in the submicromolar range. The second peak at a more positive potential, approximately − 0.4 V, may be generated by low molecular weight thiols such as Cys and its metabolites, as well as various mercapto-compounds (Forsman 1981). In studies by Vasconcelos et al. (2002) and Vasconcelos and Leal (2008), two peaks were observed during CSV in the exudates of microalgae (coccolithophores and diatoms) and macroalgae (green algae), located at approximately − 0.45 V and − 0.55 V, and were identified as Cys-like and glutathione-like compounds, respectively. Nonetheless, thiols such as Cys and glutathione accounted for about 87% of the total dissolved organic Cu ligands, and the statistically significant correlation between dissolved organic Cu ligands and thiols suggests that thiol-like compounds released by eukaryotic algae play a crucial role in Cu binding (Vasconcelos et al. 2002).

Redox speciation of Cu in the Krka River estuary

The depth profile of total dissolved Cu is characterised by higher Cu concentrations in the brackish water layer than in the seawater layer (Fig. 3a), consistent with previous work in the estuary (Cindrić et al. 2015; Marcinek et al. 2022, 2025; Crmarić et al. 2025). The maximum total dissolved Cu is measured at the halocline where accumulation of trace elements and organic matter may occur (Žutić and Legović 1987; Cauwet 1991; Marcinek et al. 2022). The redox speciation of Cu in the Krka River estuary (July 15 2024) revealed that the Cu(I) fraction in the total dissolved Cu pool varies between 50 and 83%, with the highest values (83% and 73%) determined in the subsurface samples (0.2 m and 1.5 m, respectively), where the Cys-like compounds were

