



University of Zagreb

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**COPPER BIOGEOCHEMISTRY IN AN
ESTUARINE ENVIRONMENT: THE
SIGNIFICANCE OF REDOX
TRANSFORMATIONS**

DOCTORAL THESIS

Zagreb, 2026



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DOCTORAL DISSERTATION

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Sveučilište u Zagrebu

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BIOGEOKEMIJA BAKRA U ESTUARIJSKOM OKOLIŠU – VAŽNOST REDOKS TRANSFORMACIJA

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COPPER BIOGEOCHEMISTRY IN AN ESTUARINE ENVIRONMENT: THE SIGNIFICANCE OF REDOX TRANSFORMATIONS

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Copper (Cu) is both an essential micronutrient and a toxic element for marine microorganisms, and its bioavailability depends on its speciation. Nevertheless, Cu redox speciation is often overlooked, as Cu is generally assumed to occur predominantly as Cu(II) under oxic conditions. This thesis investigates the occurrence of Cu(I) in an estuarine environment and the processes governing its formation and stabilisation. The research comprised investigations of Cu–thiol interactions, adaptation of a method for Cu redox speciation, and its application in the Krka River estuary. Particular emphasis was placed on biologically produced organic ligands and photochemical processes in Cu(I) stabilisation, followed by chloride complexation. Laboratory experiments showed that thiols promote Cu(II) reduction and stabilise Cu(I) through complex formation. An adapted solid-phase extraction (SPE) method, coupled with differential pulse anodic stripping voltammetry (DP-ASV), enabled determination of Cu(I), Cu(II), and total dissolved Cu (Cu_T). Field measurements revealed that Cu(I) constituted 20.7–88.7% of Cu_T throughout the vertical water column of the estuary, with pronounced seasonal and vertical variability. High Cu(I) fractions in surface waters coincided with indicators of recent primary production and detection of cysteine (Cys)-like compounds, while the high conditional stability constant of the Cu(I)–Cys complex supported its potential contribution to Cu(I) stabilisation. Diel experiments demonstrated the contribution of photochemical processes to Cu redox transformations. Overall, the results show that Cu(I) is an environmentally relevant component of Cu_T and should therefore be considered in studies of Cu speciation and bioavailability, as this may improve understanding of Cu biogeochemical cycling and acquisition in natural waters.

Keywords: Copper, redox, speciation, estuary, organic ligands, thiols

This work contains 180 pages, 13 figures, 9 tables and 281 references. Original work is written in English.

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Bakar (Cu) je esencijalni mikronutrijent i toksični element za morske mikroorganizme, pri čemu njegova bioraspoloživost ovisi o specijaciji. Ova doktorska disertacija istražuje pojavnost Cu(I) u estuarijskom okolišu te procese koji određuju njegovo nastajanje i stabilizaciju. Istraživanje je obuhvatilo ispitivanje interakcija Cu i tiola, prilagodbu metode za određivanje redoks specijacije Cu te njezinu primjenu u estuariju rijeke Krke. Posebna pozornost posvećena je organskim ligandima biološkog podrijetla i fotokemijskim procesima u nastajanju i stabilizaciji Cu(I), uz kompleksiranje kloridom. Laboratorijski eksperimenti pokazali su da tioli reduciraju Cu(II) i stabiliziraju Cu(I) u obliku kompleksa. Prilagođena metoda ekstrakcije na čvrstoj fazi (engl. *solid-phase extraction*, SPE), u kombinaciji s diferencijalno-pulsnom voltametrijom anodnog otapanja (engl. *differential pulse anodic stripping voltammetry*, DP-ASV), omogućila je određivanje Cu(I), Cu(II) i ukupnog otopljenog Cu (Cu_T). Terenska mjerenja pokazala su da je Cu(I) činio 20,7–88,7 % Cu_T duž vertikalnog vodenog stupca estuarija, uz izraženu sezonsku i vertikalnu varijabilnost. Visoki udjeli Cu(I) u površinskim vodama podudarali su se s pokazateljima recentne primarne proizvodnje i detekcijom cisteinu (Cys)-nalik spojeva, dok je visoka uvjetna konstanta stabilnosti kompleksa Cu(I)–Cys potvrdila njegov potencijalni doprinos stabilizaciji Cu(I). Eksperimenti provedeni tijekom dnevno-noćnog ciklusa pokazali su doprinos fotokemijskih procesa redoks transformacijama Cu. Sveukupno, rezultati pokazuju da Cu(I) predstavlja okolišno relevantnu sastavnicu Cu_T te ga stoga treba uzeti u obzir u istraživanjima specijacije i bioraspoloživosti Cu, čime se može unaprijediti razumijevanje biogeokemijskog kruženja Cu i njegova staničnog unosa u prirodnim vodama.

Ključne riječi: bakar, redoks, specijacija, estuarij, organski ligandi, tioli

Izvorni rad je napisan na engleskom jeziku i sadrži 180 stranica, 13 slika, 9 tablica i 281 literaturnih navoda.

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INFORMATION ABOUT THE SUPERVISOR

Dr. Elvira Bura-Nakić is a Senior Scientist at the Ruđer Bošković Institute, Division for Marine and Environmental Research, Laboratory for Physical Chemistry of Traces, with research activities based at the Marine Research Station Martinska Station in Šibenik and in Zagreb, Croatia.

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Dr. Bura-Nakić joined the Ruđer Bošković Institute in 2001 as a Professional Associate. She subsequently worked as a Research Assistant, Senior Assistant, Research Associate and Senior Research Associate at the Ruđer Bošković Institute, with a postdoctoral position at ETH Zürich, Switzerland, from 2014 to 2016. For her postdoctoral research at ETH Zürich, she received competitive Marie Curie funding for project “*Using lakes to develop isotopic tools for understanding ocean redox through Earth history*”. She was appointed Senior Scientist at RBI in 2023.

Her research focuses on trace element biogeochemistry, redox speciation, and the development of analytical methodologies for investigating redox-sensitive elements in aquatic and marine environments. Dr. Bura-Nakić has led several Croatian Science Foundation projects as Principal Investigator. She currently leads the 2025–2028 RHEMAR project, “*Evaluation of the importance and formation of rhenium-containing particles in the marine environment*”. Previously, she led the 2021–2024 SeCuTrans project “*Understanding copper speciation and redox transformations in seawater*”, and the 2018–2022 REDOX project “*Geochemistry and redox proxies signature under the diverse environmental conditions: towards better understanding of the past redox*”.

Her methodological contributions are centered on establishing analytical protocols that significantly enhance resolution in trace element and redox speciation knowledge, and include implementation of the isotope dilution method for determining low element concentrations, introduction of ion chromatography and online derivatisation for the determination of vanadium redox species, implementation of solid-phase extraction for the separation of copper redox species in marine matrix and introduction of organic phase extraction as a tool for element speciation.

She has experience in academic mentorship, having served as the main mentor of three PhD candidates, one of whom has successfully defended their PhD thesis, while two are currently pursuing their doctoral studies. In addition, she has supervised and mentored three postdoctoral researchers at the Ruđer Bošković Institute. Dr. Bura-Nakić has participated in international research programmes and scientific networks, where she has served as co-convenor, plenary lecturer and invited lecturer at international scientific conferences, and has organised international scientific workshops. She is a regularly invited reviewer for leading high-impact (Q1/D1) international journals, including Nature, Earth and Planetary Science Letters, Geochimica et Cosmochimica Acta and Chemical Geology.

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PROŠIRENI SAŽETAK

Bakar (Cu) je element u tragovima koji ima važnu ulogu u biogeokemijskim procesima u morskom i estuarijskom okolišu. Esencijalni je mikronutrijent za morske mikroorganizme jer sudjeluje u brojnim metaboličkim procesima, no pri povišenim koncentracijama, osobito u obliku slobodnih hidratiziranih iona (Cu^{2+}), može djelovati toksično (e.g. Brand i sur., 1986). Bioraspoloživost i biološki učinci Cu stoga ne ovise samo o ukupnoj koncentraciji, već i o njegovoj kemijskoj specijaciji, odnosno raspodjeli između različitih kemijskih vrsta koja uvjetuje reaktivnost, bioraspoloživost i/ili toksični učinak te transport. U morskom i estuarijskom okolišu više od 99 % ukupnog otopljenog Cu (Cu_T) nalazi se u kompleksima s organskim ligandima, koji se s obzirom na vrijednost uvjetne konstante stabilnosti kompleksa najčešće dijele na jaki (L_1) i slabi (L_2) razred liganada te se najčešće određuju pomoću kompetitivne izmjene liganada–adsorptivne voltametrije katodnog otapanja (engl. *competitive ligand exchange–cathodic stripping voltammetry*, CLE-AdCSV) (Ruacho i sur., 2022). Unatoč važnosti specijacije Cu, njegova se redoks specijacija, odnosno raspodjela između oksidacijskih stanja +I i +II često zanemaruje. U oksičnim prirodnim vodama Cu(II) termodinamički je povoljnija vrsta (Turner i sur., 1981), zbog čega se uobičajeni pristupi specijacijske analize Cu uglavnom temelje na pretpostavci da je Cu(II) dominantna vrsta. Međutim, istraživanja redoks specijacije Cu u prirodnim vodama pokazala su da Cu(I) može činiti značajan udio Cu_T (Buerge-Weirich i Sulzberger, 2004; Moffett i Zika, 1988; Kieber i sur. 2004). Prisutnost Cu(I) upućuje na postojanje procesa koji omogućuju redukciju Cu(II) i stabilizaciju nastalog Cu(I), a među mogućim procesima posebno se ističu fotokemijske reakcije otopljene organske tvari (OOT) i interakcije Cu s organskim ligandima. Fotokemijska transformacija OOT može dovesti do nastanka reaktivnih kisikovih vrsta koje sudjeluju u redoks reakcijama metala, dok organski ligandi također mogu utjecati na redoks transformaciju i stabilnost nastalih kompleksa. U redoks specijaciji Cu posebno su važni tioli, odnosno organski spojevi koji sadržavaju sulfhidrilnu skupinu ($-\text{SH}$), zbog njihove sposobnosti redukcije Cu(II) i sklonosti prema kompleksiranju Cu(I) (Pearsons, 1963). Međutim, uloga tiola u nastajanju i stabilizaciji Cu(I) u prirodnim vodama još uvijek nije dovoljno razjašnjena. Najčešće korištene analitičke metode u redoks specijaciji Cu u prirodnim vodama su ekstrakcija tekuće-tekuće (engl. *liquid–liquid extraction*, LLE) i ekstrakcija krutom fazom (engl. *solid-phase extraction*, SPE), kombinirane s određivanjem Cu u frakcijama pomoću atomske

apsorpcijske spektrometrije (AAS). Ipak, postojeće metode za određivanje redoks specijacije Cu u prirodnim vodama ograničene su nepotpunim iskorištenjem i mogućim promjenama izvorne raspodjele redoks vrsta Cu tijekom pohrane i obrade uzoraka, što naglašava potrebu za razvojem metoda koje osiguravaju kvantitativno iskorištenje, niske granice detekcije i očuvanje izvorne redoks specijacije Cu.

Estuariji su osobito važni za istraživanje ovih procesa jer predstavljaju dinamična sučelja slatke i morske vode, u kojima se istodobno mijenjaju fizikalno-kemijski uvjeti i biološka aktivnost. Estuarij rijeke Krke je mikroplimni estuarij koji karakterizira izražena vertikalna stratifikacija i postojanje halokline, na kojoj se mogu akumulirati organske tvari, hranjive soli i mikroorganizmi (e.g. Žutić i Legović, 1987). Unos elemenata u tragovima i otopljene organske tvari iz rijeke Krke u estuarij je nizak, a zabilježene povišene koncentracije elemenata u tragovima uglavnom su rezultat antropogenih aktivnosti (Cukrov i sur., 2008; Cindrić i sur., 2015, 2020; Carić i sur., 2021). Posebno se ističe sezonski porast koncentracije Cu tokom ljetnih mjeseci, čiji je uzrok nautički turizam, gdje se Cu koristi u protuobraštajnim bojama na brodovima i plovilima. Unatoč brojnim istraživanjima biogeokemije elemenata u tragovima u estuariju, redoks specijacija Cu dosad nije bila istražena.

Glavni cilj doktorskog istraživanja bio je utvrditi pojavnost Cu(I) u estuarijskom okolišu te istražiti procese koji utječu na njegovo nastajanje i stabilizaciju, s posebnim naglaskom na ulogu organskih liganada i fotokemijskih procesa. Istraživanje je obuhvatilo tri glavna cilja:

- (C1) ispitati interakcije Cu s tiolima u modelnim otopinama,
- (C2) optimizirati metodu za određivanje redoks specijacije Cu te primijeniti u prirodnim vodama,
- (C3) utvrditi čimbenike koji utječu na redoks specijaciju bakra u prirodnim vodama.

Postavljene su tri hipoteze:

- (H1) Tioli reduciraju Cu(II) i stabiliziraju Cu(I) u modelnim otopinama čak i u prisutnosti kisika.
- (H2) U estuarijskom okolišu, Cu(I) čini značajan udio ukupnog otopljenog Cu,
- (H3) Nastajanje Cu(I) u prirodnim vodama potaknuto je biološkom aktivnošću i fotokemijskim procesima.

U prvom dijelu istraživanja (Znanstveni članak I) ispitane su interakcije Cu s biološki i okolišno važnim tiolima: cisteinom (Cys), glutationom (GSH), 3-merkaptopropionskom kiselinom (3-MPA) i tiooctenom kiselinom (TAA). Eksperimenti su provedeni u kontroliranim

uvjetima kako bi se utvrdila sposobnost navedenih spojeva da reduciraju Cu(II) i stabiliziraju Cu(I) stvaranjem kompleksa. Posebna pozornost posvećena je utjecaju strukture i koncentracije tiola odnosno omjera koncentracija tiola i Cu na tijek redoks reakcija.

Drugi dio istraživanja obuhvatio je prilagodbu SPE metode za određivanje redoks specijacije bakra u prirodnim vodama (Znanstveni članak II). Metoda se temelji na selektivnom kompleksiranju Cu(I) s batokuproin disulfonatom (BCS), a Cu(II) s etilendiamintetraoctenom kiselinom (EDTA). Nakon razdvajanja Cu(I)- i Cu(II)-frakcija na koloni, koncentracije Cu određene su diferencijalno-pulsnom voltametrijom anodnog otapanja (engl. *differential pulse-anodic stripping voltammetry*, DP-ASV). Adaptacijom metode nastojalo se poboljšati iskorištenje ekstrakcije i smanjiti mogućnost promjene izvorne redoks specijacije tijekom obrade i skladištenja uzoraka.

Treći dio istraživanja obuhvatio je određivanje redoks specijacije bakra u estuariju rijeke Krke na postajama koje se razlikuju prema stupnju antropogenog utjecaja, kao i istraživanje sezonske i vertikalne varijabilnosti Cu(I) (Znanstveni članci II i III te dodatna uzorkovanja u estuariju Krke). Analizirani su uzorci prikupljeni tijekom različitih godišnjih doba i na različitim dubinama, uključujući površinski riječni/bočati sloj, haloklinu i morski sloj. Redoks specijacija istražena je i u kišnici uz paralelno određivanje u površinskom sloju estuarija. Dobiveni rezultati uspoređeni su s fizikalno-kemijskim parametrima, uključujući salinitet i koncentraciju otopljenog organskog ugljika (DOC), pokazateljima biološke aktivnosti te optičkim svojstvima OOT. Dodatno je istražena promjena redoks specijacije pod različitim svjetlosnim režimima (dan/noć). Nadalje, okolišna relevantnost rezultata iz Znanstvenog članka I, tj. tiolima uzrokovana redukcija Cu(II) i stabilizacija Cu(I) ispitivana je voltametrijskom analizom uzoraka estuarija kojom je ispitana prisutnost liganada nalik tiolima u estuariju, te određivanjem uvjetne konstante stabilnosti kompleksa Cu(I)–Cys spektrofotometrijskim i voltametrijskim reverznim titracijama (Znanstveni članak III).

Laboratorijski eksperimenti pokazali su da svi ispitani tioli, Cys, GSH, 3-MPA i TAA, mogu sudjelovati u redukciji Cu(II) i stabilizaciji nastalog Cu(I) stvaranjem kompleksa, čak i u prisutnosti kisika. Učinak je ovisio o kemijskoj strukturi i koncentraciji pojedinog tiola tj. o njegovu omjeru prema koncentraciji Cu. Reducirajuća snaga ispitivanih tiola može se sažeti kako slijedi: Cys≈GSH>MPA>TAA. Dobiveni rezultati potvrđuju da tioli mogu omogućiti postojanje Cu(I) u oksigeniranim uvjetima te upućuju na njihov potencijalni značaj u redoks ciklusu bakra u morskim i estuarijskim vodama.

Prilagođena SPE metoda, povezana s DP-ASV, omogućila je određivanje koncentracija Cu(I), Cu(II) i Cu_T. Postignuto je kvantitativno iskorištenje ekstrakcije za oba oksidacijska stanja, čime je unaprijeđen analitički pristup određivanju redoks specijacije Cu u prirodnim vodama. Primjena metode omogućila je istraživanje redoks specijacije Cu estuariju rijeke Krke i kišnici. Određivanjem redoks specijacije Cu ustanovljeno je da je Cu(I) bio prisutan u svim istraženim estuarijskim uzorcima te je činio 20,7–88,7 % Cu_T duž vertikalnog vodenog stupca. Utvrđena je izražena sezonska i vertikalna varijabilnost, što upućuje na snažan utjecaj fizikalno-kemijskih i bioloških uvjeta na njegovu raspodjelu. Visoki udjeli Cu(I) zabilježeni su u površinskom sloju i na haloklini, a često su se podudarali s pokazateljima pojačane biološke aktivnosti, uključujući koncentraciju klorofila *a* i optičke pokazatelje OOT povezane s biološki proizvedenom organskom tvari. Ova podudarnost upućuje na moguću ulogu biološki proizvedenih organskih liganada u nastajanju i stabilizaciji Cu(I), iako se njihov doprinos ne može u potpunosti razdvojiti od utjecaja drugih procesa tj. fotokemijskih procesa i kompleksiranja kloridom.

U većini analiziranih uzoraka kišnice detektiran je Cu(I), ali je udio Cu(I) u Cu_T bio niži nego u estuarijskim uzorcima. Ovaj rezultat upućuje na to da atmosferski unos Cu(I) ne može objasniti visoke udjele Cu(I) u estuariju. Naprotiv, atmosferske lebdeće čestice morskog podrijetla vjerojatno doprinose stabilizaciji Cu(I) u kišnici.

Istraživanje je dodatno pokazalo moguću važnost spojeva nalik Cys u stabilizaciji Cu(I). Njihova prisutnost utvrđena je u estuarijskim uzorcima u kojima su zabilježeni najviši udjeli Cu(I). Određena uvjetna konstanta stabilnosti kompleksa Cu(I)–Cys bila je unutar raspona karakterističnog za L₁ razred liganada u istraživanom sustavu. Zajedno, ovi rezultati podupiru pretpostavku da Cys i slični tioli mogu pridonositi stabilizaciji Cu(I) u estuariju.

Istraživanje dnevno-noćne dinamike pokazalo je promjene udjela Cu(I) tijekom 24-satnog ciklusa, pri čemu se njegov udio povećavao od jutra prema večeri. Istodobne promjene optičkih svojstava OOT upućuju na moguću povezanost redoks transformacija Cu s fotokemijskim procesima i transformacijama organske tvari. Fotokemijske reakcije pridonose redukciji Cu(II) i nastajanju Cu(I) u površinskom sloju estuarija. Ipak, opažena postojanost Cu(I) i u mraku upućuje na doprinos ostalih procesa, uključujući potencijalnu redukciju tiolima.

Sveukupno, rezultati doktorskog istraživanja pokazuju da Cu(I) predstavlja značajnu i okolišno relevantnu sastavnicu Cu_T u estuariju rijeke Krke. Laboratorijski eksperimenti potvrdili su sposobnost ispitivanih tiola da reduciraju Cu(II) i stabiliziraju Cu(I) u oksičnim uvjetima, dok

je adaptirana SPE metoda omogućila određivanje redoks specijacije Cu u prirodnim vodama uz kvantitativno iskorištenje ekstrakcije. Utvrđena sezonska, vertikalna i dnevno-noćna varijabilnost Cu(I) upućuje na važnost bioloških i fotokemijskih procesa u redoks ciklusu Cu. Rezultati istraživanja kompleksa Cu(I)–Cys dodatno podupiru moguću ulogu liganada nalik tiolima u stabilizaciji Cu(I) u estuarijskom okolišu. Međutim, relativni doprinos pojedinih liganada i mehanizama još nije moguće jednoznačno utvrditi, jer se ispitivani procesi odvijaju istovremeno. Ovo istraživanje pokazuje da je redoks specijaciju potrebno uključiti u cjelovito razumijevanje biogeokemijskog kruženja Cu u estuarijskim i morskim sustavima. Budući da se uobičajenim metodama određivanja organske specijacije ne razlikuju oksidacijska stanja Cu, zanemarivanje Cu(I) može ograničiti interpretaciju biogeokemije Cu, uključujući njegovu bioraspoloživost i toksičnost. Naime, Cu(I) je prepoznat kao ključna vrsta u određivanju toksičnosti Cu (Wang i Wang, 2021). Dobiveni rezultati stoga predstavljaju doprinos razumijevanju čimbenika koji kontroliraju nastajanje i stabilizaciju Cu(I) te pružaju osnovu za daljnja istraživanja njegove uloge u biogeokemijskim procesima u prirodnim vodama.

1. INTRODUCTION

Copper (Cu) is a trace metal in the marine environment and an essential micronutrient that plays a key role in regulating cellular metabolism and growth in phytoplankton (Raven et al., 1999; Twining and Baines, 2013), while its bioavailability largely depends on its chemical speciation. Despite its role as a micronutrient, Cu becomes toxic at elevated concentrations of free (hydrated) Cu^{2+} ions (Cu^{2+}), which, together with labile Cu complexes, are considered the bioavailable forms of Cu (Tercier-Waeber et al., 2012 and references therein). However, organically complexed Cu may also be bioavailable (Semeniuk et al., 2015; Walsh et al., 2015). It is well established that more than 99% of total dissolved Cu (Cu_T) occurs in complexes with organic ligands, whose exact structures are still under investigation and which are commonly classified, based on conditional stability constants, into a stronger (L_1 , $\log K'_{\text{Cu}L1} > 13.5$) and a weaker ligand class (L_2 , $\log K'_{\text{Cu}L2} < 13.5$), although this boundary is not strictly defined and depends on the system under investigation (Ruacho et al., 2022 and references therein). The most commonly used methodology in organic speciation is competitive ligand exchange–adsorptive cathodic stripping voltammetry (CLE-AdCSV).

Since Cu(II) is the thermodynamically favoured oxidation state of Cu in oxic seawater (Turner et al., 1981), the redox speciation of Cu has largely been overlooked. However, significant fractions of Cu(I) relative to Cu_T (hereafter noted as Cu(I) fractions ($\text{Cu(I)}/\text{Cu}_T$) and expressed in percentages) have been detected in natural waters (Buerge-Weirich and Sulzberger, 2004; Kieber et al., 2004; Moffett and Zika, 1988; Xue et al., 1991). Methods for determining Cu(I) are limited by low natural concentrations and the risk of altering speciation during sample handling and storage. Moffett et al. (1985) developed a simple spectrophotometric method based on the complexation of Cu(I) with bathocuproine disulfonate (BCS; disodium 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline-4,7-disulfonate) and Cu(II) with ethylenediamine (EN). Using a similar approach, Xue et al. (1991) measured Cu(I) fractions of 4–90% in fog water, which they attributed to the presence of sulfite and the reduction of Cu(II) by organic compounds. Nevertheless, the high detection limit restricts the applicability of these methods (Moffett et al., 1985; Xue et al., 1991). Moffett and Zika (1988) subsequently developed a new method using neocuproine and EN as Cu(I) and Cu(II) ligands, respectively, with methylene chloride extraction, and Cu concentrations were determined by atomic absorption spectroscopy (AAS). Cu(I) fractions of 5–10% were measured in the western Atlantic Ocean and the Gulf of

Mexico, with a surface maximum, while Cu(I) was not detected below the photic zone. The presence of Cu(I) has been attributed to photochemical processes involving hydrogen peroxide and organic reductants such as humic substances and biologically produced ligands (Moffett and Zika, 1988). Photochemical oxidation of dissolved organic matter (DOM) generates superoxide and peroxide radicals capable of reducing complexed Cu(II) (Moffett and Zika, 1983; Voelker et al., 2000). Using an adapted extraction method (Moffett and Zika, 1988), Voelker et al. (2000) determined the concentrations of Cu(I) formed during radiolysis of seawater spiked with Cu and found that measured concentrations were lower than expected, indicating the presence of organically complexed Cu(I). Kieber et al. (2004) determined Cu(I) concentrations in rainwater using the extraction method of Moffett and Zika (1988), reporting Cu(I) fractions of 22–32%, which were linked to photochemical reduction of Cu(II) complexes, reduction of Cu(II) by organic matter, and stabilisation of Cu(I) by inorganic species such as sulfite. Buerge-Weirich and Sulzberger (2004) developed a solid-phase extraction (SPE) method using Cu(I) and Cu(II) ligands, BCS and EN, respectively, which requires smaller volumes of organic solvents. Copper(I), eluted with methanol, is determined by AAS. Cu(I) fractions of 5–80% were measured in the Scheldt estuary and the North Sea. High Cu(I) fractions were associated with photochemical reduction of Cu(II) complexes and chloride complexation in high-salinity regions, and with thermal reduction of Cu(II) and stabilisation by sulphur-containing species (e.g. thiols) in low-salinity regions. Low Cu(I) fractions in regions of intermediate salinity likely results from oxidation of Cu(I) in the presence of strong Cu(II)-binding ligands. However, Cu(I) measurements were performed on previously frozen samples that were exposed to artificial light of higher intensity than sunlight after thawing, meaning the results do not reflect in situ Cu redox speciation. Furthermore, these methods did not achieve complete recovery, and the results were therefore corrected for the measured recovery (Buerge-Weirich and Sulzberger, 2004; Kieber et al., 2004; Moffett and Zika, 1988; Xue et al., 1991).

The literature indicates that dissolved organic matter (DOM) is an important factor in the redox speciation of Cu, with humic substances and biologically produced ligands such as thiols, chalkophores, and exopolysaccharides being particularly relevant (Croot et al., 2000; Moffett and Brand, 1996; Whitby et al., 2018, 2017). Allochthonous humic substances are mainly derived from aromatic lignins and tannins, whereas autochthonous humics are primarily produced by phytoplankton. Humic substances generally belong to L₂, although they can also be part of L₁, especially in estuarine environments (Marcinek et al., 2025; Muller and Batchelli,

2013), while most biological ligands constitute L_1 (Marcinek et al., 2025; Ruacho et al., 2022; Whitby et al., 2018, 2017; Whitby and van den Berg, 2015). Many marine organisms produce Cu-binding ligands, such as thiols and phytochelatins, which are mostly low-molecular-weight (LMW) DOM (e.g. Biddanda and Benner 1997; Leal et al. 1999; Croot et al. 2000; Swarr et al. 2016). In the stabilisation of Cu(I), thiols (e.g. cysteine, Cys) and other reduced sulphur species containing –SH groups are particularly important (e.g. Croot et al., 2000; Leal and van den Berg, 1998). While intracellular interactions between Cu(I) and thiols are well established (Maiti et al., 2018), their interactions in natural waters are poorly understood, although Cys and other thiols have been detected there (e.g. Swarr et al., 2016) and, given their high affinity for Cu(I), are potential candidates for Cu(I) complexation. Whitby et al. (2017) observed in the Duplin River estuary a correlation between thiourea-like thiols and L_1 , and between humic substances and L_2 . As most Cu was complexed by L_1 resembling thiols, it is possible that Cu(I) accounted for up to 90% of Cu_T . The correlation between L_1 and thiourea-like species has also been observed in the northeastern Pacific (Whitby et al., 2018), with other identified ligands including glutathione (GSH)-like thiols and humic substances. However, most ligands were likely composed of other thiols, such as Cys and 3-mercaptopropionic acid (3-MPA), possibly incorporated into Cu(I)-binding chalkophores. A correlation between L_1 and protein-like substances (primarily thiols and their derivatives), and between L_2 and humic-like substances, has also been observed in the Krka River estuary (Marcinek et al., 2025), although the redox speciation of Cu was not investigated. Furthermore, Leal and van den Berg (1998) observed that the addition of Cys and GSH to seawater leads to the reduction of Cu(II) and the formation of a complex between Cu(I) and the added thiol, suggesting that some previously detected Cu(II)-binding ligands may in fact complex Cu(I). Conversely, some authors have concluded that Cys, due to its low conditional stability constant, is not a candidate for Cu(I) complexation in seawater (Walsh et al., 2015; Walsh and Ahner, 2013), which contrasts with previous calculations (Çakir et al., 2000; van den Berg et al., 1988).

The literature review indicates that knowledge of Cu(I) in marine and estuarine environments remains limited. Further research and the development of methods enabling in situ detection and quantification of Cu(I) are required, along with a better understanding of the factors controlling its formation and stabilisation and its role in the biogeochemical cycling of Cu. Therefore, this thesis addresses the occurrence and environmental significance of Cu(I) in an estuarine environment, with emphasis on processes governing its formation and stabilisation. The research includes investigation of Cu interactions with biologically and environmentally

relevant thiols: Cys, GSH, 3-MPA and thioacetic acid (TAA) in model solutions under oxic conditions. Furthermore, an adaptation of SPE methodology for Cu redox speciation is presented, which enabled Cu redox speciation in the Krka River estuary, including both estuarine water and rainwater. Combined with physico-chemical parameters, optical characterisation of DOM, organic Cu speciation, detection of thiol-like compounds in the estuary, and diel experiments, a new dataset on the occurrence of Cu(I) and the environmental factors controlling its formation and stabilisation is delivered.

The research undertaken in this thesis, and published within Research Papers I, II and III on which the thesis is based, advanced the understanding of Cu redox speciation in estuarine environments, with particular emphasis on the role of organic ligands in Cu redox processes. The results provided novel insights into the processes governing Cu redox transformations and contributed to a more comprehensive understanding of Cu biogeochemical cycling in estuarine and marine systems.

2. LITERATURE OVERVIEW

2.1. Concentration and distribution of elements in marine environment

Elements in seawater are commonly classified according to their concentration levels. The International Union of Pure and Applied Chemistry (IUPAC) classifies elements in natural waters as major, minor, and trace constituents. Throughout this work, concentrations are expressed using molar units, with mmol dm^{-3} (mM), $\mu\text{mol dm}^{-3}$ (μM), nmol dm^{-3} (nM), or pmol dm^{-3} (pM) used according to the concentration range. Major constituents include chlorine (Cl), sodium (Na), magnesium (Mg), sulphur (S), calcium (Ca), and potassium (K), which exhibit conservative behaviour in the water column; that is, they do not participate in chemical or biological reactions to an extent that would affect their concentration. These elements control seawater salinity and ionic strength. In contrast, elements present at levels below $1 \mu\text{M}$ are considered minor and trace constituents (Bruland and Lohan, 2003). These are prone to participate in chemical and biological processes and therefore generally exhibit non-conservative behaviour in the water column. Minor constituents are bromine (Br), boron (B), strontium (Sr), silica (Si), and fluorine (F). Trace constituents include metals such as copper (Cu), iron (Fe), zinc (Zn), cadmium (Cd), nickel (Ni), cobalt (Co), lead (Pb), and manganese (Mn). Although the concentration of trace metals is most often at nanomolar levels or below,

they are key components in marine biogeochemistry. According to their distribution and chemical behaviour in seawater, trace elements can be classified as conservative, nutrient, scavenged, hybrid, and mixed, among which Cu exhibits a hybrid-like distribution.

2.1.1. Concentration and distribution of copper in marine and estuarine environment

In marine and estuarine environments, Cu is distributed between particulate and dissolved forms. This work focuses on the dissolved form (Cu_T). The distribution of Cu_T in the open ocean is generally characterised by lower surface concentrations that increase with depth (Heller and Croot, 2015; Liang et al., 2023; Wong et al., 2021; Zheng et al., 2024). While this distribution shares features with both nutrient-like and scavenged profiles, Cu concentration throughout the ocean water column increases almost linearly. The depth profile of Cu_T is influenced by biological uptake and remineralisation; other factors include reversible scavenging of Cu onto sinking particles and benthic sources of Cu. The model developed by Little et al. (2013) showed that reversible scavenging explains the approximately linear vertical distribution of Cu_T , which was further supported by Richon and Tagliabue (2019). In addition to reversible scavenging, a model by Takano et al. (2014), based on both the concentration and isotopic composition of Cu, suggested benthic flux as the missing source of Cu to deep water. Roshan et al. (2020) further elaborated that Cu removal by scavenging is balanced by benthic source of Cu_T , resulting in a gradual increase in Cu_T with depth.

However, the concentration and distribution of Cu_T vary seasonally and spatially, reflecting differences in biological productivity, water mass circulation, and the magnitude of external inputs. For example, the Pacific Ocean exhibits higher concentrations of Cu_T than the Atlantic Ocean, which is attributed to older waters and longer accumulation of regenerated trace metals along the global thermohaline circulation pathway (Bruland, 1980; Roshan et al., 2020; Takano et al., 2014). Furthermore, seasonal variations in Cu_T and other trace elements in the open ocean have been observed, with higher Cu_T concentrations during winter possibly resulting from mixing with deep waters that are slightly more enriched in Cu (Cloete et al., 2019; Tagliabue et al., 2014). During spring and summer, increased biological productivity leads to greater particle production and enhanced scavenging onto sinking particles, resulting in lower surface Cu_T concentrations. The largest seasonal Cu_T fluctuations have been observed in the Southern Ocean and the Mediterranean, and are linked to changes in surface physical and biogeochemical conditions as well as external nutrient sources (Richon and Tagliabue, 2019). Varying atmospheric deposition of Cu can also contribute to differences in Cu_T in the surface

ocean and coastal waters, underscoring the susceptibility of marine ecosystems to anthropogenic Cu-rich aerosols (Chance et al., 2015; Jordi et al., 2012; Yang et al., 2020). For example, due to northern Asian sources, anthropogenic aerosols dominate in the North Pacific, while Saharan dust supplies most natural aerosols to the equatorial Atlantic. However, these differences are more pronounced in coastal environments, where anthropogenic inputs locally elevate Cu_T concentrations to 50 nM or higher (e.g. Buck and Bruland, 2005; Moffett et al., 1997). For example, a multi-fold increase in Cu_T compared to open ocean has been observed in San Francisco Bay (Buck and Bruland, 2005). The lowest Cu_T in San Francisco Bay was found at the site most influenced by the Pacific Ocean, characterised by low Cu_T , while the highest Cu_T occurred at sites with restricted water exchange and higher levels of urban and industrial inputs. A similar increase in Cu_T was observed in harbours on the south coast of Cape Cod, where freshwater input and tidal flushing with coastal waters exhibiting lower Cu_T concentrations are limited (Moffett et al., 1997). Although increases in Cu_T have been observed in heavily utilised, urban estuaries such as Chesapeake Bay, the Gironde estuary, the estuaries of the Tumenjiang River and other north-west Pacific rivers (Audry et al., 2007; Dryden et al., 2007; Yu et al., 2025), seasonal increase has also been found in more pristine estuarine systems, including the Krka River estuary, the study site of this work (Cindrić et al., 2020, 2015). Considering vertical distribution of Cu_T in coastal waters, the variations may be more pronounced than in the open ocean and, depending on the dominant driver, several types of vertical profiles are observed (Bruland and Lohan, 2003). The surface enrichment is driven by riverine inputs, wastewaters discharge, atmospheric deposition and nautical activity, i.e. antifouling paints. On the other hand, processes like near-bottom remineralisation of organic matter and resuspension of Cu from the sediment may result in near-bottom maxima of Cu_T . Uniform Cu_T concentrations throughout the water column are usually observed in regions with strong physical mixing. However, estuaries show the largest vertical gradient of Cu_T , due to the interactions between freshwater, seawater and suspended particles (Sholkovitz, 1978).

Understanding these patterns requires assessing the sources and sinks of Cu in marine and estuarine environments, while recognizing the role of speciation on its distribution and behaviour.

2.2. Sources and sinks of copper in marine and estuarine environment

Sources of Cu in the marine environment are both natural and anthropogenic. Most Cu enters the oceans through riverine discharge and atmospheric deposition, while other significant sources include rock weathering, soil erosion, sedimentary fluxes, hydrothermal inputs, and anthropogenic activities. The relative importance of these sources, as well as the processes controlling Cu distribution and cycling, varies spatially and depends strongly on regional environmental conditions (Bruland and Lohan, 2003; Richon and Tagliabue, 2019). The major external sources, internal cycling pathways, and removal processes controlling marine Cu distribution discussed below are summarized in **Figure 1**.

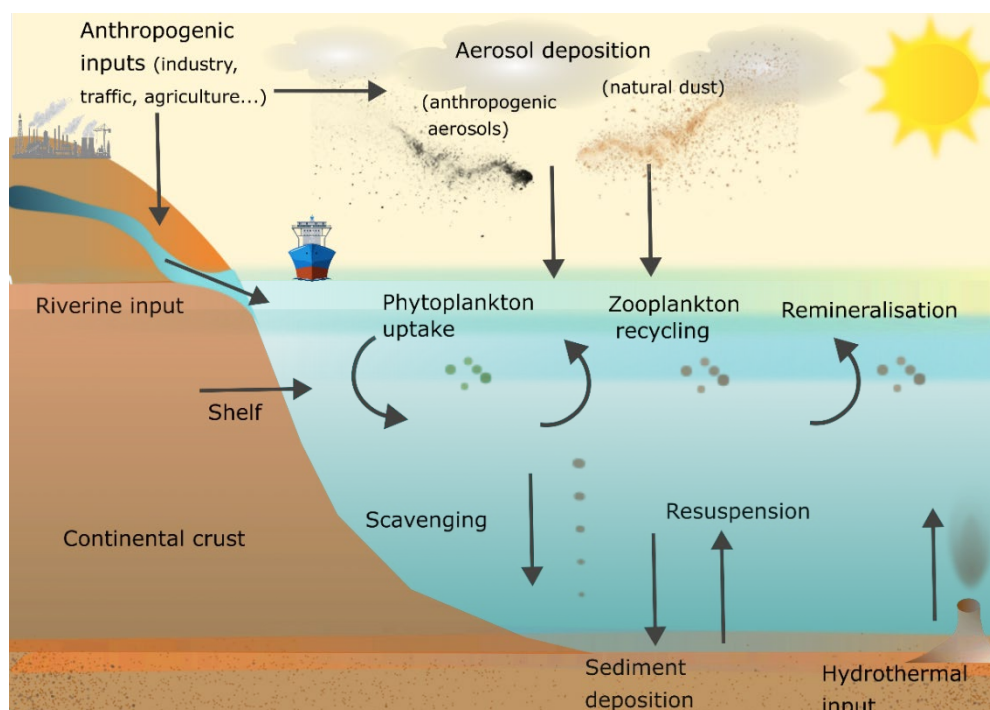


Figure 1. Schematic of the main sources, cycling pathways, and sinks of Cu_T in the marine environment.

Although estimates of riverine Cu fluxes differ among studies due to variations in datasets, sampling coverage, and methodological approaches, rivers are consistently identified as the dominant external source of Cu to the ocean (Bruland and Lohan, 2003; Little et al., 2014; Richon and Tagliabue, 2019; Vance et al., 2008). Concentrations of Cu_T in rivers span a wide range, reflecting differences in lithology, hydrological conditions, and the degree of anthropogenic influence across river basins. Pristine rivers, such as those draining the Kamchatka Peninsula and the Kalix River, typically exhibit low Cu_T concentrations (<10 nM)

(Vance et al., 2008). Similarly, despite increasing anthropogenic pressure over the past century, Cu_T concentrations in the Amazon River remain similar to those measured decades ago, likely because the enormous river discharge minimises the impact of Cu pollution (Hollister et al., 2021). The Krka River, the focus of this study, is also considered a relatively pristine system (e.g. Cukrov et al. 2008). In contrast, substantially elevated Cu_T concentrations have been reported in anthropogenically impacted rivers, including the Rhine (>40 nM), Mississippi River (>70 nM), Yangtze (>100 nM), and the Ganges (~ 700 nM) (Bussan et al., 2017; Mitra et al., 2018; Vega and Weng, 2013; Wang et al., 2020). Under extreme anthropogenic disturbance, Cu_T can reach concentrations several orders of magnitude higher, as exemplified by Río Tinto, an acid mine drainage endmember (Olías et al., 2020).

Atmospheric deposition represents another major external Cu source and includes both natural and anthropogenic particulate inputs. Although estimates of atmospheric Cu deposition vary due to differences in modelling approaches and assumptions regarding deposition fluxes and Cu solubility, studies generally agree that natural aerosols dominate atmospheric Cu inputs (Little et al., 2014; Richon and Tagliabue, 2019). Natural aerosols include mineral dust, volcanic emissions, and biogenic particles, whereas anthropogenic aerosols derive mainly from road traffic, fossil fuel and biomass combustion, and industrial processes (Duan and Tan, 2013; Moreno et al., 2009). Unlike riverine inputs, which can be substantially modified in estuaries and coastal waters, atmospheric deposition directly influences the surface ocean.

Emerging evidence further indicates that hydrothermal systems represent a significant source of Cu, challenging the traditional view that vent-derived metals were removed locally through sulphide and oxide precipitation and therefore contributed negligible fluxes to the open ocean (Sander and Koschinsky, 2011; Stüeken, 2020). Complexation with organic ligands associated with hydrothermal fluids stabilises metals, including Cu, and facilitates their transport into the deep ocean. Models estimate that hydrothermal vents contribute approximately 2–20% (Stüeken et al., 2020) or 5–14% (Sander and Koschinsky, 2011) of the oceanic Cu_T inventory.

Anthropogenic Cu inputs to the marine environment include industrial activities (e.g. electrical equipment manufacturing), mining, agriculture (fertilisers and pesticides), and transportation (Nriagu and Pacyna, 1988; Rauch and Graedel, 2007). Although Cu has been used as an antifouling agent for centuries, Cu-based antifouling paints have become particularly important over the last two decades following the global ban of tributyltin (TBT) in 2008 (Yebra et al., 2004). Recent studies from the Baltic Sea indicate that Cu inputs from maritime shipping and

leisure boating account for more than one-third of the annual Cu load, exceeded only by rivers (Lagerström et al., 2026; Ytreberg et al., 2022). Their relative contribution depends on vessel density, paint composition, and salinity. For example, Cu leaching generally increases with salinity because cuprous oxide (Cu_2O), a common antifouling pigment, forms soluble chloride complexes. In contrast, temperature effects on Cu leaching are more complex and depend on coating formulation and release mechanisms (Kiil et al., 2002; Singh and Turner, 2009).