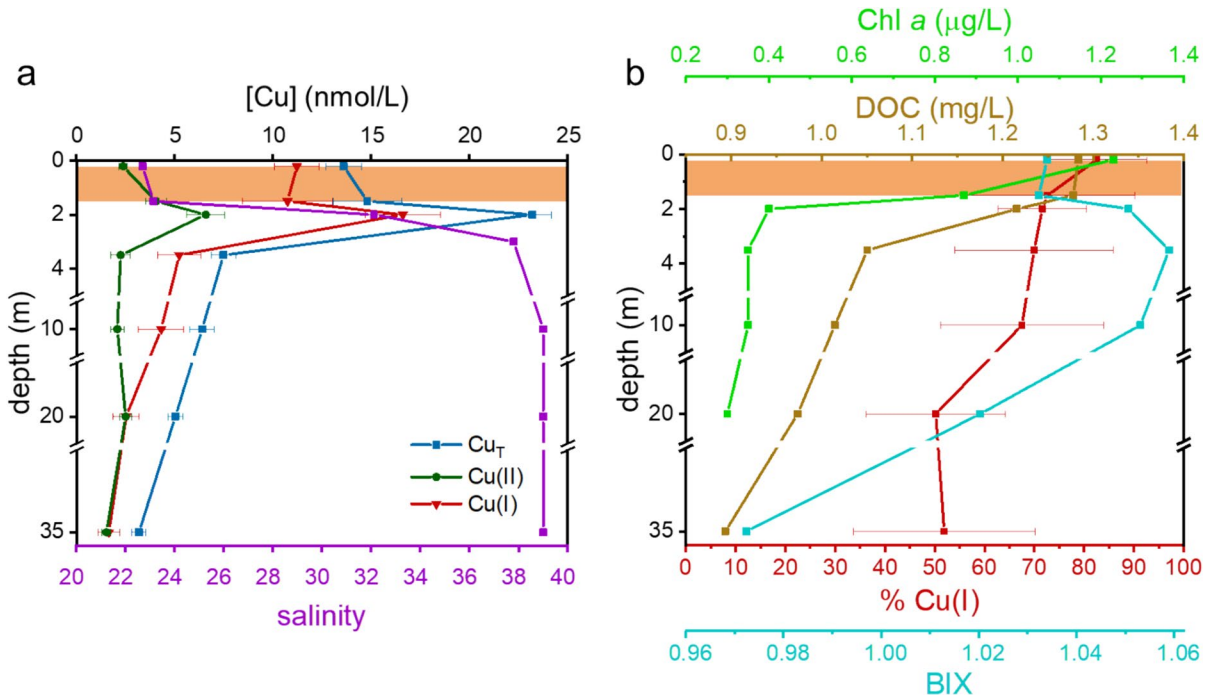

Fig. 3 Depth profile of a copper(I) (Cu(I), red line), copper(II) (Cu(II), dark green line), total dissolved copper (Cu_T, blue line) and salinity (purple line), and b) copper(I) presented as the fraction of Cu(I) in Cu_T (red line), chlorophyll *a* (Chl *a*, green line), dissolved organic carbon (DOC, brown line), and biological index (BIX, turquoise blue line) in the Krka River estuary. Samples were collected in the estuary on July 15 2024 at the sampling site marked in Fig. 1. The area between the depths where cysteine-like compounds were detected is shaded orange

detected together with the maximum of Chl *a* and DOC (Fig. 3b). The peak of primary production in the estuary may occur either at the halocline or in the surface layer, depending on season, physico-chemical and environmental factors (Viličić et al. 1989; Svensen et al. 2007), while near-bottom maxima have also been observed during the summer months (Marcinek et al. 2025). Although BIX is highest at the halocline, where the highest Cu(I) concentration was measured (16.6 nmol/L at 2 m), it remains high in the surface layer where the maximum fraction of Cu(I) (83%) was observed, indicating recent autotrophic productivity (Fig. 3a, b). Previous work on the characterisation of dissolved organic matter (DOM) and its dynamics in this estuary has revealed that, although the DOM in the surface water of the Krka estuary has a terrestrial signature, this is significantly lower in July compared to the winter months, while the fluorescence of protein-like substances of biological origin is higher (Marcinek et al. 2020). This is due to low river discharge during the summer months, which, combined with high temperatures, favours primary production of DOM. In addition, DOM accumulates in the surface layer due to the decoupling between DOC production (phytoplankton bloom) and removal (consumption by prokaryotic heterotrophs), as well as the existence of a sharp halocline, which prevents DOM mixing throughout the water column (Marcinek et al. 2020). In the study by Svensen et al. (2007), the maximum concentration of Chl *a* was observed in the brackish surface layer above the halocline (as also seen in this study, Fig. 3b) and corresponded to the highest abundance of marine diatoms, dinoflagellates, and coccolithophores Additionally, peaks in coccolithophore abundance were recorded in the estuarine surface water during the summer months, while in the autumn months their peak occured at the halocline (Skejić et al. 2021). These organisms may contribute to the pool of sulphur-containing organic ligands in estuarine waters (e.g. Leal et al. 1999; Duffy et al. 2022), either through direct production or cell lysis, which may bind Cu(I) rather than Cu(II). Furthermore, it is important to note that solar irradiation, which is most intense in the surface layer, may have a photo-bleaching effect on complex organic molecules, transforming them into lighter and simpler compounds (Vidali et al. 2010). Photochemical processes may result in the formation of radicals such as hydrogen peroxide and contribute to the stabilisation of Cu(I) (Moffett and Zika 1983, 1988). Thiols such as Cys can undergo transformation when exposed to solar irradiation, but once complexed, they are less susceptible to photochemical changes than humic complexes, which are the first to undergo photobleaching (Wang et al. 2021). This may result in a lower binding capacity for Cu(II) (Vidali et al. 2010). Specifically, humic complexes are strong chromophores in the region of solar irradiation (Zhang et al. 2025), whereas thiol complexes do not absorb in that region (Rigo et al. 2004). Together with the high stability constant, this makes Cu(I)-Cys more resistant to photochemical transformations, which is consistent with field findings showing that Cu-saturated L_1 (most likely thiol-like ligands of biological origin) are photodegraded more slowly than L_2 (most likely humic-like ligands) (Laglera and van den Berg 2006).