When riverine Cu_T reaches estuaries, non-conservative processes such as flocculation and scavenging during increasing salinity may substantially modify and remove Cu_T before transport into the open ocean (Section 2.4). Once in the ocean, Cu is further regulated by internal cycling processes, including biological uptake, passive scavenging, water-column recycling, and benthic exchange, as illustrated in **Figure 1**. In the upper 100 m, phytoplankton accounts for most biological Cu uptake, while some Cu is also removed as sinking particulate matter (Richon and Tagliabue, 2019). Much of the biologically assimilated Cu is recycled through zooplankton grazing and bacterial remineralisation, while the remainder sinks as particulate organic matter (POM). Approximately one-half to two-thirds of Cu burial in oxic sediments is associated with Fe–Mn oxyhydroxides, while the remainder is likely linked to sulphides and POM (Little et al., 2014). Although sediments are generally regarded as the ultimate sink for trace metals in seawater, they may also act as a source through resuspension, diffusion, and bioturbation (Libes, 2009). The mobility of Cu in sediments depends on sediment composition, physicochemical conditions in both sediments and overlying waters, biogeochemical reactions, and physical and biological transport mechanisms. Decreasing pH, changing redox conditions, and elevated salinity can promote Cu remobilisation from sediments.

2.3. Copper speciation in marine and estuarine environments

2.3.1. Fundamental terminology in trace metal speciation

To properly understand the concept of speciation, it is necessary to define chemical species, speciation analysis, speciation, and fractionation. A chemical species is a form of an element defined by its isotopic composition, electronic or oxidation state, and/or molecular or complex structure (Templeton and Fujishiro, 2017). Isotopic composition can provide information on the sources and biogeochemical transformations of an element (e.g. Araújo et al., 2017; Gao, 2016; Tu et al., 2023). The oxidation state influences the chemical and biological behaviour of an element. For example, Cr occurs mainly as Cr(III) and Cr(VI) in marine systems, with

Cr(III) generally being less soluble and more particle-reactive, while Cr(VI) is favoured under oxic conditions and is generally more toxic and bioavailable (Geisler and Schmidt, 1991). Similarly, Fe(II) is more soluble than Fe(III), although it can rapidly oxidise under oxic conditions; organic complexation can substantially increase the solubility and bioavailability of dissolved Fe (Leigh-Smith et al., 2018; Lin et al., 2018). Speciation is defined as the distribution of an element among its defined chemical species within a system (Templeton and Fujishiro, 2017). In marine waters, this distribution is controlled primarily by ligand availability, pH, redox conditions, and ionic strength, and can be further modified by biological and photochemical processes. The complexity of seawater, with numerous species occurring at low concentrations in a high-ionic-strength matrix, presents substantial analytical challenges (Bruland and Lohan, 2003). Speciation analysis therefore refers to the analytical procedures used to identify and quantify individual chemical species, using approaches such as spectrometric, electroanalytical, chromatographic, and extraction-based techniques (Templeton and Fujishiro, 2017). Fractionation involves separating or classifying an analyte or groups of analytes according to physical or chemical properties, such as size, solubility, binding affinity, or reactivity. Because individual species may be unstable, difficult to determine separately, or altered during analysis, environmental studies often characterise operationally defined groups rather than individual species. Thus, speciation describes the distribution of chemically defined species, whereas fractionation separates groups according to defined properties or analytical procedures. For example, soluble/insoluble or particle-size fractions provide information on the distribution of an element but do not necessarily identify the individual chemical species within each fraction. These distinctions provide the conceptual basis for examining the speciation of Cu in natural waters.

2.3.2. Copper speciation with respect to natural organic and inorganic ligands

Copper speciation in seawater is influenced by complexation with both inorganic and organic ligands. Inorganic Cu speciation is relatively well characterised and can be described using established thermodynamic equilibrium constants. For Cu(II), the principal inorganic ligands in seawater are carbonate (CO_3^{2-}) and hydroxide (OH^-), with CuCO_3 representing the most important inorganic Cu(II) species under typical seawater conditions (Turner et al., 1981). Complexes between Cu(II) and chloride are negligible in seawater conditions, but are particularly important for Cu(I), which forms various cuprous chloride complexes (Turner, 1996; Zirino and Yamamoto, 1972). While inorganic Cu speciation can be described relatively well, the organic Cu-binding ligand pool in natural waters is considerably more complex and

heterogeneous. It is well established that more than 99% of Cu_T occurs in complexes with organic ligands (Ruacho et al., 2022 and references therein). Organic complexation also contributes substantially to the speciation of several other trace metals, including Fe, Zn, Co, and Cd. For Cu, organic complexation generally decreases the concentration of Cu^{2+} and can therefore reduce Cu toxicity and influence its bioavailability. However, organically complexed Cu is not necessarily biologically unavailable, as some Cu complexes can contribute to biological Cu uptake (Section 2.5.3). Natural organic Cu-binding ligands are heterogeneous and include humic substances, thiol-containing compounds, and other organic compounds with different Cu-binding affinities (Section 2.6.3). The molecular structures of some Cu-binding ligands are well defined, whereas many Cu-binding sites within natural DOM cannot be assigned to individual compounds because of their chemical heterogeneity. In the absence of structural characterisation of individual Cu-binding ligands, their complexation is commonly described using conditional stability constants and ligand concentrations obtained from speciation measurements. Unlike thermodynamic stability constants, which describe the equilibrium between a defined metal ion and ligand, conditional stability constants account for the chemical conditions under which the complexation is measured, including pH and competition from other ions (Gledhill and Gerringa, 2017). Because natural Cu-binding ligands are heterogeneous and their individual molecular identities are often unknown, these conditional constants provide an operational measure of their Cu-binding strength rather than a universal thermodynamic constant for a single defined compound. The exact structure of Cu-binding organic ligands is still under investigation; therefore, they are commonly classified, based on conditional stability constants, into a stronger ligand class (L_1 , $\log K'_{\text{Cu}L_1} > 13.5$) and a weaker ligand class (L_2 , $\log K'_{\text{Cu}L_2} < 13.5$), although this boundary is not strictly defined and depends on the system under investigation (Ruacho et al., 2022). Progressively higher L_i classes represent weaker ligand pools. However, these classes are operational rather than chemically defined and do not necessarily correspond to individual compounds. The L_1 , L_2 , and L_3 notation has been used in marine and estuarine Cu studies for decades, and the number and strength of ligand classes resolved depend partly on the analytical method, detection window, and characteristics of the samples (Ruacho et al., 2022). Consequently, conditional stability constants and ligand concentrations obtained for different ligand classes should be interpreted in relation to the analytical method and experimental conditions used for their determination. The most commonly used methods are anodic stripping voltammetry (ASV) and competitive ligand exchange-adsorptive cathodic stripping voltammetry (CLE-AdCSV).

2.3.3. Redox speciation of copper

2.3.3.1. Thermodynamic insight into copper redox speciation

Speciation models usually assume that the only significant oxidation state of Cu in seawater is +II (e.g. Turner et al., 1981; Zirino and Yamamoto, 1972; Zuehlke and Kester, 1983). Under oxic conditions, +II is indeed the thermodynamically favourable oxidation state of Cu. This conclusion follows from equilibrium calculations performed on the assumption that seawater is controlled by a sufficiently oxidising redox couple. For example, assuming equilibrium with atmospheric O₂ through the O₂/H₂O couple gives a negative logarithm of electron activity (pE) of approximately 12.5 at seawater pH, corresponding to a redox potential (E_h , the potential in seawater sample relative to standard hydrogen electrode (SHE)) of about +0.74 V. On the other hand, Breck (1972) suggested that the O₂/H₂O₂ couple may be more relevant to the redox potential of oxygenated natural waters, with substantially lower potentials than predicted from the O₂/H₂O couple. Even under these less oxidising conditions, Cu(II) remains thermodynamically favoured over Cu(I). However, these values represent a thermodynamic equilibrium potential and should not be regarded as the actual redox potential of seawater, because in seawater many kinetically slow redox reactions occur, complicating redox equilibrium measurements (Lindberg and Runnells, 1984).

The thermodynamic relationship between the Cu oxidation states can be examined more directly using the standard electrode potentials (E°) of the simple aqueous Cu species (Pfennig, 2015):



The values in Eqs. (1)–(3) are standard potentials for the simple aqueous Cu species and should not be applied directly to seawater, where Cu is strongly complexed (Section 2.3.2). For the uncomplexed Cu species, however, the potentials demonstrate why Cu(I) is prone to disproportionation. Combining Eqs. 1 and 3 (reversing Eq 3. to represent oxidation of Cu(I)) gives:

$$E^\circ_{\text{disproportionation}} = E^\circ_{\text{Cu}^+/\text{Cu}} - E^\circ_{\text{Cu}^{2+}/\text{Cu}^+} = 0.520 \text{ V} - 0.159 \text{ V} = 0.361 \text{ V} \quad \text{Eq. 4}$$

This indicates that disproportionation is thermodynamically favoured under standard conditions. Nonetheless, Cu(I) formation may occur through non-equilibrium dynamic

biochemical, photochemical or thermochemical processes (Moffett and Zika, 1983), discussed further in the following sections. Consequently, the occurrence of Cu(I) in oxic seawater reflects a balance between its continuous production and its removal, rather than thermodynamic equilibrium alone.

2.3.3.2. *Evidences for copper(I) occurrence in natural waters*

Copper redox speciation in natural waters has largely been neglected; consequently, speciation models generally assume Cu(II) to be the only environmentally relevant oxidation state of Cu. Nevertheless, significant fractions of Cu(I) have been detected in the open ocean, estuaries, and rainwater (Buerge-Weirich and Sulzberger, 2004; Kieber et al., 2004; Moffett and Zika, 1988). These observations demonstrate that Cu(I), despite its thermodynamic instability under oxic conditions, can persist in natural aquatic environments and may represent an environmentally relevant fraction of the Cu_T pool. Moffett and Zika (1988) reported Cu(I) fractions of 5–10% in the western Atlantic Ocean and the Gulf of Mexico. The highest fractions were measured at the surface and decreased with depth, while Cu(I) was not detected below the photic zone. The authors attributed the formation and stabilisation of Cu(I) to various photochemical processes involving hydrogen peroxide, as well as to the presence of organic reductants such as humic substances and biologically derived organic matter. The significant influence of photochemical processes on the redox speciation of trace metals results from the photo-oxidation of DOM in the photic zone, which leads to the formation of superoxide and peroxide radicals capable of reducing complexed Cu(II) (Moffett and Zika, 1983; Voelker et al., 2000). Voelker et al. (2000) investigated the interaction of superoxide radicals with naturally occurring organic Cu complexes and observed that CuL₂ complexes reacted with superoxide anions to a nearly equal extent as inorganic Cu complexes, whereas biologically produced CuL₁ complexes were less reactive. Their observations demonstrated that the chemical nature of the organic ligand can influence the susceptibility of complexed Cu(II) to reduction and, consequently, may affect the persistence of Cu(I) in natural waters. Furthermore, the concentrations of Cu(I) formed during the radiolysis of Cu-amended seawater were substantially lower than expected based on the proposed role of superoxide as the major reductant. This discrepancy suggested that a fraction of the Cu(I) produced was associated with organic complexes. Cu(I) has also been detected in atmospheric deposition, where Kieber et al. (2004) reported Cu(I) fractions of 22–32% in rainwater. The formation and stabilisation of Cu(I) in rainwater were associated with the photochemical reduction of Cu(II) complexes, reduction of Cu(II) by organic matter, and stabilisation of Cu(I) by inorganic species such as

sulphite. These observations further demonstrate that Cu(I) formation is not restricted to marine waters and that both organic and inorganic components can contribute to its stabilisation. Buerge-Weirich and Sulzberger (2004) reported Cu(I) fractions ranging from 5 to 80% in the Scheldt estuary (the Netherlands/Belgium) and the North Sea. The presence of Cu(I) was associated with light-induced reduction of Cu(II) complexes and chloride complexation in high-salinity regions, as well as with thermal reduction of Cu(II) and subsequent stabilisation by sulphur-containing species, such as thiols, in low-salinity regions. In contrast, the low Cu(I) fractions observed at intermediate salinities were most likely attributed to the rapid oxidation of Cu(I) due to the presence of strong Cu(II)-binding ligands. These findings highlight the strong influence of salinity, ligand composition, redox-active species, and photochemical transformations on Cu redox speciation. However, the available observations of Cu(I) in natural waters should be interpreted with caution because methodological constraints can affect the measured redox distribution (Section 2.7).

2.4. Estuaries - key interfaces for trace metal speciation

Speciation of trace metals, including Cu, depends on various physical, chemical, and biological parameters, such as pH, salinity, redox potential, hydrodynamic conditions, the presence of microorganisms, and complexation by autochthonous and allochthonous organic ligands (Libes, 2009). Consequently, in estuarine systems, where pronounced physical and chemical gradients occur, significant changes in trace metal partitioning and speciation arise, making the behaviour of trace metals in estuarine waters more complex than in other natural aquatic systems (Millward, 1995). Estuaries are semi-enclosed coastal water bodies where freshwater from land drainage mixes with marine water via a free connection to the open sea (Cameron and Pritchard, 1963). Estuaries can be classified according to water balance, geomorphology, vertical salinity structure, and hydrodynamics (Valle-Levinson, 2010). Based on vertical salinity structure, which is governed by the relative intensities of tidal mixing and river discharge, estuaries can be classified as salt wedge, highly stratified, weakly stratified, and vertically mixed (homogeneous) estuaries. Salt wedge estuaries, such as the Mississippi River (USA), Río de la Plata (Argentina), Rhône River (France), and Krka River (Croatia), form under conditions of high river discharge and weak tidal forcing (Ibañez et al., 1997; Valle-Levinson, 2010; Viličić et al., 1989). Salt wedge estuaries are strongly stratified during flood tides, with a wedge-shaped intrusion of seawater, a sharp halocline, dominant outflow in most of the water column, and weak near-bottom inflow due to limited mixing, though this structure may weaken during dry periods. A highly stratified estuary forms where tidal range is small

and river flow dominates, producing a sharp, persistent halocline with increasing surface salinity seaward and a relatively unchanged deep layer. In contrast, weakly stratified estuaries result from weak to moderate river discharge and moderate to strong tidal forcing. The mean salinity profile exhibits weak or continuous stratification (except near the bottom mixed layer), with relatively strong exchange flow driven by mixing between river and ocean waters. Examples include the Tamar River (Australia), Chesapeake Bay (USA), and Hudson River (USA). Finally, strong tidal forcing combined with low river discharge produces vertically mixed estuaries, where salinity is nearly uniform with depth and flow is relatively uniform throughout the water column. Examples of vertically mixed estuaries are the Scheldt (the Netherlands/Belgium) and Weser (Germany).

Metal concentrations in estuarine waters can be assessed using the relationship between metal concentration and salinity, schematically illustrated as a simplified two-endmember system consisting of riverine and marine waters (Wen et al., 1999). A linear relationship between metal concentration and salinity indicates conservative behaviour, whereas deviations from linearity reflect non-conservative behaviour, resulting in either enrichment or removal of a given trace metal within the estuary. Particle-reactive metals such as Fe and Pb typically show non-conservative removal due to aggregation and sedimentation of colloidal and particulate phases during estuarine mixing. In contrast, Cu and Zn often exhibit more variable behaviour because their speciation is strongly influenced by complexation with organic ligands, adsorption–desorption reactions, and biological activity. Both conservative and non-conservative behaviour of Cu have been reported in the literature; importantly, anthropogenic inputs may substantially contribute to non-conservative behaviour (Ackroyd et al., 1986; Boyle et al., 1982; Cindrić, 2015; Hollister et al., 2021; Roux et al., 1998).

The sorption and desorption of metals onto particles, together with the behaviour of these particles in estuarine environments, strongly influence the fate and transport of metals in estuaries. In river waters characterised by low salinity and ionic strength, suspended colloidal particles are kinetically more stable because their gravitational settling velocity is lower than the random motion caused by collisions with surrounding water molecules (Brownian motion) (Mosley and Liss, 2020; Tercier-Waeber et al., 2012). Large biopolymers are particularly prone to aggregation, so colloidal aggregation and sedimentation rates depend strongly on the concentration of biologically produced organic macromolecules (Chen et al., 2005). In contrast, in estuarine and marine waters, where salinity and ionic strength are substantially higher, changes in colloidal surface charge occur, most markedly within the so-called critical salinity

of 5–8 (Telesh and Khlebovich, 2010). Riverine particles entering estuaries are highly heterogeneous, but their surfaces are commonly coated with natural organic matter, resulting in predominantly negatively charged particles that remain stable in low-salinity waters due to electrostatic repulsion (Mosley and Liss, 2020 and references therein). As salinity increases during estuarine mixing, seawater cations such as Ca^{2+} and Mg^{2+} compress the electrical double layer surrounding the particles. Once the double layer is sufficiently compressed, van der Waals attractive forces dominate over the electrostatic interactions prevailing in river water, reducing repulsive forces and promoting coagulation and flocculation. Consequently, colloidal particles rapidly aggregate into larger flocs that can settle from the water column, strongly influencing particle dynamics and trace metal transport in estuaries. Conversely, sediment resuspension in estuarine waters may release trace metals back into the water column (e.g. Simpson et al. 1998; Cukrov 2021). Estuarine pH typically decreases slightly at low salinities, increases at intermediate salinities, and stabilises at higher salinities as a result of changes in ionic strength and carbonate system equilibria during freshwater–seawater mixing (Mosley et al., 2010). The extent and pattern of these pH changes depend on river water composition, including alkalinity and dissolved inorganic carbon concentrations. In addition, biological activity, sediment processes, eutrophication, and climate-driven increases in carbon dioxide (CO_2) can further modify estuarine pH and consequently influence trace metal speciation and sorption behaviour.

2.4.1. Krka River estuary

The Krka River is located on the eastern coast of the Adriatic Sea in Croatia and is a medium-sized river that drains predominantly carbonate terrains. The catchment is largely underlain by limestone, resulting in karstification and the formation of characteristic tufa barriers. The freshwater section is 49 km long, while the salt-wedge 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 of between 1.5 and 3.5 m, while its position and thickness depend on river flow, weather conditions and location. Seawater renewal time ranges from 50 to 250 days and also depends on river flow (Legović, 1991). The average annual flow is approximately $50 \text{ m}^3/\text{s}$, with a minimum as low as $5 \text{ m}^3/\text{s}$ and a maximum as high as $450 \text{ m}^3/\text{s}$ (Bonacci et al., 2017).

A sharp halocline at the FSI accumulates sinking particles; consequently, it is enriched with trace elements, organic matter, nutrients and microorganisms of both freshwater and seawater origin (Cauwet, 1991; Louis et al., 2009; Viličić et al., 1989; Žutić and Legović, 1987). Riverine

phytoplankton from Visovac Lake die in the estuary due to increased salinity, and the dead phytoplankton sink to the bottom, supplying 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 with other European rivers and estuaries (Marcinek et al., 2020), and generally show low levels of trace metals. The pristine Krka estuary has been the focus of research interest for decades, and numerous articles have been published on the distribution, speciation and dynamics of trace metals (e.g. Cindrić et al., 2015; Cukrov et al., 2008; Elbaz-Poulichet et al., 1991; Marcinek et al., 2022; Marcinek et al., 2025; Omanović et al., 2006). Nevertheless, only a few studies have examined the redox speciation of trace metals (Knežević et al., 2021; Pađan et al., 2019), and Cu redox speciation has not yet been investigated. Although Cu concentrations in the estuary are relatively low, a multifold increase in Cu concentrations has been reported during the summer season, due to Cu emissions from antifouling coatings (Carić et al., 2021; Cindrić et al., 2020, 2015). For this reason, studies on Cu speciation in the estuary are of considerable research interest.

Within the scope of this thesis, sampling campaigns were conducted at the E1, E2 and E3 sampling sites (**Figure 2**). Sampling sites E1 and E2 represent a “clean” and a “polluted” site, respectively. Site E2 is located in the marina and is therefore significantly influenced by anthropogenic activities than site E1. At both sites, samples were taken at different depths in spring and summer 2023 and 2024 to determine Cu redox speciation in the brackish surface layer, at the halocline and in the seawater layer. In addition, four samples were taken from the surface layer at E2 every 5–7 h over an 18 h period in summer 2023 to determine the effects of solar radiation on Cu redox speciation. The diel changes in Cu redox speciation were further investigated in the vertical water column at E1 in summer 2025. Additionally, an automatic rainwater sampler was placed at the E3 sampling site, at the Marine Research Station Martinska, Ruđer Bošković Institute, and rainwater samples were periodically collected from October 2023 to September 2024, in parallel with estuarine surface water samples at the same site.

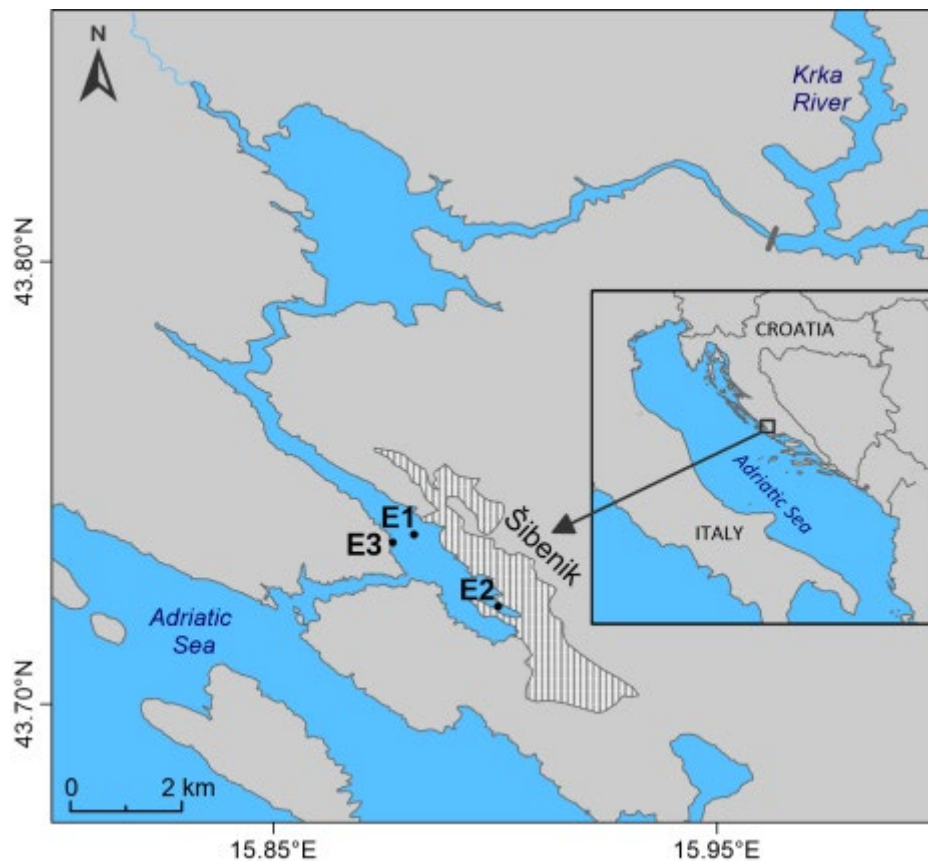


Figure 2. Map of the study section of the Krka River estuary with indicated sampling sites: E1, E2 and E3.

2.5. Interactions of copper with marine biota

2.5.1. Copper as an essential micronutrient

As an essential trace metal, Cu is required for the growth and metabolism of marine microorganisms. Due to its ability to alternate between Cu(I) and Cu(II) oxidation states, Cu participates in numerous enzymatic redox reactions and electron transfer processes. It acts as a cofactor in enzymes involved in photosynthesis, respiration, iron acquisition, nitrogen metabolism and protection against oxidative stress. One of the most important roles of Cu in marine phytoplankton is its participation in electron transport through the plastocyanin. Plastocyanin is Cu-containing protein where single Cu atom is coordinated by two imidazole nitrogens, a thioether sulfur, and a thiolate sulfur, which stabilize Cu(I) oxidation state (Redding and Santabarbara, 2023). Some diatoms use plastocyanin in photosynthesis instead of Fe-containing cytochrome *c6*, since Cu is usually more abundant than Fe in the open ocean (Peers and Price, 2006). Cu deficiency in *Thalassiosira oceanica* likely impairs the production of functional plastocyanin, resulting in reduced efficiency of photosynthetic electron transfer.

In many algae, Cu limitation can induce the synthesis of cytochrome *c6* as an alternative electron carrier; however, observations for diatom *Thalassiosira oceanica* indicate that this compensatory mechanism is probably absent, as variations in Fe availability did not affect photosynthetic electron transport under low Cu conditions. Copper is also an important structural and catalytic component of several enzymes, including Cu/Zn-superoxide dismutase (Cu/Zn-SOD), multicopper oxidases, amine oxidases, and nitrite reductases. Copper/Zn-SOD is an antioxidant metalloenzyme containing Cu and Zn metal centres that catalyses the dismutation of superoxide radicals into molecular oxygen and hydrogen peroxide, thereby representing one of the primary enzymatic defence mechanisms against oxidative stress in marine organisms (Zeinali et al. 2015 and references therein). In Cu/Zn-SOD, Cu cycles between Cu(I) and Cu(II) states. Multicopper oxidases catalyse the four electron reduction of molecular oxygen to water while simultaneously oxidising a broad range of substrates, therefore they contain at least four Cu atoms organised into three Cu centers (Nikolaivits et al., 2021). Certain low-molecular-weight proteins and peptides present in algal cells, such as metallothioneins and phytochelatins, are also capable of binding Cu, specifically in Cu(I) form, since they are rich in thiol groups which bind Cu(I) over Cu(II) (Pearson, 1963). However, these compounds are not considered cuproproteins or cuproenzymes because Cu is not required for their biological activity as a catalytic cofactor; instead, Cu binding occurs primarily as a detoxification and metal homeostasis mechanism (Cobbett and Goldsbrough, 2002; Palacios et al., 2011). Amine oxidases in marine phytoplankton are cell-surface Cu-dependent enzymes that catalyse the oxidative deamination of primary amines, producing extracellular hydrogen peroxide, aldehydes, and ammonium. The released ammonium is subsequently utilised by phytoplankton as a nitrogen source, indicating that these enzymes contribute to nitrogen acquisition under nitrogen-limited conditions (Palenik and Morel, 1991). A Cu dependent metalloenzyme, Cu-containing nitrite reductase, catalyses the reduction of nitrite to nitric oxide, has been reported in both denitrifying and non-denitrifying microorganisms (Kondo et al., 2012). Furthermore, because of its role in Fe acquisition, Cu may be an important nutrient in Fe-depleted regions of the oceans. The experiments conducted in the Bering Sea (Fe-depleted) have shown that addition of Cu doubled the phytoplankton growth, while combined with Fe addition increase the growth compared to the samples treated with Fe only (without Cu) (Peers et al., 2005). While the examples discussed above demonstrate that Cu is an essential micronutrient for marine microorganisms, the required concentrations vary among species. Moreover, the same species may exhibit different Cu requirements in oceanic compared with coastal environments. Whereas the cellular requirements for trace metals such as Fe and Zn are

generally lower in oceanic than in coastal microorganisms, Cu exhibits the opposite trend. For example, approximately 10-fold higher Cu quotas were reported for oceanic compared with coastal *Thalassiosira oceanica* (Peers and Price, 2006). In general, *Thalassiosira oceanica* has a relatively high Cu quota due to its reliance on the Cu-containing protein plastocyanin, and Cu depletion may therefore impair photosynthetic electron transport. Many algae that have plastocyanin are known to synthesize cytochrome *c6* under Cu deficiency, although that is not the case for *Thalassiosira oceanica*. Similar diatom *Thalassiosira weissflogii*, that uses cytochrome *c6* instead of plastocyanin for electron transport, has lower Cu quota. Moreover, higher Cu quotas are needed in Fe-limited Southern Ocean compared to Equatorial Pacific and North Atlantic Ocean (Kong 2022 and references therein). Marine bacteria exhibit pronounced interspecies variability in Cu requirements and Cu quotas; for example, growth and cellular Cu content of *Pseudoalteromonas* spp. remained largely unaffected by decreasing Cu availability, whereas *Dokdonia* sp. showed up to approximately 80 % growth reduction and substantially decreased Cu quotas under Cu-depleted conditions, indicating markedly higher physiological Cu demand (Posacka et al., 2019). On the other hand, exceeding cellular Cu quotas may exert toxic effects on marine microorganisms.

2.5.2. Toxicity of copper to marine organisms

The toxicity of Cu is primarily associated with the formation of reactive oxygen species (ROS) and interference with essential cellular processes. Early studies by Brand et al. (1986) demonstrated that sensitivity to Cu toxicity among marine microorganisms is strongly phylogenetically dependent, with cyanobacteria being the most sensitive group, followed by dinoflagellates and coccolithophores, whereas diatoms are among the least sensitive taxonomic groups. Reduced reproduction rates of cyanobacteria were observed at Cu^{2+} concentrations as low as 1 pM, and Cu^{2+} concentrations of 10 pM did not affect the reproduction rates of most eukaryotic algae, among which *Emiliana huxleyi*, *Skeletonema costatum*, *Thalassiosira pseudonana* and *Thalassiosira oceanica* were the most resistant to Cu (Brand et al., 1986). While most data correlating phytoplankton growth and Cu^{2+} are included in the above-mentioned study, several other studies have confirmed and expanded insights into Cu-induced inhibition of marine microorganism growth by including additional species and discussion of mechanisms. Many of these are summarised in the review article by Lopez et al. (2019), which found no additional trends in Cu toxicity with respect to microalgal biogeography, finer taxonomic divisions, cell size, or surface area to volume ratios. However, cross-study comparison was severely limited because of methodological discrepancies between the studies,

resulting in many of the studies reporting only Cu_T , although Cu^{2+} is considered more relevant (Leal et al., 2016; Lopez et al., 2019 and references therein). Control of trace metal speciation in culture media is the most overlooked parameter, and it is essential for producing reproducible and comparable results in Cu toxicity studies. An increase in Cu_T concentration in the diatom *Ditylum brightwellii* resulted in greater adsorption to the cell surface and induced oxidative stress, as evidenced by depletion of reduced GSH and increased SOD activity. This led to impaired photosynthesis and cell division, reduced chlorophyll content, and caused cellular deformation and breakage (Rijstenbil et al., 1994). Similar conclusions regarding Cu-induced oxidative stress arise from studies on *Chaetoceros calcitrans*, which further demonstrated that Cu adsorption is rapid, while intracellular uptake is the rate-limiting step (Anu et al., 2016). Additionally, several antioxidant enzymes were examined, and enzymatic inactivation was observed under prolonged exposure to elevated Cu_T . These studies represent only a few examples of Cu-induced cellular damage, as numerous additional examples have been reported (e.g. Cervantes-Cervantes et al., 2005; Overnell, 1974; Pandey and Bergey, 2016; Park et al., 2020; Paytan et al., 2009). To maintain optimal intracellular Cu levels and prevent Cu toxicity, cellular Cu quotas are maintained within a narrow range regulated by Cu uptake and efflux systems.

2.5.3. Bioavailability and cellular uptake of copper

Definitions of bioavailability can vary between different fields; however in marine chemistry, it is most appropriate to use the general definition that bioavailability is “the extent of absorption of a substance by a living organism compared to a standard system” (Nordberg et al., 2010). Metal bioavailability depends on the transport of metal species to the cell surface, their interaction with membrane receptor sites, and often their internalisation into the cell, where they can affect intracellular processes and induce biological responses such as altered growth, respiration, reproduction, or photosynthesis (Tercier-Waeber et al., 2012). Environmental parameters such as pH, alkalinity, and water hardness alter metal speciation and modify competitive interactions at biological interfaces. Interactions with other trace metals may also influence uptake through competition for transporters and cellular binding sites. Furthermore, anthropogenic ligands, including ethylenediaminetetraacetic acid (EDTA), can substantially modify metal speciation and thus regulate metal bioavailability and toxicity. Generally, increasing water hardness lowers metal bioavailability because Ca^{2+} and Mg^{2+} compete with trace metals for uptake and surface binding sites. Variations in pH can influence metal uptake by altering metal speciation, proton competition, and membrane surface charge.

However, all of these factors ultimately influence trace metal speciation, which remains the primary determinant of Cu bioavailability and, consequently, Cu toxicity.

2.5.3.1. Extracellular copper speciation as a determinant of copper toxicity

The pioneering work demonstrated that Cu toxicity and cellular Cu uptake are controlled by Cu^{2+} activity rather than Cu_T concentration (Anderson and Morel, 1978; Jackson and Morgan, 1978; Sunda, 1975; Sunda and Guillard, 1976). Naturally occurring organic ligands reduced Cu toxicity by complexing Cu and decreasing cellular uptake, supporting the importance of Cu speciation in determining bioavailability and toxicity. This was further supported by Sunda and Lewis (1978) and Brand et al. (1986). These observations led to the development of the free ion activity model (FIAM), which was later extended into the biotic ligand model (BLM) (Brown, 2000; Morel, 1983; Paquin et al., 2002). Both models propose that divalent metals must first bind to transport or receptor sites on the organism surface prior to internalisation, meaning that metal uptake and toxicity are primarily controlled by the concentration of free metal ions in solution. However, this relationship should not be interpreted to imply that the Cu^{2+} itself is the species transported across the cell membrane. Rather, free Cu activity represents the chemical reactivity of Cu at the biological interface, where reduction, ligand exchange and interactions with membrane transport systems occur before cellular uptake. Free metal ion is considered to be in equilibrium with cell surface binding sites (Morel, 1983). Metal species that do not readily react with these binding sites are therefore considered biologically inactive. Accordingly, metal-binding ligands present in seawater DOM are expected to reduce the bioavailability of metals such as Cu. In seawater, the inorganic Cu pool comprises Cu^{2+} and inorganic Cu complexes (CuX_i), which together constitute the labile Cu pool (Cu') (Hudson, 1998). These inorganic complexes rapidly dissociate near the cell surface and can replenish Cu^{2+} during uptake, thereby contributing to Cu bioavailability and toxicity. However, more recent work implied that the Cu^{2+} diffusion cannot be the only mechanism governing Cu uptake. Calculations demonstrated that diffusive flux of inorganic Cu alone could not explain the observed Cu uptake, suggesting that organically complexed Cu may be bioavailable through rapid dissociation of these complexes within the cell boundary layer (Hudson, 1998; Quigg et al., 2006; Sunda and Huntsman, 1995). Furthermore, Annett et al. (2008) found that Cu uptake correlated better with Cu_T than with Cu^{2+} , indicating the bioavailability of at least part of organically complexed Cu. Similarly, Semeniuk et al. (2009) noted that the oxidation state of Cu and the Cu:ligand ratio are more important for Cu uptake than Cu^{2+} concentration in solution, providing the first evidence of the bioavailability of organically complexed Cu. In

subsequent work, the authors proposed that Cu uptake may occur via enzymatic reduction of Cu(II) complexes at the cell surface to Cu(I), which is then transported into the cell through high-affinity Cu(I) transporters (Semeniuk et al., 2015). Another possible mechanism is ligand exchange between weak Cu-binding organic ligands and cellular Cu transport sites, enabling transfer of Cu from organic complexes to the cell membrane. Enhanced uptake after addition of weak Cu(I) ligands further supported the importance of Cu(I)-mediated transport pathways. Many marine microorganisms possess Cu uptake transporters (CTR) that take up Cu(I), following its reduction at the cell surface. The marine diatom *Thalassiosira oceanica* showed that extracellular reduction of Cu(II) is the first step in Cu(I) uptake, as suppression of Cu(II) reduction decreased Cu uptake by 97%, suggesting that this pathway enables diatoms to utilise naturally occurring Cu-organic complexes (Kong and Price, 2020). Expression of CTR increased under Cu limitation, enabling efficient Cu uptake in Cu-depleted marine environments (Kong and Price, 2019). Although transport of Cu(II) through low-affinity transporters, including ZIP family transporters and divalent metal transporter (DMT1), has also been proposed (Blaby-Haas and Merchant, 2012; Rosakis and Köster, 2005), experimental evidence suggests that Cu(I)-mediated uptake is the dominant pathway in phytoplankton. Barber-Lluch et al. (2023) showed that the bioavailability of Cu complexed with DOM depends strongly on Cu_T . At the highest tested Cu_T (100 nM), additions of fulvic acid (FA) and natural marine DOM reduced Cu internalisation by the marine dinoflagellate *Prorocentrum micans*, consistent with ASV measurements of Cu' and predictions of the BLM. In contrast, at a lower and more environmentally relevant Cu_T (20 nM, corresponding to 1–10 nM Cu'), organically complexed Cu appeared partially bioavailable, resulting in greater cellular Cu accumulation than expected from Cu' alone. The authors proposed that this could result either from dissociation of organic Cu complexes near the cell surface, enhancing diffusion-limited uptake, or from reduction of complexed Cu(II) to Cu(I), followed by transport through high-affinity Cu(I) transporters. Similar conclusions were drawn from large-scale ocean biogeochemical simulations using the NEMO/PISCES model, which suggested that modelled inorganic Cu concentrations alone are insufficient to meet phytoplankton Cu demand, implying that at least part of the organically complexed Cu pool must be bioavailable to phytoplankton (Richon and Tagliabue, 2019).

On the other hand, increasing evidence suggests that biological effects are governed more by intracellular Cu speciation than by bulk Cu accumulation.

2.5.3.2. Intracellular copper redox speciation as a determinant of copper toxicity

While the concepts described above emphasise the role of extracellular Cu speciation in regulating Cu uptake, they do not account for the intracellular transformations of Cu following cellular uptake. The reducing conditions within cells favour the stabilisation of Cu(I), whereas Cu(II) is generally considered the predominant oxidation state of Cu in oxygenated aquatic environments. However, Cu(II) may also exist intracellularly, and the intracellular transformations of Cu(II) and Cu(I), and the toxicity of both species, remain important research questions. With the development of different colourimetric and fluorescent probes for intracellular and extracellular detection of Cu(I) and Cu(II) (e.g. Xiang et al., 2006; Xiao et al., 2018), progress has been made in this field. Pioneering work on the importance of Cu redox speciation for determining Cu toxicity to phytoplankton was conducted by Wang and Wang (2021). The authors demonstrated that Cu toxicity in the model freshwater alga *Chlamydomonas reinhardtii* correlated better with the intracellular accumulation of the labile Cu(I) pool than with total intracellular Cu; specifically, intracellular accumulation of labile Cu(I) correlated with toxicity in algal cells under Fe-deficient conditions. More specifically, high intracellular Cu(I) levels, which can result from intracellular biotransformations of Cu(II)/Cu(I), putatively via mechanisms involving thiol species, can damage Fe–S clusters in proteins or catalyse Fenton-like reactions (Wang and Wang, 2021 and references therein). Conversely, normal and Zn-deficient cells showed high contents of Cu(II) after Cu exposure, yet this was not associated with toxicity. Further studies demonstrated that the heterogeneous response of *Chlamydomonas reinhardtii* to Cu stress was related to the cell cycle; increases in labile Cu(I) corresponded to cell division and fluctuated with the diurnal cycle and cell cycle (Deng and Wang, 2023; Wang and Wang, 2023). Subsequent studies provided insight into the dynamic regulation of the intracellular labile Cu(I)/Cu(II) cycle, and showed that labile Cu(I) and Cu(II) exhibit different intracellular distributions, while Cu stress disrupted the Cu(I)/Cu(II) cycle and its balance (Deng and Wang, 2024; Gui and Wang, 2024). A GSH-dependent system is activated to scavenge excess ROS and stabilise internal labile Cu(I), while the formation of lysosome-related organelles is suggested as a coping mechanism for elevated intracellular Cu(I) (Deng and Wang, 2025). Overall, the available evidence suggests that intracellular labile Cu(I) is the Cu species most closely associated with toxicity in phytoplankton.

2.6. Role of organic matter in copper complexation in marine and estuarine environment

2.6.1. Definition and classification of organic matter

Organic matter comprises all organic compounds present in the marine environment, varying in structure, composition, and size, and includes both living and non-living natural organic material. The most abundant elements in organic matter are carbon (C), hydrogen (H), nitrogen (N), oxygen (O), phosphorus (P), and sulphur (S), while trace metals are also present in minor quantities. Organic matter exists as POM and DOM, which are operationally separated by filtration using pore sizes of 0.7 μm , 0.45 μm , or 0.2 μm , with this classification considering colloids as part of the dissolved pool (Denis et al., 2017; Verdugo et al., 2004). Furthermore, DOM and POM are not discrete pools but are interconnected through continuous transformation processes. DOM can be converted into POM through abiotic processes, including the destabilisation of colloidal DOM induced by increasing salinity in estuarine environments (Libes, 2009). In addition to the operational separation of DOM and POM, DOM can be further fractionated into low- and high-molecular-weight fractions (LMW and HMW, respectively). These fractions are commonly separated using a molecular-weight cut-off of 1 kDa and may exhibit markedly different trace metal-binding properties (e.g. Liu et al. 2023). DOC is the parameter most commonly used to describe DOM in seawater and encompasses a wide range of organic species. Analogous to DOM, DOC is defined as organic carbon that passes through a filter with a pore size varying from 0.2 to 0.7 μm , thereby including both colloidal and dissolved organic material. It accounts for the majority of DOM in natural waters, although the exact percentage depends on the characteristics of the studied natural water. The average DOC concentration in coastal waters, defined as waters extending from estuaries (salinity >1) to continental shelf break (depth <200 m) is approximately 2.19 mg L^{-1} (Lønborg et al., 2024), exceeding that of the open ocean, which varies from an average of approximately 0.42 mg L^{-1} in the deep ocean to 0.96 mg L^{-1} in the surface ocean (Hansell et al., 2009). Among natural waters, the highest DOC is measured in rivers, with a global average of 10.4 mg L^{-1} (Liu and Wang, 2022). Once introduced into the marine environment, organic matter serves as a food source for heterotrophic organisms. Through the consumption and transformation of organic matter into new biomass or various dissolved and particulate forms of detrital material, organic matter participates in the global carbon cycle. In addition to serving as a nutrient source for marine organisms, organic matter is also an important electron donor and therefore

participates in numerous redox reactions, consequently exerting a significant influence on the speciation of trace metals.

Within the DOM pool, the fraction capable of absorbing radiation in the ultraviolet (UV) and visible (Vis) regions is termed chromophoric or coloured DOM (CDOM), while the fraction of CDOM that can emit a portion of the absorbed energy as fluorescence is referred to as fluorescent DOM (FDOM). CDOM is highly photoreactive, and exposure to sunlight can induce photochemical degradation and photomineralisation, resulting in changes in its optical properties, a reduction in molecular weight, and the formation of dissolved inorganic carbon and smaller organic compounds (Coble, 2007). Furthermore, CDOM can affect redox reactions of trace metals. The optical properties of CDOM provide information on both its abundance and the chemical properties of the bulk sample, which can be modified by chemical, biological, and physical processes. From CDOM absorbance, parameters such as the absorption coefficient at 254 nm (a_{254}), its ratio to DOC, i.e. specific UV absorbance at 254 nm (SUVA), and the spectral slope over the 275–295 nm spectral range ($S_{275-295}$) can be derived, and these are used as proxies for allochthonous DOM and the degree of humification or aromaticity within the sample (Helms et al., 2008; Stedmon and Nelson, 2015). On the other hand, using excitation–emission matrix (EEM) spectroscopy, fluorescence peaks of humic-, protein-, and pigment-like groups can be identified. Fluorescence indices Coble peaks A, C, and M can identify humic-like compounds, while Coble peaks B and T are related to protein-like compounds (tyrosine- and tryptophan-like, respectively) (Coble, 2007). Additionally, the humification index (HIX) and biological activity index (BIX) can be used as indicators of the degree of DOM humification and autotrophic productivity, respectively (Santos et al., 2016).

2.6.2. Sources of organic matter in marine and estuarine environments

Most natural OM in the marine environment is of biological origin and is produced in situ by phytoplankton; this is referred to as autochthonous organic matter (Libes, 2009). Living organisms may release organic matter in dissolved or particulate form (e.g. faecal pellets), while organic matter is also introduced into the environment following organism death through lysis of cellular membranes. Phytoplankton is considered the principal source of marine organic matter and plays a major role in marine carbon and nitrogen cycling. Experimental studies conducted on different phytoplankton taxonomic groups (*Synechococcus bacillaris*, *Phaeocystis* sp., *Emiliania huxleyi* and *Skeletonema costatum*) demonstrated a close balance between dissolved inorganic carbon uptake and total organic carbon production during growth in synthetic seawater media. DOC production represented approximately 10–32% of total

organic carbon production, indicating that a substantial fraction of photosynthetically fixed carbon is released into the dissolved phase (Biddanda and Benner, 1997). In these experiments, dissolved carbohydrates were predominantly composed of polysaccharides, while both POM and DOM generally exhibited C:N ratios close to Redfield values. However, HMW DOM showed substantially elevated C:N ratios compared to LMW DOM, indicating compositional differences between DOM fractions and selective enrichment in carbon-rich compounds. Phytoplankton primarily produces LMW DOM enriched in nitrogen, although it may also produce carbon-rich HMW DOM. It mainly consists of carbohydrates (monosaccharides and polysaccharides), followed by amino acids, lipids, and organic acids. Carbohydrates, predominantly in the form of exopolymers, account for up to 80% of DOC in seawater (Engel et al., 2012; Verdugo et al., 2004). Simultaneously, marine microorganisms are a major sink for DOM, with a substantial fraction of the organic carbon fixed by marine autotrophs being consumed within microbial food webs (Libes, 2009). On the other hand, allochthonous DOM enters the marine environment through sediment redeposition following erosion caused by currents, mechanical instability, earthquakes, and tectonic activity, as well as from terrestrial sources via atmospheric deposition, riverine input, and glacial transport (Rullkötter, 2006). Riverine inputs are particularly important in estuarine and coastal environments, where terrestrial humic substances and suspended particulate material can strongly influence DOM composition and trace metal chemistry. Humic substances may account for 50-80 % of riverine DOC, while their content in DOC decreases with an increase in salinity (Otero et al., 2003). However, humic compounds in marine and estuarine waters may also be autochthonous. Wilson et al. (1983) showed that the diatom *Phaeodactylum tricornutum* produces substances resembling marine humic matter and suggested that uronic acids may act as precursors in the formation of aromatic humic compounds. Similarly, phytoplankton species from the genera *Chaetoceros*, *Skeletonema*, *Prorocentrum*, and *Micromonas* were found to produce both protein-like and humic-like substances, with approximately 20% of humic substances in the coastal Ría de Vigo estimated to originate from in situ phytoplankton production (Castillo et al., 2010). Humic-like substances were also correlated with chlorophyll maxima in the northeastern Atlantic Ocean, indicating a close link with primary production and microbial metabolism (Lønborg et al., 2015). Bacteria additionally contribute to the production and transformation of humic-like DOM in seawater (Shimotori et al., 2009). Phytoplankton mainly produces aliphatic humic substances, which may subsequently be transformed by bacteria into more aromatic humic-like compounds (Romera-Castillo et al., 2011). Similarly, marine microorganisms are a major DOM sink. A smaller fraction of DOM is formed through abiotic

processes, such as photochemical reactions in surface waters, high-temperature reactions in hydrothermal systems, and spontaneous condensation and polymerisation of degraded biomolecules, some of which contribute to the formation of humic-like substances (Libes, 2009). On the other hand, abiotic DOM removal occurs by phototransformation, sorption of DOM onto particles, condensation of marine microgels and hydrothermal circulation (Carlson and Hansell, 2015). Photochemical transformations of DOM may alter its degree of humification, aromaticity and molecular weight, which may result in production of reactive species which further mediates trace metal speciation (Section 2.6.4). Spontaneous condensation of marine gels may form self-assembled microgels which under changes in experimental conditions can sink. Similarly, sorption of DOM onto sinking particles removes it from dissolved pool. High temperature circulation of seawater through ridge flank basalts can also remove DOM from solution, although it represents a minor global DOM sink.

2.6.3. Copper complexation by organic matter

Owing to its high chemical complexity, the DOM pool encompasses a broad range of compound classes, including hydrocarbons; simple sugars and complex carbohydrates; lipids; nucleotides and nucleic acids; amino acids and proteins; pyrroles and porphyrins; LMW nitrogenous compounds, carboxylic acids, and P- and S-containing compounds; organohalogenes; and humic substances (Libes, 2009). The following discussion focuses on DOM components of particular relevance to Cu speciation, including humic substances, carbohydrates and associated extracellular polymeric substances, amino acids and proteins and thiol-containing compounds, owing to the presence of functional groups capable of metal coordination. These groups partially overlap; for example, some thiol-containing ligands are amino acids or peptides. In addition, special metal-binding metabolites, such as siderophores and chalkophores, may contribute to Cu complexation.

2.6.3.1. *Complexes with humic substances*

The basic structure of humic substances consists of aliphatic chains and aromatic rings with attached functional groups such as hydroxyl and carboxyl groups, which determine their reactivity (Cook and Langford, 1998; Yang and van den Berg, 2009). In the water column they mainly occur as humic and fulvic acids (HA and FA, respectively). HA is operationally defined as soluble in alkaline solutions but insoluble under strongly acidic conditions, whereas FA is soluble throughout the entire pH range. These fractions are commonly distinguished based on their solubility and adsorption behaviour on XAD-8 resin (McKnight and Aiken, 1998).

Humic-like DOM is generally characterised by higher aromaticity, molecular weight, and chromophoric content compared with freshly produced biological DOM. Its molecular weight spans a broad range from approximately 500 Da for dissolved FA to over 100,000 Da for sedimentary HA. Allochthonous humic substances are primarily derived from highly aromatic lignins and tannins originating from terrestrial plants, whereas autochthonous humic substances are mainly produced by phytoplankton and are generally enriched in carboxylic and aliphatic groups with lower aromaticity (Harvey et al., 1983; Hertkorn et al., 2006; Shevchenko and Bailey, 1996). The composition of humic substances depends on their source as well as on microbial activity in both the original and receiving environments. Consequently, investigations of phytoplankton intracellular and extracellular compounds, together with microbial processes, are essential for understanding the formation of marine humic substances (Wilson et al. 1983). Complexation by humic substances has been most extensively studied for Cu and Fe, although humic substances are also known to bind other trace metals, including Zn, Co, Ni, Cd, and Hg (Millero, 2001; Millero and Pierrot, 2002; Yang and van den Berg, 2009). Whitby and van den Berg (2015) reported conditional stability constant for Cu–HA complexes that fall within the range of $\log K'_{\text{CuL}_2}$. Although stronger Cu-binding ligands have been identified, such as thiols (Section 2.6.3.4), L_2 ligands also contribute to Cu complexation, particularly in environments where the concentration of L_1 ligands is low, such as estuaries (Abualhaija et al., 2015; Whitby and van den Berg, 2015). Yang and van den Berg (2009) demonstrated competition between Fe and Cu for binding to humic ligands, with Fe forming more stable complexes than Cu, consistent with previously reported conditional stability constants for complexes between various trace metals and humic substances. Similarly, Vachet and Callaway (2003) concluded that humic substances constitute a component of the L_2 , whereas Kogut and Voelker (2001) suggested that humic substances may contribute to both the L_1 and L_2 , depending on the other ligands present, with their strong Cu-binding behaviour attributed to the random arrangement of humic functional groups into multidentate Cu-binding sites. Muller and Batchelli (2013) argue that L_1 ligands are indeed terrestrial humic substances, while L_2 ligands are produced in situ in coastal waters.

Humic substances not only complex Cu but also participate in its redox cycling. The above listed literature assumes Cu(II) specie in humic complexes and in seawater in general. Nonetheless, Cu(II) reduction upon complexation with HA has been reported (Maurer et al., 2013; Pham et al., 2012). Pham et al. (2012) reported reduction to Cu(I) of up to 75% of Cu_T , both in the presence and absence of oxygen. Although the exact reaction mechanism remains

uncertain, both proposed models agree that FA complexes Cu(II) and reduces it through two distinct electron-donating groups differing in their reactivity: a relatively labile donor that rapidly reduces Cu(II) and a less labile donor that reduces Cu(II) more slowly. Fulda et al. (2013) investigated Cu redox transformations upon addition of both Cu(II) and Cu(I) to reduced HA in the presence and absence of oxygen, and reported Cu(I)–HA complexes in anoxic conditions at low Cu_T , while higher Cu_T resulted in Cu(0) formation. On the other hand, Cu(II)–HA complexes prevailed under oxic conditions. Nonetheless, part of Cu pool was stabilized as Cu(I)–HA in oxic conditions. X-ray absorption spectroscopy revealed that Cu(I) in Cu(I)–HA complexes was mainly 2-fold coordinated, with part being 3- to 4-fold coordinated, and including at least one N- and/or S-containing ligand, while Cu(II) was in the form of five-membered ring chelates (Fulda et al., 2013 and references therein). The S-containing groups are most likely thiolates, thioethers or disulphide groups, while nitrogen containing group is most likely related to nitrogen in heterocyclic aromatic compounds which are known for their high affinity to Cu(I) (Maurer et al., 2013 and references therein). Overall, HA is expected to reduce portion of Cu even in oxic environments. This is further addressed in the authors second part of the study, where they focused on thermodynamical explanation for the observed Cu(I) binding with HA (Maurer et al., 2013). The authors also showed that Cu(I) binds strongly to HA, with a higher affinity than Ag(I) and Cd(II), and concluded that the different complexation behaviour of Cu(I) and Cu(II) modifies Cu redox equilibria by affecting the thermodynamic stability of the different Cu oxidation states. Similarly, reduction of Cu(II) in the presence of FA has been reported under both oxic and anoxic conditions, with significantly higher Cu(II) reduction and slower Cu(I) oxidation in the absence of oxygen (Xing et al., 2020). The presence of chloride affects Cu speciation through both chloride complex formation and increased ionic strength, the latter reducing the affinity of FA for Cu(I) and Cu(II). However, further experiments with materials other than HA and FA, and under different experimental conditions, are needed in order to assess Cu redox behaviour in the natural environment. Nonetheless, humic substances are part of the chromophoric CDOM pool and therefore they are prone to photodegradation (Section 2.6.4).

2.6.3.2. *Complexes with polysaccharides*

Carbohydrates are formed by condensation of simple sugars, and are often referred to as polysaccharides. Associated extracellular polysaccharides (EPS) are HMW extracellular polymers produced by phytoplankton, bacteria, and other microorganisms in marine and estuarine environments. They mainly consist of polysaccharides, with smaller proportions of

proteins and uronic acids, and are produced as protective compounds under environmental stress conditions such as nutrient limitation, unsuitable pH and temperature, and elevated trace metal concentrations (Comte et al., 2008; Gupta and Diwan, 2017; Lau et al., 2005). Due to the presence of functional groups such as carboxyl, hydroxyl, sulfate, and amino groups, EPS can bind trace metals and reduce their uptake into microbial cells. These EPS can rapidly assemble into polymer gels and transparent exopolymer particles (TEP), which play an important role in aggregation processes, marine snow formation, microbial interactions, and vertical carbon export to deeper ocean layers (Verdugo et al., 2004). TEP may represent a substantial fraction of particulate organic carbon, particularly following phytoplankton blooms, highlighting the importance of carbohydrate-rich organic matter in seasonal carbon cycling and sedimentation processes (Engel et al., 2012). Complexation of trace metals by polysaccharides has been studied more extensively for Fe, whereas Cu also forms complexes with some polysaccharides, such as Na-alginate and chondroitin sulphate (Plavšić and Strmečki, 2016). The equilibrium expression used to determine the apparent stability constants in this work is comparable with the one defined in Ruacho et al. (2022) and references therein, allowing the qualitative comparison of Cu-binding affinities. The reported logarithm of apparent stability constants of 8.1–8.3 are consistent with the L_3 ligand class ($\log K'_{CuL_3} < 10$), indicating that polysaccharides are relatively weak Cu-complexing ligands and are unlikely to account for the strongest Cu-binding ligands present in natural waters (Lombardi et al., 2005; Plavšić and Strmečki, 2016; Ruacho et al., 2022). Nevertheless, due to their high abundance in productive coastal and estuarine waters, they may influence Cu speciation. Consistent with their functional group composition, polysaccharides are also expected to preferentially bind Cu(II) rather than Cu(I) (Pearson, 1963; Smith et al., 2002).

2.6.3.3. Complexes with amino acids and proteins

Amino acids contain at least one amine group and a carboxyl group, and they are the building blocks of proteins, which play a crucial role as components of various enzymes and structural parts in marine organisms (Libes, 2009). Proteins provide multiple potential metal-binding sites and can therefore form Cu complexes with coordination environments that differ from those of individual amino acids, as protein conformation and the accessibility of individual binding sites are important factors in trace metal binding. Dissolved proteins are continuously introduced into seawater through biological processes and are subject to microbial and enzymatic degradation. Nevertheless, some proteins can persist sufficiently long to be detected in oceanic waters. Tanoue (1995) identified dissolved proteins in Pacific Ocean, including a bacterial

membrane-protein homologue, while Yamada and Tanoue (2003) demonstrated the widespread occurrence of dissolved glycoproteins in oceanic waters. Protein degradation occurs through extracellular hydrolysis, generating peptide intermediates that can undergo further degradation to smaller peptides and amino acids, which may subsequently be assimilated or transformed by microorganisms (Hollibaugh and Azam, 1983; Nunn et al., 2003). Combined with analytical constraints which complicate the exact structural characterisation of natural organic ligands in seawater, their contribution to the bulk pool of naturally occurring Cu-binding ligands in seawater remains poorly constrained (e.g. Marcinek et al., 2025; Ruacho et al., 2022; Sander and Koschinsky, 2011). Marcinek et al. (2025) reported a correlation between protein-like CDOM (fluorescence of peak T) and L_1 during summer months of increased primary production. The authors proposed that these protein-like substances may comprise thiols and thiol-like substances, which is in accordance with previous evidences (Laglera and van den Berg, 2003; Marcinek et al., 2025; Whitby et al., 2017). In contrast with hard donor ligands (-O and -N containing), which are in general characteristic for Cu(II) binding (Pearson, 1963; Smith et al., 2002), such as complexes of Cu(II) with histidine, glutamic acid, asparagine and arginine (Baffi et al., 1992; Deschamps et al., 2005), thiol groups are prone to form Cu(I) complexes. Therefore, thiol-like compounds and thiol-rich proteins are discussed separately in the following section.

2.6.3.4. *Complexes with thiols and other reduced sulfur species*

Phytoplankton is an important source of thiols in marine environments, where numerous LMW thiols have been detected, including Cys, GSH, 3-MPA, homocysteine, *N*-acetylcysteine, ethanethiol, and mercaptoethanesulfonate, usually in the nanomolar concentration range (Al-Farawati and van den Berg, 2001; Dryden et al., 2007; Liem-Nguyen et al., 2015; Swarr et al., 2016; Tang et al., 2000). Thiols readily complex numerous trace metals; however they exhibit particularly strong binding towards Cu followed by Ni and Zn (Zheng et al., 2008). Its chemical reactivity arises from the thiol (-SH) group, which upon deprotonation forms the highly reactive thiolate anion (RS^-). Thiolate groups readily participate in oxidation reactions, nucleophilic substitution, and trace metal complexation (Poole, 2015). They are soft sulphur donors, thereby expressing high affinity towards Cu(I) (Pearson, 1963; Smith et al., 2002).

GSH is a tripeptide composed of glutamate, Cys, and glycine, with the Cys residue providing the reactive thiol group responsible for metal binding. GSH has been detected in many prokaryotic organisms and in most mammalian cells at intracellular concentrations ranging from 0.2 to 10 mM (Anderson, 1998). Because intracellular GSH is generally present in large

excess relative to Cu, it plays a central role in cellular Cu homeostasis by binding Cu predominantly as Cu(I)–GSH complexes and facilitating its transfer to metallothioneins and Cu chaperones (Anderson, 1998; Maryon et al., 2013). Cell lysis and active exudation may release GSH and other LMW thiols into the surrounding water column (Dupont and Ahner, 2005; Leal et al., 1999). Although intracellular GSH occurs at mM concentrations, dissolved GSH in seawater is typically present at nM levels, where it has been proposed to contribute to the L₁ ligands (Hu et al., 2006; Liem-Nguyen et al., 2015; Tang et al., 2005, 2004; Whitby et al., 2018). Elevated Cu concentrations may stimulate GSH release by phytoplankton (Tang et al., 2005); however, the exact chemical speciation of GSH in seawater and the mechanisms governing the formation of Cu–GSH complexes remain incompletely understood. Phytochelatins are oligomers of glutathione synthesised by phytoplankton and other organisms in response to elevated trace metal concentrations (Ahner et al., 1997, 1995, 1994; Ahner and Wei, 2005). Their production correlates strongly with the concentration of free Cu, Pb, Ni and Zn, but primarily with Cd. In addition to GSH, other LMW thiols, particularly Cys, have been proposed as important Cu(I)-binding ligands in natural waters, although the mechanisms governing Cu(I) complexation remain incompletely understood (Leal and van den Berg, 1998; Swarr et al., 2016). Cys is a non-essential amino acid that plays an important role in protein synthesis and stabilisation through the formation of disulphide bonds (Takagi and Ohtsu, 2017). They may form mononuclear Cu(I)–thiolate complexes, mixed N-S coordination complexes, and multinuclear Cu(I)-thiolate clusters (Pushie et al., 2012). Under Cu-limiting conditions, Cys may facilitate Cu acquisition by reducing Cu(II) complexes to Cu(I). *Emiliania huxleyi* has been shown to acquire Cu in the form of Cu(I)–Cys complexes via a specific Cys transporter (Walsh et al., 2015). Metallothioneins are a family of LMW, Cys-rich proteins produced by many marine organisms in response to elevated trace metal concentrations. They bind metals including Ag, Cd, Cu, Zn, and Hg and are widely used as biomarkers of trace metal contamination in marine environments (Amiard et al., 2006; Ge et al., 2020; Wang and Rainbow, 2010). In addition to their role in detoxification, metallothioneins are essential for the homeostasis of biologically important trace metals such as Cu and Zn. Their expression and metal-binding kinetics depend strongly on species-specific detoxification mechanisms and environmental conditions. Consequently, metallothioneins may influence trace metal redox speciation. For example, binding of Cu(II) to a metallothionein containing four Cys residues results in reduction of Cu(II) to Cu(I), oxidation of two Cys residues to a disulphide, and coordination of the resulting Cu(I) by the remaining thiolate groups (Carpenè et al., 2007). Besides GSH and Cys, 3-MPA is also widespread in natural waters and may originate from

both biological and abiotic processes (Kiene and Taylor, 1988). Biologically, 3-MPA may be produced during the metabolic degradation of other thiols and has also been proposed as a possible alternative to coenzyme M in *Methanocaldococcus jannaschii* (Allen and White, 2016). In aquatic environments, 3-MPA undergoes rapid oxidation and readily complexes Fe and Cu (Kiene and Taylor, 1988). Furthermore, TAA is a thiocarboxylic acid that has been proposed as a potential precursor of coenzyme A in prebiotic chemistry (Battin and Brumaghim, 2008; Chandru et al., 2016; Sanden et al., 2020). Oxidation of TAA and ethanethiol by Fe(III) produces Fe(II), while thioacetate is oxidised to disulphides with concomitant formation of thioesters and Fe sulphide.

2.6.3.5. *Specific copper-binding ligands*

Unlike humic substances, polysaccharides, amino acids, proteins, and thiols, which bind Cu as part of their broader chemical functionality, the compounds discussed in this section are characterised by highly specific interactions with Cu that are directly linked to their biological function. Chalkophores are Cu-binding ligands produced by methanotrophic bacteria to facilitate copper acquisition under Cu-limiting conditions, analogous to the role of siderophores in Fe acquisition (Kenney and Rosenzweig, 2018). Methanobactin is a well-characterised example of a chalkophore. It is a ribosomally synthesised and post-translationally modified peptide secreted by methanotrophs to ensure sufficient Cu uptake for particulate methane monooxygenase, a key Cu-dependent enzyme involved in methane oxidation. Methanobactins are multidentate ligands that coordinate Cu primarily through nitrogen atoms of imidazolone (or related heterocyclic) rings and sulphur atoms of oxazolone/thioamide-derived donor groups, resulting in exceptionally high Cu-binding affinities. With a reported logarithm of conditional stability constant of 18.8, methanobactin is among the strongest naturally occurring Cu-binding ligands identified in marine and estuarine environments (El Ghazouani et al., 2012). Furthermore, Whitby et al. (2018) suggested that chalkophores may contribute more substantially to Cu complexation in seawater than previously recognised. Using CLE-AdCSV, the authors identified sulphur-containing ligands with thiourea-like electrochemical behaviour that correlated with the L₁. Because thiocarbonyl donor groups are characteristic structural features of methanobactins and other chalkophores, these compounds have been proposed as potential contributors to the strong Cu-binding ligand pool in natural waters. Methanobactin exhibits a pronounced preference for Cu(I) over Cu(II) (El Ghazouani et al., 2012), making it, together with thiol-containing compounds, a strong candidate for the stabilisation of dissolved Cu(I) in natural waters.

2.6.4. Effect of photochemical transformation of dissolved organic matter on copper speciation

Photochemical transformation of DOM is an important process in sunlit marine and estuarine waters. It may occur directly and indirectly, i.e. by direct absorption of photons by chromophores or by indirect pathway where it is mediated by radicals produced (Li et al., 2024; Vione et al., 2014). CDOM absorbs photons and may form singlet or triplet state DOM ($^1\text{DOM}^*$ and $^3\text{DOM}^*$, respectively) and produce dissolved inorganic carbon and LMW acids. On the other hand, the indirect pathway includes generation of ROS such as hydroxyl radical ($\bullet\text{OH}$), singlet oxygen ($^1\text{O}_2$) and superoxide radical ($\text{O}_2^{\bullet-}$), which can subsequently react with DOM and cause its transformation. Li et al. (2024) found that phototransformation of highly humic DOM was mainly associated with ROS-induced oxidation, whereas for algal-derived DOM with high protein content, autooxidation of excited-state $^3\text{DOM}^*$ appeared to be more important than indirect ROS-mediated oxidation. $\bullet\text{OH}$ are highly reactive intermediates in natural-water photochemistry and can be generated through several pathways, including the photolysis of nitrate (NO_3^-) and nitrite (NO_2^-). DOM photochemistry can also contribute to $\bullet\text{OH}$ production through both H_2O_2 -dependent and H_2O_2 -independent pathways (Page et al., 2011). Photochemical processing can additionally alter the molecular and optical characteristics of DOM; irradiation may decrease the degree of humification, aromaticity and molecular weight of allochthonous DOM and preferentially remove highly humified components, with the extent of transformation depending on DOM source and composition (Zhu et al., 2023). Such photochemical processing can therefore modify the relative abundance and properties of different DOM components and potentially affect the availability and characteristics of metal-binding sites and consequently modify trace metal speciation. Laglera and van den Berg (2006) demonstrated that sunlight can degrade Cu-complexing ligands including thiols in estuarine waters, with Cu-complexing ligand concentrations decreasing by 2–60% and thiol concentrations by 30–50% after one day of sunlight exposure. Under simulated daylight, natural Cu-complexing ligands exhibited half-lives of approximately 20–45 h, whereas thiols were degraded more rapidly, with a half-life of approximately 3 h. Importantly, Cu complexation increased the photochemical half-life of thiols by nearly an order of magnitude (Laglera and van den Berg, 2006). Similarly, Shank et al. (2006) observed a 15–33% loss of strong Cu-complexing ligands in organic-rich estuarine waters after 1–2 days of natural sunlight exposure, while 94 h of exposure to a full-spectrum solar simulator, equivalent to slightly more than two weeks of natural summer sunlight, resulted in losses exceeding 90%.

Such loss of strong Cu-binding ligands can reduce the capacity of DOM to complex Cu and may allow weaker ligands to become more important in Cu speciation, while increasing the proportion of Cu^{2+} . However, photochemical transformation does not necessarily decrease Cu-binding capacity. In laboratory experiments using algal- and soil-derived DOM, Xu et al. (2018) found that Cu(II)-binding capacity increased during photodegradation of algal-derived humic- and fulvic-like DOM, whereas soil-derived DOM showed a slight decrease. Thus, the effect of DOM phototransformation on Cu binding depends on the origin and chemical characteristics of the DOM and cannot be described simply as a loss of Cu-binding capacity.

Photochemical processing of DOM can also affect Cu redox speciation by promoting the reduction of Cu(II) to Cu(I). Moffett and Zika (1987) demonstrated that sunlight influences Cu speciation in the upper water column, including the formation of Cu(I) during sunlight exposure, while in their earlier work the authors showed that Cu(I) generated through non-equilibrium processes could accumulate to significant steady-state levels, depending on its rate of oxidation (Moffett and Zika, 1983). One proposed pathway involves photoexcitation of Cu(II)-organic complexes followed by ligand-to-metal charge transfer (LMCT), resulting in reduction of Cu(II) to Cu(I) and oxidation of the ligand and. The authors demonstrated sunlight-induced Cu(I) formation using Cu(II) complexes with nitriloacetic acid (NTA), oxalate, glycine and alanine in seawater, although they also noted that the direct importance of LMCT reactions involving natural seawater Cu complexes remained uncertain. Photochemically generated reducing species provide additional pathways for Cu reduction. Moffett and Zika (1983) proposed that photochemical oxidation of DOM generates hydrated electrons and subsequently reducing species including $\text{O}_2^{\bullet-}$ and H_2O_2 , both of which can reduce Cu(II) to Cu(I). Sharma and Millero (1988) subsequently considered this pathway in seawater and explicitly described the reduction of Cu(II) by photochemically generated $\text{O}_2^{\bullet-}$ and H_2O_2 . The subsequent oxidation of Cu(I) by dissolved O_2 in seawater is strongly affected by ligand availability, pH, temperature and salinity, with chloride-dependent effects attributed to the formation and oxidation of Cu(I)-chloride complexes. H_2O_2 can also participate in Cu redox cycling: it can reduce Cu(II) to Cu(I) and oxidize Cu(I) back to Cu(II). Sharma and Millero (1989) quantified the latter reaction in seawater and sodium chloride (NaCl) solutions. Importantly, at typical surface-seawater concentrations of approximately $200\ \mu\text{M}$ O_2 and $0.1\ \mu\text{M}$ H_2O_2 , they calculated that O_2 oxidation of Cu(I) was about 130 times faster than H_2O_2 oxidation. Thus, although H_2O_2 can participate in Cu redox cycling, O_2 is expected to be the dominant Cu(I) oxidant under typical surface-seawater conditions. Together, these processes indicate that sunlight can contribute to a

dynamic Cu redox cycle in surface waters, although the quantitative contribution of individual photochemical pathways to Cu(I) formation and overall Cu redox speciation under natural conditions remains poorly constrained.

2.7. Analytical approaches to copper speciation

The determination of individual chemical species of trace metals in natural waters presents a number of analytical challenges. When selecting an appropriate method, several factors should be considered, including (I) minimising manipulation of the natural sample, (II) keeping the time between sampling and analysis as short as possible, and, where this is not feasible, establishing appropriate sample storage conditions, (III) sensitivity, (IV) selectivity, and (V) cost-effectiveness of the method (Nürnberg, 1977). These considerations are particularly important for trace metal speciation in marine and estuarine waters, where concentrations of trace metals are generally very low and the complex composition of seawater can further limit the range of suitable analytical approaches (Bruland and Lohan, 2003). In particular, the presence of high concentrations of major ions and DOM can complicate the selective determination of individual metal species.

Methods that can be used for the determination of Cu^{2+} are usually equilibrium methods such as CLE-AdCSV (e.g. Campos and van den Berg, 1994), ion-selective electrodes (ISE) (e.g. Marcinek et al., 2021), ion-exchange/chelating resin methods (e.g. Noël et al., 2006; Sweileh et al., 1987), permeation liquid membrane (PLM) (e.g. Gunkel-Grillon and Buffle, 2008), absence of gradients and Nernstian equilibrium stripping (AGNES) (e.g. Galceran et al., 2004), and the Donnan membrane technique (DMT) (e.g. Kalis et al., 2006). For measuring the operationally defined labile fraction of Cu, ASV and diffusive gradients in thin films (DGT) technique can be used (e.g. Cindrić et al., 2020). In complexation studies, direct approaches such as ASV and CLE-AdCSV are the most common methods (Ruacho et al., 2022). (Worsfold, 2019)

In ASV, during the first stage, the metal is cathodically reduced and deposited onto the working electrode, most commonly a hanging mercury drop electrode (HMDE). This deposition step, also referred to as preconcentration, is performed during the accumulation time under continuous stirring. The applied deposition potential is typically 300–400 mV more negative than the polarographic half-wave potential of the metal. After stirring is stopped and the solution is allowed to equilibrate, the potential is scanned in the positive direction, causing

anodic oxidation and dissolution of the deposited metal back into solution (Scholz, 2015). The peak current occurs when the applied potential reaches the characteristic oxidation potential of the electroactive species. While ASV detects labile metal complexes, inert metal complexes may also partially dissociate on the electrode during the preconcentration step.

In AdCSV, in the presence of the added competing ligand, the metal of interest, forms a surface-active complex with a known stability constant, which accumulates at the surface of working electrode. The adsorption potential is typically more positive than the reduction potential of the metal-ligand complex. The complex is accumulated for a defined period, after which it is reduced by scanning the potential in the negative direction. CLE-AdCSV involves successive additions of the metal of interest to the sample, enabling its equilibrium partitioning between the added ligand and naturally occurring organic ligands to be determined. In the case of Cu, the most commonly used ligand is salicylaldoxime (SA). In stripping voltammetry, a desorption step, i.e. a short switch to very negative potentials where desorption of surface-active substances from the electrode surface occurs, can reduce interferences caused by fouling of the electrode by organic substances (Louis, 2008). The low detection limits required for the determination of trace metals in natural waters can be achieved through the use of pulsed potential modulation and appropriate accumulation times. Common approaches include square-wave (SW) and differential-pulse (DP) techniques, which, with accumulation times of 1–30 min, can enable the detection of trace metals at concentrations as low as 1 pM (Worsfold, 2019).

However, these methods do not allow the oxidation state of Cu to be determined directly in marine and estuarine samples.

2.7.1. Analytical approaches to redox speciation of copper

Determining Cu redox speciation in natural waters is analytically challenging because Cu(I) is typically present at very low concentrations and can undergo rapid oxidation or ligand exchange during sample handling. Analytical procedures must therefore selectively distinguish Cu(I) from Cu(II) while minimising disturbance of the original redox state.

One of the earliest approaches for determining Cu(I) in natural waters was developed by Moffett et al. (1985). The method was based on selective complexation of Cu(I) with BCS and Cu(II) with EN, followed by spectrophotometric determination. The $[\text{Cu(I)BCS}_2]^{3-}$ complex exhibits an absorption maximum at a wavelength of 483 nm (A_{483}), and A_{483} increased linearly

with Cu(I) concentration between 10 and 2,000 nM in a 0.7 M NaCl medium at a seawater pH of 8.1. However, a decrease in complex stability is observed at pH values below 6. Although the method provided a relatively simple means of differentiating Cu(I) from Cu(II) and high yield (>97%), its application to natural waters was limited by a relatively high detection limit of approximately 10 nM.

Moffett and Zika (1988) subsequently developed a more sensitive, extraction-based method suitable for determining Cu(I) at concentrations encountered in natural waters. Cu(I) was selectively complexed with neocuproine (2,9-dimethyl-1,10-phenanthroline), a ligand similar to BCS that forms a sufficiently water-soluble Cu(I) complex, which is readily extracted into the solvents of interest, while Cu(II) was complexed with EN as in the spectrophotometric assay. After ligand addition to seawater, the Cu(I) complex was extracted into methylene chloride and subsequently back-extracted with nitric acid (HNO₃). Cu(I) concentrations were determined by AAS. The extraction yield was around 80%, and the data obtained were therefore corrected. Voelker et al. (2000) subsequently adapted the extraction procedure of Moffett and Zika (1988) to investigate Cu(I) formation during the radiolysis of seawater enriched with Cu. Neocuproine and pentetic acid were used as Cu(I)- and Cu(II)-selective ligands, respectively, and the concentration of Cu(I) generated during the experiment was determined using a similar extraction procedure. The recovery obtained was 51–55%, significantly lower than in the original work by Moffett and Zika, and the values were corrected accordingly. Overall measured Cu(I) values were significantly lower than expected, and several reasons may explain this; namely organic complexation or rapid oxidation of Cu(I) species by oxygen or hydrogen peroxide. Kieber et al. (2004) applied the extraction procedure developed by Moffett and Zika (1988) to rainwater. The yield was 48% in rainwater samples and 44% in seawater samples, which is lower than in the previous study. The study demonstrated the applicability of the extraction procedure to atmospheric waters but also highlighted the broader challenge of preserving Cu redox speciation during sample collection and processing.

Buerge-Weirich and Sulzberger (2004) developed an alternative SPE approach based on selective complexation of Cu(I) and Cu(II) with BCS and EN, respectively. Following selective complexation, the Cu(I) fraction was retained on the SPE column and subsequently eluted with methanol, while Cu concentrations were determined by AAS. The column contained 60 mg of a cross-linked polymeric sorbent with hydrophobicity comparable to that of conventional C8 sorbents. During passage of Cu(I)-containing samples, a distinct, narrow orange band formed within the column, indicating efficient retention of the [Cu(I)BCS₂]³⁻ complex by the sorbent.

Compared with liquid–liquid extraction procedures, this method avoided the use of large volumes of toxic organic solvents.

Despite their usefulness, these methods share several important limitations. First, none of the environmentally applicable approaches achieved 100% analytical recovery, so the measured Cu(I) concentrations had to be corrected for the recoveries obtained (Buerge-Weirich and Sulzberger, 2004; Kieber et al., 2004; Moffett and Zika, 1988; Voelker et al., 2000). Second, sample preservation and manipulation can represent a significant source of uncertainty. In particular, Buerge-Weirich and Sulzberger (2004) analysed samples that had been stored at -18°C and subsequently exposed to artificial light after thawing. The intensity of the artificial radiation was considerably higher than that of solar radiation within the water column, potentially altering the original Cu redox distribution. Consequently, although the method provided valuable information on Cu(I) occurrence, the resulting measurements cannot necessarily be considered representative of *in situ* Cu redox speciation. Overall, existing analytical approaches demonstrate that Cu(I) can be quantified in natural waters, but they also reveal a substantial methodological gap. The development of methods that minimise sample handling, provide quantitative recovery, achieve sufficiently low detection limits, and preserve the original Cu redox speciation is therefore essential for resolving Cu redox speciation under environmentally relevant conditions.

In this work, an adapted SPE method based on Buerge-Weirich and Sulzberger (2004) was applied to determine Cu redox speciation in estuarine and rainwater samples. The adaptations to the original procedure are described in Section 5.1 and Research Paper II.

3. OBJECTIVES AND HYPOTHESES OF THE WORK

The doctoral thesis has three main objectives:

- (O1) to investigate the interactions between Cu and thiols in model solutions,
- (O2) to optimise a method for determining Cu redox speciation and apply it to natural waters,
- (O3) to assess the factors controlling Cu redox speciation in natural waters.

The thesis addresses three main hypotheses:

- (H1): Thiols reduce Cu(II) and stabilise Cu(I) in model solutions even in the presence of oxygen.
- (H2): In estuarine environments, Cu(I) constitutes a significant fraction of total dissolved Cu.
- (H3): Cu(I) generation in natural waters can be attributed to biological activity and photochemical processes.

The proposed research is expected to advance the understanding of Cu redox speciation in estuarine environments, primarily by defining the role of organic ligands in Cu redox processes. Optimisation of analytical protocols for the determination of Cu(I) and new datasets on Cu(I) and the factors controlling its formation and stabilisation in the environment are anticipated. Overall, the study should introduce novel insights into the current understanding of Cu biogeochemical cycling in estuarine and marine systems.

4. RESEARCH PAPERS

This doctoral thesis is based on three scientific publications:

Research Paper I (Appendix A.1): Crmarić, D., Bura-Nakić, E. 2023. Interaction between copper and thiols of biological and environmental importance: case study using combined spectrophotometric/bathocuproine disulfonate disodium salt (BCS) assay. *Molecules* 28, 5065. <https://doi.org/10.3390/molecules28135065>

Research Paper II (Appendix A.2): Crmarić, D., Marcinek, S., Cindrić, A.-M., Omanović, D., Bura-Nakić, E. 2025. Redox speciation of copper in the estuarine environment: Towards better understanding of copper water chemistry. *Mar. Chem.* 268, 104471. <https://doi.org/10.1016/j.marchem.2024.104471>

Research Paper III (Appendix A.3): Crmarić, D., Iranzo, O., Mlakar, M., Stipanović, N., Bura-Nakić, E. 2026. Stability of copper(I)-cysteine complex assessed using reverse titrations: implications for estuarine and seawater copper biogeochemistry. *Biogeochemistry* 169, 5. <https://doi.org/10.1007/s10533-025-01299-4>.

The research initially focused on understanding the interaction between Cu and thiols under controlled conditions in model solutions. Research Paper I showed that thiols such as Cys, GSH, 3-MPA and TAA can reduce Cu(II) to Cu(I) and contribute to Cu(I) stabilisation through complex formation. The next step was to adapt a method for Cu redox speciation in an estuarine environment and to assess whether Cu(I) occurs, and which factors may lead to its formation and stabilisation. Research Paper II showed that Cu(I) represents a significant fraction of dissolved Cu in the Krka River estuary and that its distribution is related to biological activity, organic complexation and photochemical reactions. In particular, the high Cu(I) fractions observed at the surface and the halocline during periods of high biological production suggested a possible role for biologically produced ligands in Cu(I) stabilisation. This led to the next research step, presented in Research Paper III, in which Cys-like compounds were detected directly in the estuary and their interaction with Cu(I) was further investigated. The co-occurrence of the highest Cu(I) fractions where the Cys-like compounds were detected, together with the conditional stability constant of the Cu(I)–Cys complex being within the range reported for the strong Cu-binding ligand class, supports its possible role in Cu(I) stabilisation and Cu redox cycling.

5. DISCUSSION

5.1. Analytical challenges and methodological advances in copper redox speciation

5.1.1. Copper(I) stability and chloride effects

Working with Cu(I) in aqueous media is challenging because of its inherent susceptibility to oxidation and disproportionation. Therefore, Cu(I) experiments should preferably be conducted under anaerobic conditions, ideally in a glovebox, using deoxygenated solvents. However, as access to a glovebox is not always feasible, preparation of Cu(I) stocks in highly concentrated, acidified NaCl provides a simpler and more practical alternative for improving Cu(I) stability over the timescale required for analytical measurements. Two Cu(I) stock-preparation procedures were used in this work: dissolution of tetrakis(acetonitrile)copper(I) hexafluorophosphate in deoxygenated acetonitrile (ACN) under anaerobic conditions in a glovebox (Research Paper III), and dissolution of CuCl in deoxygenated NaCl/HCl, following a modified procedure of Moffett et al. (1985) (Research Papers I, II and III). Both stock solutions were subsequently checked for Cu(I) content by spectrophotometry based on the absorbance of the $[\text{Cu(I)BCS}_2]^{3-}$ complex (Xiao et al., 2004), and their concentrations were corrected accordingly. For the Cu(I)-ACN procedure, the stock solution remained stable for several days under anoxic conditions inside the glovebox (data not shown). On the other hand, the stability of the CuCl stock solution varied with the NaCl concentration in the medium (Appendix A4). The stock solution with the highest NaCl concentration (4 M) was the most resistant to oxidation. Therefore, the NaCl concentration used for stock preparation was increased from the 1 M proposed by Moffett et al. (1985) to 4 M, as significant Cu(I) oxidation was not observed during the experimental period. This observation is consistent with the strong influence of Cl^- on Cu(I) speciation and oxidation kinetics reported previously. In particular, several studies in seawater and NaCl solutions have demonstrated that Cu(I) oxidation by O_2 decreases with increasing Cl^- concentration and ionic strength, an effect attributed to changes in Cu(I)-Cl speciation and the different oxidation reactivities of the resulting Cu(I) species (González-Dávila et al., 2009; Moffett and Zika, 1983; Sharma and Millero, 1988). At 4 M NaCl, Cu(I) is expected to be predominantly present as higher-order Cu(I)-Cl complexes, particularly CuCl_2^- and CuCl_3^{2-} (Mei et al., 2013; Sherman, 2007). These observations were subsequently reflected in recommendations for working with Cu(I) in aqueous media developed within the framework of the NECTAR COST Action 18202 (Iranzo et al., 2024).

On the other hand, while Cl^- stabilises Cu(I) against oxidation and is therefore among the potential factors contributing to Cu(I) stabilisation in natural waters, it can pose an analytical challenge for Cu(I) determination using BCS. As discussed in Research Paper III, Cl^- can affect $[\text{Cu(I)BCS}_2]^{3-}$ formation. In Cl^- -free solutions, Cu(I) concentrations calculated from the absorbance of the $[\text{Cu(I)BCS}_2]^{3-}$ agreed well with the concentrations expected from the added Cu(I). However, in Cl^- -containing solutions, lower Cu(I) concentrations were obtained when BCS was not present in sufficient excess, and the absorbance of the $[\text{Cu(I)BCS}_2]^{3-}$ decreased systematically with increasing NaCl concentration, potentially through the formation of mixed Cu(I)–BCS–Cl complexes. Under the conditions tested, i.e. 0.55 M NaCl and pH 8.2, a BCS:Cu(I) ratio of 4:1 or higher was necessary to suppress the chloride effect. Although Cu(I) shows improved stability in acidic compared with basic media, this effect is relatively small compared with that of ionic strength, i.e. NaCl concentration, indicating that acidic or basic species are not main drivers of Cu(I) oxidation (González-Dávila et al., 2009). However, formation of the $[\text{Cu(I)BCS}_2]^{3-}$ requires $\text{pH} > 6$; therefore, the use of a buffer, such as borate buffer, is necessary to support complex formation. Without a buffer, the acidic Cu(I) stock solution lowers the pH of the Cu(I)–BCS solution, contributing to protonation of BCS and hindering Cu(I)–BCS complex formation.

5.1.2. Methodological advancements in copper redox speciation

An adapted SPE approach was developed to address the analytical challenge of separating Cu(I) and Cu(II), based on the work of Buerge-Weirich and Sulzberger (2004). BCS was used to stabilise and selectively retain Cu(I) as the $[\text{Cu(I)BCS}_2]^{3-}$ complex, while EDTA was added to complex Cu(II) and prevent its retention on the column. A BCS:EDTA ratio of 1:5 was applied, resulting in final concentrations of 1 μM BCS and 5 μM EDTA in the samples. The Cu(I)–BCS complex was retained on the polymeric SPE sorbent, whereas Cu(II)–EDTA passed through the column. The SPE procedure is illustrated in **Figure 3**, which shows columns after passage of a model solution; the visible orange band corresponds to the retained $[\text{Cu(I)BCS}_2]^{3-}$ complex, which is subsequently eluted with methanol.