Overall, the correspondence between high Cu(I) levels and Cys-like peaks in the surface layer of the Krka estuary indicates that Cys-like compounds may be involved in the Cu redox cycle, which could have broader implications for the biogeochemical cycling of Cu. The stabilisation of Cu(I) by biologically derived thiol-like ligands may therefore influence the distribution, chemical speciation and bioavailability of Cu in natural waters.

Conditional stability constant of the Cu(I)-Cys complex: spectrophotometric and electrochemical results

The conditional stability constants for L_1 and L_2 Cu-binding classes (log K'_{L1} and log K'_{L2} from 12.13 to 15.51), determined in the recent study in the Krka River estuary (Crmarić et al. 2025), are consistent with those reported by other authors for estuarine systems (e.g. Whitby et al. 2017; Wong et al. 2018). The presence of Cys-like compounds in the Krka River estuary together with high fractions of Cu(I), led us to assess the K''_{CuL} and compare it with reported log K'_{L1} and log K'_{L2}. Previous literature suggests that Cys is a candidate for the L_1, consistent with the findings of Leal and van den Berg (1998), who also concluded that CLE-AdCSV, used to determine conditional stability constants of Cu complexes in seawater, cannot distinguish between Cu(I) and Cu(II) oxidation states. Specifically, Cu(II) added during CLE-AdCSV can be reduced to Cu(I), producing responses indistinguishable from those of Cu(II), highlighting a limitation of

CLE-AdCSV (Leal and van den Berg 1998). On the other hand, Walsh et al. (2013, 2015) determined the conditional stability constant of the Cu(I)-Cys complex, log $K'_{Cu(I)\text{-}Cys}$, of 11 and stated that under seawater conditions (assuming 0.2 nmol/L of Cu(I) and 10 nmol/L of Cys) 90% of Cu(I) will be in the form of Cl^- complexes (Walsh and Ahner 2013; Walsh et al. 2015). Nonetheless, the authors state that a much stronger Cu(I)-binding ligand, with a log $K'_{Cu(I)\text{-}Cys}$ of 15, is needed to buffer free Cu to less than 1 pmol/L. In this study, we performed reverse titrations using a known Cu(I) probe (BCS), followed by spectrophotometric and electrochemical analysis of $[CuBCS_2]^{3-}$ and Cu(I)-Cys complex removal in different media, i.e. Cl^--free and Cl^--containing media. These analyses were used to estimate the log K''_{CuL} of Cu(I)-Cys, as explained in the Supplementary Information (section S3), and to compare it with the log K'_{L1} in natural waters.

Stability of $[CuBCS_2]^{3-}$ complex in Cl^--containing medium

Prior to the titrating of $[CuBCS_2]^{3-}$ with Cys, we evaluated the stability of the $[CuBCS_2]^{3-}$ in both Cl^--free and Cl^--containing media, using different BCS:Cu(I) molar ratios. The concentration of $[CuBCS_2]^{3-}$ was estimated from A_{483} according to the Beer-Lambert law and the ε_{483} reported by Xiao et al. (2004). In Cl^--free solutions, the Cu(I) concentrations derived from A_{483} agreed within 10% with those calculated from the mass of Cu(I) salt (CuACN or CuCl) added to the stock solution, across both lower (2.5:1, 2.9:1) and higher (4.2:1) BCS:Cu(I) ratios. In contrast, in Cl^--containing solutions, observed Cu(I) concentrations were than 10% lower than expected when using lower BCS:Cu(I) ratios (2.2:1 and 2.5:1), whereas at a higher ratio (4.0:1), the Cu(I) concentration matched the expected value (within 5%). Furthermore, a systematic decrease in A_{483} was observed with increasing NaCl concentration (0.08–0.55 mol/L) when using a BCS:Cu(I) ratio of 2:1 (Fig. S2a).