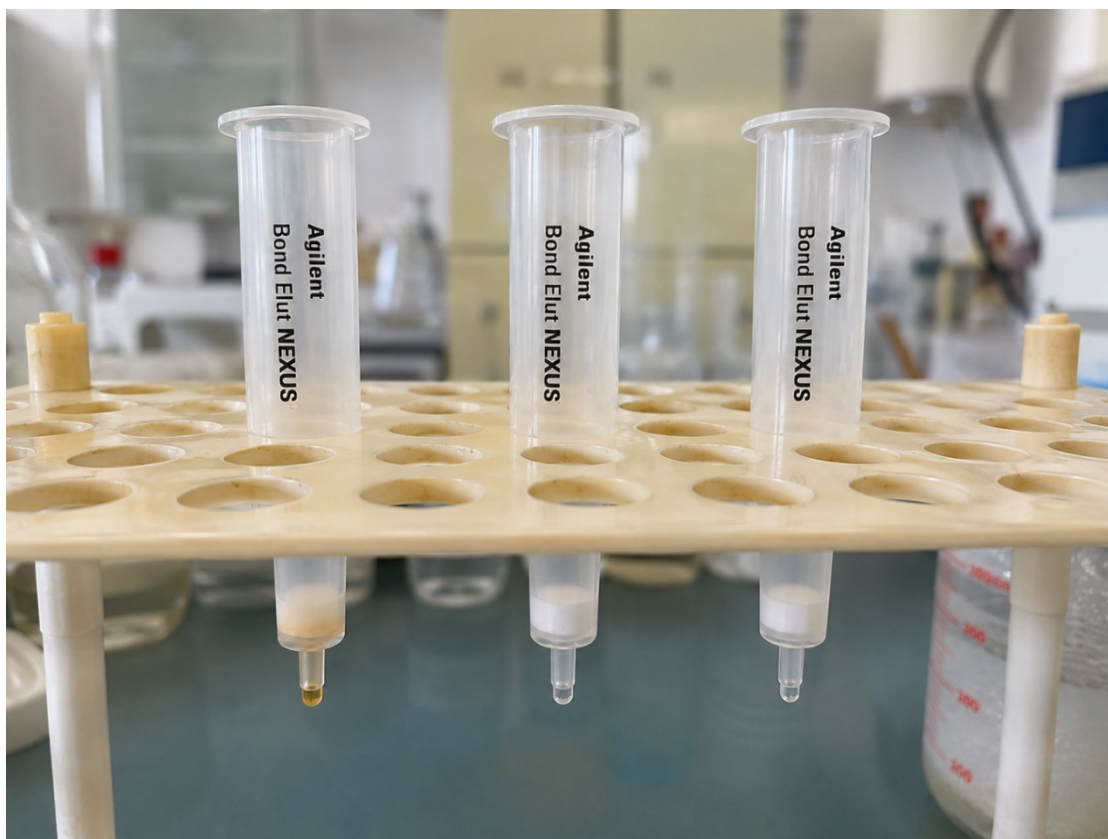


Figure 3. An adapted SPE procedure on columns used for Cu redox speciation in Research Papers II and III.

Following elution of Cu(I)–BCS with acidified methanol, the methanolic eluate was evaporated and the residue was redissolved in ultrapure water prior to voltammetric determination. This step enabled the Cu(I) fraction to be analysed by differential pulse anodic stripping voltammetry (DP-ASV), replacing the AAS used in the original approach. Cu(I), Cu(II) and Cu_T were subsequently quantified by DP-ASV using the standard addition method. The Cu(I) fraction was pre-concentrated during SPE, whereas Cu(II) and Cu_T were analysed without pre-concentration. Importantly, pre-concentration of Cu(I) extends the applicability of the method beyond the concentration range encountered in this work to systems where Cu(I) occurs at multi-fold lower concentrations. The method was validated with respect to the recovery of Cu(I) and Cu(II) from an estuarine sample. Quantitative recovery after SPE was achieved, allowing the determination of Cu redox speciation in the Krka River estuary without further modifications. Nonetheless, in the present method, 50 mL of sample was passed through the 60 mg polymeric SPE column over approximately 2 h (0.4 mL min^{-1}), considerably more slowly than the $1\text{--}2 \text{ mL min}^{-1}$ loading rate reported by (Buerge-Weirich and Sulzberger, 2004). The slower flow rate, together with the other modifications introduced in the present procedure,

may have contributed to the quantitative Cu recovery observed in the present recovery experiments, compared with the mean recovery of approximately 70% reported for the original method. Moreover, in the former study, samples were frozen on board and subsequently thawed and irradiated before Cu(I)/Cu(II) separation, whereas the adapted procedure applied in the Krka River estuary introduced the Cu(I)- and Cu(II)-binding ligands immediately after filtration and enabled Cu redox speciation to be determined shortly after sampling.

In Research Paper III, the analytical approach was further extended from determining Cu redox speciation in estuarine samples to the voltammetric investigation of ligands that are potential candidates for Cu(I) stabilisation, providing an experimental basis for further investigating the mechanisms responsible for Cu(I) occurrence in natural waters. Based on these analytical approaches, the following sections examine the environmental occurrence and distribution of Cu(I) in the Krka River estuary, followed by a discussion of the role of thiols and photochemical reactions in Cu redox transformations. Finally, the broader biogeochemical implications of Cu(I) occurrence in estuarine waters are discussed.

5.2. Environmental occurrence of copper(I) in the Krka River estuary

To assess the occurrence of Cu(I) in the Krka River estuary, an adapted SPE method was applied along the vertical water column at two sites (E1 “clean site”, E2 “polluted” site in the nautical marina) during four sampling campaigns (spring 2023, summer 2023, spring 2024, summer 2024). Additionally, seasonal sampling of the surface estuarine layer and rainwater was conducted periodically at the Marine Research Station Martinska (E3) (autumn 2023–summer 2024). The sampling campaigns conducted in spring and summer 2023 are presented in Research Paper II, while the sampling conducted at E1 in summer 2024 is presented in Research Paper III. These results are briefly discussed in the following sections, together with unpublished results from the additional sampling campaigns.

5.2.1. Seasonal redox speciation of copper in the Krka River estuary: E1 „clean“ site

At station E1, Cu(I) represented a substantial fraction of Cu_T throughout the water column. In 2023, the Cu(I) fraction ranged from 36.1–88.7% of Cu_T in spring and 50.9–83.4% in summer. The vertical distribution of Cu(I) differed markedly between seasons. In spring, the highest Cu(I) fraction was observed in the low-salinity surface layer, decreasing towards the deeper

seawater layer, where the lowest values were measured. The surface maximum occurred under conditions favourable for freshwater biological activity, with a relatively elevated temperature for the spring period, and may therefore reflect the production of organic Cu(I)-binding ligands by riverine-derived biota. In contrast, the summer profile showed the lowest Cu(I) fraction at the surface and the highest values around the halocline. The surface layer was characterised by low salinity, higher SUVA and stronger humic-like fluorescence (A, C and M), indicating a greater contribution of terrestrial, more aromatic and humified DOM. The lower Cu(I) fraction in this layer may therefore be related to the predominance of humic-like organic matter, which is generally associated with Cu(II) complexation. At the halocline, where Cu(I) fraction was highest, DOC was elevated and BIX reached its maximum, indicating a greater relative contribution of recently produced, protein-like DOM, consistent with a potential contribution of biologically derived organic ligands. In the seawater layer, Cu(I) remained the dominant Cu redox form despite lower biological activity, and the higher salinity and associated chloride complexation may contribute to its persistence. The observed association between Cu(I) and biologically influenced organic matter points to an important role of organic ligands in Cu(I) stabilisation, while photochemical reactions may additionally influence organic ligands and Cu redox transformations.

The subsequent spring 2024 sampling at E1 revealed that Cu(I) accounted for 43.3–72.4 % of Cu_T (**Figure 4**). Detailed information on the sampling campaign is provided in Appendix A.5.1. The spring 2024 profile again revealed a surface maximum in Cu(I)/ Cu_T , similar to the pattern observed at the same station in spring 2023. The maximum Cu(I) share found in the surface layer was followed by similar shares of Cu(I) at the halocline. The highest abundance of protein-like fluorescent material (peak T) was at the halocline and in the surface layer. In contrast, the seawater layer showed lower protein-like fluorescence and Cu(I) fractions; accumulation of protein-like fluorophores in the estuary is common above the halocline, reflecting production and degradation of protein-like compounds, while primary production peaks at the halocline, where the highest BIX is observed. However, low DOC but relatively high SUVA and low $S_{275-295}$, together with high humic-like fluorescence, indicate that the surface layer was strongly influenced by riverine water (Marcinek et al., 2020). Nevertheless, the co-occurrence of the highest Cu(I) shares and fluorescence of protein-like substances, together with indications of the role of biologically derived ligands in Cu(I) formation and stabilisation already suspected in summer 2023, prompted an investigation of thiol-like ligands in parallel with Cu redox speciation in summer 2024.

Besides the data published in Research Paper III, additional data regarding DOM characterisation in the summer 2024 campaign may be found in Appendix A.5.2. The summer 2024 sampling at E1 further confirmed the substantial occurrence of Cu(I) in the Krka River estuary, with Cu(I) accounting for 50.2–82.5% of Cu_T. The highest Cu(I) fractions were observed in the surface/subsurface brackish layer, where the highest DOC and chlorophyll *a* (Chl *a*) were recorded. Cys-like compounds were also detected in this layer, along with high fluorescence of protein-like substances. Although the contribution of riverine waters was still most pronounced in the surface layer, a notably stronger contribution of recently produced organic matter was observed in summer (Table A2), in contrast to the more pronounced terrestrial humic signature observed in the spring surface layer (Table A1). Overall, this co-occurrence of maxima in Cu(I) fraction, DOC and Chl *a*, together with the presence of Cys-like compounds and protein-like fluorophores, provides a link between Cu(I) occurrence and biologically derived organic ligands. However, in contrast to summer 2023, when the Cu(I) maximum at the halocline coincided with the highest BIX, the July 2024 Cu(I) maximum occurred above the halocline, whereas BIX reached its maximum at the halocline. Nonetheless, the highest Cu(I) concentration occurred at the halocline, although the highest fraction with respect to Cu_T occurred at the surface.

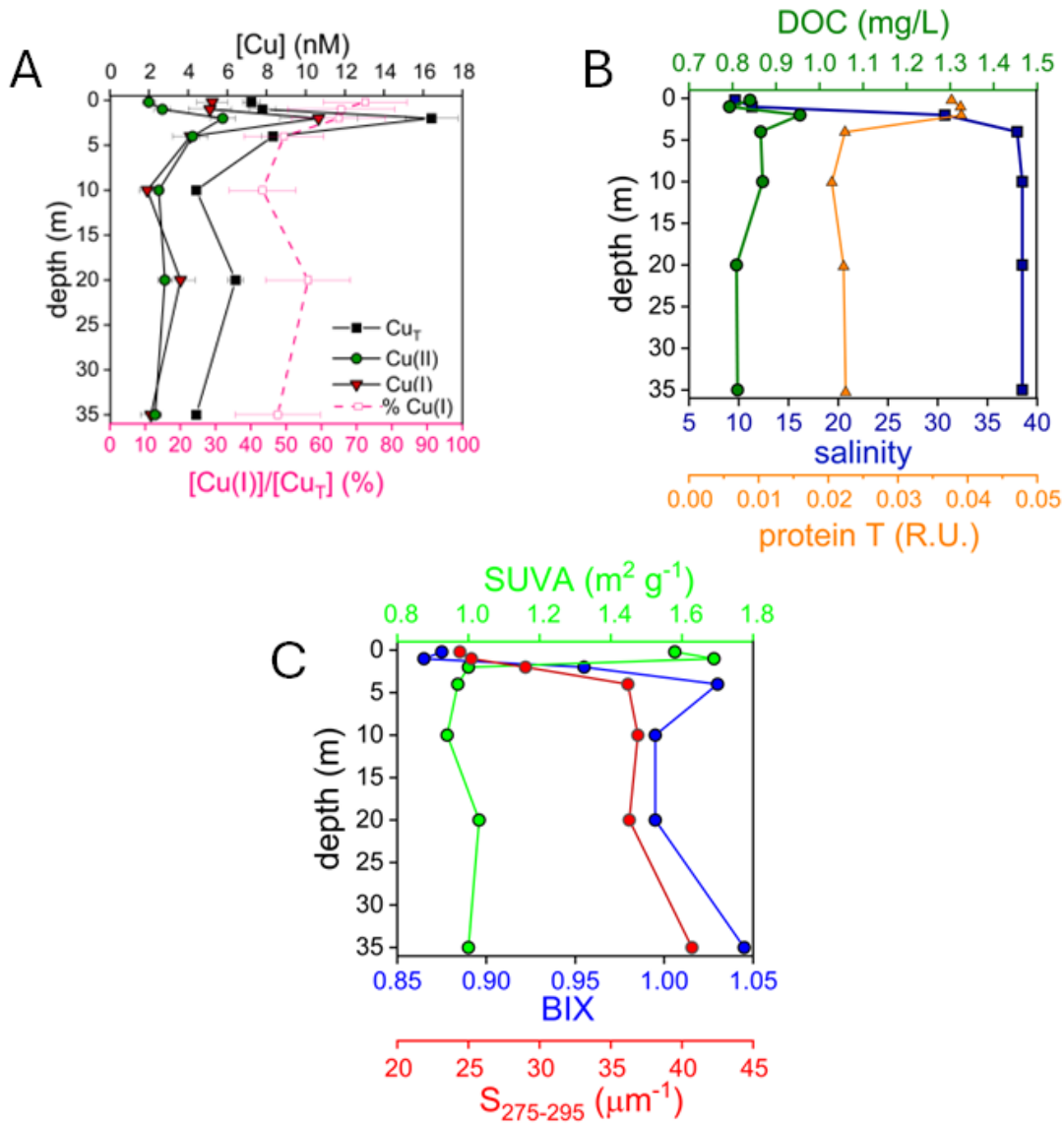


Figure 4. Vertical distribution of: Cu_T , $Cu(II)$, $Cu(I)$ and the fraction of $Cu(I)$ at E1 in spring 2024 (A); DOC (green, top x-axis), salinity (blue; middle/lower x-axis) and protein T (orange, bottom x-axis) (B); SUVA (green, top x-axis), BIX (blue; middle/lower x-axis) and $S_{275-295}$ (red; bottom x-axis) (C).

To further evaluate the relationship between biologically derived DOM and $Cu(I)$, Spearman rank correlations were calculated between BIX and both $Cu(I)$ fraction and $Cu(I)$ concentration. In summer 2023 at E1, BIX showed a moderate positive correlation with the $Cu(I)$ fraction ($\rho=0.486$, $p=0.356$), although this was not statistically significant, and a very strong statistically significant positive correlation with $Cu(I)$ concentration ($\rho=0.943$, $p=0.017$). This relationship was largely driven by the halocline, where the highest BIX value (1.0092) coincided with both the highest $Cu(I)$ fraction (83.4%) and the highest $Cu(I)$ concentration (7.76 nM). The statistical relationships were less consistent in 2024. Considering all depths sampled at E1 during 2024,

BIX showed no significant correlation with either the Cu(I) fraction or Cu(I) concentration. In contrast, protein T showed a moderate positive correlation with the Cu(I) fraction ($\rho=0.591$, $p=0.026$), while its correlation with Cu(I) concentration was positive but did not reach statistical significance ($\rho=0.499$, $p=0.069$). This was mainly due to the strong positive correlation between the Cu(I) fraction and fluorescence of protein-like substances observed in spring 2024 ($\rho=0.857$, $p=0.024$). The statistical analysis should nevertheless be regarded as exploratory, as the number of sampling campaigns, depth observations and available DOM parameters was limited. However, considering these four sampling campaigns, some differences were noted in Cu redox speciation between seasons. Values of Cu_T were relatively similar across the E1 campaigns, whereas the Cu(I) fraction exhibited greater seasonal variability, with a higher median in summer (71.6%) than in spring (60.6%) (Appendix A.5.3, Table A3). This observation could further point to the role of biologically produced organic ligands in Cu(I) formation and stabilisation, as biological activity in summer was higher throughout the vertical water column compared with spring, and the contribution of humic-like ligands, presumably with higher affinity towards Cu(II) than Cu(I), to the estuarine DOM pool is lower than in spring. Nonetheless, longer duration of insolation and higher UV irradiation in summer compared with spring (Croatian Meteorological and Hydrological Service, 2023/2024) suggests greater photochemical activity, which could potentially result in more Cu(II) reduction. The role of thiol-like compounds in Cu(I) stabilisation is discussed in Section 5.3, whereas the role of photochemical processes, along with an additional diurnal sampling of surface layer at E2, is addressed in Section 5.4.

5.2.2. Seasonal redox speciation of copper in the Krka River estuary: E2 “polluted” site

At the E2 “polluted” site, located in a nautical marina, Cu(I) also represented a substantial fraction of Cu_T , accounting for 63.2–73.8% in spring 2023. The Cu(I) fraction was relatively uniform throughout the water column, with only a slight decrease towards the bottom, despite increasing DOC with depth. Compared with E1, the absence of a pronounced surface maximum in spring 2023 may be related to the higher surface salinity and consequently lower freshwater biological activity. In summer 2023, however, Cu_T increased several-fold compared with spring, consistent with increased Cu inputs from antifouling coatings in the marina (Cindrić et al., 2020, 2015). Cu_T was also significantly higher at E2 than at E1. At the same time, the Cu(I)/ Cu_T ratio decreased to 20.7–59.3%, with the highest value at the halocline and the lowest in the bottom seawater layer. The halocline maximum coincided with the highest BIX,

suggesting a contribution from biologically produced organic ligands, whereas the low Cu(I) fraction at the bottom corresponded to low DOC and BIX and high salinity. At the surface, high DOC and stronger humic-like fluorescence (A, C and M) were associated with a lower Cu(I) fraction. Photochemical reduction may nevertheless contribute to the persistence of Cu(I) in the illuminated surface layer, as reduction of Cu(II) complexes with HA may occur via photochemical pathways. Overall, the E2 results indicate that anthropogenic Cu enrichment during summer was accompanied by a shift towards a greater Cu(II) contribution. This contrasts with E1, where Cu(I) remained the predominant redox form throughout the summer water column. Although complexation parameters were not determined at E2 due to the high Cu_T , the Cu(I)-binding ligand pool was likely insufficient to complex the high Cu_T concentrations, resulting in a greater proportion of Cu present as Cu(II). Moreover, it is possible that other anthropogenic organic compounds, which bind Cu(II) over Cu(I), were present at the site.

In addition to the sampling campaigns conducted in spring and summer 2023 at E2, two additional sampling campaigns at E2 were conducted in spring and summer 2024; details may be found in Appendices A.5.1 (spring 2024) and A.5.2 (summer 2024). The fraction of Cu(I) in Cu_T ranged from 47.4 to 79.2% in spring, and from 37.9 to 63.9% in summer (**Figure 5**). An increase in Cu_T in spring 2024 in the surface layer and the halocline compared with spring 2023 can be explained by a later sampling date (May 2024 compared with March 2023), as nautical tourism is more pronounced in May than in March (Carić et al., 2021). In spring, the maximum Cu(I) fraction was found in the surface layer, while in summer it was at the halocline. The spring profile was characterised by higher Cu(I) fractions in the surface layer, characterised by low salinity and high DOC and Chl *a*. However, the relatively modest Chl *a* gradient and the lack of additional DOM characterisation for spring do not allow a direct attribution of the Cu(I) distribution to biologically produced ligands. The summer profile was substantially different with respect to Cu_T , which markedly increased compared with spring, as anthropogenic input of Cu related to nautical tourism reaches its peak during the summer months. Unlike at E1, where Cu(I) correlated with parameters pointing to increased primary production/concentration of biologically produced organic ligands, no direct correlation was found between the fraction of Cu(I) and DOC, Chl *a*, salinity (**Figure 5**) or optical characteristics of DOM (Table A2). In summer, the maximum Cu(I) fraction was found at the halocline, while the highest BIX, protein-like fluorescence and DOC occurred in the surface layer. As already discussed for summer 2023 at the same site, this decoupling could be explained by exceptionally high Cu_T

in the surface layer, probably exceeding the concentration of Cu(I)-binding ligands. Nonetheless, decoupling was observed between DOC and Chl *a*, as Chl *a* increases with decreasing DOC, emphasising that DOC dynamics can be decoupled from phytoplankton biomass, i.e. that DOC and Chl *a* content responded to different controlling processes; for example, riverine-derived allochthonous DOM may have a stronger influence on DOC than phytoplankton biomass (Lee and Kim, 2026; Sánchez-Pérez et al., 2020). Consequently, other factors such as chloride complexation (Section 5.1.1 and Appendix A.4) and photochemical processes may (Section 5.4) play a more pronounced role at E2 than complexation with ligands of biological origin.

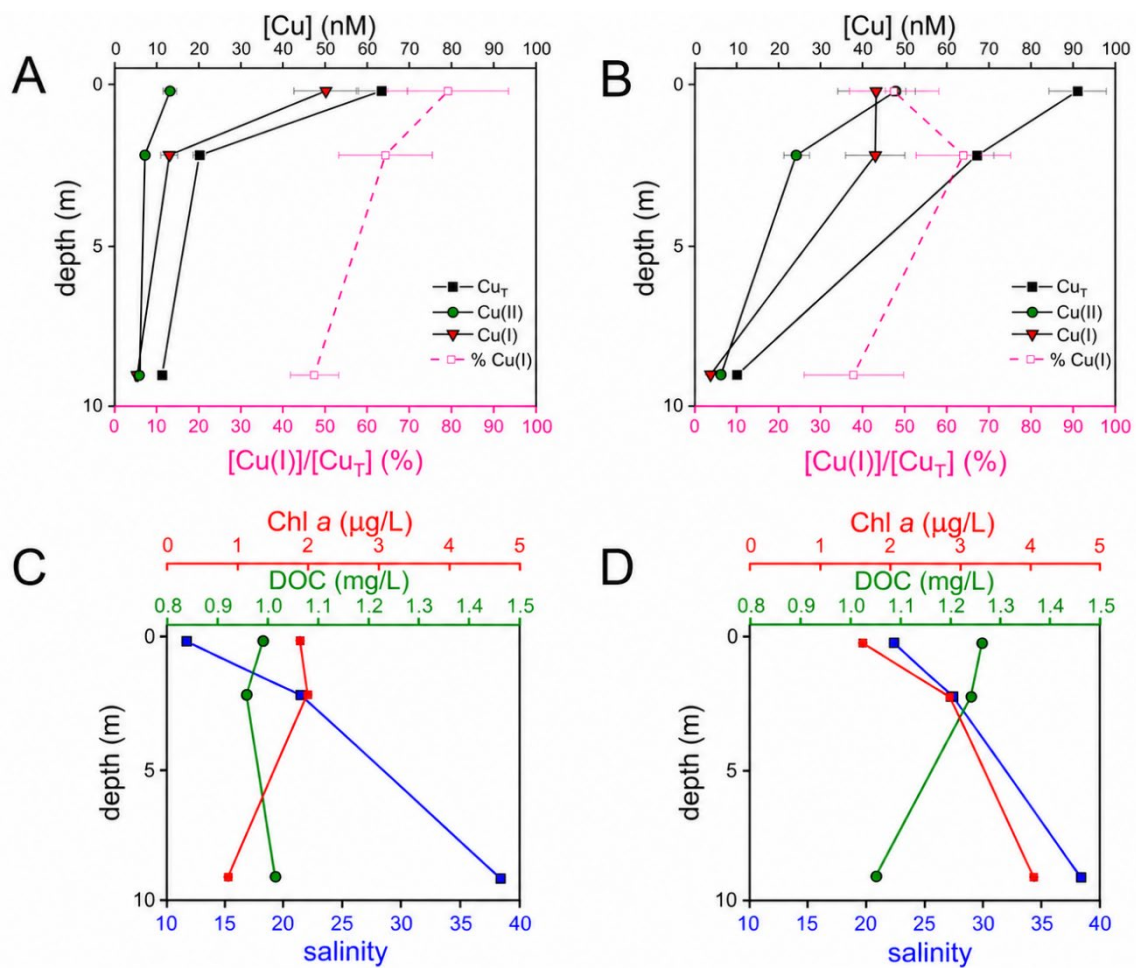


Figure 5. Vertical distribution of Cu_T, Cu(I), Cu(II) (top x-axis) and fraction of Cu(I) (bottom x-axis) at E2 in spring 2024 (A) and summer 2024 (B); Chl *a* (red, top x-axis), DOC (green, middle/lower x-axis), and salinity (blue, bottom x-axis) at E2 in spring 2024 (C) and summer 2024 (D).

Further statistical analysis at E2 was not considered relevant, as fewer depths ($n=3$) were sampled per sampling campaign, and optical characterisation of DOM was available only for the summer campaigns. However, the observations from the spring and summer 2023 sampling campaigns at E2, namely that the summer increase in Cu_T was accompanied by a decrease in the Cu(I) fraction, are further supported by the 2024 sampling campaigns. Across the two spring campaigns, the median Cu_T was 17.11 nM, compared with 50.37 nM across the two summer campaigns, while the median Cu(I) fraction decreased from 68.6% in spring to 42.7% in summer (Table A3). This consistent inverse relationship between Cu_T and the Cu(I) fraction supports the hypothesis that elevated Cu_T concentrations during summer may exceed the available pool of Cu(I)-binding ligands, thereby limiting Cu(I) stabilisation and resulting in a greater relative contribution of Cu(II).

5.2.3. Seasonal redox speciation of copper in rainwater and the estuarine surface layer: E3 site

In addition to the seasonal sampling of the vertical profiles at E1 and E2, rainwater and surface estuarine layer sampling at E3 were conducted periodically from October 2023 to September 2024. The resulting dataset is provided in Appendix A.5.4, together with detailed information on the applied methodologies. The combined rainwater and estuarine dataset demonstrates substantial variability in Cu concentrations and redox speciation (**Figure 6A and B**). In rainwater, Cu_T ranged from 3.95 to 227.4 nM, while Cu(I) ranged from not detected to 41.67 nM and represented 0–47.0% of Cu_T (**Figure 6A**). The median fraction of Cu(I) was 6.7%. The concentration of Cu(I) was significantly positively correlated with Cu_T ($\rho=0.797$, $p=0.003$), whereas no significant relationship was observed between Cu_T and the fraction of Cu(I) ($\rho=0.269$, $p=0.424$). Therefore, although events with higher Cu_T generally contained higher concentrations of Cu(I), an increase in Cu_T did not result in a proportional increase in the Cu(I) fraction, indicating that Cu loading and Cu redox partitioning are not controlled by the same factors.

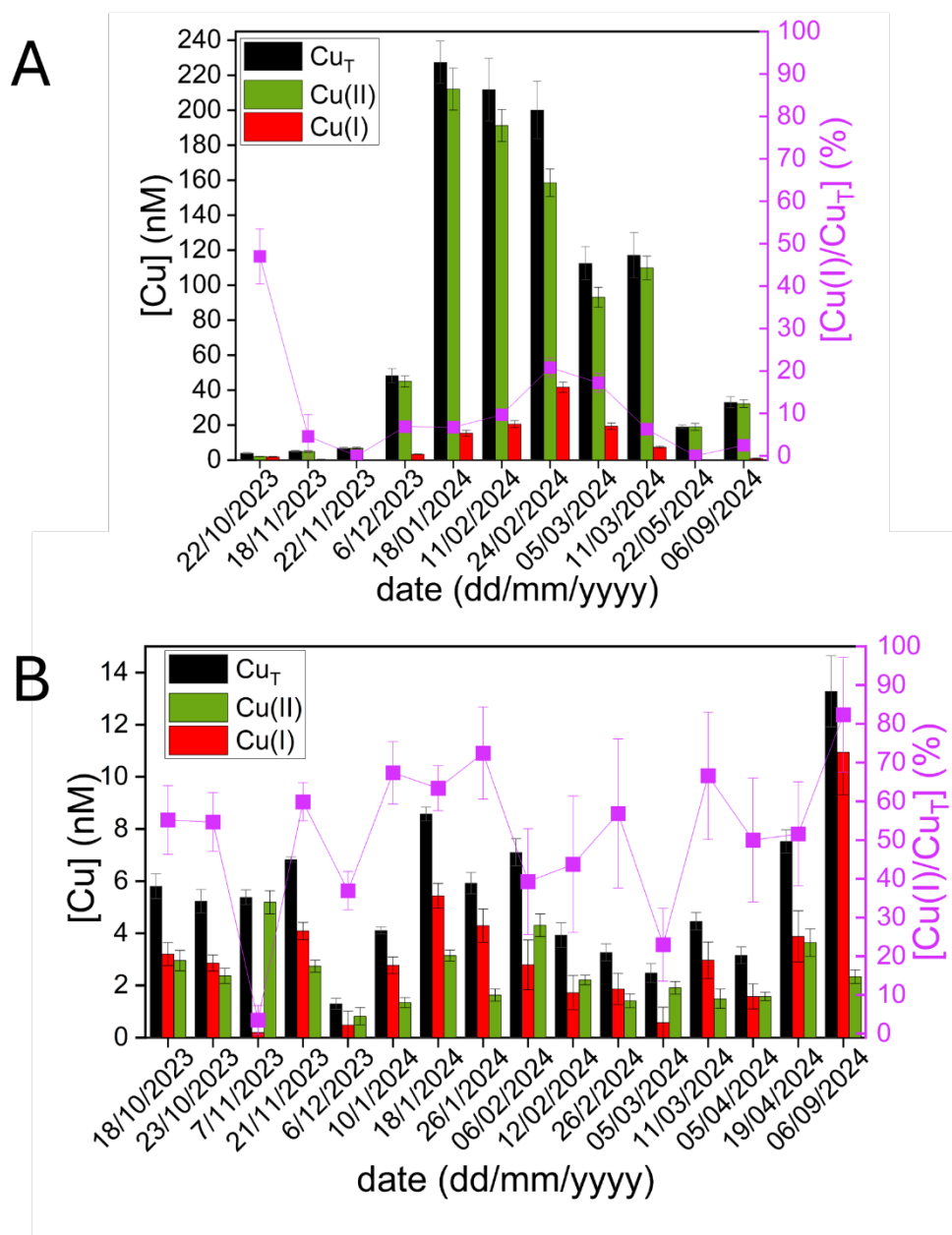


Figure 6. Temporal variation in Cu_T, Cu(II), and Cu(I) concentrations and the Cu(I) fraction in the rainwater (A) and estuarine surface water (B) at E3. Bars show Cu_T, Cu(II), and Cu(I) concentrations (left y-axis), while magenta squares and lines show the Cu(I) fraction (right y-axis).

In rainwater samples, Na was positively correlated with Cu(I) ($\rho=0.679$, $p=0.022$) and the Cu(I) fraction ($\rho=0.606$, $p=0.048$), as was Mg, which showed positive correlations with Cu(I) ($\rho=0.706$, $p=0.015$) and the Cu(I) fraction ($\rho=0.724$, $p=0.012$). Neither Na nor Mg showed a significant relationship with Cu_T. Since Na and Mg are related to marine aerosols, these relationships indicate that marine-influenced atmospheric conditions may have contributed to the occurrence and persistence of Cu(I). However, marine and continental/anthropogenic

influences were not necessarily mutually exclusive, as the concentrations of major ions and trace metals indicate that individual precipitation events were influenced simultaneously by multiple sources (Appendix A.5.4; Table A4 and **Figure A3**). Nonetheless, strong relationships with the Cu(I) fraction were observed for Cl^- , which is also commonly associated with marine aerosol input (**Figure 7A**). Chloride was significantly positively correlated with both Cu(I) ($\rho=0.788$, $p=0.004$) and the Cu(I) fraction ($\rho=0.815$, $p=0.002$), whereas no significant relationship was observed between Cl^- and Cu_T ($\rho=0.436$, $p=0.180$). Therefore, Cl^- was associated specifically with the concentration and fraction of Cu present as Cu(I), rather than with Cu_T . This observation is consistent with Kieber et al. (2004), who identified chloride, sulfite and organic Cu-complexing ligands as potential contributors to Cu(I) stabilisation in rainwater. A similar distinction was observed for sulphate (SO_4^{2-}). SO_4^{2-} was not significantly correlated with Cu_T ($\rho=0.251$, $p=0.457$), and its relationship with the concentration of Cu(I) was positive but not statistically significant ($\rho=0.573$, $p=0.065$). In contrast, the Cu(I) fraction was significantly positively correlated with the measured SO_4^{2-} concentration ($\rho=0.696$, $p=0.017$) (**Figure 7B**). Interpretation of this relationship requires particular caution because of the analytical behaviour of the ion chromatographic method used in this study. Standard additions of SO_4^{2-} and sulfite (SO_3^{2-}) resulted in peaks at the same retention time under the applied analytical conditions, possibly due to oxidation of SO_3^{2-} to SO_4^{2-} before or during analysis (data not shown). The observed relationship with the Cu(I) fraction may consequently also include a contribution from SO_3^{2-} . This possibility is particularly relevant because Kieber et al. (2004) identified SO_3^{2-} as a potential Cu(I)-stabilising ligand in rainwater, while Xue et al. (1991) demonstrated SO_3^{2-} -mediated Cu(II) reduction and formation of complexes between Cu(I) and SO_3^{2-} in fogwater. However, because SO_3^{2-} was not independently quantified, the data cannot demonstrate that SO_3^{2-} was responsible for the observed relationship.

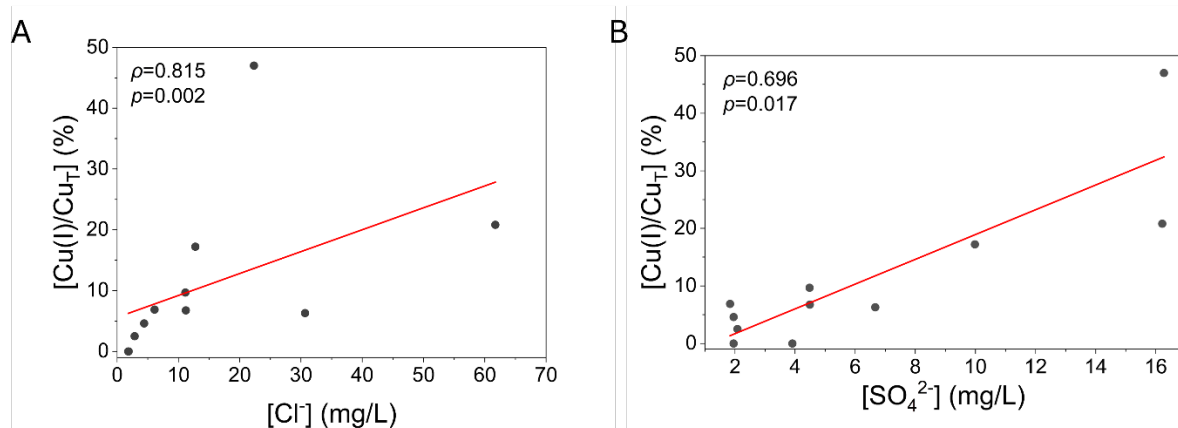


Figure 7. Relationships between the Cu(I) fraction and chloride (Cl⁻) (A) and sulphate (SO₄²⁻) (B) concentrations in rainwater at E3 (autumn 2023–summer 2024). Spearman's rank correlation coefficients (ρ) and corresponding p-values are shown in each panel. Red lines represent linear regression fit.

Specific atmospheric events provide additional information on the factors affecting Cu redox speciation. The pollen period at the site starts in March and peaks at the end of March and beginning of April, and has previously been associated with an increase in allochthonous Cu-binding ligands such as proteins (Strmečki et al., 2024). The 5 and 11 March 2024 events therefore occurred during the pollen period and may have been affected by pollen-derived organic material, although no sampling occurred during the peak pollen period. The two events had similar Cu_T concentrations, 112.5 and 117.2 nM, respectively, but differed substantially in Cu(I), with 19.38 nM (17.2%) on 5 March and 7.39 nM (6.3%) on 11 March. Pollen-derived Cu-binding ligands therefore represent a possible contributing factor, although the available observations are insufficient to demonstrate a specific pollen effect.

The Saharan dust events documented at the site in April, May and June (Milinković et al., 2026; under review) are compatible with the sampling on 22 May 2024. This event was characterised by strong mineral aerosol input, yet no Cu(I) was detected, while Cu_T was 19.02 nM. The absence of Cu(I) during this event indicates that enhanced mineral aerosol input did not necessarily favour Cu(I) formation or persistence. Considering all rainwater samples, among the elements associated with mineral dust, significant correlation with Cu (either the concentration of Cu(I), its fraction, or Cu_T) was observed only for Fe, which may origin from anthropogenic sources as well (Matsui et al., 2018). Fe showed significant negative relationships with Cu_T ($\rho=-0.609$, $p=0.047$) and Cu(I) ($\rho=-0.697$, $p=0.017$), whereas its relationship with the Cu(I) fraction was negative but not statistically significant ($\rho=-0.533$,

$p=0.091$). Considering other trace elements analysed in rainwater, a significant positive correlation was observed between Cu_T and Zn ($\rho=0.691$, $p=0.019$), while Cu(I) showed an even stronger positive relationship with Zn ($\rho=0.743$, $p=0.009$). In contrast, no significant relationship was observed between the fraction of Cu(I) and Zn ($\rho=0.305$, $p=0.361$). Therefore, Zn was associated with the magnitude of the Cu_T , and the concentration of Cu(I), but not with the redox speciation of Cu. Zn is frequently associated with anthropogenic atmospheric particles (Wei et al., 2025), and has previously been positively correlated with a group of metals originating from road traffic emissions (Cu, Pb, Sb, As, Co, Mo) (Orlović-Leko et al., 2020), although no statistically significant correlations with other group members apart from Cu were observed in this work.

Another event of interest was associated with biomass burning on 6 September 2024 (Milinković et al., 2026; under review). This event had the highest DOC concentration among the rainwater samples (1.781 mg/L), but only 0.83 nM Cu(I), corresponding to Cu(I) fraction of 2.5%. No statistically significant relationship was observed between DOC and Cu(I) fraction ($\rho=0.068$, $p=0.842$), nor between DOC and Cu(I) concentration ($\rho=0.360$, $p=0.277$). This does not exclude an important role of organic ligands, because DOC represents a heterogeneous pool of compounds with different Cu-binding properties and different abilities to participate in redox reactions. As already proposed, the identity and reactivity of individual ligands may therefore be more important than the overall DOC concentration.

The estuarine samples showed similarly pronounced variability in Cu redox speciation (**Figure 6B**). Cu_T ranged from 1.30 to 13.28 nM, while the Cu(I) fraction ranged from 3.5 to 82.4%. A significant positive correlation was observed between Cu_T and concentration of Cu(I) ($\rho=0.821$, $p<0.001$), whereas Cu_T was not significantly correlated with the Cu(I) fraction ($\rho=0.456$, $p=0.076$). Direct comparison between rainwater and estuarine samples requires caution because of the sampling timing. Rainwater was collected from the sampler either on the day of precipitation or on the following day, and an estuarine surface-layer sample was collected directly from the estuary on the same day as the rainwater collection. Thus, the two samples represent closely temporally associated conditions, although the rainwater had been collected over the preceding precipitation event and the estuarine sample represents the conditions in the receiving environment at the time of collection. An estuarine sample may therefore reflect not only the most recent precipitation but also dilution, mixing, particle

interactions, biological uptake and chemical transformations occurring between deposition and estuarine sampling. Direct date-to-date correlations between rainwater and estuarine redox speciation should therefore not be interpreted as direct measurements of atmospheric Cu transfer. Nevertheless, both Cu_T and Cu(I) concentrations were substantially lower in estuarine water than in rainwater, while the fraction of Cu(I) increased (**Figure 6**). The median fraction of Cu(I) observed in the estuarine samples was 54.9%, compared with 6.7% in rainwater. The higher Cu(I) fraction in the estuary may result from preferential removal of Cu(II) , changes in ligand binding, particle association, biological uptake, dilution and/or redox transformations. The available data do not allow these processes to be distinguished, but the observation clearly indicates that the atmospheric Cu redox composition was not transferred conservatively to the estuarine environment. Given the observed correlation between marine aerosols and the Cu(I) fraction in rainwater, the data instead suggest that the more likely direction of this relationship is from marine aerosols to rainwater.

DOC showed no significant relationship with Cu redox speciation in the estuary. DOC was not significantly correlated with either the concentration of Cu(I) ($\rho=0.312$, $p=0.240$) or the Cu(I) fraction ($\rho=0.335$, $p=0.204$). Optical characterisation of DOM was available for six estuarine samples and provides some additional information. BIX showed a positive relationship with Cu(I) fraction ($\rho=0.657$, $p=0.156$), but this relationship was not statistically significant. No significant relationship was observed between BIX and the concentration of Cu(I) ($\rho=0.200$, $p=0.704$). However, this positive trend may further support indications from previous samplings in the estuary (spring and summer of 2023 and 2024 at E1 and E2), but the limited number of observations does not allow a statistically supported relationship between BIX and Cu redox speciation to be established. Moreover, no significant relationship was observed between protein-like substances and fraction of Cu(I) . The fluorescence intensity of protein-like substances observed in these six samples (autumn 2023–spring 2024, Appendix A.5.4, Table A8) was approximately 70% lower than that observed in the surface brackish layer at station E1 during summer 2024 (Appendix A.5.2, Table A2). This was accompanied by an approximately 35–50% decrease in the fluorescence intensity of humic-like substances from winter to summer. A similar seasonal pattern was reported by Marcinek et al. (2020), who observed a 75% higher intensity of protein-like substances in summer compared with winter, while the fluorescence intensity of humic-like substances decreased by approximately 40–50% during summer compared with winter. The ligand measurements provide stronger evidence for an association between a specific ligand class and Cu redox partitioning. Stronger ligand class

(L₁) was not significantly related to the Cu(I) fraction ($\rho=0.179$, $p=0.702$), whereas weaker ligand class (L₂) showed a significant negative relationship with Cu(I) fraction ($\rho=-0.786$, $p=0.036$). Thus, higher L₂ concentrations were associated with a lower fraction of Cu(I). L₂ in the Krka River estuary has previously been characterised as being dominated by humic-like substances (Marcinek, 2025), whose functional groups may preferentially bind Cu(II) over Cu(I).

The absence of a significant relationship between the fraction of Cu(I) and Cl⁻ in the estuarine samples, in contrast to the strong relationship observed in rainwater, indicates differences in the relative importance of the factors governing Cu redox speciation in estuarine waters and rainwater. However, this does not exclude a contribution of Cl⁻ to Cu(I) stabilisation in the estuarine environment. Rather, the lack of a significant relationship suggests that Cl⁻ is unlikely to be the main parameter governing Cu redox speciation.

5.3. Thiol mediated formation and stabilisation of copper(I)

The potential role of thiols in Cu(I) formation and stabilisation in estuarine waters emerges from the combined evidence obtained in Research Papers I, II and III. In Research Paper I, the interaction between Cu and thiols is investigated using experiments in buffered model solutions (pH 8.3) containing Cu(II) and four biologically and environmentally relevant thiols: GSH, Cys, 3-MPA and TAA. The addition of Cu(II) to thiol solutions resulted in rapid Cu(II) reduction and the formation of Cu(I)–thiol complexes under oxic conditions. The extent of Cu(II) reduction, its kinetics and the stability of the Cu(I)–thiol complex against oxidation depended on the structure and concentration of the thiol, i.e. the Cu:thiol ratio. Cys and GSH exhibited the greatest reducing capacity among the investigated thiols. Increasing thiol availability generally promoted Cu(I) formation and, under conditions of thiol excess, could increase its persistence through complexation. However, the experiments also showed that the reaction kinetics could change during the course of the experiment as additional redox processes became important. Thus, the presence of a thiol does not necessarily result in persistent Cu(I); rather, its effect on Cu redox speciation depends on the balance between Cu(II) reduction, Cu(I) complexation and subsequent oxidation reactions. For example, changes in reaction kinetics over time can be attributed to a Fenton-like process, where the reverse reaction of Cu(II) with generated ROS occurs (Kachur et al., 1998; Moffett and Zika, 1983). Nonetheless, this process may be manifested by the formation of a complex between Cu and oxidised thiol. This coupling is important for interpreting Cu(I) in oxygenated natural waters, as the formation of Cu(I) alone would not necessarily lead to its accumulation because Cu(I) remains susceptible to disproportionation (Section 2.3.3.1).

The potential relevance of the investigated interactions between Cu and thiols to natural waters was subsequently considered in the Krka River estuary in spring and summer 2023 (Research Paper II). Cu(I) constituted a substantial and highly variable fraction of Cu_T , ranging from 20.7 to 88.7%. Importantly, in summer, maximum Cu(I) fractions occurred at the halocline and coincided with the highest biological production, leading to the hypothesis that biologically produced ligands such as thiols contributed to Cu(I) complexation. Moreover, the highest Cu(I) fractions in summer were associated with both the highest ligand concentrations and the highest conditional stability constants, while the strong L_1 ligand class in the estuary had previously been associated with recent biological production (Marcinek et al., 2025). While these field observations suggested that biologically derived ligands, including potentially thiol-containing

compounds, could contribute to the high Cu(I) fractions observed, the potential contribution of thiol-like ligands was not investigated at that stage.

The subsequent detection of Cys-like compounds in the Krka River estuary within the scope of Research Paper III provides an additional indication that thiol-containing ligands may be involved. In the summer 2024 sampling, a voltammetric signal attributed to Cys-like compounds was detected at 0.2 and 1.5 m, where the highest Chl *a* concentrations were also observed. At these same depths, the maximum of Cu(I) was observed and accounted for 82.5% and 72.6% of Cu_T, respectively. While the voltammetric signal corresponds to Cys (as confirmed with the standard addition method), it is not possible to assess its exact form (possibly within a larger molecule). However, the possibility of Cys as a Cu(I) ligand in estuarine and marine waters is further supported by the determination of the conditional stability constant of the Cu(I)–Cys complex, by reverse titration experiments. In short, the spectrophotometric analytical approach involved titration of the [Cu(I)BCS₂]^{3–} complex with Cys, and the decrease in *A*₄₈₃ of the [Cu(I)BCS₂]^{3–} was followed. The obtained conditional stability constant of 15.35 falls within the range of *L*₁ measured in the estuary within the scope of Research Paper II. Notably, in the summer 2023 sampling campaign at E1, the highest Cu(I) concentration (7.76 nM) and the highest fraction of Cu(I) (83.4%) occurred at the halocline (2.5 m) and coincided with a high total ligand concentration (26.23 nM) and the highest measured log *K'*_{CuL1} (15.50), despite a relatively low *L*₁ concentration (4.43 nM).

Taken together, the three studies provide complementary evidence that thiols can influence Cu redox chemistry at several levels. The laboratory experiments demonstrate that thiols can reduce Cu(II) and form Cu(I)–thiol complexes under oxic conditions. The estuarine observations show that high Cu(I) fractions can occur in biologically productive waters, while the detection of Cys-like compounds at depths characterised by high Cu(I) provides a possible link between thiol-containing ligands and Cu(I) speciation. Finally, the conditional stability constant demonstrate that such complexation can be sufficiently strong to be relevant to the strong Cu-binding ligand pool. The combined evidence therefore supports thiol-mediated Cu(I) formation and complexation as a plausible contributor to Cu redox cycling in biologically active estuarine waters, rather than establishing it as the dominant mechanism controlling Cu(I). Other processes identified in the Krka River estuary, particularly photochemical reactions (Section 5.4) and chloride complexation (Section 5.1.1 and Appendix A.4), may operate simultaneously. The relative contribution of these pathways, including the extent to which naturally occurring thiol-like compounds control the persistence of Cu(I) in estuarine

waters, is difficult to resolve due to their simultaneous occurrence, and is expected to vary with the physical, chemical and biological conditions of the system.

5.4. Advances in understanding the effect of photochemical reactions in Cu(I) formation and stabilisation

Previous studies have shown that photochemical reactions can contribute to Cu(I) formation in sunlit natural waters (Section 2.6.4). Photochemically generated reactive species can reduce Cu(II), while simultaneous transformation of DOM may alter the organic ligands involved in Cu(I) stabilisation. Moffett and Zika (1988) demonstrated that the distribution of Cu(I) in the Atlantic Ocean and the Gulf of Mexico was consistent with photochemical Cu(II) reduction, but the importance of this process relative to other concurrent biological and chemical processes in complex natural systems remains difficult to assess. To address this, and to provide the first data on the diel Cu redox cycle in estuarine waters, changes in Cu redox speciation at the E2 site in the Krka River estuary were monitored from 05:00 to 23:00 h during a summer 2023 sampling campaign, together with characterisation of DOM (Research Paper II). The Cu(I) fraction increased during the day, in the absence of substantial changes in DOC and salinity. The early-morning minimum in Cu(I) occurred after the longest period without solar irradiation, whereas Cu(I) increased during the subsequent daylight period. At 17:00, the DOM characteristics also suggested substantial photochemical processing: the SUVA reached its lowest value and the $S_{275-295}$ reached its highest value, while the fluorescence of humic-like fluorophores A, C and M was lowest. These changes were interpreted as photo-bleaching of humic-like DOM and potentially photochemical reduction of Cu(II) complexes. Moffett and Zika (1987) showed that most Cu(II) complexes with carboxylate and amino functional groups are photoreactive. Moreover, they showed that all complexes with LMCT bands above 300 nm are photoreactive, which includes a substantial part of the CDOM pool. Photooxidation of CDOM and the associated production of superoxide provide a plausible pathway for the reduction of organically complexed Cu(II) to Cu(I). Cu within L_2 complexes, which in the Krka River estuary are mostly associated with photoreactive humic compounds (Marcinek et al., 2025), was found to be highly reactive towards superoxide (Voelker et al., 2000). However, the highest Cu(I) fraction would be expected to occur around peak solar irradiation, whereas the observed pattern did not represent an instantaneous response of Cu(I) to solar irradiation. This observation indicates that Cu(I) formed during daylight could persist after the cessation of direct irradiation, or that processes continuing after sunset contribute to its further formation. In laboratory experiments involving irradiation of Cu(II)–salicylate complexes, Moffett and

Zika (1987) observed that Cu(II) reduction continued after the irradiation period, suggesting the involvement of a subsequent second-order reaction. Nonetheless, the reported half-life of photochemically produced Cu(I) is approximately 12 hours (Moffett and Zika, 1987), which may explain its persistence but not the subsequent increase in Cu(I). The high BIX and enhanced fluorescence of the fluorophore M at 23:00 indicated elevated biological activity at this time. Consequently, biologically mediated production of ROS, including $O_2^{\bullet-}$ and H_2O_2 , was considered an additional possible contributor to Cu(I) formation after sunset. Moreover, the diurnal changes were not limited to Cu redox speciation, and significant fluctuations in Cu_T were also observed. A similar pattern was reported by Tercier-Waeber et al. (2009), who observed an increase in Cu_T during the daytime at the Joanis site on the Riou-Mort River in France, an area impacted by mining and ore-processing activities. In contrast, the dynamic Cu fraction decreased during the day, reaching a minimum in the late afternoon. As the particulate Cu fraction remained relatively constant, the authors proposed that the release of Cu-binding EPS from biofilms could explain these observations, as newly available ligand sites on EPS could outcompete weaker ligands associated with dynamic Cu complexes, thereby shifting Cu towards the non-labile fraction. Additional biological processes may occur concurrently with changes in Cu(I) during the day–night cycle. Dupont et al. (2004) found that GSH concentrations in cultures of *Thalassiosira pseudonana* and *Emiliana huxleyi* reached a maximum during the light period, whereas Cys concentrations reached a maximum early in the dark period. As both compounds are potential Cu(I)-binding ligands in natural waters, these observations suggest that the observed diel Cu(I) pattern may result from coupled photochemical and biological processes. Nonetheless it should be emphasised that the study site was located in a nautical marina, where Cu was continuously released from antifouling coatings on ship hulls, complicating the assessment of potential biological pathways.

These findings prompted further investigation of the extent to which photochemical reactions affect Cu redox speciation in the estuarine environment. In summer 2025, Cu redox speciation was investigated at three depths at station E1, with samples analysed immediately after sampling and after 48 h of storage under two light conditions: dark conditions and ambient day/night conditions. Details of the sampling campaign are provided in Appendix A.5.5, and the results are shown in **Figure 8**. Copper(I) accounted for a substantial fraction of Cu_T in all the investigated samples, i.e. at the surface, halocline and in the seawater layer, with a maximum at the surface and a minimum in the seawater layer. Cu(I) correlated with DOC and Chl *a*, implying that biological factors may affect its distribution. However, Cu redox

speciation changed markedly over 48 h, with different trends in the surface layer compared with the halocline and seawater layer. In the surface layer, dark exposure resulted in a decrease from 64.1% to 20.9% of Cu(I) in Cu_T, while a smaller decrease to 50.3% was observed in the sample kept under ambient day/night conditions. A decrease in Cu(I) in the absence of light was expected, although one might expect the shift towards Cu(II) to be more pronounced after a prolonged dark period. Therefore, it is assumed that additional non-photochemical processes partially govern Cu redox speciation in the surface sample. The importance of non-photochemical processes in governing Cu redox speciation was evident at the halocline and in the seawater layer, where opposite responses to dark exposure were observed. At the halocline, an increase from 52.2% to 65.1% of Cu(I) in Cu_T occurred, and an increase to 59.3% was also observed in the ambient day/night sample, although to a lesser extent. A similar pattern was observed in the seawater layer, where an increase from 46.1% to 70.4% of Cu(I) in Cu_T was recorded, with an increase to 61.8% also observed in the ambient day/night sample. This indicates that Cu(I) formation and/or stabilisation continued in the dark, potentially through non-photochemical processes. One possible pathway is the reduction of Cu(II) by thiols, which are known to reduce Cu(II) through non-photochemical pathways, i.e. without requiring photons for the reaction to proceed (Buerge-Weirich and Sulzberger, 2004; Krezel et al., 1999), as already discussed in detail in Research Paper I. Although the presence of thiols was not investigated within the scope of this sampling campaign, the results are indicative of mixed contributions to Cu redox speciation.

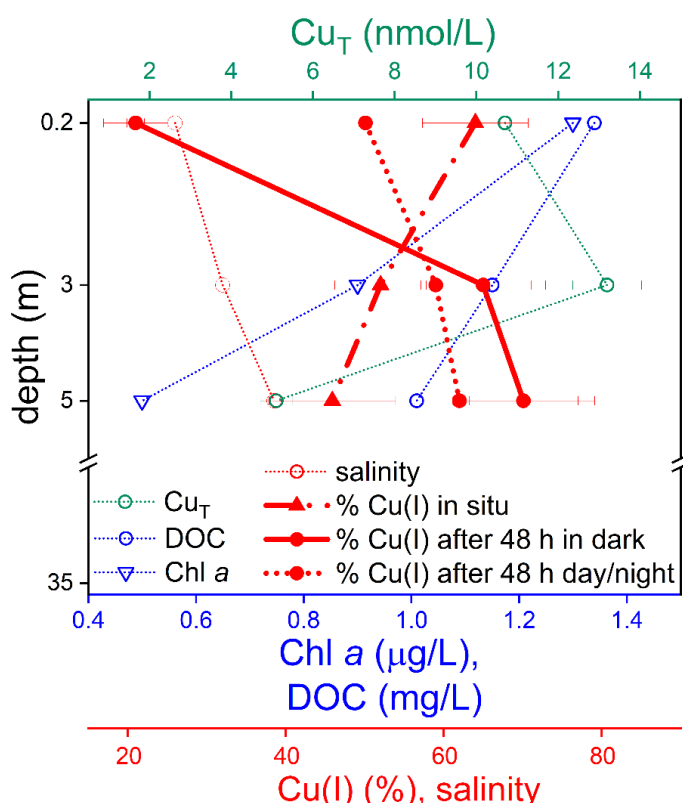


Figure 8. Vertical distributions of Cu(I) fractions at E1 in July 2025 measured in situ (red dash-dotted line, filled triangles; bottom x-axis), after 48 hours in dark (red solid line, filled circles; bottom x-axis) and 48 hours under diel light-dark cycle (red dotted line, filled circles; bottom x-axis). Vertical profiles of Cu_T (green dotted line, top x-axis), DOC (dotted blue line, open circles; lower/middle x-axis), Chl *a* (blue dotted line, open triangles, lower/middle x-axis), and salinity (red dotted line, open circles; bottom x-axis).

In an attempt to further investigate the possible mechanisms of Cu(II) photoreduction and Cu(I) stabilisation, model experiments were conducted in solutions containing Cu(II) and/or histidine (His) and BCS at pH 8.3, under dark conditions (absence of light) and light conditions (UV lamp, $\lambda=365$ nm), which exhibited significantly different rates of Cu(II) reduction depending on the ligands present and the light regime (**Figure A5**). Experimental procedure may be found in Appendix A.6. The reported logarithmic formation constant (β_2') for the Cu(I)–BCS₂ complex with a ligand:metal ratio of 2:1 is 19.8, while for the complex with Cu(II) it is 11.9 (Xiao et al., 2004; Xiao and Wedd, 2010), and for the complex of Cu(II) with His is 17.5 (Deschamps et al., 2005). The E_h of Cu(II)–BCS₂ is around +0.45 V, and any species in a sample that can be oxidised by Cu(II)–BCS₂ will yield Cu(I)–BCS₂ (provided there are no stronger Cu(II)-binding ligands in the solution) (Chen et al., 2016). Under these experimental conditions, Cu(I)–BCS does indeed form spontaneously, in both light (UV lamp) and dark

conditions. However, under light conditions the reduction is much greater. In contrast, in the system containing only Cu(II) and His, no reduction was observed under either dark or light conditions (data not shown). The absence of measurable Cu(I) accumulation in the Cu(II)–His system may therefore reflect the lack of a strong Cu(I)-stabilising ligand and subsequent reoxidation if any Cu(I) is photogenerated, due to its high excess over Cu(II) and high value of stability constant. Nonetheless, in a system containing Cu(II), His and BCS, almost no reduction was observed in the dark, while reduction was significant under light conditions, exceeding that observed in a system containing Cu(II) and BCS under light, suggesting that His introduced an additional pathway for photochemical Cu(II) reduction. It is known that Cu(II) complexes with amino acids can undergo intramolecular LMCT upon irradiation. Photochemical reduction of Cu(II)–amino acid complexes through LMCT processes has previously been demonstrated, including carboxylate-to-Cu(II) charge transfer resulting in Cu(I) formation (Lin et al., 2021). The substantially greater Cu(I) formation observed upon irradiation in the presence of His and BCS, compared with Cu(II)–BCS alone, suggests that His-containing Cu(II) species may participate in the light-induced reduction. However, the present experiments do not establish whether the photochemical step involves direct LMCT from His, a mixed Cu(II)–His–BCS complex, or another photochemically generated reducing species. The strong Cu(I)-binding capacity of BCS would favour accumulation of any Cu(I) generated photochemically by rapidly forming the stable Cu(I)–BCS complex. Importantly, these observations suggest that a ligand involved in the reduction of Cu(II) does not necessarily have to be the ligand responsible for stabilising the resulting Cu(I).

These conclusions may be relevant for explaining the potential role of photochemical reactions in Cu(I) occurrence in natural waters. If irradiation of Cu(II) complexes with photochemically reactive ligands (e.g. complexes in which Cu(II) is coordinated by ligands containing carboxylate, amine or imidazole functionalities) induces LMCT, the Cu(II)-binding ligand could act as an electron donor to Cu(II), reducing it to Cu(I). The photogenerated Cu(I) could subsequently be stabilised by other Cu(I)-binding ligands, such as Cys, which were detected in the Krka River estuary. Importantly, the results obtained in the model systems suggest that such photochemical Cu(II) reduction may occur even when the Cu(II)-binding ligand is present in substantial excess relative to both Cu(II) and the Cu(I)-binding ligand.

5.5. Biogeochemical implications of copper(I) in estuarine environment

The occurrence and substantial contribution of Cu(I) to Cu_T observed throughout the Krka River estuary have implications beyond redox speciation, indicating that redox transformations may represent an active component of the estuarine Cu cycle. High Cu(I) fractions were observed across all sampling campaigns and were associated with biological and photochemical processes, including the occurrence of biologically derived organic ligands and photochemical transformations, while Cu(I) partitioning was not necessarily coupled to Cu_T inputs. Thus, although the total dissolved Cu pool may remain relatively stable, its distribution between Cu(I) and Cu(II) can be altered by environmental conditions, including ligand composition and availability and light exposure. Copper redox speciation should therefore complement conventional approaches to Cu speciation, particularly because commonly used approaches do not explicitly resolve the presence and role of Cu(I) in natural waters. In estuarine and marine environments, Cu_T is predominantly organically complexed, yet the oxidation state of Cu associated with these ligand pools is generally unresolved. CLE-AdCSV, one of the principal approaches used to characterise natural Cu-binding ligands, cannot distinguish between Cu(I) and Cu(II) (Leal and van den Berg, 1998; Whitby et al., 2017). Consequently, conditional stability constants and ligand concentrations obtained using this approach should not necessarily be interpreted as describing Cu(II) complexes alone. This limitation becomes particularly relevant in light of the substantial Cu(I) fractions observed in the present study, as the composition of L_1 may include Cu(I)-binding compounds, particularly thiol-like ligands. The implications therefore extend beyond speciation terminology: if a proportion of the ligand pool stabilises Cu(I), estimates of Cu^{2+} , and consequently assessments of Cu bioavailability and fluxes, may differ substantially from those based on an exclusively Cu(II)-dominated system. The inorganic complexation of Cu should also be considered when evaluating these implications. In seawater, inorganic anions occur at concentrations far exceeding those of trace metals and can substantially influence their speciation. At pH 8.3, the inorganic side-reaction coefficient, α' , for Cu(II) mainly reflects complexation with carbonate and hydroxide, whereas for Cu(I) chloride complexation is particularly important. These differences result in α' values that differ by several orders of magnitude: approximately 36 for Cu(II) compared with 1.4×10^5 for Cu(I) in seawater (Leal and van den Berg, 1998). Consequently, accounting for Cu(I) when correcting conditional ligand stability constants can substantially alter estimates of Cu^{2+} , with potential consequences for the interpretation of Cu

uptake, toxicity and fluxes. These calculations should nevertheless be treated cautiously because free Cu(I) in equilibrium with organic and inorganic Cu(I) complexes can undergo disproportionation to Cu(II) and Cu(0), meaning that these species also need to be considered when evaluating Cu(I) speciation.

The possible involvement of biologically derived ligands provides a further link between Cu redox speciation and biological Cu acquisition. Although Cu²⁺ has traditionally been considered the principal bioavailable form in Cu toxicity models, organically complexed Cu can also contribute to biological uptake. Walsh et al. (2015) demonstrated that Cys enhanced Cu uptake by marine phytoplankton through reductive processes, although the Cu(I)–Cys stability constant they obtained was considered insufficient for Cys to substantially stabilise Cu(I) under natural conditions. In contrast, logarithm of the conditional Cu(I)–Cys stability constant determined in the present study exceeds value 15, a value previously suggested as a threshold for environmentally relevant Cu(I) stabilisation. This suggests that Cys-like ligands may contribute to Cu(I) stabilisation under estuarine conditions. Current models indicate that organically bound Cu(II) can undergo extracellular reduction before Cu(I) is transported into cells through high-affinity Cu(I) transporters. If Cu(I) is already present in the surrounding waters, potentially through association with thiol-like compounds, Cu acquisition may instead involve uptake from pre-existing Cu(I) complexes rather than requiring reduction of Cu(II) immediately prior to uptake. This represents a potentially different pathway of Cu acquisition and links environmental Cu redox cycling directly to biological Cu transfer. Nevertheless, its relevance under natural estuarine conditions remains to be demonstrated.

The biological significance of environmental Cu(I) should also be distinguished from intracellular Cu redox speciation. While extracellular redox transformations influence the chemical forms available for uptake, intracellular Cu undergoes a separate and dynamically regulated Cu(I)/Cu(II) cycle. Wang and Wang (2021) showed that Cu toxicity in *Chlamydomonas reinhardtii* was more closely associated with intracellular labile Cu(I) than with total intracellular Cu. Subsequent studies demonstrated that intracellular Cu(I) and Cu(II) occupy distinct cellular pools and are dynamically regulated in response to Cu stress, with labile Cu(I) also varying with cell division and the diurnal cycle (Wang and Wang 2023; Deng and Wang 2024; Gui and Wang 2024). More recent work suggests that GSH-dependent processes and lysosome-related organelles contribute to the regulation of elevated intracellular labile Cu(I) (Deng and Wang 2025). While intracellular Cu(I) accumulation is primarily related to the intracellular biotransformation of Cu(II) to Cu(I), rather than to the direct uptake of

extracellular Cu(I) (Wang and Wang, 2021), further investigation of the relationship between extracellular and intracellular Cu redox speciation is warranted, particularly in natural waters where Cu(I) constitutes a substantial fraction of Cu_T and thiol-like ligands have been detected.

6. CONCLUSIONS

This doctoral thesis investigated Cu redox speciation in an estuarine environment, with emphasis on factors contributing to formation and stabilisation of Cu(I) and its significance for Cu biogeochemistry. The research combined investigation of interactions between Cu and thiols in model solutions, methodological advancements in SPE methodology for Cu redox speciation, and field observations in the Krka River estuary. The key findings and contributions can be summarised as follows:

- 1) Thiols can reduce Cu(II) and stabilise Cu(I) under oxic conditions. Laboratory experiments have demonstrated that biologically and environmentally relevant thiols, including Cys, GSH, 3-MPA and TAA, can reduce Cu(II) and stabilise it in the form of a Cu(I) complex, even in the presence of oxygen. The extent of this effect and its susceptibility to oxidation depended on the structure and concentration of the thiol and on its ratio to Cu. These results imply that thiols, which are present in marine and estuarine environments, may contribute to the formation and stabilisation of Cu(I), even though Cu(II) is thermodynamically more favourable in oxic environments.
- 2) An adapted methodological approach enabled the determination of Cu redox speciation in natural waters with improved recovery and minimal sample manipulation. The adapted SPE method, based on complexation of Cu(I) with BCS and Cu(II) with EDTA, was coupled with DP-ASV for the determination of Cu concentrations in Cu(I), Cu(II) and Cu_T fractions. Quantitative recovery of Cu(I) and Cu(II) was achieved, and the method was applied to determine Cu redox speciation in the Krka River estuary.
- 3) Copper(I) constitutes a significant fraction of Cu_T in the Krka River estuary. Cu(I) was present in all investigated estuarine samples, reaching up to 88.7% of Cu_T . Moreover, Cu(I) was detected in the majority of the rainwater samples, although it contributed to Cu_T to a lesser extent than in the estuarine samples. The results therefore support the hypothesis that Cu(I) is an environmentally relevant component of Cu_T in estuarine waters.
- 4) Biologically produced organic ligands may contribute to Cu(I) formation and stabilisation in the estuary. High Cu(I) concentrations and fractions frequently occurred in

waters characterised by indicators of biological production, including elevated Chl *a*, BIX and protein-like fluorescence. Nonetheless, detection of Cys-like compounds at depths with high Cu(I) fractions, together with the experimentally determined conditional stability constant of the Cu(I)–Cys complex, provides complementary evidence for the potential involvement of thiol-like ligands. The obtained conditional stability constant falls within the range characteristic of the strong Cu-binding ligand class observed in the estuary. However, the relative contribution of biologically produced organic ligands to Cu(I) formation and stabilisation is hard to distinguish from contribution of other processes, such as photochemical reduction of Cu(II).

5) Photochemical processes contribute to Cu redox transformations in the estuary. The diel study in the estuary showed an increase in the fraction of Cu(I) from morning to evening, together with simultaneous changes in the optical properties of DOM. These observations support a contribution of photochemical reduction of Cu(II) and/or photobleaching of DOM to changes in Cu redox speciation. However, it has been demonstrated that Cu(I) can persist in the dark, pointing to the contribution of non-photochemical processes, including potentially thiol-mediated reduction.

6) Copper redox speciation should be considered an integral component of biogeochemical cycling of Cu. The significant occurrence of Cu(I) in the estuary demonstrates that Cu cycling in oxygenated estuarine waters may involve continuous redox transformations rather than the previously assumed dominance of Cu(II). This challenges conventional Cu speciation, as the commonly applied CLE-AdCSV approach cannot distinguish between different Cu oxidation states. Nonetheless, the presence of Cu(I) may affect the current understanding of Cu bioavailability and toxicity.

Overall, the results support the central premise of this thesis that Cu(I) is an environmentally relevant component of Cu_T in estuarine environments, although the relative contribution of the individual mechanisms of Cu(I) formation and stabilisation remains difficult to resolve, as they operate simultaneously.

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LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviation

AAS	Atomic absorption spectroscopy
ACN	Acetonitrile
ASV	Anodic stripping voltammetry
BCS	Bathocuproine disulfonate
BIX	Biological index
CDOM	Chromophoric dissolved organic matter
Chl <i>a</i>	Chlorophyll <i>a</i>
CLE-AdCSV	Competitive ligand exchange–adsorptive cathodic stripping voltammetry
CTR	Copper uptake transporter
Cys	Cysteine
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
DP-ASV	Differential-pulse anodic stripping voltammetry
DGT	Diffusive gradients in thin films
DMT	Donnan membrane technique
EEM	Excitation–emission matrix
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substances
FA	Fulvic acid
FDOM	Fluorescent dissolved organic matter
FSI	Freshwater-seawater interface
GSH	Glutathione
HA	Humic acid
HIX	Humification index
HMW	High molecular weight
HMDE	Hanging mercury drop electrode
ICP-MS	Inductively coupled plasma mass spectrometry
ISE	Ion-selective electrode
LMCT	Ligand-to-metal charge transfer
LMW	Low molecular weight
MPA	3-Mercaptopropionic acid
NTA	Nitrilotriacetic acid
OM	Organic matter
POM	Particulate organic matter
PLM	Permeation liquid membrane
ROS	Reactive oxygen species
SHE	Standard hydrogen electrode

SPE	Solid-phase extraction
SWV	Square-wave voltammetry
TAA	Thioacetic acid
TEP	Transparent exopolymer particles
UV	Ultraviolet
Vis	Visible

Symbol

α	Side reaction coefficient
a_{254}	Absorption coefficient at 254 nm
Cu_T	Total dissolved copper
Cu^{2+}	Free copper ion
Cu'	Labile copper (sum of free and inorganically complexed copper)
CuL_i	Copper bound to organic ligands of i^{th} class
CuX_i	Copper bound to inorganic anions
E_h	Redox potential expressed relative to the standard hydrogen electrode
E°	Standard electrode potential
K'_{CuLi}	Conditional stability constant of a organic ligands of i^{th} class
M	Molar concentration; mol dm^{-3}
ρ	Spearman's rank correlation coefficient
$S_{275-295}$	Spectral slope over the 275–295 nm wavelength range
SUVA	Specific absorbance at 254 nm

APPENDICES

A.1. Research Paper I

Crmaric, D., Bura-Nakić, E. 2023. Interaction between copper and thiols of biological and environmental importance: case study using combined spectrophotometric/bathocuproine disulfonate disodium salt (BCS) assay. *Molecules* 28, 5065.
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Article

Interaction between Cu and Thiols of Biological and Environmental Importance: Case Study Using Combined Spectrophotometric/Bathocuproine Sulfonate Disodium Salt Hydrate (BCS) Assay

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Abstract: Considering the biological and ecological importance of Cu–thiol interactions and the discrepancies in previous research, this study focuses on Cu interactions with biologically and ecologically relevant thiols: glutathione (GSH), L-cysteine (L-cys), 3-mercaptopropionic acid (MPA), and thioacetic acid (TAA) in aqueous solution. The addition of Cu(II) to a thiol-containing solution led to a rapid reduction of Cu(II) and the formation of a Cu(I)–thiol complex. The mechanism of Cu(II) reduction and Cu(I) complex formation as well as the kinetics of Cu(I) oxidation strongly depend on the structural properties of the individual thiols investigated. The reducing power of the investigated thiols can be summarized as follows: L-cys $\cong$ GSH > MPA > TAA. The reaction order, with respect to Cu(I) oxidation, also changes over the time of the reaction course. The deviation of the reaction kinetics from the first order with respect to Cu(I) in the later stages of the reaction course can be attributed to a Fenton-like reaction occurring under low thiol concentration conditions. At high Cu:thiol ratios, in the case of GSH, L-cys, and MPA, the early stage of the reaction course is characterized by high Cu(I) stability, most likely as a result of Cu(I) complexation by the thiols present in excess in the reaction mixture.

Keywords: copper; thiols; reduction; oxidation; kinetics

1. Introduction

As one of the most abundant trace elements in living organisms, copper (Cu) is an essential metal in the biochemistry and physiology of all organisms [1]. Because of its ability to undergo redox changes between the oxidation states of Cu(I) and Cu(II), Cu is an important catalytic cofactor in redox reactions involved in electron transfer [2,3]. Thiol compounds are known to interact strongly with Cu and can inhibit Cu-containing lysyl oxidase and tyrosinase enzymes [4–7]. Furthermore, biological thiols containing Cu in the active sites are an example of substrates used by copper–zinc superoxide dismutase (Cu/Zn-SOD), which catalyzes the dismutation of superoxide radicals to oxygen and hydrogen peroxide [8,9]. However, Cu(II) reduced to Cu(I) by hydrogen peroxide, superoxide, or thiol compounds can catalyze the formation of reactive oxygen species (ROS) that can lead to cell damage, with Cu acting as a pro-oxidant in this scenario [10,11]. Winterbourn et al. reported that thiols, especially aminothiols, such as cysteine, enhance the reduction of the Cu active site of CuZn-SOD and lead to the formation of hydrogen peroxide and disulfide, though CuZn-SOD is less reactive than free Cu(II) [12]. Many authors attribute Cu toxicity to the oxidative damage caused by free radicals, which leads to DNA damage, increased lipid peroxidation, or decreased enzymatic activity [13–16]. Murakami et al. showed that Cu ions inhibit GSH reductase activity, with Cu(I) ions acting more strongly than Cu(II) ions, which is consistent with previous studies by Xiao et al. [17,18]. Considering Cu toxicity, it is

crucial to maintain Cu homeostasis in cells. To this end, cells have evolved a sophisticated machinery that includes multiple Cu ion transporters and chaperones that transport Cu to proteins [19]. Thiols are widely distributed in Cu-rich proteins and Cu chaperones. In eukaryotic organisms, Cu transporter 1 (CTR1) is responsible for binding Cu(II) via the N-domain of CTR1, reducing Cu(II) ions to Cu(I) and transferring Cu(I) ions to the cytoplasmic domain—a complex process involving the amino acids methionine, histidine, and cysteine in Cu binding [20,21]. Once Cu has entered cells, it binds to Cu chaperones, e.g., antioxidant 1 (ATOX1), which is the Cu chaperone for super-oxide dismutase (CCS), and the cytochrome C oxidase Cu chaperone (COX17), whose homologues in yeast are found in bacteria and thiol molecules [22]. Hatori et al. found that the cellular glutathione/glutathione disulfide pair (GSH/GSSG) prevents the oxidation of cysteines of ATOX1 and thereby regulates Cu binding to ATOX1 [23]. Furthermore, the importance of Cu–thiol interactions has been demonstrated in yeast CuCOX17, where Cu atoms are trigonally coordinated with thiolates, and in human COX17, where cysteine and glutathione are involved in Cu(I) binding and transfer to the cochaperones SCO1 and COX1 [24,25].

In addition, thiol compounds may play an important role in binding Cu in marine waters and detoxifying Cu in marine phytoplankton. It is known that marine cyanobacteria produce strong Cu-binding ligands in response to Cu pollution. Consequently, biologically produced ligands may contribute significantly to the pool of strong binding ligands classified as L1 [26,27]. Although the exact composition of Cu-binding ligands in seawater is not yet known, Whitby et al. suggested that thiourea (TU)- and glutathione (GSH)-like thiols contribute significantly to the Cu-binding ligand pool, resulting in strong Cu complexation and, consequently, femtomolar concentrations of free toxic Cu [28]. The authors also suggested that other thiols, such as cysteine, 3-mercaptopropionic acid, and 2-mercaptoethanol, may play an important role in Cu binding in marine waters. Tang et al. reported an increased GSH release in the diatom *Thalassiosira weissflogii* after exposure to elevated inorganic Cu concentrations [29]. However, they suggested that this was due to cell membrane damage caused by the increased Cu rather than a cellular mechanism that counteracts Cu toxicity [29]. However, they suggested that GSH release by diatoms may contribute significantly to the Cu-binding ligand pool in marine waters [29]. Because of the biological and ecological importance of Cu–thiol complexes, Cu interactions with thiols have been of interest for many years, though there are discrepancies in the understanding of the exact reaction mechanisms and Cu oxidation state in such reactions [30]. Nevertheless, Smith et al. reported that the stabilization of the free radical intermediate in Cu-catalyzed thiol oxidation varies as a result of structural differences between thiols affecting the oxidation rate, which is further discussed in their paper [31]. Considering the biological and ecological importance of Cu–thiol interactions, as well as the discrepancies in the reaction mechanisms proposed so far, this study focuses on Cu interactions with biologically relevant thiols: glutathione (GSH), L-cysteine (L-cys), 3-mercaptopropionic acid (MPA), and thioacetic acid (TAA).

The tripeptide GSH contains the amino acids glycine, cysteine, and glutamic acid, with the cysteine component providing the reactive thiol group. GSH has been found in many prokaryotic and most mammalian cells in a concentration range of 0.2–10 mM [32]. Since GSH is present in at least a twofold excess over Cu, it plays an important role in cellular Cu uptake, where Cu binds to GSH as a Cu(I) complex shortly after entering the cell and is transferred in complexed form to metallothionein [33,34]. The Cu-buffering role of GSH was demonstrated in *Streptococcus pyogenes*, where only GSH that was strongly protected against Cu toxicity and excess Cu showed no toxic effects when GSH was abundant [33]. Endogenous GSH also plays an important role in the antioxidant defense of plants against abiotic stress by scavenging ROS [35]. In aquatic environments, processes such as cell lysis and exudation can lead to the release of low molecular weight (LMW) thiols, including GSH, into natural waters [36,37]. Although GSH is abundant in micromolar concentrations in many organisms, GSH concentrations in natural waters are usually in the nanomolar range [38–40]. Increasing the concentration of Cu in water promotes the release of GSH,

but the exact form of GSH in water and the mechanisms of Cu–GSH complex formation are still under investigation [39]. It has been suggested that GSH measured in water may be in its oxidized form, i.e., glutathione disulfide (GSSG), or in the form of complexes with trace metals [39]. In addition to GSH, other LMW thiols, such as cysteine, have also been detected in lakes, estuaries, and marine waters and have been proposed as Cu(I)-binding ligands, though the exact mechanisms of Cu(I) complexation with thiols are still under investigation [41,42]. L-cysteine is a non-essential amino acid that plays an important role in protein synthesis and stability through the formation of disulfide bonds [43]. The reactivity of L-cys is due to the sulfhydryl group (-SH), and L-cys serves as a ligand for the binding of trace metals, including Cu, and for active sites in enzymes. Deprotonation of the sulfhydryl group produces a negatively charged and reactive thiolate anion that can participate in oxidation or alkylation by electrophiles [44]. The thiolate anion is known to form mononuclear complexes with Cu(I), including mononuclear mixed thiolate-nitrogen-bonded species as well as Cu(I)–thiolate clusters [45]. In addition to glutathione and L-cysteine, 3-mercaptopropionic acid (MPA) is widely distributed in freshwater and marine environments, where it may be of biological and abiotic origins [38]. Biologically produced MPA has been addressed as a metabolite product of other thiol species, but it is also found in the methanogens *M. jannaschii*, where its presence is associated with the coenzyme M rather than the metabolism of other thiols [46,47]. Abiotically generated MPA may originate from the abiotic transformation of sulfur species, such as hydrogen sulfide (H₂S) [46,47]. Although interaction with Cu ions has been reported in marine and freshwater systems, it has not been thoroughly investigated because of the rapid oxidation of MPA in natural environments and the presence of other possible MPA-binding trace metals, such as iron, Fe [38]. Finally, thioacetic acid (TAA) is a thiocarboxylic acid that has been implicated in the origin of life as a potential acetyl-CoA analogue in prebiotic chemistry [48–50]. The oxidation of TAA and ethanethiol (ET) by Fe(III) has already been studied, and the mechanism by which the Fe(III) is reduced to Fe(II) while oxidizing thioacetate to diacetyl disulfide, leading to the formation of thioester and iron sulfide (FeS), has been proposed [50]. However, similar interactions of Cu with TAA are not known so far.

In this manuscript we present a kinetic study of Cu(II) reduction with thiols of biological and ecological importance (GSH, L-cys, MPA, and TAA). The kinetics of Cu(II) reduction and the Cu(I) oxidation formed are evaluated under different experimental conditions, i.e., different Cu and thiol concentration ratios. Thiols such as GSH are present in excess in many prokaryotic and mammalian cells, as well as in the aquatic environment; therefore, we decided to investigate Cu(II) reduction and Cu(I) oxidation with excess thiol in relation to Cu [32–34,38–40]. However, previous research on the interactions of Cu with thiols, including GSH, lacks data on Cu(II) reduction when Cu(II) and GSH are present in equimolar ratios. Therefore, we decided to additionally investigate Cu(II) reduction in the presence of thiols when Cu:thiol = 1:1.

2. Results and Discussion

2.1. Interaction of Cu and GSH

The interactions of Cu(II) with GSH were studied at Cu:GSH ratios of 1:1 and 1:10 over the course of 100 min at pH = 8.4 in 0.1 M borate buffer under oxic conditions (Figures 1 and 2). For both Cu:GSH ratios studied, Cu(II) reduction occurred immediately after the addition of Cu(II) to the buffered GSH solution, and Cu(I) concentrations were monitored using a BCS assay as described in Section 3.2. The kinetics of Cu(II) reduction and Cu(I) oxidation are shown in Figure 1. At a ratio of Cu:GSH = 1:10, all the Cu present in the solution was in its reduced form at the beginning of the reaction (the added Cu(II) was equal to Cu(I) detected using the BCS assay), implying the rapid reduction of Cu(II) in the presence of excess GSH. Oxidation of Cu(I) under the conditions of a tenfold excess of GSH with respect to the initial Cu(II) started after 60 min, and after 100 min, Cu(I) was no longer present in the solution. The UV–Vis spectra of Cu:GSH = 1:10 show peaks indicative of the ligand charge transfer band of a Cu(I)–GSH complex, with λ_{max} values observed at

255 nm and 300 nm [51,52]. After 60 min, the oxidation of Cu(I) began and a decrease in A_{255} and A_{300} was also observed, while a peak characteristic of the Cu(II) complex with oxidized glutathione, Cu(II)–GSSG, appeared with an $A_{\max}$ of 625 nm [52,53]. A peak with an $A_{\max}$ of 250 nm was also observed in the solution with Cu:GSH = 1:1, but a peak at 300 nm was not present. A peak with an $A_{\max}$ of 625 nm, which can be attributed to the Cu(II)–GSSG complex, was present from the beginning of the reaction, which is consistent with Cu(I) accounting for a maximum of 52% of the Cu present in the solution. An examination of reaction kinetics ($\ln$ Cu(I) and $1/\text{Cu(I)}$ concentration–time) shows significant differences between the reaction kinetics of mixtures with Cu:GSH = 1:1 and those of mixtures with Cu:GSH = 1:10 (Figure S1 in the Supplementary Materials). In the mixture with Cu:GSH = 1:1, linearity with time can be observed in the $\ln$ Cu(I) concentration over the whole course of the reaction (100 min), implying first-order kinetics, with respect to Cu(I), with the reaction rate constant, k , reaching 0.0024 min^{-1} . The plots of $\ln$ Cu(I) and $1/\text{Cu(I)}$ concentration–time in the case of a mixture with Cu:GSH = 1:10 in the first 60 min of the reaction course show reaction kinetics independent of the Cu(I) concentration. The later phase of the reaction is characterized by rapid Cu(I) oxidation. The rapid Cu(I) oxidation is enhanced by the formation of the Cu(II)–GSSG complex. The formation of the Cu(II)–GSSG complex inhibits the Fenton-like reversal reaction, as explained in the case of L-cysteine (see below) and in Kachur et al. and Moffet and Zika [54,55]. The early stage of the reaction, in the case of GSH excess, can be explained by strong interactions between the Cu(I) and GSH present in excess, which prevent fast Cu(I) oxidation with molecular O_2 . Ngamchuea et al. proposed a two-phase mechanism in which a Cu(II)–GSH complex with a stoichiometry of 1:2 is formed in the first phase, and the second phase comprises the Cu-catalyzed oxidation of GSH to GSSG [56]. In general, our results are in agreement with the two-phase mechanism of GSH oxidation proposed by Ngamchuea et al. [56]. However, while Ngamchuea et al. proposed the formation of a Cu(II) complex with GSH as an intermediate species in the first reaction phase, our results support the formation of a Cu(II) complex with oxidized GSH in the early as well as later stages of the reaction, depending on the Cu(II)-to-GSH ratio [57,58]. In the solution containing Cu:GSH = 1:1, a peak with an $A_{\max}$ of 625 nm was present from the very beginning of the reaction, indicating the presence of a Cu(II)–GSSG complex even at the early reaction stage. In contrast, for Cu:GSH = 1:10, a peak with an $A_{\max}$ of 625 nm was not present in the first reaction stage, since 100% of the Cu present in the solution was in its reduced form in the first reaction stage. For Cu:GSH = 1:10, a peak with an $A_{\max}$ of 625 was formed when Cu(I) oxidation began.

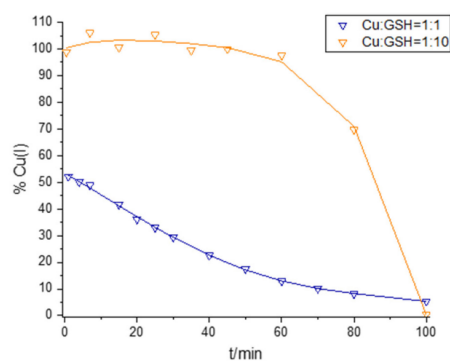


Figure 1. Kinetics of Cu(II) reduction and Cu(I) oxidation in solution containing 100 μM Cu(II) and either 100 μM GSH (blue line) or 1000 μM GSH (orange line).