Similar trends have been reported previously in the literature. Xiao et al. 2013 demonstrated that Cu(I) complexes with Ferrene S (Fs) and Ferrozine (Fz) are sensitive to substitution by weak Cu(I) ligands such as Cl^- and ACN. At a 2:1 ligand to Cu(I) ratio, increasing Cl^- or ACN concentrations caused a decrease in absorbance at 484 and 470 nm (A_{484} and A_{470}, respectively), whereas an excess of Fs or Fz ($\geq$4:1 ratio) was required to suppress this effect. Earlier studies by Hall et al. (1962, 1963) similarly found that Cu(I) complexes such as Cu(dmp)X (X = Cl, Br, I; dmp = 2,9-dimethyl-1,10-phenanthroline) exhibited unstable and time-dependent absorbance at 454 nm (A_{454}) unless a large ligand excess (sixfold) was present. The authors concluded that an excess of dmp is essential for reproducible spectrophotometric determination of Cu(I), ensuring independence from halide concentration. The low absorbance values observed for halide complexes in chloroform were attributed to dissociation equilibria of the form $Cu(dmp)_2X \leftrightarrow Cu(dmp)X + dmp$.

Therefore, to assess whether Cl^- affects the course of the reverse titration of $[CuBCS_2]^{3-}$ with Cys, the titrations were performed in both Cl^--containing and Cl^--free media and monitored by UV–Vis spectrophotometry.

Reverse titrations of $[CuBCS_2]^{3-}$ with Cys in absence and presence of chloride: spectrophotometric approach

The experimental data shown in Fig. 4 enabled the determination of the K''_{CuL} of Cu(I)-Cys in Cl^- free (Fig. 4a) and Cl^--containing medium (Fig. 4b). Data fitting is described in detail in the Supplementary Information (section S3). The derived log K''_{CuL} values were 15.35 ± 0.11 and 14.36 ± 0.11 for Cl^- free and Cl^- solution, respectively. The standard deviation for log K''_{CuL} was calculated after averaging the log K''_{CuL} values from the individual titration points. The derived log K''_{CuL} for the Cu(I)-Cys complex falls within the range of the log K'_{L1} values determined in natural waters (Ruacho et al. 2022 and references therein), as well as the values obtained in a recent study in the Krka River estuary (Crmarić et al. 2025), suggesting that Cys is a candidate Cu(I)-binding ligand in estuarine waters. A literature review of the logarithmic values of the constants obtained for Cu(I) complexes with Cys is provided in the Supplementary Information (Table S2), along with a discussion of the evaluation of the constants, the experimental setup, and a comparison of the literature values with those determined in this work (section S4). However, it should be noted that direct comparison of the obtained stability constants with literature values is difficult due to the differences in procedures, medium

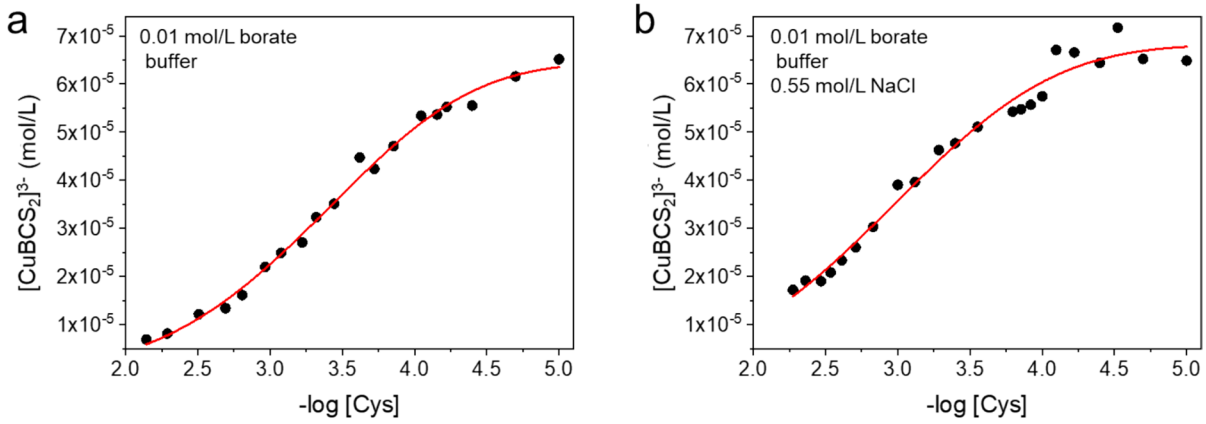

Fig. 4 Experimental data (black circles) versus fitting curve (red lines). Solution composition: **a** c(copper(I), Cu(I)) = 70 µmol/L, c(bathocuproine disulfonic acid disodium salt, BCS) = 175 µmol/L, c(borate buffer) = 0.01 mol/L b) c(Cu(I)) = 70 µmol/L, c(BCS) = 154 µmol/L, c(borate buffer) = 0.01 mol/L, c(sodium chloride, NaCl) = 0.55 mol/L. The solution pH in both a) and **b** was 8.3

composition and calculations (i.e. which side reactions are considered, if any) (Stricks and Kolthoff 1951; Vortisch et al. 1976; van den Berg et al. 1988; Çakir et al. 2000; Königsberger et al. 2015). In 0.55 mol/L NaCl medium, the titration curve is shifted towards a higher Cys concentration, indicating that a greater Cys concentration is required to titrate the same $[CuBCS_2]^{3-}$ concentration. This suggests the possible presence of a yet uncharacterised mixed complex involving BCS, Cu(I) and Cl^-, as previously discussed. The difference between the derived K''_{CuL} values in Cl^--free and Cl^--containing media is approximately an order of magnitude, which warrants further evaluation of the influence of Cl^- on the determined K''_{CuL} using an electrochemical approach to avoid spectral overlap between the $[CuBCS_2]^{3-}$ complex and putative ternary complexes of BCS, Cu, and Cl^- (Hall et al. 1962).

Reverse titrations of Cu(I)-Cys with BCS in presence of Cl^-: electrochemical approach

To further evaluate the effect of Cl^- on the K''_{CuL}, an electrochemical titration of a solution containing Cu(I) and Cys with increasing BCS concentration in a medium of 0.55 mol/L NaCl and 0.01 mol/L borate buffer was performed, in addition to the spectrophotometric titrations (a detailed investigation of the electrochemical behaviour of solutions containing Cys and Cu(I) is provided in the Supplementary Information, section S5). Although the composition of the medium was the same as in the spectrophotometric study, the concentration range was lower to avoid saturation of the electrode surface. Figure 5a and b show the results of Cu(I)-Cys complex (C3) titration with increasing BCS concentration at a fixed accumulation time of 30 s and deposition potential of 0.0 V. In agreement with our previous work, the peaks labelled C4 and C5 can be assigned to the reduction/desorption of the BCS ligand at the Hg electrode surface (Bura-Nakić et al. 2025). The addition of BCS to the solution led to a decrease in the C3 peak (Fig. 5a) as competition between the added BCS and the adsorbed Cu(I)-Cys for Cu(I) progressed. At higher BCS concentrations, two new peaks appeared, which can be assigned to the reduction of Cu(I) and Cu(II) complexes with BCS, in agreement with the literature (Bura-Nakić et al. 2025). Consequently, the C3 peak shifted towards more positive potentials as the competition between Cys and BCS for Cu(I) increased with further additions of BCS during the titration (blue line in Fig. 5a, b), and we were unable to follow the titration course with respect to the C3 peak with further increases in BCS. The experimental data shown in Fig. 5 allowed determination of the K''_{CuL} in solutions containing Cu(I) (Fig. 5c) and Cu(II) (Fig. 5d) as starting Cu salts in Cl^-/borate buffer medium. The derived log K''_{CuL} values were 14.70 ± 0.47 and 13.95 ± 0.21 for solutions containing Cu(I) and Cu(II), respectively. The standard