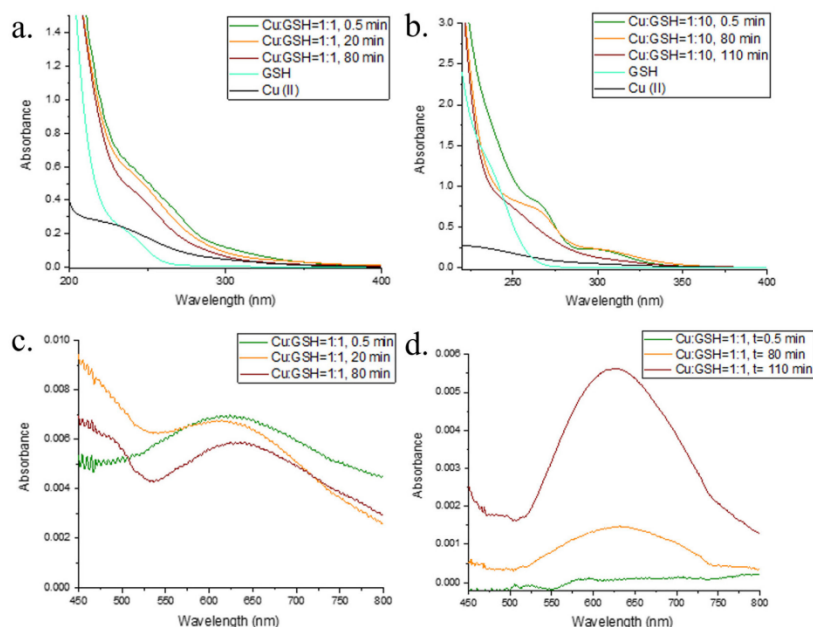


Figure 2. UV-Vis spectra of (a) Cu:GSH = 1:1 at $t = 0.5$ min (green line), $t = 20$ min (orange line), and $t = 80$ min (red line). UV-Vis spectra of a solution containing $100 \mu\text{M}$ GSH without Cu (cyan line) and $100 \mu\text{M}$ Cu without GSH (black line) are also shown. (b) Cu:GSH = 1:10 at $t = 0.5$ min (green line), $t = 80$ min (orange line), and $t = 110$ min (red line). The UV-Vis spectra of the solution with $1000 \mu\text{M}$ GSH without Cu (cyan line) and $100 \mu\text{M}$ Cu without GSH (black line) are also shown. (c) and (d) are as in (a) and (b), respectively, with the UV-Vis output wavelength changed to show changes during the course of the reaction at wavelengths above 450 nm.

2.2. Interaction of Cu and L-cys

The kinetics of Cu(II) reduction and Cu(I) oxidation over time at Cu:L-cys ratios of 1:1, 1:2.5, 1:5, and 1:10 are shown in Figures 3 and 4. Immediately after the addition of Cu(II) to the L-cys solution, the reduction of Cu(II) to Cu(I) was observed, as in the study of the interaction of Cu and GSH presented in this manuscript (Section 2.1.). The presence of Cu(I) was detected in all Cu:L-cys ratios studied during the 180 min, indicating the formation of a Cu(I) complex with L-cys that was resistant to oxidation with molecular O_2 . The amount of reduced Cu(II) and the stability of Cu(I) to oxidation increased with increasing Cu:L-cys ratios, as shown in Figure 3. The UV-Vis spectrum of L-cys alone in 0.1 M borate buffer is characterized by a peak with an A_{max} around 235 nm, which is consistent with the literature data [59]. Furthermore, Battin et al. reported A_{max} values at the same wavelength for all sulfur-mediated compounds, which prevented Cu-mediated DNA damage [51]. Immediately after the addition of Cu(II) to a solution containing L-cys and borate buffer, the previously observed peak at 230 nm disappeared, indicating the loss of free L-cys. At the same time, two new peaks appeared with A_{max} values of 260 nm and 336 nm. Similar peaks were also observed in studies by Pecci et al. and Rigo et al. and were attributed to Cu(I)-cys complexes, with a difference in the position of the second peak (300 nm), which could be due to the different reaction conditions (different buffers and pH values, and different oxic and anoxic reaction conditions) [60,61]. Cavalini et al. reported a compound with an A_{max} of 330 nm at a pH of >8 as an intermediate Cu(II)-cysteine

complex with a stoichiometry of 1:2, which disappeared after cysteine oxidation during the reaction [59]. Also in our case, the peaks at 260 and 330 nm disappeared during the course of the reaction when L-cys was oxidized and cystine was formed, which was confirmed by an increase in A in the 200–400 nm region where cystine absorbs [61].

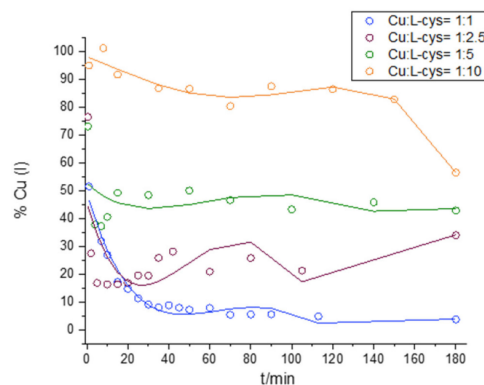


Figure 3. Kinetics of Cu(II) reduction and Cu(I) oxidation in solution containing 100 μM Cu(II) and 100 μM L-cys (blue line), 250 μM L-cys (wine red line), 500 μM L-cys (green line), or 1000 μM L-cys (orange line).

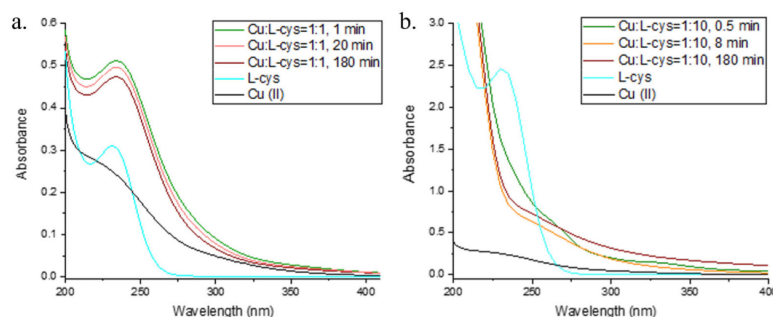


Figure 4. UV-Vis spectra of (a) Cu:L-cys = 1:1 at $t = 1$ min (green line), $t = 20$ min (orange line), and $t = 180$ min (red line). The UV-Vis spectra of a solution containing 100 μM L-cys without Cu (cyan line) and 100 μM Cu without L-cys (black line) are also shown. (b) Cu:L-cys = 1:10 at $t = 0.5$ min (green line), $t = 8$ min (orange line), and $t = 110$ min (red line). The UV-Vis spectra of the solution containing 1000 μM L-cys without Cu (cyan line) and 100 μM Cu without L-cys (black line) are also shown.

An analysis of reaction kinetics ($\ln \text{Cu(I)}$ and $1/\text{Cu(I)}$ concentration–time plots) in all four model solutions containing a fixed Cu(II) concentration and in which L-cys was varied showed strong changes in kinetics during the 180 min reaction course (Supplementary Materials, Figure S2). In the model solutions containing equimolar L-cys and Cu(II), linearity was observed in the $\ln \text{Cu(I)}$ concentration–time plots during the first minutes of the reaction course (up to 20 min), which is characteristic of first-order reaction kinetics. As the L-cys concentration increased, reaching a tenfold excess over the initial Cu(II) concentration, the linearity disappeared even in the early stages of the reaction course, and the reaction course showed a pattern that was independent of the Cu(I) concentration. A comparison of the calculated rate constants (k , Table S1 in the Supplementary Materials) for $\ln \text{Cu(I)}$ concentration–time indicates faster Cu(I) oxidation in the presence of L-cys compared

to GSH (under the conditions of an equimolar Cu:thiol ratio and under the conditions of linearity in $\ln$ Cu(I) concentration–time). The changes in the reaction kinetics over time in the solution with equimolar L-cys and Cu can be attributed to the Fenton-like process. The same reaction pattern was previously observed by Moffet and Zika for Cu(I) oxidation in solutions containing NaCl [55]. The pattern is explained by the existence of a reverse reaction in which formed Cu(II) is reduced with ROS. It is known that the absence of free thiol in the solution produces H_2O_2 , and Fenton-like reactions between Cu(I)–thiol and ROS occur [54]. During the reverse reaction, Cu(I) was formed and additionally influenced the reaction kinetics, as shown by a change in the reaction slope (k) as well as the reaction order. When L-cys was present in excess relative to the added Cu, the reaction kinetics were independent of the Cu(I) concentration alone, indicating a complex reaction scheme involving oxidation of the free L-cys and the Cu(I)–cys complex, followed by a competition between cystine and L-cys for Cu(I) [60,62,63].

2.3. Interaction of Cu and 3-MPA

The kinetics of Cu(II) reduction and Cu(I) oxidation in solutions containing Cu and MPA in metal:ligand ratios of 1:1, 1:2.5, 1:5, and 1:10 and under oxic conditions at pH = 8.4 are shown in Figures 5 and 6. The complete reduction of Cu(II) took place in all the solutions studied in which MPA was present in excess, while Cu:MPA = 1:1 resulted in a maximum reduction of 52% Cu(II). The maximum reduction occurred within 30 s after adding Cu to a solution containing MPA in 0.1 M borate buffer. In the sample containing Cu:MPA = 1:10, a peak with an $A_{\max}$ of 260 nm and a shoulder at 300 nm appeared immediately after the addition of Cu to a solution containing MPA. Similar peaks were observed in Cu:GSH = 1:10 and are addressed as Cu(I)–thiol complexes [52,53]. The peaks disappeared after the start of Cu(I) oxidation, i.e., about 45 min after the start of the reaction course in the mixture with Cu:MPA = 1:10.

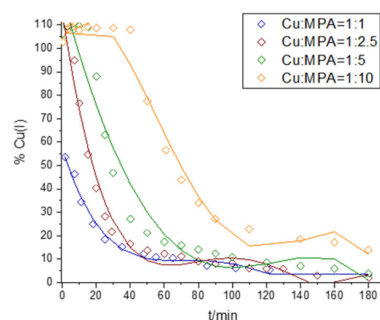


Figure 5. Kinetics of Cu(II) reduction and Cu(I) oxidation in solutions containing 100 μ M Cu(II) and 100 μ M MPA (blue line), 250 μ M MPA (wine red line), 500 μ M MPA (green line), or 1000 μ M MPA (orange line).

Similar to what has already been described in this manuscript, we investigated the reaction kinetics in more detail (Supplementary Materials, Figure S3). The reaction course allowed us to analyze reaction kinetics with respect to the Cu(I) concentration at all four investigated Cu:MPA ratios. An analysis of k for the plots of $\ln$ Cu(I)–time (in the parts where linearity was observed) shows the dependence of k on MPA concentration, as shown in Table S1 (Supplementary Materials). The first-order reaction rate (with respect to the Cu(I) concentration) for the Cu(I) oxidation reaction decreased with an increasing MPA concentration. This observed pattern again points to the importance of the experimental conditions used, i.e., the initial ratio of Cu(II) to thiol, as already observed in the case of Cu and GSH and L-cys. When thiol (in this case, MPA) is present in a tenfold excess with respect to the initial concentration of Cu(II), the formation of Cu(I)–MPA is favored and

the oxidation of Cu(I) by O_2 is strongly inhibited as a result of the Cu(I) complexation (Figure 6a). As was already observed (in Section 2.2, where the interaction of Cu with L-cys is investigated), a shift in the reaction kinetics from first to second order occurred over the course of the reaction, which in turn can be explained by the fact that more than just Cu(I) was involved in the reaction mechanism, namely ROS and Cu(II).

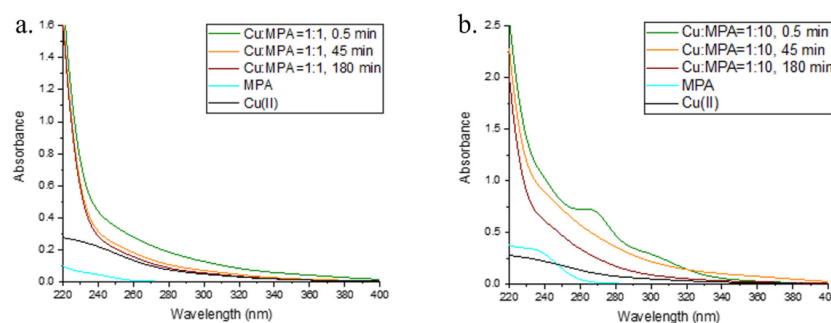


Figure 6. UV-Vis spectra of (a) Cu:MPA = 1:1 at $t = 0.5$ min (green line), $t = 45$ min (orange line), and $t = 180$ min (red line). The UV-Vis spectra of a solution containing 100 μ M MPA without Cu (cyan line) and 100 μ M Cu without MPA (black line) are also shown. (b) Cu:MPA = 1:10 at $t = 0.5$ min (green line), $t = 45$ min (orange line), and $t = 180$ min (red line). The UV-Vis spectra of the solution containing 1000 μ M MPA without Cu (cyan line) and 100 μ M Cu without MPA (black line) are also shown.

2.4. Interaction of Cu with TAA

The interaction of Cu(II) with TAA was studied by adding an aliquot of Cu(II) to solutions with increasing TAA in 0.1 M borate buffer at pH 8.4, as shown in Figures 7 and 8. Four different ratios of Cu to TAA were studied over 180 min: Cu:TAA = 1:1, Cu:TAA = 1:2.5, Cu:TAA = 1:5, and Cu:TAA = 1:10. Immediately after the addition of Cu(II) to TAA, a reduction of Cu(II) took place, but at a much slower rate than when Cu(II) was mixed with GSH, L-Cys, or MPA. For Cu:TAA = 1:1, the Cu(I) maximum was reached after about 100 min, while for Cu:TAA = 1:2.5, Cu:TAA = 1:5, and Cu:TAA = 1:10, the maximum of Cu(I) was reached after 80, 40, and 7 min, respectively. Both Cu:TAA = 1:5 and Cu:TAA = 1:10 resulted in the 100% reduction of Cu(II), but the maximum was reached faster with a higher Cu:TAA ratio. In addition to monitoring Cu(I) with the BCS assay, UV-Vis spectra of the Cu:TAA = 1:5 solution were recorded because the 1:5 ratio resulted in the 100% reduction of Cu(II), and the reduction was slow enough to monitor with UV-Vis spectrophotometry. The UV-Vis spectrum of TAA in 0.1 M borate buffer without Cu addition resulted in a peak with an $A_{\max}$ of 246 nm and a lower peak with an $A_{\max}$ of 300 nm. After the addition of Cu(II) to the TAA solution, the peak with an $A_{\max}$ of 246 decreased while the Cu(I) concentration increased, indicating a loss of free thiol due to its complexation with Cu(I) and its oxidation after reaction with Cu(II). After 40 min, the concentration of Cu(I) began to decrease (Figure 7), followed by an increase in the peak with an $A_{\max}$ of 246 nm (Figure 8), indicating the release of the thiol group bound to Cu. These results are consistent with our recent study on the interaction of vanadates with thiols, which also showed the lower reducing ability of TAA compared to L-cysteine and MPA [64]. It seems very likely that, because of the different structural properties of the thiols studied (different chelating properties, i.e., monodentate vs. bidentate), TAA is not able to stabilize the formed Cu(I) against oxidation and thus exhibits a lower reduction of Cu(II) at lower Cu:TAA ratios. Moreover, the SH group in the TAA molecule is located next to the C=O group, resulting in a resonant structure, which is not the case for any other thiol studied (GSH, L-cys, or MPA), affecting the electron-donating properties of the sulfur atom of TAA [65].

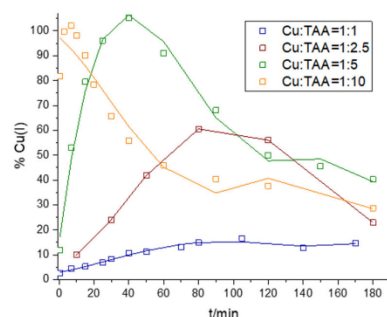


Figure 7. Kinetics of Cu(II) reduction and Cu(I) oxidation in solutions containing 100 μM Cu(II) and 100 μM TAA (blue line), 250 μM TAA (wine red line), 500 μM TAA (green line), or 1000 μM TAA (orange line).

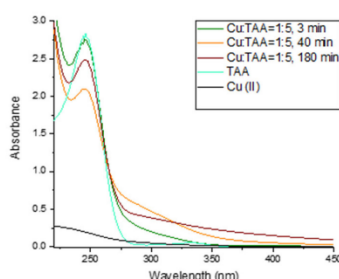


Figure 8. UV-Vis spectra of Cu:TAA = 1:5 at $t = 3$ min (green line), $t = 40$ min (orange line), and $t = 180$ min (red line). The UV-Vis spectra of a solution containing 500 μM TAA without Cu (cyan line) and 100 μM Cu(II) without TAA (black line) are also shown.

After closely inspecting the reaction kinetics (Supplementary Materials, Figure S4) for Cu:TAA = 1:1, we were able to determine the k value of the reduction reaction of Cu(II) with TAA (Table S1, Supplementary Materials). The figure in the Supplementary Materials (Figure S4a–h) also indicates rapid changes in the reaction sequence as well as in the course of the reaction (reduction vs. oxidation). Unfortunately, we were able to determine k for the Cu(I) oxidation reaction only in the cases where TAA was in five- and tenfold excess with respect to the added Cu (Table S1, Supplementary Materials). However, as noted in the case of L-cys and MPA, the initial reduction of Cu(II) was followed by the Cu(I) oxidation reaction, which, in turn, shows changes in the reaction order from first to second that, again, can be explained as earlier.

3. Materials and Methods

3.1. Materials

All solutions were prepared with deionized water from the Milli-Q (MQ) system (18.2 M Ω , Millipore, Burlington, MA, USA), and all chemicals were of analytical grade. The Cu(I) standard solution used for the calibration curve of the Cu(I)–bathocuproine complex (see Section 3.2) was prepared by dissolving copper(I) chloride (CuCl; Thermo Fisher Scientific, Waltham, MA, USA) in a solution containing 1 M sodium chloride (NaCl; Grammol, North Salt Lake, UT, USA) and 0.1 M hydrochloric acid (HCl; Roth, Newport Beach, CA, USA), which was previously purged with high purity nitrogen to remove oxygen [66]. The bathocuproine sulfonate disodium salt hydrate (BCS; Thermo Fisher Scientific), was prepared by dissolving BCS in MQ water to a concentration of 1000 μM .

The Cu(II) standard solution was prepared by dissolving copper(II) sulfate (CuSO_4 ; VWR BDH Prolabo Chemicals, Radnor, PA, USA) in MQ water to a final concentration of 0.01 M Cu(II). Thiol solutions of L-cysteine (L-cys), reduced glutathione (GSH), thioacetic acid (TAA), and 3-mercaptopropionic acid (MPA) were purchased from Thermo Fisher Scientific and prepared fresh daily by dissolving the thiols in MQ water to a final concentration of 0.01 M. A constant ionic strength and a pH = 8.4, relevant to the environmental and physiological conditions, in the model solutions containing Cu and thiol were achieved using 0.1 M borate buffer. The borate buffer was prepared from ortho-boric acid (VWR BDH Prolabo Chemicals), and its pH was adjusted to pH = 8.4 with sodium hydroxide (NaOH; Lach-ner Chemicals, Neratovice, Czech Republic).

Copper reduction by individual thiol species was investigated by measuring the Cu(I) concentration with a UV-Vis spectrophotometer (Analytik Jena, Jena, Germany) in a 1 cm quartz cuvette.

3.2. Procedure

The interaction of Cu with thiols was studied by adding aliquots of a Cu(II) stock solution to a solution containing thiol (L-cysteine, glutathione, 3-mercaptopropionic acid, or thioacetic acid) buffered to pH = 8.4 with 0.1 M borate buffer. The Cu concentration of 100 μM was the same in all experiments, while the thiol concentrations ranged from 100 to 1000 μM . The kinetics of the reduction of Cu(II) and the oxidation of Cu(I) were studied by adding aliquots of the Cu–thiol solution to the mixture of BCS and EDTA (BCS assay). Previous studies have shown that BCS effectively binds Cu(I) in an orange complex with an absorption maximum (A_{max}) of 484 nm, while a masking ligand is necessary to avoid Cu(II) interference [66,67]. In this study, EDTA was used as the masking ligand for Cu(II) with a fivefold excess of EDTA over BCS, which has been found to be optimal for ensuring Cu(II) complexation while avoiding Cu(I) oxidation [66,67]. A volume of 1 mL of the Cu–thiol model solution was added to the mixture containing 3 mL of BCS (1000 μM) and 0.15 mL of EDTA (0.1 M), resulting in a dilution factor (DF) of 4.15 in the Cu–thiol model solution and final concentrations of 723 μM BCS and 0.00361 M EDTA. The addition of the Cu–thiol solution to the mixture of BCS and EDTA resulted in the formation of an orange-colored solution with an absorption maximum (A_{max}) of 484 nm, indicating the presence of Cu(I) in the solution. Cu(I) concentrations were determined by measuring the A_{484} solution, calculating the Cu(I) concentration from the Cu(I)–BCS calibration curve, and correcting the Cu(I) concentration by a dilution factor of 4.15. For the Cu(I)–BCS calibration curve, one blank and five standard additions of Cu(I) were prepared. The blank solution contained 3 mL of 1000 μM BCS, 0.15 mL of 0.1 M EDTA, 0.1 mL of 1 M borate buffer, and MQ water with a final volume of 4.15 mL. The final concentrations of 723 μM BCS and 0.00361 M EDTA were the same as those used to measure the absorbance of Cu–thiol solutions. The Cu(I) standard solutions were prepared in the same way as the blank solution, adding five different Cu(I) aliquots that resulted in a linear absorbance over the concentration range studied, from 1.1 to 35.2 μM . The Cu(I) standard solution was freshly prepared before the experiment, following the procedure described in Section 3.1. To better understand the reaction mechanism, in addition to monitoring the Cu(I) concentration, UV-Vis spectra of solutions containing Cu and L-cys were recorded at the same reaction time as was the Cu(I) determination.

4. Conclusions

The study of the interactions of Cu with GSH (glutathione), L-cys (L-cysteine), MPA (3-mercaptopropionic acid), and TAA (thioacetic acid) revealed a complex reaction scheme with a rapid Cu(II) reduction in the case of GSH, L-cys, and MPA. The reduction reaction was rapid (within seconds), and we could instead follow the kinetics of Cu(I) oxidation under conditions where the thiol compound being studied was varied in stoichiometry with respect to the Cu(II) added. An examination of the $\ln \text{Cu(I)}$ and $1/\text{Cu(I)}$ concentrations over time shows that the reaction kinetics changed over the course of the reaction. Only

the solution with Cu:GSH = 1:1 showed first-order reaction kinetics with respect to Cu(I) oxidation, and the kinetics did not change during the 180 min of the reaction course. During the reaction courses of the Cu:L-cys = 1:1 and Cu:MPA = 1:1 mixtures, changes in the reaction kinetics for the Cu(I) oxidation process were observed in the later stages of the reaction course. In the later stages of the reaction, the H₂O₂ formed during Cu(I) oxidation with O₂ accumulated and triggered a Fenton-like reaction. The addition of Cu(I) to the already-ongoing reaction and the involvement of further reactants (Cu(II) and ROS) changed the reaction kinetics, as can be observed. Increasing the thiol concentration also increased the stability of Cu(I) against oxidation, which is probably due to the formation of stable Cu(I) complexes with the investigated thiols. Only in the case of TAA were we able to follow the reduction kinetics of added Cu(II), and the reducing power of the thiols studied can be summarized as follows: L-cys $\cong$ GSH > MPA > TAA. The lower reducing ability of TAA towards Cu(II) can be explained by its monodentate nature, as well as by the SH group being located next to the C=O group, with respect to the GSH, L-cys, and MPA.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/molecules28135065/s1>, Figure S1: Plots of reaction kinetics for (a) first order reaction, Cu:GSH = 1:1 (b) second order reaction, Cu:GSH = 1:1 (c) first order reaction, Cu:GSH = 1:10 (d) second order reaction, Cu:GSH = 1:10; Figure S2: Plots of reaction kinetics for (a) first order reaction, Cu:L-cys = 1:1 (b) second order reaction, Cu:L-cys = 1:1 (c) first order reaction, Cu:L-cys = 1:2.5 (d) second order reaction, Cu:L-cys = 1:2.5 (e) first order reaction, Cu:L-cys = 1:5 (f) second order reaction, Cu:L-cys = 1:5 (g) first order reaction, Cu:L-cys = 1:10 (h) second order reaction, Cu:L-cys = 1:10; Figure S3: Plots of reaction kinetics for (a) first order reaction, Cu:MPA = 1:1 (b) second order reaction, Cu:MPA = 1:1 (c) first order reaction, Cu:MPA = 1:2.5 (d) second order reaction, Cu:MPA = 1:2.5 (e) first order reaction, Cu:MPA = 1:5 (f) second order reaction, Cu:MPA = 1:5 (g) first order reaction, Cu:MPA = 1:10 (h) second order reaction, Cu:MPA = 1:10; Figure S4: Plots of reaction kinetics for (a) first order reaction, Cu:TAA = 1:1 (b) second order reaction, Cu:TAA = 1:1 (c) first order reaction, Cu:TAA = 1:2.5 (d) second order reaction, Cu:TAA = 1:2.5 (e) first order reaction, Cu:TAA = 1:5 (f) second order reaction, Cu:TAA = 1:5 (g) first order reaction, Cu:TAA = 1:10 (h) second order reaction, Cu:TAA = 1:10; Table S1: Reaction rate constants (*k*) for reactions of Cu(II) reduction and Cu(I) oxidation.

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Supplementary Material

Interaction between Cu and Thiols of Biological and Environmental
Importance: Case Study Using Combined Spectrophotometric/Bathocuproine
Sulfonate Disodium Salt Hydrate (BCS) Assay

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Figure S1 shows plots of $\ln$ absorbance ($\ln A$) for Cu(I) as a function of time (Figure S1a,c), representative of the first order reaction, and plots for $1/A$ as a function of time, representative of the second order reaction (S1b, S1d), for solutions containing Cu and GSH in different ratios. For Cu:GSH=1:1, linearity of $\ln A$ with respect to time is observed for most of the reaction course, indicating a first-order reaction (Figure S1a). In the case of GSH excess with respect to the initial Cu(II) concentration, the Cu(I) concentration was constant for the first 60 min of the reaction course, when it was replaced by rapid Cu(I) oxidation. It can be seen from the diagrams in Figure 1c,d that the reaction kinetics within the first 60 minutes do not follow either first or second order reaction kinetics. The black line (Figure S1a) represents the linearity of $\ln A$ versus time used to calculate the reaction rate constant (k) which is displayed in Table S1.

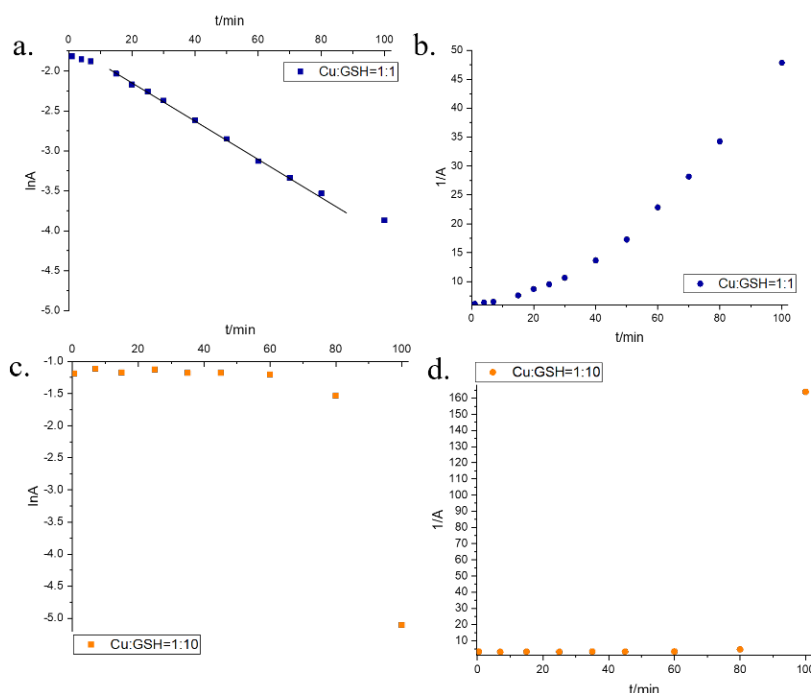


Figure S1. Plots of reaction kinetics for a) first order reaction, Cu:GSH=1:1, b) second order reaction, Cu:GSH=1:1, c) first order reaction, Cu:GSH=1:10, d) second order reaction, Cu:GSH=1:10.

Figure S2 shows plots of $\ln A$ for Cu(I) as a function of time (Figures S2a, S2c, S2e, S2g) representative of the first order reaction and plots for $1/A$ as a function of time representative of the second order reaction (Figures S2b, S2d, S2f, S2h) for solutions containing Cu and L-cys in different ratios. For Cu:L-cys=1:1, a linearity of $\ln A$ is observed compared to the time at the beginning of the reaction, which is characteristic of the first order reaction (Figure S2a). However, the second reaction phase is characterized by a linearity of $1/A$ with respect to time, implying a shift from the first order reaction to the second order reaction in the later reaction phase (Figure S2b). In the case of the L-cys excess with respect to the initial Cu concentration, a non-linearity of both $\ln A$ with respect to time and $1/A$ with respect to time is observed, indicating that the reaction kinetics change over the time of the reaction course (Figure S2c-S2f). The black lines represent the

linearity of $\ln A$ versus time and $1/A$ versus A , where the linearity of $\ln A$ versus time is used to calculate the reaction rate constant (k).

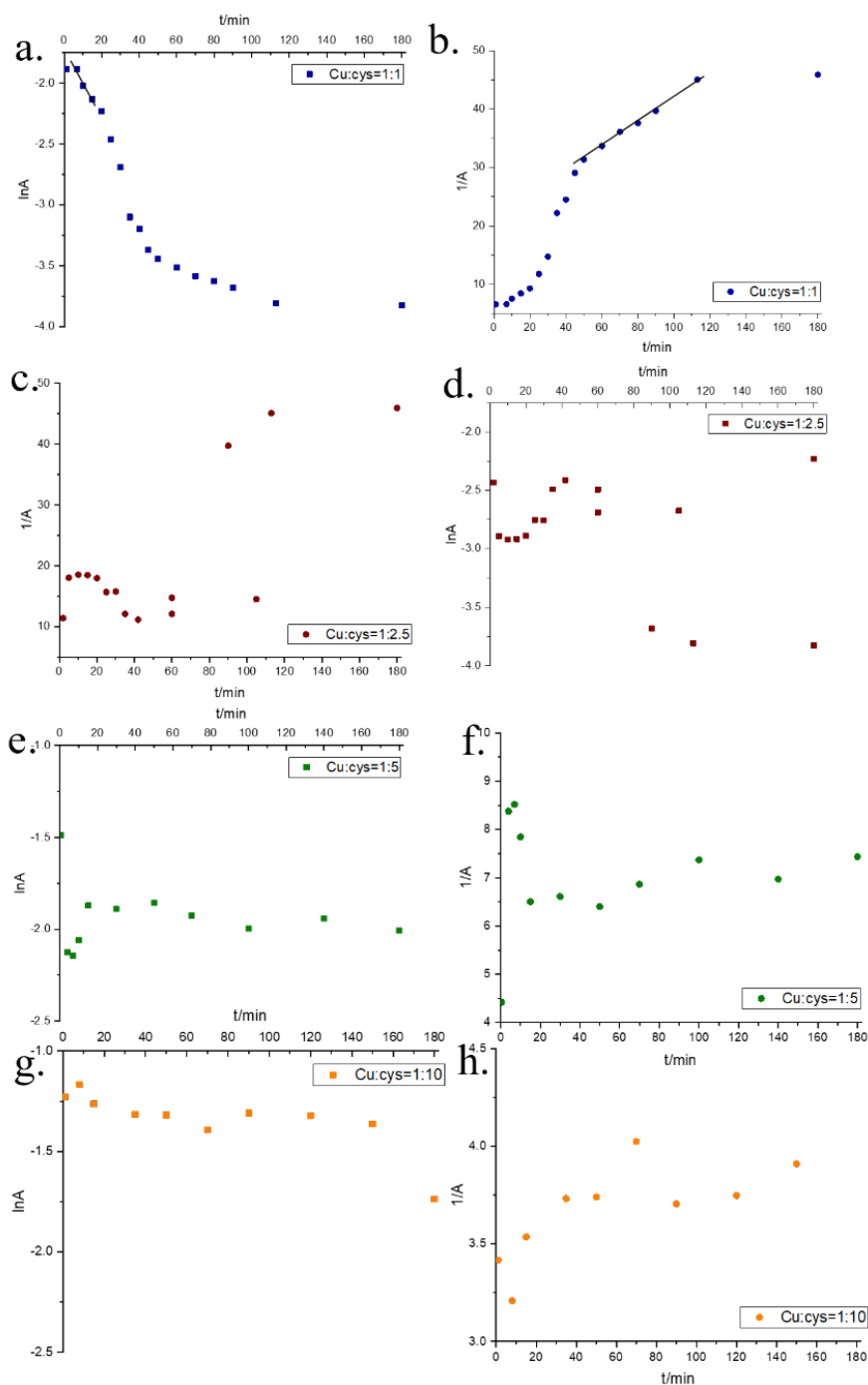


Figure S2. Plots of reaction kinetics for a) first order reaction, Cu:L-cys=1:1, b) second order reaction, Cu:L-cys=1:1, c) first order reaction, Cu:L-cys=1:2.5, d) second order reaction, Cu:L-cys=1:2.5, e) first order reaction, Cu:L-cys=1:5, f) second order reaction, Cu:L-cys=1:5, g) first order reaction, Cu:L-cys=1:10, and h) second order reaction, Cu:L-cys=1:10.

Figure S3 shows plots of $\ln A$ for Cu (I) as a function of time (Figures S3a, S3c, S3e, S3g), representative of the first order reaction, and plots for $1/A$ as a function of time, representative of the second order reaction (Figures S3b, S3d, S3f, S3h), for solutions containing Cu and MPA in different ratios. For Cu:MPA=1:1, the linearity of $\ln A$ is not observed (Figure S3a). Instead, linearity between $1/A$ and time is observed, indicating a second order reaction at the beginning of the reaction course (Figure S3b). In the case of the MPA excess with respect to the initial Cu concentration, linearity versus time is observed for both $\ln A$ and $1/A$, indicating that the reaction kinetics change over time of the reaction course (Figure S3c-S3f). The black lines represent the linearity of $\ln A$ versus time and $1/A$ versus A , where the linearity of $\ln A$ versus time is used to calculate the reaction rate constant (k).

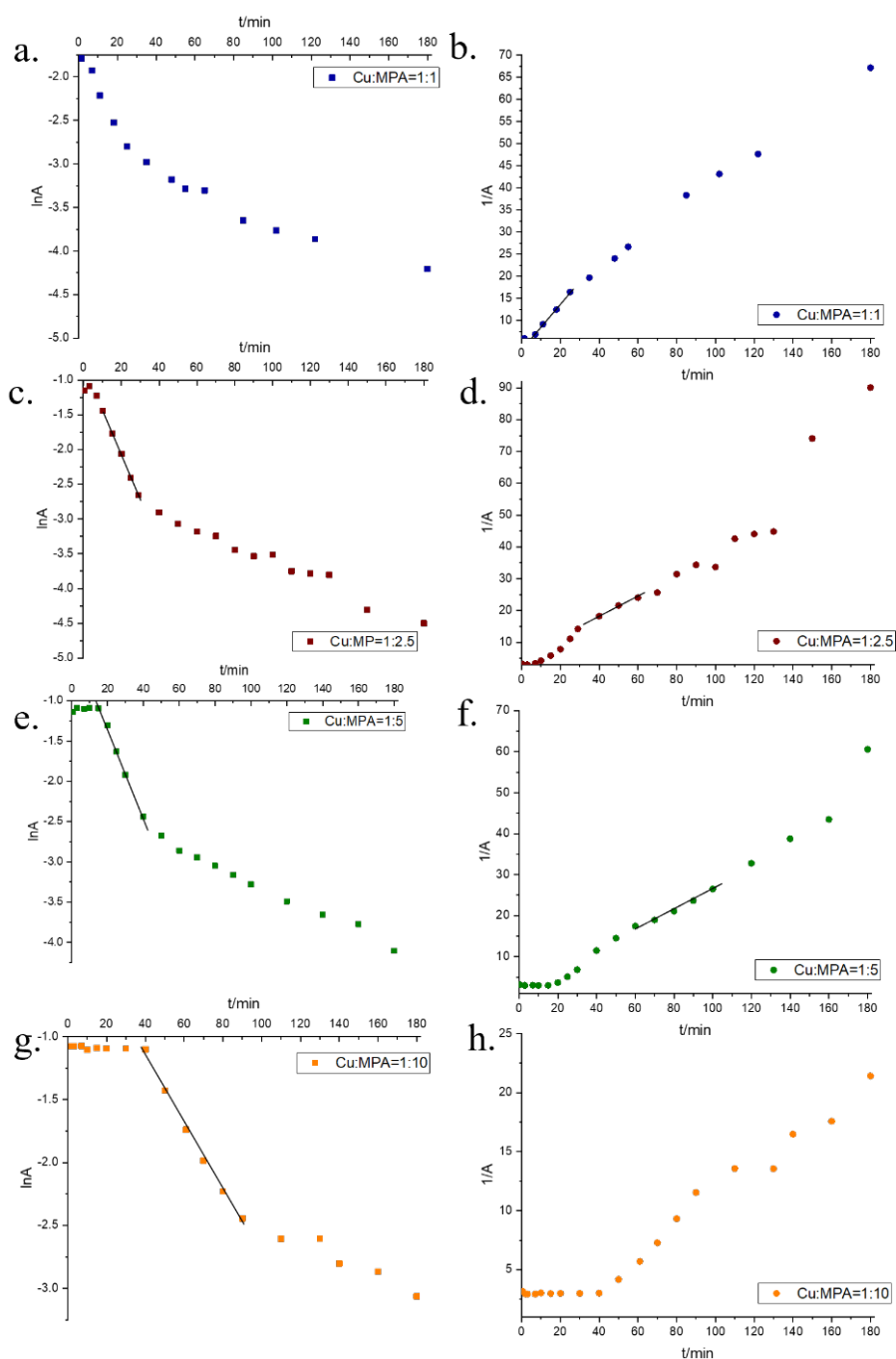


Figure S3. Plots of reaction kinetics for a.) first order reaction, Cu:MPA=1:1 b.) second order reaction, Cu:MPA=1:1 c.) first order reaction, Cu:MPA=1:2.5 d.) second order reaction, Cu:MPA=1:2.5 e.) first order reaction, Cu:MPA=1:5 f.) second order reaction, Cu:MPA=1:5 g.) first order reaction, Cu:MPA=1:10 h.) second order reaction, Cu:MPA=1:10.

Figure S4 shows plots of $\ln$ absorbance ($\ln A$) for Cu (I) as a function of time (Figures S4a, S4c, S4e, S4g), representative of the first-order reaction, and plots of $1/A$ as a function of

time, representative of the second-order reaction (Figures S4b, S4d, S4f, S4h), for solutions containing Cu and TAA in different ratios. In contrast to the results in Figures S1, S2 and S3 in the Supplementary Material of the manuscript, the nature of the interaction between Cu and TAA allows us to follow the reduction rates in the early stages of the reaction (up to 80 minutes). For Cu: TAA =1:1, the linearity of $\ln A$ is observed as a function of time in the first 40 minutes of the reaction course (Figure S4a), which allows the determination of k for the reduction reaction (Table S1, Supplementary Material). Due to the rapid changes in the reaction order as well as in the reaction course (reduction vs. oxidation), we were able to determine k for the Cu(I) oxidation reaction for Cu: TAA =1:5 and Cu: TAA =1:10 (Table S1, Supplementary Material). The black lines represent the linearity of $\ln A$ versus time and $1/A$ versus A , where the linearity of $\ln A$ versus time is used to calculate the reaction rate constant (k).

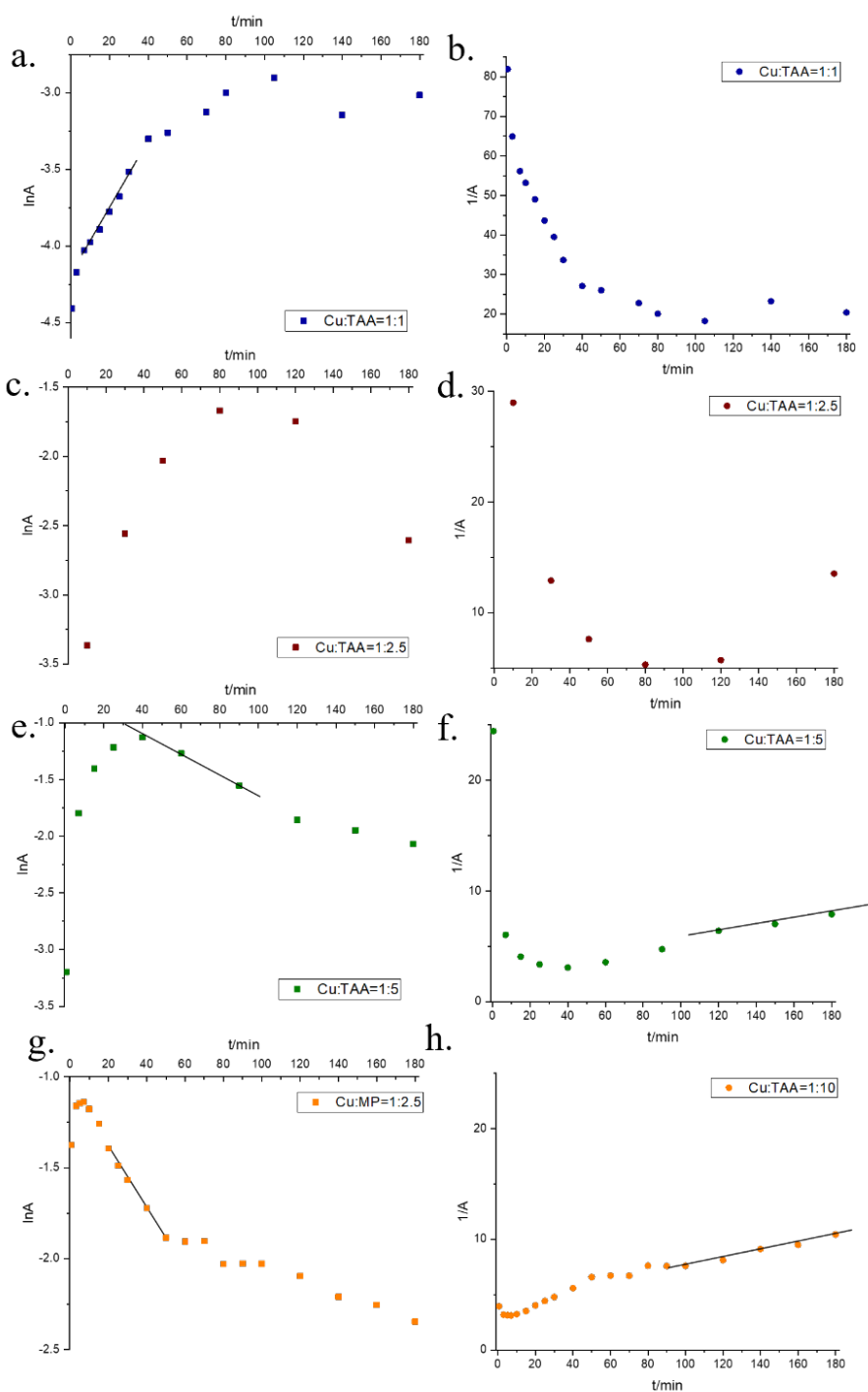


Figure S4. Plots of reaction kinetics for a) first order reaction, Cu:TAA=1:1, b) second order reaction, Cu:TAA=1:1, c) first order reaction, Cu:TAA=1:2.5, d) second order reaction, Cu:TAA=1:2.5, e) first order reaction, Cu:TAA=1:5, f) second order reaction, Cu:TAA=1:5, g) first order reaction, Cu:TAA=1:10, and h) second order reaction, Cu:TAA=1:10.

Table S1. Reaction rate constants (*k*) for reactions of Cu(II) reduction and Cu(I) oxidation.

Thiol	Reaction	Cu:thiol ratio	k/min ⁻¹
L-cys	Cu(I) oxidation	1:1	0.0040
GSH	Cu(I) oxidation	1:1	0.0024
MPA	Cu(I) oxidation	1:2.5	0.0646
	Cu(I) oxidation	1:5	0.0551
	Cu(I) oxidation	1:10	0.0269
TAA	Cu(II) reduction	1:1	0.0196
	Cu(I) oxidation	1:5	0.0098
	Cu(I) oxidation	1:10	0.0161

A.2. Research Paper II

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Redox speciation of copper in the estuarine environment: Towards better understanding of copper water chemistry

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ABSTRACT

An adapted solid-phase extraction method was used for Cu redox speciation in the dissolved fraction in the stratified Krka River estuary in March and July 2023 at a “clean” and a “polluted” site, while anodic stripping voltammetry in differential pulse mode was used for Cu determination in the Cu(I), Cu(II) and Cu_T fractions. Overall, [Cu(I)]/[Cu_T] was significant in the Krka River estuary and varied between 20.7 and 88.7 %. At the “clean” site E1, the surface maximum of [Cu(I)]/[Cu_T] was observed in March. This was assumed to be related to the biological activity of the freshwater biota, as the temperature rose in March (compared to the previous winter months) and the salinity was low. On the other hand, at E1 in July, the maximum of [Cu(I)]/[Cu_T] was observed at the halocline and corresponded to the highest biological production thus we hypothesised it to be the result of Cu(I) complexation with ligands of biological origin. The minimum of [Cu(I)]/[Cu_T] was observed at the surface at E1 in July where terrestrial humic-like substances were present with higher affinity for Cu(II) than for Cu(I). At the “polluted site” E2 high [Cu(I)]/[Cu_T] values were observed at both the surface and the halocline in March. However, [Cu(I)]/[Cu_T] maximum was observed at the halocline at E2 in July and was associated with the highest biological production, as also observed at E1 in July. Nevertheless, the stabilisation of Cu(I) by chloride should not be disregarded as it maintained relatively high [Cu(I)]/[Cu_T] in the seawater layer at both stations in March and July. The fluctuations in [Cu(I)]/[Cu_T] occurred during the day at the surface at E2 in July, with the minimum of [Cu(I)]/[Cu_T] observed in the early morning, followed by an increase in [Cu(I)]/[Cu_T] during the day, indicating the importance of photochemical reactions in the Cu(I) formation and stabilisation.

1. Introduction

Copper (Cu) plays an important role in photosynthesis, iron acquisition and denitrification by marine organisms (Kong, 2022; Peers et al., 2005; Peers and Price, 2006; Zumft and Kroneck, 2006), while availability of Cu to marine organisms depends strongly on its chemical speciation. Nevertheless, Cu has a dual role as a both, micronutrient at low and as a toxicant at high concentrations of free Cu ion ([Cu_{free}]) (Lopez et al., 2019; Mann et al., 2002; Pérez et al., 2010), which necessitates a better understanding of its chemical speciation in natural waters. It is known that over 99 % of dissolved Cu (hereafter referred to as [Cu_T]) in seawater is complexed by organic ligands (Buck et al., 2007; Moffett and Dupont, 2007), but Cu redox speciation is overlooked. Since Cu(II) in seawater is thermodynamically favoured, speciation models usually assume that Cu(II) is the only significant Cu oxidation state in seawater (Turner et al., 1981; Zirino and Yamamoto, 1972; Zuehlke and Kester, 1983). Nevertheless, significant Cu(I) concentrations have been

found in marine and estuarine waters, as well as in rain and in fog waters (Buerge-Weirich and Sulzberger, 2004; Kieber et al., 2004; Moffett and Zika, 1988; Xue et al., 1991). Moffett and Zika (1988) measured Cu(I) concentrations of 5–10 % of [Cu_T] in the Atlantic Ocean and Gulf of Mexico, with the highest Cu(I) concentrations observed at the surface and decreasing with depth, while Cu(I) was not detected below the photic zone. The authors attributed the formation and stabilisation of Cu(I) to various photochemical processes involving hydrogen peroxide (H₂O₂), as well as to the presence of organic reductants such as humic compounds and organic compounds of biological origin (Moffett and Zika, 1988). Moreover, in the other works by Moffett and Zika (Moffett and Zika, 1983; Moffett and Zika, 1987), Paul Hansard et al. (2010) and Rusak et al. (2011), the authors reported the considerable importance of photochemical processes for the redox equilibria of transition metals, such as the photooxidation of dissolved organic matter (DOM) in the photic zone, leading to the formation of Cu(II)-reducing superoxide (O₂^{•−}) radicals and H₂O₂ radicals (mainly from the protonation of O₂^{•−}), both of

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which were measured at the sunlit seawater surface in the picomolar and nanomolar range. It is hypothesised that organically bound Cu may react with O_2 , a product of DOM oxidation in sunlit surface waters, and lead to the formation of Cu(I) (Voelker et al., 2000). Furthermore, Buerge-Weirich and Sulzberger (2004) reported Cu(I) concentrations between 5 and 80 % of $[Cu_T]$ in the Scheldt estuary and the North Sea. The measured Cu(I) concentrations varied with dissolved organic carbon (DOC) values and salinity and were attributed to light-induced reduction of Cu(II) complexes, stabilisation of Cu(I) by chloride at higher salinities, thermal reduction of Cu(II) by sulphide-containing compounds at low salinities and reoxidation of Cu(I) due to the stabilisation of Cu(II) by strong organic ligands at intermediate salinities (Buerge-Weirich and Sulzberger, 2004). Significant concentrations of Cu(I) in rainwater, ranging from 22 to 32 % of $[Cu_T]$ were reported by Kieber et al. (2004) and contributed to the various mechanisms of photoreduction, reduction of Cu(II) by organic compounds, and stabilisation of Cu(I) by inorganic compounds such as sulphite (SO_3^{2-}). In addition, Xue et al. (1991) measured Cu(I) concentrations of 4–90 % of $[Cu_T]$ in fog water where Cu(I)-sulphite and Cu(II)-oxalate complexes predominated, with Cu(I) most probably being formed by various reduction reactions including organic compounds. Overall, it is evident that DOM plays an important role in the redox chemistry of Cu in addition to photochemical processes. The importance of humic substances, thiol-like substances and amino-acids/proteins in Cu binding has been reported in numerous works (Dulaquais et al., 2020; Gibbon-Walsh et al., 2012; Muller and Batchelli, 2013; Ruacho et al., 2022; Whitby et al., 2018; Whitby and van den Berg, 2015). Within the complex DOM pool in natural waters Cu ligands are usually categorised into classes L_1 and L_2 according to the value of conditional stability constant $\log K'$, with L_1 representing the class of strong ligands with $\log K'_{L_1}$ values >12 and L_2 the class of weak ligands with $\log K'_{L_2}$ values <12 (Coale and Bruland, 1990; Croot et al., 2000; Moffett and Brand, 1996; Whitby et al., 2018; Zitoun et al., 2021). Stronger class of ligands (L_1) are most likely produced by microorganisms in response to increased $[Cu_{free}]$ and correlate well with concentrations of thiol compounds such as glutathione (GSH) (Croot et al., 2000; Fernanda et al., 1998; Moffett and Brand, 1996; Tang et al., 2004, 2005; Whitby et al., 2018). It is suggested that in addition to GSH, other thiols such as cysteine, 3-mercaptopropionic acid and 2-mercaptoethanol, all of which are strong Cu(I)-binding ligands, may contribute to L_1 ligand class, as well as other yet unidentified compounds such as methanobactin (Whitby et al., 2017, 2018). In addition, Leal and Van Den Berg (1998) pointed out the presence of stable Cu(I) in seawater, which is attributed to the high stability of Cu(I) complexes with GSH and cysteine, both of which are abundant in seawater. Although not directly measured, Whitby et al. suggested that up to 90 % of $[Cu_T]$ in the salt marsh estuary may be present as Cu(I), since 82–99 % of $[Cu_T]$ in the region is bound to the ligand class L_1 , which consists of Cu(I)-binding thiols (Whitby et al., 2017). The weaker class of ligands (L_2) usually consists of humic-like compounds, originating from sea or land (Whitby et al., 2018; Whitby and van den Berg, 2015). Nevertheless, terrestrial humic substances have also been suggested as a source of strong Cu-binding ligands (Muller and Batchelli, 2013). Extensive research on copper speciation has shown that biologically produced proteinaceous substances have higher affinity for Cu than humic substances, with $\log K'$ in the range of 12 to 15 (Ruacho et al., 2022). Similar observations were made in the Krka River estuary, the study site of this work, where L_1 class of ligands is mainly derived from phytoplankton production, while humic substances dominate in L_2 class of ligand (Marcinek, 2021). On the other hand, the competitive effect of Fe over Cu for humic-like ligands has been reported, leading to in situ production of Cu-binding ligands (Muller and Cuscov, 2017). Active release of Cu-binding organic ligands by phytoplankton, until $[Cu_{free}]$ reached sufficiently low levels, has also been recognized in the work by Muller et al. (Muller et al., 2003).

Methods for the measurement of Cu(I) usually consist of the complexation of Cu(I) with bathocuproine (BCP) or its disodium salt

(bathocuproine disulfonic acid disodium salt, BCS) or complexation of Cu(I) with neocuproine, while Cu(II) interference is inhibited by masking ligands such as ethylenediamine, ethylenediamine tetraacetic disodium salt (EDTA) or diethylenetriamine pentaacetate (DTPA) (Buerge-Weirich and Sulzberger, 2004; Moffett et al., 1985; Moffett and Zika, 1988; Voelker et al., 2000). Subsequently, the Cu(I) concentration is determined either by spectrophotometry or liquid-liquid solid-phase extraction with atomic absorption spectroscopy.

Although the literature cited indicates the presence of Cu(I) in marine and estuarine waters, there are few articles that have measured Cu(I) in natural waters, and it is clear that Cu redox speciation is overlooked. The aim of this work was therefore to determine Cu redox speciation in estuarine waters and to identify the factors leading to its formation/stabilisation. The solid-phase extraction method (SPE) was adapted to determine Cu redox speciation at “clean” and “polluted” sites in Krka River estuary. The formation and stability of Cu(I) specie is discussed in terms of photochemical reactions, biological production, DOM properties and chloride complexation.

2. Site description: Krka River estuary

The Krka River (Croatia), with a freshwater section length of 49 km, is a medium-sized karstic river that flows into the east coast of Adriatic Sea and drains carbonate terrains. It is characterized by extremely low natural concentrations of trace metals, apart from anthropogenic point source pollution registered downstream of the town of Knin. These concentrations decrease again in the rest of the freshwater stream due to the self-purification in the lakes formed by tufa barriers and groundwater input (Cukrov et al., 2008; Filipović Marijić et al., 2018). Ranging from 0.42 mg/L to 1.78 mg/L, concentrations of DOC in the Krka River and its estuary are very low (Marcinek et al., 2020). The Krka estuary begins after the Skradinski buk waterfall and stretches 23.5 km to the Šibenik channel (Fig. 1). Due to the freshwater discharge and low tides, the estuary is permanently stratified. Consequently, there is a sharp halocline between the freshwater and seawater layers, the thickness of which varies between a few cm to approximately 1.5 m depending on the river flow and weather conditions. As a result of the salinity and shear gradient, organic matter accumulates at the freshwater/seawater interface (Zutić and Legović, 1987). The pristine Krka estuary has been the subject of research interest for decades and numerous articles have been published on the distribution, speciation and dynamics of trace metals (Cindrić et al., 2015a; Cindrić et al., 2015b; Cukrov et al., 2008; Elbaz-Poulichet et al., 1991; Marcinek, 2021; Marcinek et al., 2022; Omanović et al., 2006). Nevertheless, only a few research articles have been published on the redox speciation of trace metals (Knežević et al., 2021; Pađan et al., 2019), whereas Cu redox speciation has not been studied. Although Cu concentrations in the estuary are relatively low, a multi-fold increase in Cu concentrations have been reported during the summer season, due to Cu emissions from antifouling coatings (Carić et al., 2021; Cindrić, 2015). For this reason, studies on Cu speciation in the estuary are of great research interest.

Two sampling sites were selected for this study: E1 and E2, representing a “clean” and a “polluted” site, respectively. As can be seen in Fig. 1, site E2 is located in the marina, thus is significantly more anthropogenically influenced than site E1. At both sites, samples were taken at different depths in March and July 2023 to determine the Cu redox speciation in the brackish water surface layer, at the halocline (the freshwater/seawater interface) and in the seawater layer. In addition, four samples were taken from surface layer at E2 every 5–7 h over an 18-h period (5:00 h, 10:00 h, 17:00 h, 23:00 h) in July to determine the effects of solar radiation on Cu redox speciation. These samples are further labelled as E2_{5h}, E2_{10h}, E2_{17h} and E2_{23h}. In addition to Cu redox speciation, the values for DOC and salinity are also reported for all sampling sites as well as the determination of Cu-binding ligands and the characterisation of chromophoric/coloured DOM (CDOM) at E1 in July.

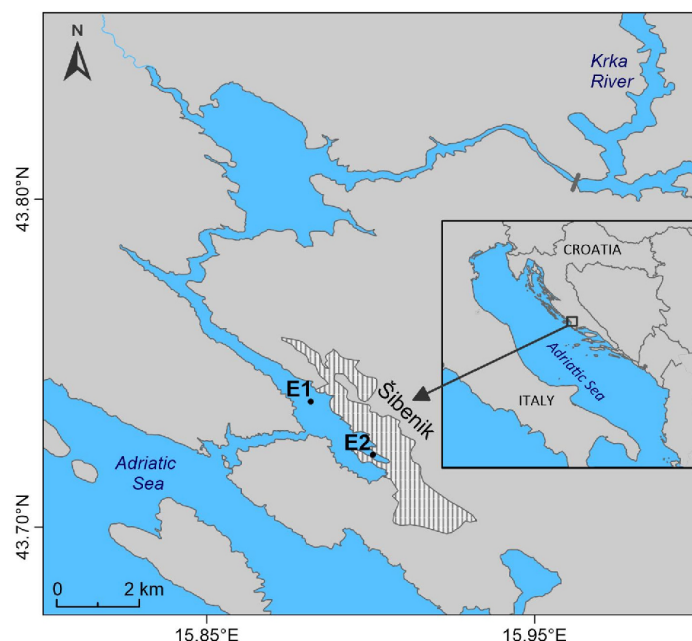


Fig. 1. Map of the study section of the Krka River estuary with indicated sampling sites, E1 and E2.

3. Experimental section

3.1. 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. Copper(I) chloride (CuCl; Thermo Fisher Scientific, MA, USA) and copper(II) sulphate (CuSO₄, VWR BDH Prolabo Chemicals, PA, USA) were used as Cu(I) and Cu(II) salts, respectively. Copper(I) standard solution was prepared according to a procedure modified from that published by Moffet and Zika (Moffet et al., 1985), and consisted of the dissolution of CuCl in a solution with 4 M sodium chloride (NaCl; Grammol, UT, USA) and 0.1 M hydrochloric acid (HCl s.p.; Roth, CA, USA), which was previously purged with high-purity nitrogen to remove oxygen. Copper(I) stock solution was freshly prepared for each experiment and used within 30 min. Copper(II) stock solution was prepared by dissolving CuSO₄ in dilute HNO₃ s.p. (Merck, Germany), pH ~2, to a concentration of 9.4 mM. In this study, bathocuproine disulfonic acid disodium salt (BCS; Thermo Fisher Scientific, MA, USA), a strong Cu(I) chelator, was used as a water-soluble analogue of bathocuproine. Solution of BCS was prepared daily by dissolving BCS in UP water to a concentration of 1 mM. To avoid the reduction of Cu(II) by BCS, ethylenediaminetetraacetic acid disodium salt (EDTA disodium salt; Carlo Erba Reagents S.A.S., France) was used as a masking ligand. Stock solution of EDTA was prepared by dissolving EDTA salt in UP water to a final concentration of 100 mM. Copper contribution from the BCS and EDTA stock solutions was negligible considering their final concentrations used in this work. In column cleaning procedure, borate buffer, HCl and acidified methanol were used. Acidified methanol was prepared by adding 100 μL of HCl s.p. to 50 mL of methanol s.p. (VWR BDH Prolabo Chemicals, Belgium). For preparation of 1 M borate buffer, ortho-boric acid (VWR BDH Prolabo Chemicals, Belgium) and sodium hydroxide

(NaOH; Lach-ner Chemicals, Czech Republic) were dissolved in UP water, while HCl was prepared by adding HCl s.p. to UP water to a concentration of 25 mM.

3.2. Sampling and sample handling

The bottles used for sample storage were made of fluorinated polypropylene (FEP, Nalgene) or polycarbonate (Nalgene). All bottles, tubes and syringes used were previously cleaned by soaking in 1.0 % v/v HNO₃ for at least 24 h, and then rinsed three times with UP water. Glass tubes used for DOC analysis were cleaned with chromium-sulfuric acid, rinsed with UP water, and precombusted for 4 h at 450 °C.

Sampling campaigns were conducted on March 29, 2023 (10:00 h) and July 19, 2023 (11:00 h). Samples were taken using the home-built 4.5 L „butterfly“-type water sampler, with the exception of four surface samples collected every 5–7 h at E2 site on July 19 which were collected by classic grab sampling. All the samples were filtered with 0.45 μm syringe filters made from cellulose acetate (Minisart, Sartorius, Germany) or under the nitrogen pressure through 0.45 μm cellulose-nitrate membrane filters (Sartorius, Germany), either on board or immediately afterwards at the marine research station, in each case less than 2 h after sampling. Immediately after filtration, BCS and EDTA were added to the samples for Cu redox speciation according to the procedure described in section 3.3. The samples for Cu organic speciation, DOC, CDOM and fluorescence analysis were filtered and stored in the refrigerator until the measurements were performed (within two weeks from the sampling).

3.3. Adaption of solid-phase extraction method

The SPE method for selective accumulation of Cu(I), as well as the concentrations of BCS and EDTA were adapted from Buerge-Weirich and Sulzberger (Buerge-Weirich and Sulzberger, 2004). Bathocuproine was

used to stabilise Cu(I) species, while, to avoid Cu(II) interference, EDTA was used. A ratio of BCS:EDTA of 1:5 was used. First, aliquots of BCS and EDTA were mixed in a tube and then added to the sample in the FEP bottle, with final concentrations of 1 μM BCS and 5 μM EDTA in the samples. A 100 mL sample was prepared, of which 50 mL was used for the determination of total dissolved Cu (referred to as $[\text{Cu}_\text{T}]$) and the remaining 50 mL sample was used for SPE. One hour after the addition of BCS and EDTA to the filtered seawater sample, SPE was performed by sequentially passing aliquots of 5 mL of the sample through the column to a final volume of 50 mL. The columns used for SPE were Bond Elut NEXUS LRC columns (Agilent, CA, USA) and were pre-cleaned according to the published procedure (Buerge-Weirich and Sulzberger, 2004). The columns contain 60 mg of polymeric sorbent that retains the Cu(I)-BCS complex (referred to as $[\text{Cu(I)}]$) in the column, while the Cu(II)-EDTA complex (referred to as $[\text{Cu(II)}]$) passes through the column. The Cu(I)-BCS was eluted with 1 mL of acidified methanol. The methanolic Cu(I)-BCS solution was pre-concentrated 50-fold, based on 50 mL of the sample that originally passed through the column and final volume of 1 mL. Prior to UV digestion, the methanolic Cu(I)-BCS solution was diluted 10-fold with UP water, resulting in an overall 5-fold pre-concentration. Therefore, 5-fold pre-concentration was taken in consideration while preparing samples for voltammetric measurements. After SPE, all fractions (Cu(I), Cu(II) and Cu_T) were acidified and UV digested as described in section 3.4.

While we were able to determine the Cu(I) and Cu(II) concentrations in situ in the sunlit water column using the method described in this section, in the work by Buerge-Weirich and Sulzberger (2004) the water samples used for Cu(I) determination were deep frozen on board. After thawing, the samples were irradiated before the addition of Cu(I) and Cu(II) binding reagents and passed through the column using the SPE method. The authors used the same procedure for the dark control experiments, except that the samples were not irradiated prior to SPE. Although the irradiation experiments can provide important insight into Cu(I) formation, the light intensity used in the irradiation experiments is higher than in sunlit water, so the measured Cu(I) concentrations may not represent a natural steady state. On the other hand, the sample treatment described in this section allowed us the determination of Cu(I) and Cu(II) equilibrium concentrations instantly after the sampling. Further method adaptation is discussed in section 3.4.

3.3.1. Validation of solid-phase extraction method

3.3.1.1. Determination of the column blank. To determine the column blank, a mixture of BCS and EDTA was added to 100 mL of UP water, with final concentrations of 1 μM BCS and 5 μM EDTA in UP water, and SPE was performed as described in section 3.3. After UV digestion and differential pulse-anodic stripping voltammetry (DP-ASV) measurements, both as described in section 3.4., the measured Cu concentrations were below 0.2 nM, indicating that Cu release from the column was negligible. Thus, no blank-correction was performed.

3.3.1.2. Recovery of Cu after solid-phase extraction. For the Cu recovery experiment, an aliquot of the Cu(I) or Cu(II) stock solution was added to the mixture of BCS and EDTA (total volume of prepared stock solution was 4.15 mL). The mixture of Cu, BCS and EDTA was then added to a 100 mL seawater sample with low Cu content ($[\text{Cu}_\text{T}] = 3.8 \text{ nM}$, salinity = 35) from the Krka estuary and these model solutions were labelled as M1 and M2. The concentrations of the reagents were the same as described in section 3.3, while the concentration of added Cu was 72 nM and 71 nM for M1 and M2, respectively. At the same time, a mixture of BCS and EDTA without Cu was added to another 100 mL of the same seawater sample to determine the Cu redox speciation in the natural water sample without standard addition of Cu stock solution. Copper concentrations in Cu(I) and Cu(II) fractions of Cu_T in M1 and M2 model samples were corrected for the values determined in the sample without

Cu addition, and further used for calculation of Cu(I) and Cu(II) recoveries, as presented in Table 1. As all Cu was recovered after SPE, the method could be applied to natural samples from the estuary without further corrections.

3.4. Determination of Cu concentration

Prior to voltammetric measurements, all samples were acidified with HNO_3 s.p. to $\text{pH} < 2$ and UV digested for at least 42 h. For UV oxidation, a homebuilt system with 250 W high-pressure mercury lamp (Hanau, Germany) was used. In order to avoid interferences in the voltammetric measurements, the sample with methanolic Cu(I) fraction was firstly evaporated almost to dryness by heating it to 100 $^\circ\text{C}$ and redissolved in UP water to the same volume (10 mL), whereas in the case of the Cu(II) and Cu_T fractions, which did not contain methanol, no evaporation was necessary. Copper concentrations in all fractions ($[\text{Cu(I)}]$, $[\text{Cu(II)}]$ and $[\text{Cu}_\text{T}]$) were measured by DP-ASV using the standard addition method ($n = 4$). In brief, an aliquot of 10 or 15 mL in the case of the Cu(II) and Cu_T fractions was deaerated by purging with high-purity nitrogen for at least 4 min. While the Cu(II) and Cu_T fractions were not pre-concentrated during the SPE, the Cu(I) fraction was pre-concentrated in the column (as described in section 3.3.), eluted with acidified methanol and evaporated and redissolved in UP water after UV digestion. In the case of Cu determination in Cu(I) fraction, an electrolyte consisting of 0.55 M NaCl was acidified to $\text{pH} < 2$ and deaerated before an aliquot of the Cu(I) fraction was added. To determine the copper concentrations in all fractions using the standard addition method, the standard solution of Cu(II) was added after UV digestion of the samples, which ensures the oxidation of the organic substances/ligands and consequently also the oxidation of the Cu(I) present. Each standard addition measurement was performed twice. Measurements were performed using the Autolab PGSTAT204 instrument (Metrohm-Autolab, The Netherlands) controlled by NOVA 2.1 software in a three-electrode cell (VA 663 Stand; Metrohm, Swiss). The three-electrode system used for the measurements consisted of a hanging mercury drop electrode (HMDE) with a drop surface of 0.40 mm^2 as the working electrode, a platinum wire as the counter electrode and an Ag/AgCl (3 M KCl) electrode as the reference electrode. The voltammograms were processed using the ECDSoft (ElectroChemical Data SOFTware) software developed in our laboratory (<https://sites.google.com/site/daromasoft/home/ecdsft>). The peak height was used as the signal value. The parameters used for the voltammetric measurements were based on earlier work on DP-ASV measurements of Cu in the estuary (Cindrić, 2015; Marcinek, 2021; Omanović et al., 2006). The deposition potential was set to -0.85 V and the deposition time depended on the Cu concentration in the sample and varied between 60 and 600 s. An additional desorption step was performed by switching to a negative potential of -1.3 V for 3 s at the end of the deposition time to avoid potential interferences from surface active substances (Cindrić, 2015; Louis et al., 2009). After 5 s of equilibration, the potential was scanned from -0.55 V to 0 V with following the scanning parameters: step of 0.005 V, modulation time of 0.05 s, interval time of 0.5 s and modulation amplitude of 0.025 V. The uncertainty (error bars in the plots) of the concentrations of determined Cu species ($[\text{Cu(I)}]$, $[\text{Cu(II)}]$ and $[\text{Cu}_\text{T}]$) are expressed as 95 % confidence interval based on the standard addition method of unknown concentration determination. The uncertainty associated with $[\text{Cu(I)}]/[\text{Cu}_\text{T}]$ percentage are calculated based on propagation of uncertainty rule.

Table 1
Recovery of Cu after solid-phase extraction.

sample	Cu(I) added/ nM	Cu(II) added/ nM	retained at column	not retained at column
M1	72	/	101 %	5 %
M2	/	71	<1 %	97 %

3.5. Dissolved organic matter characterisation

Additional measurements of DOC, absorbance and fluorescence were performed. Procedures are described in detail in Marcinek et al. (2020). Briefly, DOC was measured by high temperature catalytic oxidation using the Shimadzu On-line TOC-L carbon analyser. UV/Vis absorbance spectra were measured using a Specord 200 Plus (Analytik Jena GmbH) spectrophotometer with a 10 cm Suprasil quartz cuvette and processed using ASFit software (Omanović et al., 2019). Excitation-emission matrices (EEMs) were recorded using the HORIBA Aqualog® fluorescence spectrometer in 1 cm quartz cuvettes and processed using TreaEEM software (Omanović et al., 2023).

Absorption coefficient at 254 nm (a_{254}) expressed in Napierian units was used as representative of the CDOM pool. The specific UV absorbance at 254 nm (SUVA) was calculated by dividing the a_{254} (decadic) by DOC concentration and it was used as indicator of CDOM content in the total DOM pool (Stedmon and Nelson, 2015). The spectral slope over a 275–295 nm range ($S_{275-295}$) was calculated using the exponential model within ASFit software and used as proxy for average molecular weight, aromaticity and humification degree of CDOM (Helms et al., 2008). Fluorescence indices: Coble peaks A, C and M and biological activity index (BIX), were used as indicators of humic-like fluorescence (Coble, 2007) and biological activity (Santos et al., 2016), respectively. Fluorophores A and C indicate the fluorescence intensity at wavelengths characteristic for terrestrial humic-like signal, while fluorophore M, is an indicator of humic-like compounds formed in the sea by autochthonous biological activity (Coble, 2007).

3.6. Organic Cu speciation

The Cu organic speciation was analysed in the dissolved fraction, using the differential pulse - adsorptive cathodic stripping voltammetry (DP-AdCSV) and competitive ligand exchange (CLE) approach with salicylaldehyde (SA) as added competing ligand, according to “continuous multi detection window” (CMDW) protocol (Marcinek, 2021). The sample was divided into 16 aliquots of 12 mL. In all the aliquots 0.01 M borate/ammonia buffer was added to maintain ambient pH (8.2 ± 0.1). Aliquots were spiked with Cu, with the exception of the first two aliquots where ambient Cu concentration was sustained. The Cu additions in other 14 aliquots were equally distributed, respecting logarithmic scale, up to the maximum of 80 nM $[Cu_T]$. SA was added to each aliquot at concentrations continuously decreasing (in logarithmic scale) from 5 to 0.5 μ M. Before the measurements, aliquots were left for overnight equilibration (~ 17 h). The same voltammetric instrumentation was used as described in section 3.4, with instrumental settings as follows: deposition potential of -0.1 V, deposition time of 60 s, desorption step at -0.2 V for 1 s, potential scan from -0.1 V to -0.6 V, modulation time of 0.05 s, interval time of 0.1 s, step potential of 0.002 V and modulation amplitude of 0.04 V. For each aliquot, 3 repetitive measurements were performed, and all the data were used for the construction of the titration curves. Complexing parameters (ligand concentration and associated $\log K$) were estimated from the obtained titration curves, which were interpreted using the ProMCC software with complete complexation fitting model (Omanović et al., 2015). The concentration of detected ligand classes is expressed as equivalent of Cu. The $\log K$ presented here are based on $[Cu_{free}]$ and are conditional for experimental salinity and pH. The $[Cu_{free}]$ was calculated from ambient concentrations of $[Cu_T]$ and estimated complexing parameters.

4. Results and discussion

4.1. Dissolved organic carbon, salinity and CDOM characterisation at study site

The fluctuations in salinity and DOC at stations E1 and E2 in March and July are shown in Fig. 2, as is the characterisation of CDOM at

stations E1 and E2 in July. At station E1, the DOC content fluctuated between 0.735 and 0.741 mg/L in March and between 0.800 and 1.150 mg/L in July. The highest DOC value was observed at the halocline in both seasons. At station E2, however, the DOC maximum occurred at the bottom in March and at the surface in July, while the DOC value fluctuated between 0.727 and 0.840 mg/L in March and between 0.970 and 1.450 in July. As expected, surface salinity was lower in March than in July due to the higher river discharge during winter and spring compared to summer. Nevertheless, the salinity at the surface in July was unusually low for the summer season. In both profiles, the surface samples showed a very complex CDOM composition. It was characterized by high SUVA values, low slope values and pronounced humic signals (fluorophores A, C and M), indicating a strong terrestrial signature consistent with the unusually low salinity for the summer season. These low salinity values confirm the influence of river input on these samples, as rivers are the main source of humic substances in estuaries. CDOM with the lowest molecular weight and aromaticity was observed in both profiles at the halocline, as indicated by low SUVA and high $S_{275-295}$ values compared to other profile sections. Furthermore, BIX values peak at the halocline, a layer known for increased biological activity. This is consistent with previous research findings. For instance, studies by Viličić et al. (1989) documented the presence of both dead cells and active phytoplankton at the freshwater-saltwater boundary, while Cetinić et al. (2006) found an abundance of nanophytoplankton and picoplankton associated with the halocline (Cetinić et al., 2006; Viličić et al., 1989). The studies by Žutić and Legović (1987) and Svendsen et al. (2007) have also shown that freshwater plankton accumulate at this boundary and bacterial activity increases (Svendsen et al., 2007; Žutić and Legović, 1987).

4.2. Copper redox speciation at E1 “clean” site in March and July

Vertical distribution of Cu(I), Cu(II) and Cu_T , as well as DOC and salinity, at site E1 (“clean site”) in March and July are shown in Fig. 3, while mass balance is shown in Table 2.

The Cu redox speciation at E1 varied throughout the water column of the stratified Krka estuary, ranging from 36.1 to 88.7 % of $[Cu(I)]/[Cu_T]$ in March and 50.9–83.4 % of $[Cu(I)]/[Cu_T]$ in July, whereas $[Cu_T]$ at E1 was similar between two seasons. A simple statistical analysis using the two tailed *t*-test (Microsoft Excel) was performed on the vertical profiles of Cu_T and Cu(I) fractions at E1 site to support the interpretation of obtained results. There is a clear statistical difference in $[Cu(I)]/[Cu_T]$ between surface and bottom at both seasons ($p(\text{spring}) = 0.012$, $p(\text{summer}) = 0.039$) as well as clear statistical difference between seasons at both surface and bottom water layers ($p(\text{surface}) = 0.018$, $p(\text{bottom}) = 0.026$).

In March, the highest percentage of $[Cu(I)]/[Cu_T]$ was observed at the surface and decrease in $[Cu(I)]/[Cu_T]$ was observed at the halocline, while $[Cu(I)]/[Cu_T]$ minimum was observed in the seawater layer below the halocline. On the other hand, DOC maximum occurred at the halocline, while minimum was determined at the surface and the bottom (Fig. 2). Note that the salinity in surface layer in this period of the year was very low thus not presenting the salinity shock for riverine phytoplankton. Plausible explanation for the surface maximum of $[Cu(I)]/[Cu_T]$ is the increase in temperature (11.6 °C at the time of sampling) in this period of the year comparing to previous winter months (Čindrić, 2015; Marcinek, 2021) favouring biological activity at low-salinity surface layer and, thus, the biological production of Cu(I) binding ligands which can confidently be assigned to riverine-sourced biota. We are missing organic speciation and DOM characterisation in these samples to confirm it, however February–March production maxima in freshwater observed in previous studies supports these assumptions (Cetinić et al., 2006).

In contrast to March, in July, the lowest $[Cu(I)]/[Cu_T]$ occurred at the surface and the highest at the halocline (mid-salinity) accompanied with high DOC concentration (see Figs. 2 and S1.). There are several

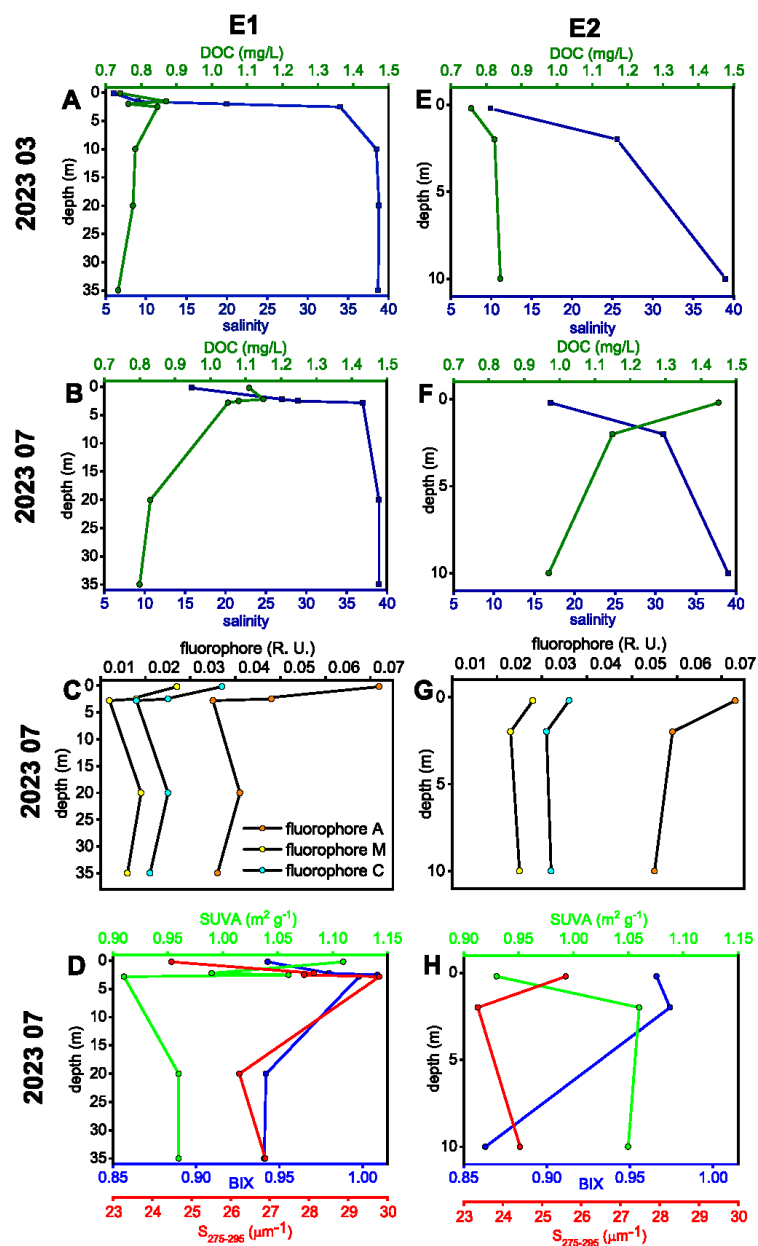


Fig. 2. DOC (upper X axis) and salinity (lower X axis) at station E1 in March (A) and in July (B). Fluorophores A, C and M at station E1 in July (C). SUVA (upper X axis), BIX (lower X axis - blue) and $S_{275-295}$ (lower X axis - red) at station E1 in July (D). DOC (upper X axis) and salinity (lower X axis) at station E2 in March (E) and in July (F). Fluorophores A, C and M at station E2 in July (G). SUVA (upper X axis), BIX (lower X axis - blue) and $S_{275-295}$ (lower X axis - red) at station E2 in July (H). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

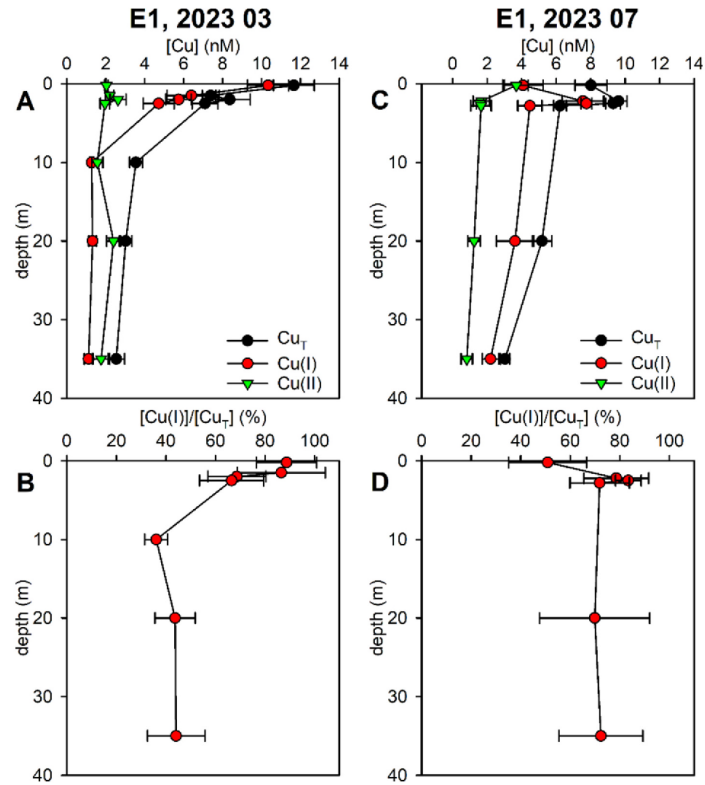


Fig. 3. Vertical distribution of [Cu(I)], [Cu(II)] and [Cu_T] at station E1 in March (A) and in July (C). Percentage of [Cu(I)]/[Cu_T] at station E1 in March (B) and in July (D).

Table 2
Percentages of [Cu(I)]/[Cu_T] and mass balance at station E1 in March and July.

month	station	depth (m)	[Cu(I)]/[Cu _T] (%)	[Cu(II)]/[Cu _T] (%)	mass balance (%)
March	E1	0.2	88.7	17.4	106.1
March	E1	1.5	86.6	29.8	116.4
March	E1	2.0	68.1	31.4	99.5
March	E1	2.5	66.5	27.6	94.1
March	E1	10.0	36.1	44.2	80.3
March	E1	20.0	43.7	79.5	123.2
March	E1	35.0	44.1	69.3	113.4
July	E1	0.2	50.9	46.0	96.9
July	E1	2.2	78.6	17.4	95.9
July	E1	2.5	83.4	17.1	100.4
July	E1	2.8	71.9	26.5	98.4
July	E1	20.0	69.9	24.1	94.0
July	E1	35.0	72.4	27.0	99.3

factors which may contribute to the lowest [Cu(I)]/[Cu_T] at the surface in July. Here, the highest fluorescence of fluorophores A, M and C was detected, along with the highest SUVA and low BIX (although inconsistent with these, high S₂₇₅₋₂₉₅ values were detected on the surface) (Fig. 2, Table S1), thus it may be presumed that terrestrial humic-like substances, with higher affinity towards Cu(II) were delivered by the river in the surface layer, while in the same time these waters were depleted from small biological organic compounds. Although humic

substances may be a source of strong Cu-binding ligands in coastal areas (Muller and Batchelli, 2013), these ligands are most likely to bind Cu(II) over Cu(I). Atmospheric deposition may also be an important source of humic compounds at the surface layer, but there is no data available of its specific input in the Krka estuary. On the other hand, increase in [Cu(I)]/[Cu_T] at the halocline was followed by the lowest fluorescence of fluorophores A, C and M and the highest BIX, as well as lower SUVA, pointing to the highest biological production (Fig. 2). It has been known for decades that highest biological activity in the Krka estuary occurs at the halocline, followed by the highest plankton abundance and bacterial activity along with significant amounts of decomposed phytoplankton (Cetinić et al., 2006; Svensen et al., 2007; Vilićić et al., 1989; Žutić and Legović, 1987). Thus, Cu(I) at the halocline in the summer was most likely complexed with biologically produced ligands. In the seawater layer there was a decrease in [Cu(I)]/[Cu_T] compared to the halocline, although Cu was still mainly present as Cu(I), while this differed from relatively low [Cu(I)]/[Cu_T] in the seawater layer in March (Fig. S1). Phytoplankton migration from surface layers in winter/spring to the bottom seawater during the summer was recently reported for the Krka River estuary, as a result of the availability of nutrients and light but low solar radiation (Marcinek et al., 2020). Although the bacterial consumption of freshly produced organic molecules should be fast in these waters, it is possible that biologically produced ligands contributed in some extent to Cu(I) stabilisation in seawater layer in summer, alongside with high salinity i.e. chloride stabilisation (Burge-Weirich and

Sulzberger, 2004; Moffett and Zika, 1983). This can be supported by relatively low SUVA and high $S_{275-295}$ values indicating the presence of lower molecular weight DOM with low aromaticity and humification degree. Considering the chloride complexation of Cu(I), which has already been reported as an important factor in the binding of Cu(I) in seawater and estuarine water (Buerge-Weirich and Sulzberger, 2004), it is important to recognise the work of Walsh and Ahner (2013), in which the authors found that in a high chloride medium (seawater), chloride dominates Cu(I) speciation when the $\log K'$ value of the organic Cu(I)-binding ligand is 12 (or lower). In such a case, Cu(II)-binding ligands with the same value of $\log K' = 12$ would have a much greater influence on Cu speciation, whereas Cu(I) would be dominated by chloride complexation (Walsh and Ahner, 2013). Therefore, we consider Cu(I) complexation with chloride to be important to stabilise Cu(I) in the seawater layer where weaker Cu-binding organic ligands (with respect to the surface layer) are present.