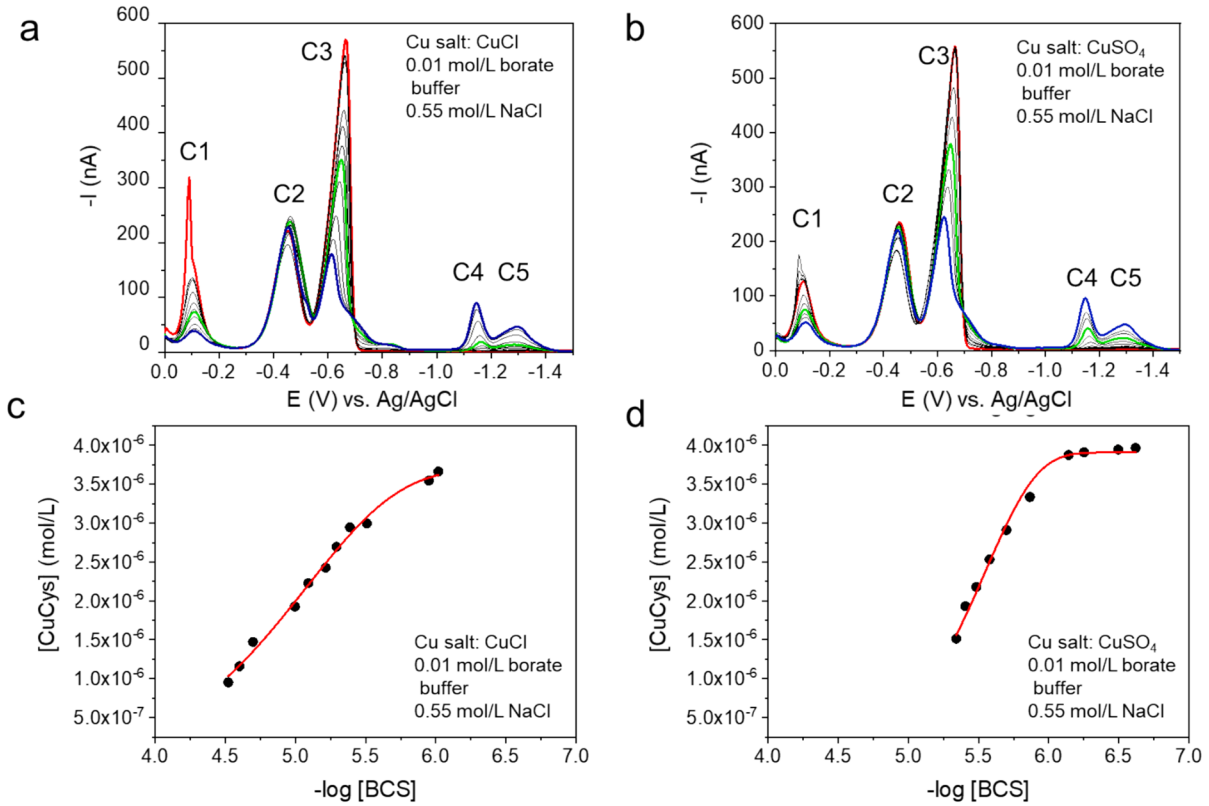

Fig. 5 Square wave (SW) voltammograms of copper(I), Cu(I) (**a**) and copper(II), Cu(II) (**b**) with cysteine (Cys) and increasing bathocuproine disulfonic acid disodium salt (BCS) concentration in cathodic stripping mode. Solution composition: **a** c(Cu(I)) = 4 µmol/L, c(Cys) = 8 µmol/L, c(borate buffer) = 0.01 mol/L, c(sodium chloride, NaCl) = 0.55 mol/L, **b** c(Cu(II)) = 4 µmol/L, c(Cys) = 8 µmol/L, c(borate buffer) = 0.01 mol/L, c(NaCl) = 0.55 mol/L. The solution pH in both a) and b) was 8.3. Experimental data (black circles) versus fitting curve (red lines) for the solution described in a (**c**) and b (**d**). Peaks C1 and C2 represent the reduction of $Hg(SR)_2$ and $Hg_2(SR)_2$, respectively. C3 represents the reduction of Cu(I)-Cys. C4 and C5 correspond to reduction/desorption of the BCS ligand

deviation for log K''_{CuL} was calculated after averaging the log K''_{CuL} values of the individual titration points, and the model used is described in the Supplementary Information (section S3.2). The derived log K''_{CuL} for the solution with Cu(I) as the starting salt (14.70 ± 0.47) is in good agreement with the values obtained from the spectrophotometric titrations of the $[Cu(BCS)_2]^{3-}$ complex with Cys in solution containing Cl^-, as described above (14.36 ± 0.11), again indicating the presence of a putative mixed complex between Cu(I), Cl^- and BCS which yields lower log K''_{CuL} in Cl^- containing solutions than in Cl^--free solutions. On the other hand, the solution with Cu(II) as the starting Cu salt gave a lower log K''_{CuL} compared to the solution containing Cu(I) as the starting Cu salt. However, if we assume that during the reaction of Cu(II) and Cys, a portion of Cys is consumed for the reduction of Cu(II), resulting in the stoichiometric production of Cys (Rigo et al. 2004), the concentration of free Cys is actually lower than assumed. This leads to a faster titration of the Cu(I)-Cys complex with BCS. All of the above, in turn, results in a lower K''_{CuL}.

Implications and impact on biogeochemical Cu cycle

High levels of Cu(I) relative to the total dissolved Cu pool were found in the Krka River estuary, consistent with previous sparse reports of high Cu(I) fractions in coastal waters. Various causes for the occurrence and stabilisation of Cu(I) in oxygenated surface waters are discussed in the literature (Moffet and Zika

1988, Buerge-Weirich and Sulzberger 2004; Crmarić et al. 2025). The correlation observed in this work between the maxima of Chl *a*, Cys-like compounds, and Cu(I) content also indicates the important role of biologically derived compounds such as thiols in the Cu redox cycle. This conclusion is supported by the reverse titrations which yielded K''_{CuL} of 15.35 ± 0.11, within the range of log K'_{L1} in natural waters. All this indicates that Cys is a candidate for Cu(I) binding and stabilisation in estuarine waters. Thus, most speciation studies assume Cu(II) as the predominant Cu oxidation state in natural waters, including toxicity studies. In terms of toxicity, the concentration of free inorganic Cu^{2+} is reported, as it has been known for decades that Cu^{2+} can be toxic to phytoplankton even at concentrations as low as pmol/L (Sunda 1975), whereas complexed Cu is considered less bioavailable and therefore less toxic (Sunda and Lewis 1978). The conditional stability constant for a complex of metal with single or group of organic binding sites (L_i), assuming a metal to ligand stoichiometry of 1:1 (Town and Filella 2000), is related to the concentrations of: 1) complexed metal, 2) unbound metal (free metal ions and inorganic species), and 3) binding sites available for complexation (Town and Filella 2000; Gledhill and Gerringa 2017). In addition to organic ligands, it is important to consider the complexation of metal with inorganic anions, which are present in seawater at much higher concentrations than metals. Therefore, an inorganic side reaction coefficient, hereafter referred to as α', is used to express the effect of inorganic ligands (Pižeta et al. 2015). In seawater at pH 8.3, α' mainly describes the complexation of Cu(II) by carbonate and hydroxide, while for Cu(I) it relates to complexation by chloride. Based on the stability constants of metal with inorganic ligands available in the literature (Turner et al. 1981) and the ambient metal concentration, α' can be easily calculated at a given pH, salinity and total dissolved inorganic carbon (Pižeta et al. 2015). The calculated α' for Cu(I) and Cu(II) varies by several orders of magnitude, namely for Cu(II), α' is in the range of 36 (in seawater at pH 8.3), while for Cu(I) it is 1.4×10^5 (Leal and van den Berg 1998). Therefore, knowledge of Cu redox speciation in the studied system is of utmost importance, as correcting K'_{L1} or K'_{L2} for the Cu(I) α'-coefficient results in a concentration of free inorganic Cu several orders of magnitude lower, which would significantly impact our understanding of Cu uptake, toxicity and fluxes. It should be noted, however, that caution is needed with these calculations, as free Cu(I) in equilibrium with organic and inorganic Cu(I) complexes disproportionates to Cu(II) and Cu^0, and these species should also be considered. In this context, further research should be conducted to determine bioavailable Cu when Cu(I) is considered the relevant species, and to investigate the significance of these results for the biogeochemical cycling of Cu. For example, due to the known affinity of Cys for Cu(I), Cu(I) should be considered the relevant species in toxicity assessments in environmental studies where Cys-like ligands have been detected, whereas current calculations are based on Cu(II) (Vasconcelos et al. 2002; Laglera and van den Berg 2003; Vasconcelos and Leal 2008). High-affinity transporters require Cu(I), which is generated by cell surface reductase, photochemical reduction of Cu(II), or possibly by reduction by thiol-like compounds (Kong and Price 2020). Although the bioavailability of organically complexed Cu has been reported (Semeniuk et al. 2015; Walsh et al. 2015; Richon and Tagliabue 2019), the possible role of Cys in Cu uptake remains to be further investigated. Walsh et al. (2015) recognised the enhancing role of Cys in Cu uptake under Cu-limited conditions and its lowering effect on Cu uptake in Cu-replete environments, although they do not consider Cys a relevant Cu(I)-binding ligand in seawater (based on a calculated log $K'_{Cu(I)\text{-}Cys}$, of 11, which is several orders of magnitude lower than in this work). However, if Cys already complexes at least part of the detected Cu(I) in natural waters, Cu uptake may follow a different pathway than previously reported, where the first step is the reduction of organically bound Cu(II) (Walsh et al. 2015).

Conclusions

The presence of Cys-like compounds in the Krka River estuary was detected by CSV and confirmed by standard addition of Cys. Although it remains uncertain whether these compounds originate from phytoplankton responses to high Cu concentrations or serve to regulate Cu toxicity and/or uptake, the correlation between Chl *a* and fractions of Cu(I) in the total dissolved Cu

pool indicates their involvement in Cu uptake by phytoplankton. Reverse titration experiments in Cl^--containing medium showed that Cl^- can interfere with $[CuBCS_2]^{3-}$ formation, likely due to ternary complexes of Cu(I), BCS and Cl^-, making the derivation of the stability constant for the Cu(I)-Cys complex questionable. To avoid this, a low Cl^- concentration or a BCS:Cu(I) ratio greater than 2:1 is recommended. The logarithmic value of the derived conditional stability constant for the Cu(I)-Cys complex ($\log K''_{CuL} = 15.35 \pm 0.11$) falls within the range of strong Cu ligands measured in estuarine and seawater environments, highlighting the potential importance of Cys-like compounds as Cu(I)-binding ligands in aquatic systems. Whether this conclusion—that thiol-like compounds indicate high Cu(I) levels—also applies to the surface waters of the oceans, where thiol-like compounds are also present in the nmol/L concentration range, remains the subject of future studies. Nonetheless, further research should be conducted to determine bioavailable Cu when Cu(I) is considered the relevant species, and to investigate the significance of these results for the biogeochemical cycling of Cu.

Supporting Information

Voltammograms of the surface sample of the Krka estuary before and after Cys additions, investigation of $[Cu(BCS)_2]^{3-}$ complex stability in medium with variable NaCl concentration and metal:ligand ratio, fitting procedure, literature review on stability constants of complexes between Cys and Cu, investigation of Cys behaviour and cuprous Cys formation on Hg electrode.

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Author contributions Conceptualisation: Elvira Bura-Nakić; Methodology: Elvira Bura-Nakić, Olga Iranzo; Formal analysis and investigation: Dora Crmarić, Narda Stipanović; Data curation- Dora Crmarić, Olga Iranzo; Data validation: Marina Mlakar; Writing-original draft preparation: Dora Crmarić, Elvira Bura-Nakić, Writing- review and editing: Marina Mlakar; Funding acquisition: Elvira Bura-Nakić; Supervision: Elvira Bura-Nakić. The authors have no relevant financial or non-financial interests to disclose.

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Data availability The data supporting the findings of this study are available at Mendeley Data repository: https://data.mendeley.com/datasets/n53xbm4vcz/1.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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