In July, the stronger class of ligand (L_1) was determined in all samples collected at E1, while the weaker class of ligand (L_2) was determined only in two samples at the halocline, as shown in Table S2. The total ligand concentrations were higher than that of $[Cu_T]$, effectively buffering the $[Cu_{free}]$ in all samples at E1, keeping it below its toxicity threshold of 10 pM (Louis et al., 2009). Finally, correlating the obtained complexation parameters with $[Cu(I)]/[Cu_T]$, it is evident that the highest $[Cu(I)]/[Cu_T]$ in July was associated with the highest ligand concentration and the highest $\log K'$ (Fig. S2), again supporting the

correlation between Cu(I) and ligands of biological origin. Namely, strong L_1 ligand class in the Krka estuary has already been described as mainly of biological origin from recent phytoplankton production, while L_2 ligand class consists predominantly of humic substances (Marcinek, 2021). This is in accordance with previous work by Whitby et al. (Whitby et al., 2018) where it has been shown that L_1 ligand class consists mainly of Cu(I)-binding thiol compounds and the implication of Cu(I) existence in estuary has been reported.

4.3. Copper redox speciation at E2 “polluted” site in March and July

Vertical distribution of $[Cu(I)]$, $[Cu(II)]$ and $[Cu_T]$, as well as DOC and salinity, at “polluted” site E2 in March and July are shown in Fig. 4 and mass balance is shown in Table 3.

Table 3
Percentages of $[Cu(I)]/[Cu_T]$ and mass balance at station E2 in March and July.

month	station	depth (m)	$[Cu(I)]/[Cu_T]$ (%)	$[Cu(II)]/[Cu_T]$ (%)	mass balance (%)
March	E2	0.0	73.8	13.1	86.9
March	E2	2.0	72.8	24.4	97.2
March	E2	10.0	63.2	30.4	93.6
July	E2	0.2	35.3	54.4	89.6
July	E2	2.0	59.3	8.5	67.8
July	E2	10.0	20.7	67.1	87.8

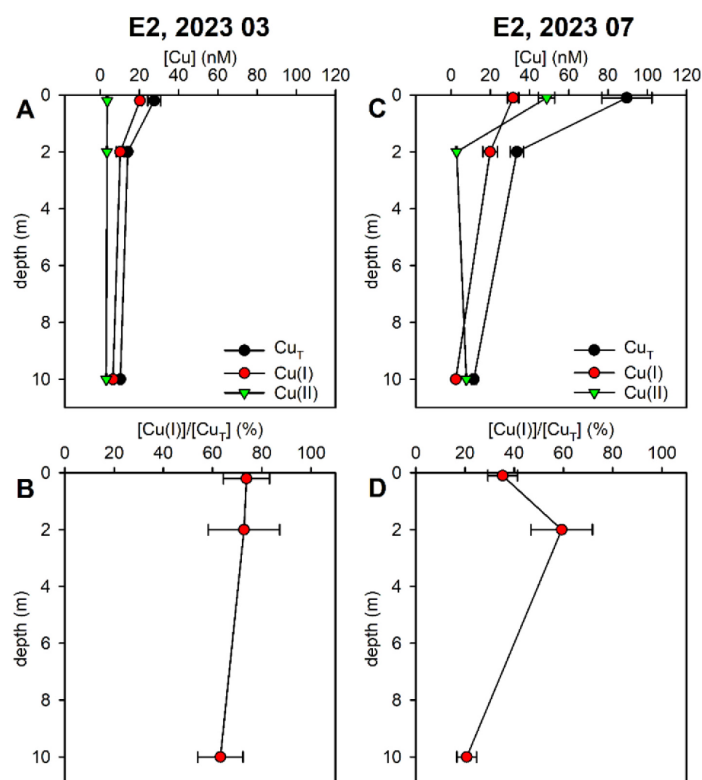


Fig. 4. Vertical distribution of $[Cu(I)]$, $[Cu(II)]$ and $[Cu_T]$ at station E2 in March (A) and in July (C). Percentage of $[Cu(I)]/[Cu_T]$ at station E2 in March (B) and in July (D).

In March, similar percentages of $[\text{Cu(I)}]/[\text{Cu_T}]$ were observed at the surface and the halocline (73.8 % and 72.8 %, respectively), with slightly lower percentage of $[\text{Cu(I)}]/[\text{Cu_T}]$ at the bottom (63.2 %), while the lowest DOC was observed at the surface and it increased with depth. This high percentage of Cu(I) throughout the whole water column might be the result of protective mechanisms of biota to metal pollution at this site, while absence of $[\text{Cu(I)}]/[\text{Cu_T}]$ surface maximum differs from the station E1 in this period of the year, due to the higher salinity in the surface layer, therefore, somewhat reduced biological activity of freshwater biota and production of Cu(I)-stabilizing ligands in surface layer comparing to station E1.

Multi-fold increase in $[\text{Cu_T}]$ was observed at E2 in July, compared to March at the same station, which can be attributed to an increase of anchored boats in the nautical marina during the summer season and reduced water exchange at E2 site position (Carić et al., 2021). As expected, $[\text{Cu_T}]$ at E2 is significantly higher than at E1. The $[\text{Cu_T}]$ in the estuary was determined previously in few articles (Cindrić, 2015; Cindrić et al., 2020), and it was clear that the variation in Cu concentration between these two sites is not due to the natural variability, but because of anthropogenic influence, as a consequence of Cu leaching from antifouling paints from the numerous boats anchored at the “polluted” E2 site. In addition, in published work, but also clearly visible from our results, similar salinity, temperature and DOC vertical profiles of these two contrasted sites could be spotted, pointing on purely seasonal and natural variability (Cindrić, 2015; Cindrić et al., 2020). Statistical test (two tailed *t*-test in Excel) additionally proved that there is a clear statistical difference between $[\text{Cu_T}]$, for both surface ($p(\text{spring}) = 0.005$), $p(\text{summer}) = 0.006$ and bottom ($p(\text{spring}) = 0.002$, $p(\text{summer}) = 0.019$) water layers.

Variations in $[\text{Cu(I)}]/[\text{Cu_T}]$ were observed in July through the water column, with $[\text{Cu(I)}]/[\text{Cu_T}]$ maximum of 59.3 % occurring at the halocline and the $[\text{Cu(I)}]/[\text{Cu_T}]$ minimum of 20.7 % at the bottom of the water column, while the highest DOC was determined at the surface, and it decreased with depth. Abundance of humic-like compounds at the surface was indicated by the highest fluorescence of fluorophores A, C and M, as well as lowest $S_{275-295}$ at the surface (Table S1). Since humic compounds are weaker Cu(II)-ligands than those of biological origin (e.g. thiols), lower $[\text{Cu(I)}]/[\text{Cu_T}]$ values occurred at the surface than at the halocline. Despite lower binding affinities of humic substances for Cu(II), it has been shown that reduction of Cu(II) complexes with humic compounds can occur (Maurer et al., 2013; Pham et al., 2012; Xing et al., 2020), especially via photochemical pathways, thereby Cu(I) was still present at the surface (section 4.4). Although the Krka River is the main input of humic-like substances to the estuary it may be possible that other anthropogenic organic compounds, which binds Cu(II) over Cu(I), are present at E2, since it is located in the nautical marina. Namely, boats and ships are recognized as a source of polycyclic aromatic hydrocarbons (PAHs) (Ytreberg et al., 2022).

Maximum of $[\text{Cu(I)}]/[\text{Cu_T}]$ has been observed at the halocline, where BIX was the highest (Fig. 2, Table S1). Again, the highest $[\text{Cu(I)}]/[\text{Cu_T}]$ at the halocline may be explained by Cu(I) stabilisation with ligands of biological origin, which were most likely produced in situ by biological activity. On the other hand, the lowest $[\text{Cu(I)}]/[\text{Cu_T}]$ were measured in the bottom seawater, with the lowest DOC and BIX and the highest salinity, indicating the absence of Cu(I)-stabilizing biological ligands in this part of the estuary, although stabilisation with chloride maintained some amount of Cu(I). Overall, it may be noted that $[\text{Cu(I)}]/[\text{Cu_T}]$ values were lower in the summer period (20.7–59.3 %) than in the winter period (63.2–73.8 %), along with higher $[\text{Cu_T}]$ in the summer compared to winter. Unfortunately, the high $[\text{Cu_T}]$ also prevents the determination of Cu organic speciation, which is impossible to perform when $[\text{Cu_T}]$ surpasses the total ligand concentration. Although complexing parameters were not determined at E2, it may be assumed that concentrations of strong Cu(I) binding ligands were not sufficient for complexing these high levels of Cu_T in Cu(I) form, thereby resulting in high Cu(II) levels, especially compared to the winter period where $[\text{Cu_T}]$

values were significantly lower. However, further investigation is necessitated to study the $[\text{Cu}_{\text{free}}]$ content and its potential toxicity to estuarine biota during the multiple increase of $[\text{Cu_T}]$ at E2 in summer.

4.4. Changes in Cu redox speciation during the day: implications for a significant role of photochemical processes

At station E2, four surface samples were collected at different times of the same day (5:00 h, 10:00 h, 17:00 h, 23:00 h), which showed significant fluctuations in $[\text{Cu_T}]$, as well as in $[\text{Cu(I)}]/[\text{Cu_T}]$, as shown in Fig. 5 and Table 4. The elevated $[\text{Cu_T}]$ and its fluctuations can be attributed to the numerous boats anchored on E2 during the samplings and the many boats entering and leaving the marina at that time.

Salinity had a constant value of 17 during the day, and no significant changes in DOC were observed (1.10–1.17 mg/L). The lowest $[\text{Cu(I)}]/[\text{Cu_T}]$ value of 24.2 % was observed at 5:00 h, followed by an increase in $[\text{Cu(I)}]/[\text{Cu_T}]$ at 10:00 h and 17:00 h, with a $[\text{Cu(I)}]/[\text{Cu_T}]$ maximum of 83.0 % at 23:00 h. This indicates that photochemical processes play an important role in Cu(I) formation, which is in accordance with previous research on Cu(I) in marine and estuarine environments (Buerge-Weirich and Sulzberger, 2004; Moffett and Zika, 1983, 1988), while the mechanism of these processes is still unknown. In addition, high SUVA and low $S_{275-295}$ values indicated the presence of DOM with high chromophoric content, high molecular weight, aromaticity and humification degree at E2_{5h}, while fluorophore values indicated the presence of terrestrial humic-like compounds (high C and A fluorescence signal) (Fig. 2, Table S1). Humic-like compounds play an important role in the binding of Cu, especially in estuarine waters, while the stability of such complexes corresponds to those of the weaker, L_2 ligand class, which most likely have higher binding affinities for Cu(II) than for Cu(I) (Vachet and Callaway, 2003; Whitby and van den Berg, 2015). However, knowing that photooxidation of CDOM produces O_2 which can reduce Cu(II) complexes, it can be assumed that the lack of sunlight leads to less reduction of Cu(II) organic complexes and consequently less Cu(I) formation at E2_{5h}. During the day, $[\text{Cu(I)}]/[\text{Cu_T}]$ increased and reached a value of 50.8 % at 17:00 h. The characterisation of DOM at E2_{17h} shows the lowest SUVA and the highest $S_{275-295}$, suggesting the presence of DOM with lower molecular weight and aromaticity. Furthermore, humic-like fluorescence (fluorophores A, C and M) showed the lowest fluorescence at E2_{17h}. Considering the not very high BIX value (similar to those at E2_{5h}) (Table S1), along with high solar radiation at this time of day, we hypothesise that photo-bleaching of humic-like substances (Catalá et al., 2013; Maie et al., 2012) occurred at E2_{17h}, leading to the reduction of Cu(II) complexes and thus to a higher $[\text{Cu(I)}]/[\text{Cu_T}]$. Unexpectedly, the highest $[\text{Cu(I)}]/[\text{Cu_T}]$ of 83.0 % occurred at E2_{23h}, about two and half hours after sunset, along with the highest $[\text{Cu_T}]$ of all samples analysed in this work. The highest BIX as well as the highest fluorescence of marine-produced fluorophore M were measured at E2_{23h}, indicating high biological activity (Coble, 2007; Santos et al., 2016) (Table S1). Furthermore, non-photochemical biological formation of reactive oxygen species (ROS), including O_2 and H_2O_2 , in the presence of pollutants is known, and while literature data suggest that the highest H_2O_2 presence should occur in the afternoon at the sunlit seawater surface, biological ROS production can also lead to high ROS concentrations at the night (Morris et al., 2022; Sutherland et al., 2020; Zhang et al., 2016). Considering the high level of $[\text{Cu_T}]$ at E2_{23h}, the possibility of biological ROS production leading to Cu(I) formation should not be ignored. Although these assumptions should be investigated in separate work, the variation of $[\text{Cu(I)}]/[\text{Cu_T}]$ during the day with the presence of early morning minimum was evident in this work.

5. Conclusion

Solid-phase extraction method followed with voltammetric determination of Cu is a simple, cost-effective method for the determination of Cu(I) and Cu(II) species in estuarine and marine waters. In Krka River

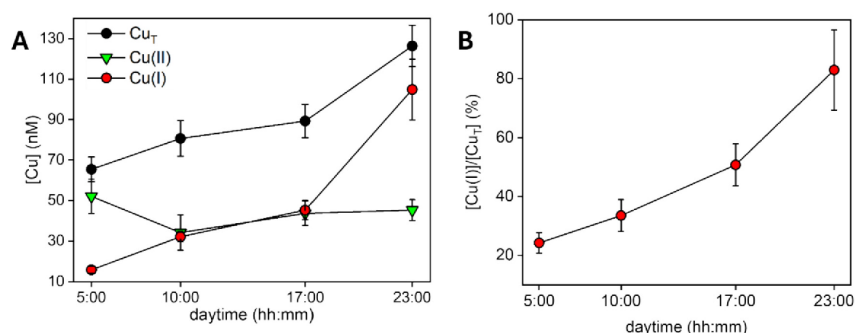


Fig. 5. A) $[Cu(I)]$, $[Cu(II)]$ and $[Cu_T]$ in the surface layer at 5:00, 10:00, 17:00 and 23:00 h. B) Percentage of $[Cu(I)]/[Cu_T]$ in the surface layer at 5:00, 10:00, 17:00 and 23:00 h.

Table 4

Percentages of $[Cu(I)]/[Cu_T]$ and mass balance at station E2 at surface at different times of the day.

month	station	depth (m)	$[Cu(I)]/[Cu_T]$ (%)	$[Cu(II)]/[Cu_T]$ (%)	mass balance (%)
July	E2 _{05:00}	0.2	24.2	79.5	103.7
July	E2 _{10:00}	0.2	31.6	33.5	65.1
July	E2 _{17:00}	0.2	50.8	48.9	99.7
July	E2 _{23:00}	0.2	83.0	35.8	118.8

estuary, $Cu(I)$ was detected in all the collected samples, ranging from 20.7 % to 88.7 % of $[Cu(I)]/[Cu_T]$. Multi-fold increase in $[Cu_T]$ occurred at E2 station (nautical marina) in July, while no significant variations in $[Cu_T]$ were noted at E1 station. Highest $[Cu(I)]/[Cu_T]$ in all profiles were associated with highest biological activity, indicating $Cu(I)$ complexation with ligands of biological origin, while complexation with chloride may contribute to $Cu(I)$ stabilisation in the seawater layer. At E1, the concentration of Cu -binding ligands was higher than $[Cu_T]$, thus, $[Cu_{free}]$ was below the toxicity threshold at all investigated depths, while $[Cu_{free}]$ levels during the multi-fold increase of $[Cu_T]$ at E2 should be assessed in future work. Additionally, variations in $[Cu(I)]/[Cu_T]$ during the day in surface waters, with early morning minimum and increase throughout the day, indicate an important role of photochemical processes in Cu redox speciation. It is likely that photoreduction of $Cu(II)$ complexes with terrestrial humic-like substances occurs in sunlit surface waters.

The impact of the significant $[Cu(I)]/[Cu_T]$ ratio observed in the estuarine system studied needs further investigation, but it could have several important implications for the biogeochemical cycling of Cu in estuarine waters, including possible influences on Cu transport to the open ocean, bioavailability, and toxicity, which remains to be determined. In particular, considering that Cu can have detrimental effects on biota, understanding the biogeochemical cycling of Cu is crucial for assessing its toxicity. This is particularly important in coastal areas where elevated Cu concentrations are observed. Furthermore, given the significant presence of $Cu(I)$ at freshwater, freshwater-seawater interface and seawater layer reported in this work, it can be assumed that significant levels of $Cu(I)$ may also occur in open waters. Whitby et al. (Whitby et al., 2018) reported the presence of both L_1 and L_2 ligand classes in the bathypelagic zone of NE Pacific and with respect to the importance of organic ligands in Cu speciation, this could imply the presence of $Cu(I)$ even below the photic zone.

CRediT authorship contribution statement

Dora Ormarić: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. Saša Marcinek: Writing – original draft, Formal analysis. Ana-Marija Cindrić: Formal analysis. Dario Omanović: Writing – review & editing, Formal analysis. Elvira Bura-Nakić: Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marchem.2024.104471>.

Data availability

Data will be made available on request.

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A.2.1. Supplementary material to Research Paper II

Supplementary Material

Redox speciation of copper in the estuarine environment: Towards better understanding of copper water chemistry

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Table S1. Values of optical parameters at stations E1 and E2 in July.

station	depth (m)	BIX	fluorophore A (R.U.)	fluorophore M (R.U.)	fluorophore C (R.U.)	S ₂₇₅₋₂₉₅ (μm ⁻¹)	a ₂₅₄ (m ⁻¹)	SUVA (m ² g ⁻¹)
E1	0.2	0.943	0.072	0.037	0.027	24.49	3.18	1.11
E1	2.2	0.980	0.048	0.026	0.018	28.13	2.64	0.99
E1	2.5	1.009	0.048	0.025	0.018	27.88	2.66	1.06
E1	2.8	0.998	0.035	0.018	0.012	29.79	2.21	0.91
E1	20	0.942	0.041	0.025	0.019	26.22	1.87	0.96
E1	35	0.941	0.036	0.021	0.016	26.89	1.76	0.96
E2	0.2	0.966	0.073	0.036	0.028	25.61	3.30	0.93
E2	2	0.974	0.059	0.031	0.023	23.36	2.98	1.06
E2	10	0.863	0.055	0.032	0.025	24.44	2.34	1.05
E2 _{05:00h}	0.2	0.989	0.078	0.041	0.030	25.65	3.18	1.25
E2 _{10:00h}	0.2	1.009	0.079	0.040	0.030	24.44	3.30	1.22
E2 _{17:00h}	0.2	0.988	0.074	0.038	0.028	27.00	3.36	1.07
E2 _{23:00h}	0.2	1.035	0.077	0.043	0.029	26.32	3.24	1.25

Table S2. Copper organic speciation and complexing parameters at E1 in July.

depth	[Cu _T] (nM)	[Cu _{free}] (pM)	[L ₁] (nM)	logK' _{L1}	[L ₂] (nM)	logK' _{L2}	[L _{total}] (nM)	[L _{total}]/DO C (μmol g ⁻¹)
0.2	7.60	0.0500	22.40	13.01	/	/	22.40	18.06
2.2	9.60	0.1790	26.00	12.52	/	/	26.00	22.37
2.5	9.40	0.0002	4.43	15.50	21.80	12.21	26.23	23.96
2.8	6.70	0.1530	3.41	14.07	20.20	12.13	23.61	22.48
20.0	5.90	0.1500	14.10	12.68	/	/	14.10	16.65
35.0	3.60	0.0916	16.00	12.50	/	/	16.00	20.09

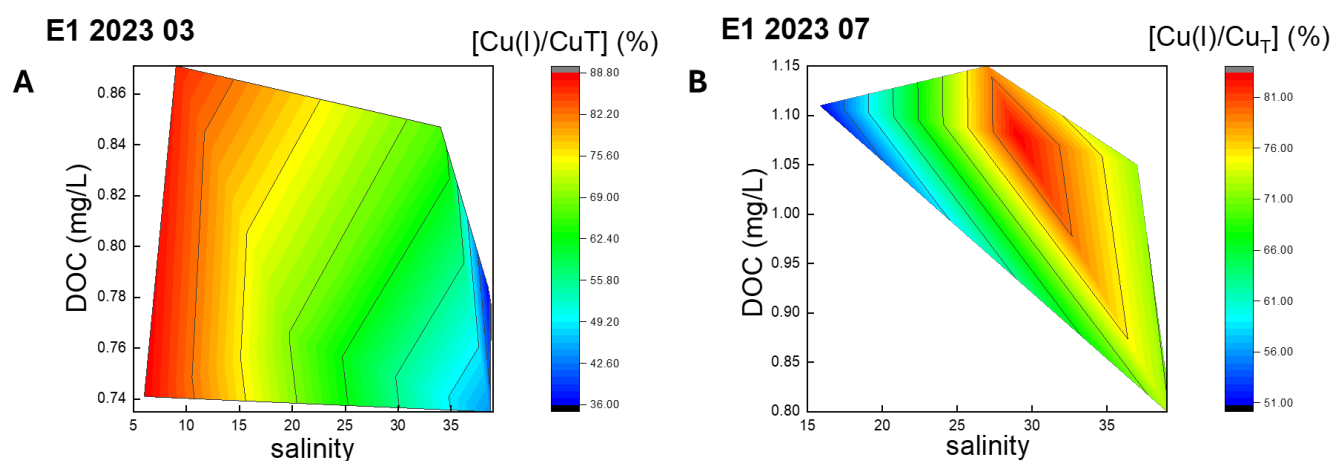


Figure S1. Influence of DOC and salinity on $[\text{Cu(I)}/\text{Cu}_T]$ at station E1 in A) March B) July.

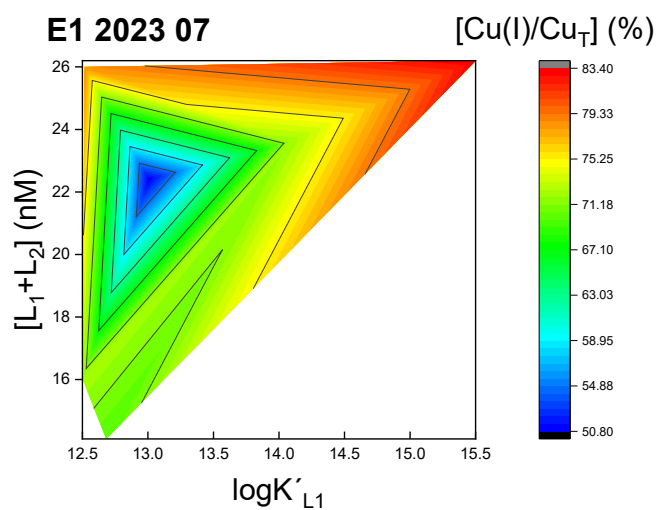


Figure S2. Influence of $[\text{L}_1 + \text{L}_2]$ and $\log K'_{L1}$ on $[\text{Cu(I)}/\text{Cu}_T]$ at station E1 in July.

A.3. Research paper III

Crmarčić, D., Iranzo, O., Mlakar, M., Stipanović, N., Bura-Nakić, E. 2026. Stability of copper(I)-cysteine complex assessed using reverse titrations: implications for estuarine and seawater copper biogeochemistry. *Biogeochemistry* 169, 5. <https://doi.org/10.1007/s10533-025-01299-4>.



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.

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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 , $\log K'_{L_1} \approx 10\text{--}13$) and weaker (L_2 , $\log K'_{L_2} \approx 13\text{--}16$) 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 (Crmaric 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''_{CuI}), 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

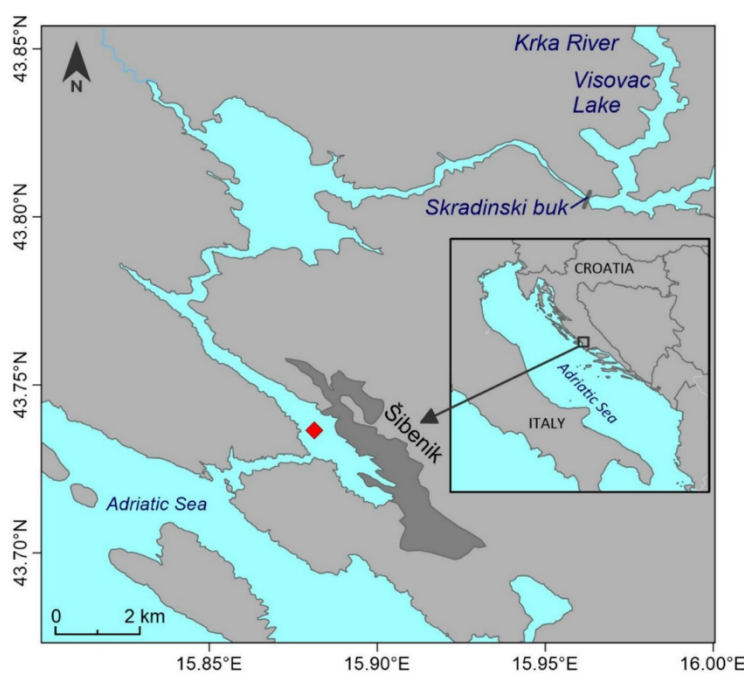
Sampling site and sampling procedure

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 $50 \text{ m}^3/\text{s}$, with a minimum as low as $5 \text{ m}^3/\text{s}$ and a maximum as high as $450 \text{ m}^3/\text{s}$ (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; Petricoli 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 pre-cleaned 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 M Ω , 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 N₂ 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 ($\epsilon_{483\text{ nm}} = 13,300\text{ L mol}^{-1}\text{ cm}^{-1}$) (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 (Crmaric 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 $5\text{ }\mu\text{mol/L}$, 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)-BCS₂ 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 (Crmaric 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. HNO₃; VWR BDH Prolabo Chemicals, Belgium) to $\text{pH} < 2$ 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/ecdssoft>), with peak height used as the signal value. Uncertainties (shown as error bars in the plots) for $[\text{Cu(I)}]$, $[\text{Cu(II)}]$, and $[\text{Cu}_T]$ are reported as 95% confidence intervals obtained from the standard addition method used for quantifying unknown concentrations. The uncertainty in the $[\text{Cu(I)}]/[\text{Cu}_T]$ 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 $[\text{Cu(I)BCS}_2]^{3-}$ 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 ($A_{483\text{nm}}$) and high value of ε_{483} . Therefore, the decrease in $A_{483\text{nm}}$ of $[\text{Cu(I)BCS}_2]^{3-}$ 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 $[\text{CuBCS}_2]^{3-}$ 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 $\mu\text{mol/L}$. The $[\text{CuBCS}_2]^{3-}$ complex was titrated with increasing concentrations of Cys, and the decrease in $A_{483\text{nm}}$ was followed. During titrations, the Cys concentration was gradually increased from 10 $\mu\text{mol/L}$ to 7200 $\mu\text{mol/L}$ and from 10 $\mu\text{mol/L}$ to 5320 $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$ to 4.56 $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$ to 30.1 $\mu\text{mol/L}$ 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) (Crmaric 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% (Crmaric 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 (Crmaric 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

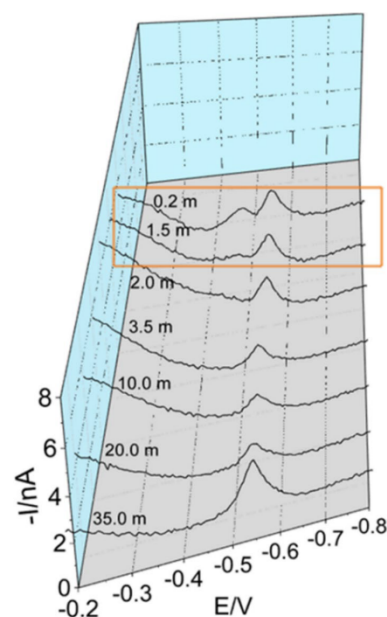


Fig. 2 Square wave (SW) cathodic stripping voltammograms of Krka River estuary samples (July 15 2024, sampling site marked in Fig. 1) after 120 s accumulation at 0.0 V vs. Ag/AgCl. The sampling depths ($n=7$) in meters are shown on the left side of the voltammograms. Additional voltammetric parameters (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

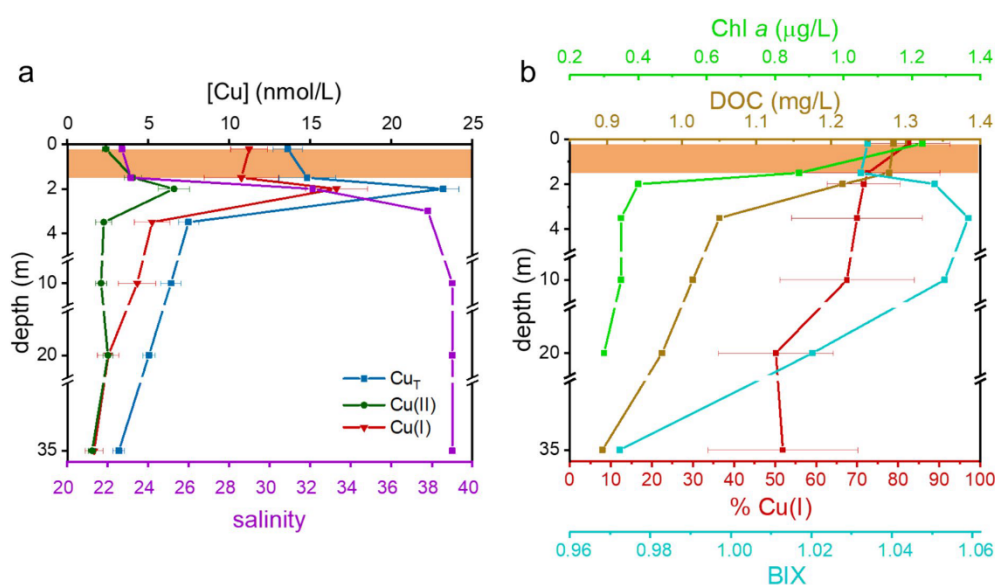


Fig. 3 Depth profile of a copper(I) (Cu(I), red line), copper(II) (Cu(II), dark green line), total dissolved copper (Cu_T, blue line) and salinity (purple line), and b) copper(I) presented as the fraction of Cu(I) in Cu_T (red line), chlorophyll *a* (Chl *a*, green line), dissolved organic carbon (DOC, 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 occurred 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 (Crmarčić 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, $\log K'_{\text{Cu(I)-Cys}}$, 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 $\log K'_{\text{Cu(I)-Cys}}$ 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 $[\text{CuBCS}_2]^{3-}$ and Cu(I)-Cys complex removal in different media, i.e. Cl^- -free and Cl^- -containing media. These analyses were used to estimate the $\log K''_{\text{CuL}}$ of Cu(I)-Cys, as explained in the Supplementary Information (section S3), and to compare it with the $\log K'_{\text{L1}}$ in natural waters.

Stability of $[\text{CuBCS}_2]^{3-}$ complex in Cl^- -containing medium

Prior to the titrating of $[\text{CuBCS}_2]^{3-}$ with Cys, we evaluated the stability of the $[\text{CuBCS}_2]^{3-}$ in both Cl^- -free and Cl^- -containing media, using different BCS:Cu(I) molar ratios. The concentration of $[\text{CuBCS}_2]^{3-}$ 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 $\text{Cu}(\text{dmp})\text{X}$ ($\text{X}=\text{Cl}, \text{Br}, \text{I}$; $\text{dmp}=2,9\text{-dimethyl-1,10-phenanthroline}$) 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 $\text{Cu}(\text{dmp})_2\text{X} \leftrightarrow \text{Cu}(\text{dmp})\text{X} + \text{dmp}$.

Therefore, to assess whether Cl^- affects the course of the reverse titration of $[\text{CuBCS}_2]^{3-}$ with Cys, the titrations were performed in both Cl^- -containing and Cl^- -free media and monitored by UV-Vis spectrophotometry.

Reverse titrations of $[\text{CuBCS}_2]^{3-}$ 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 $\log K''_{\text{CuL}}$ values were 15.35 ± 0.11 and 14.36 ± 0.11 for Cl^- free and Cl^- solution, respectively. The standard deviation for $\log K''_{\text{CuL}}$ was calculated after averaging the $\log K''_{\text{CuL}}$ values from the individual titration points. The derived $\log K''_{\text{CuL}}$ for the Cu(I)-Cys complex falls within the range of the $\log K'_{\text{L1}}$ 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 (Crmaric 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

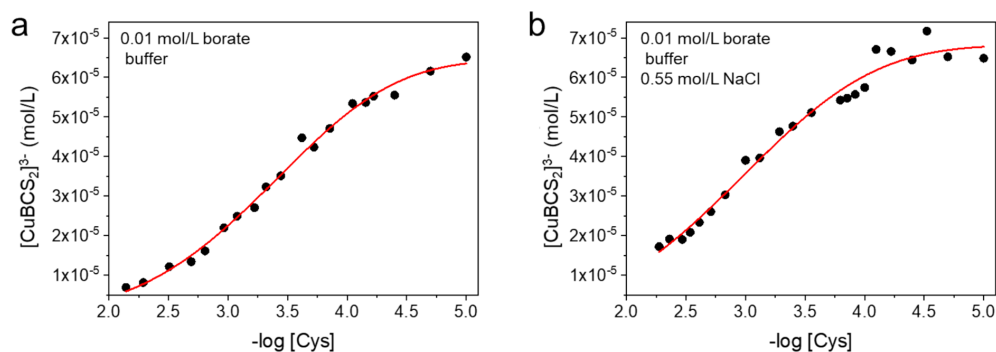


Fig. 4 Experimental data (black circles) versus fitting curve (red lines). Solution composition: **a** $c(\text{copper(I)}, \text{Cu(I)})=70 \mu\text{mol/L}$, $c(\text{bathocuproine disulfonic acid disodium salt, BCS})=175 \mu\text{mol/L}$, $c(\text{borate buffer})=0.01 \text{ mol/L}$

b $c(\text{Cu(I)})=70 \mu\text{mol/L}$, $c(\text{BCS})=154 \mu\text{mol/L}$, $c(\text{borate buffer})=0.01 \text{ mol/L}$, $c(\text{sodium chloride, NaCl})=0.55 \text{ mol/L}$. 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 $[\text{CuBCS}_2]^{3-}$ 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 $[\text{CuBCS}_2]^{3-}$ 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 $\log K''_{\text{CuL}}$ values were 14.70 ± 0.47 and 13.95 ± 0.21 for solutions containing Cu(I) and Cu(II), respectively. The standard

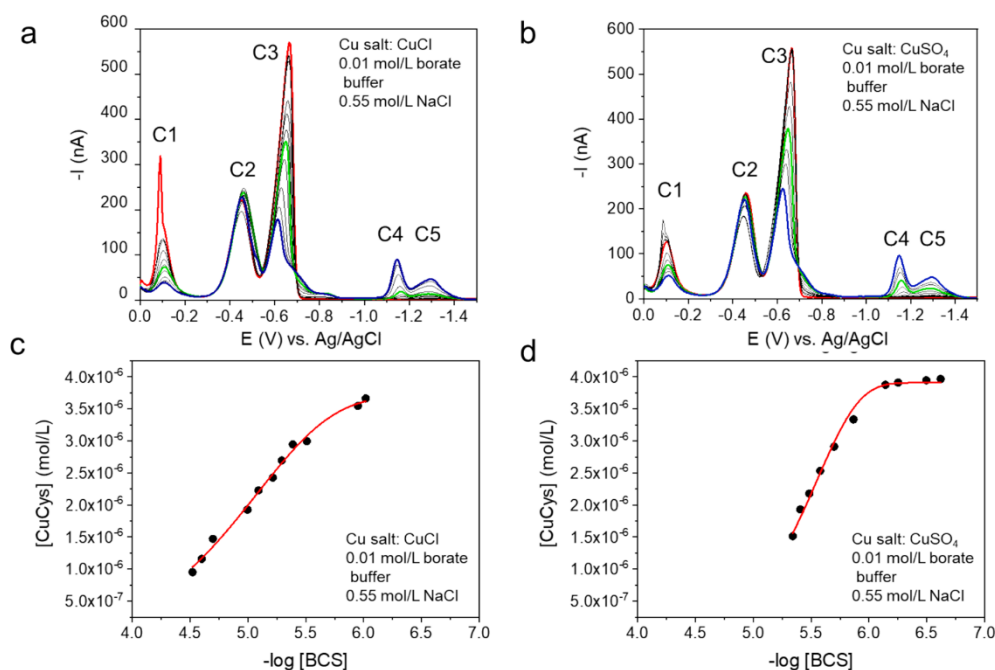


Fig. 5 Square wave (SW) voltammograms of copper(I), Cu(I) (a) and copper(II), Cu(II) (b) with cysteine (Cys) and increasing bathocuproine disulfonic acid disodium salt (BCS) concentration in cathodic stripping mode. Solution composition: a $c(\text{Cu(I)}) = 4 \mu\text{mol/L}$, $c(\text{Cys}) = 8 \mu\text{mol/L}$, $c(\text{borate buffer}) = 0.01 \text{ mol/L}$, $c(\text{sodium chloride, NaCl}) = 0.55 \text{ mol/L}$, b $c(\text{Cu(II)}) = 4 \mu\text{mol/L}$, $c(\text{Cys}) = 8 \mu\text{mol/L}$, $c(\text{borate buffer}) = 0.01 \text{ mol/L}$,

$c(\text{NaCl}) = 0.55 \text{ mol/L}$. 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 $\text{Hg}(\text{SR})_2$ and $\text{Hg}_2(\text{SR})_2$, respectively. C3 represents the reduction of Cu(I)-Cys. C4 and C5 correspond to reduction/desorption of the BCS ligand

deviation for $\log K''_{\text{CuL}}$ was calculated after averaging the $\log K''_{\text{CuL}}$ values of the individual titration points, and the model used is described in the Supplementary Information (section S3.2). The derived $\log K''_{\text{CuL}}$ 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 $[\text{Cu}(\text{BCS})_2]^{3-}$ 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 $\log K''_{\text{CuL}}$ 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 $\log K''_{\text{CuL}}$ 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 $\log K'_{\text{L1}}$ 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_r), 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 $\log K'_{\text{Cu(I)-Cys}}$ 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 $[\text{Cu}(\text{BCS})_2]^{3-}$ 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 ($\log K''_{\text{CuL}} = 15.35 \pm 0.11$) 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 $[\text{Cu}(\text{BCS})_2]^{3-}$ 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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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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A.3.1. Supplementary material to Research Paper III

Supplementary Information for

Stability of copper(I)-cysteine complex accessed using reverse titrations: implications for estuarine and seawater copper speciation

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S1. Standard addition of Cys stock solution to estuarine surface sample

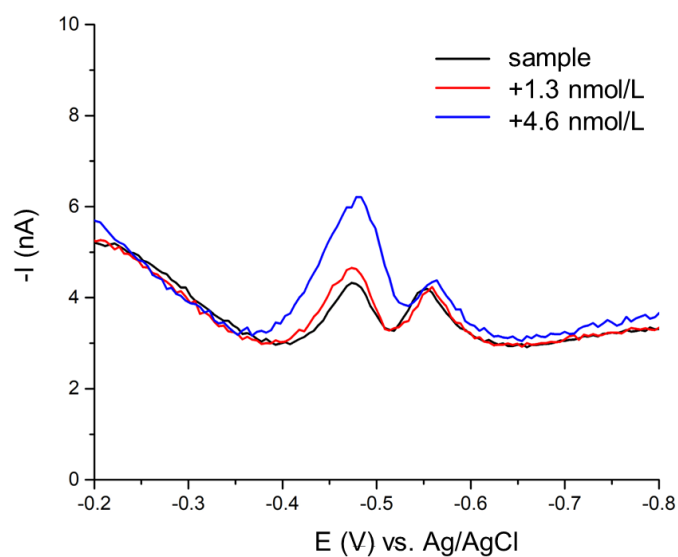


Fig. S1 Square wave (SW) voltammogram of the surface sample of the Krka estuary (0.2 m depth) (black line) after cysteine (Cys) addition of 1.3 nmol/L (red line) and 4.6 nmol/L (blue line). The accumulation potential is 0.0 V, the accumulation time is 120 s, while amplitude (a) and frequency (f) are given in the section *Determination of Cys-like compounds* in the main manuscript

S2. Effect of Cl^- on $[\text{Cu}(\text{BCS})_2]^{3-}$ stability

Table S1 Comparison between expected Cu(I) concentration and estimated Cu(I) concentration using reported molar absorption coefficient ($\epsilon_{483 \text{ nm}} = 13300 \text{ L mol}^{-1} \text{ cm}^{-1}$) in solutions with varying NaCl concentrations and Cu(I):BCS molar ratios

	Cu(I) ($\mu\text{mol/L}$)	BCS ($\mu\text{mol/L}$)	BCS:Cu(I) molar ratio	Solution composition (mol/L)	Measured absorbance $A_{483 \text{ nm}}$	Estimated Cu ($\mu\text{mol/L}$); $\epsilon_{483} = 13300 \text{ L mol}^{-1} \text{ cm}^{-1}$
1	72*	300	4.16	0.01 BB	1.0150	76.3
2	70*	175	2.50	0.01 BB	0.9245	69.5
3	70*	200	2.86	0.01 BB	0.9177	69.0
4	100*	250	2.50	0.01 BB	1.3734	103.3
5	70*	154	2.20	0.01 BB; 0.55 NaCl	0.7071	53.2

6	70*	154	2.20	0.01 BB; 0.55 NaCl	0.7033	52.9
7	70*	154	2.20	0.01 BB; 0.55 NaCl	0.6973	52.4
8	70*	280	4.00	0.01 BB; 0.55 NaCl	0.9655	72.6
9	70 ⁺	175	2.50	0.01 BB; 0.08 NaCl	0.8449	63.5
10	70 ⁺	175	2.50	0.01 BB; 0.20 NaCl	0.6899	51.9
11	70 ⁺	175	2.50	0.01 BB; 0.20 NaCl	0.6909	52.0
12	70 ⁺	175	2.50	0.01 BB; 0.35 NaCl	0.6260	47.1
13	70 ⁺	175	2.50	0.01 BB; 0.55 NaCl	0.5527	41.6
14	70 ⁺	175	2.50	0.01 BB; 0.55 NaCl	0.5539	41.7
15	70 ⁺	175	2.50	0.01 BB; 0.55 NaCl	0.5541	41.7

*The Cu(I) stock solution was prepared according to procedure 1 as described in the experimental section of the main manuscript. ⁺The stock solution used for the preparation of other test solutions was prepared according to procedure 2 as described in the experimental section of the main manuscript. BB-borate buffer.

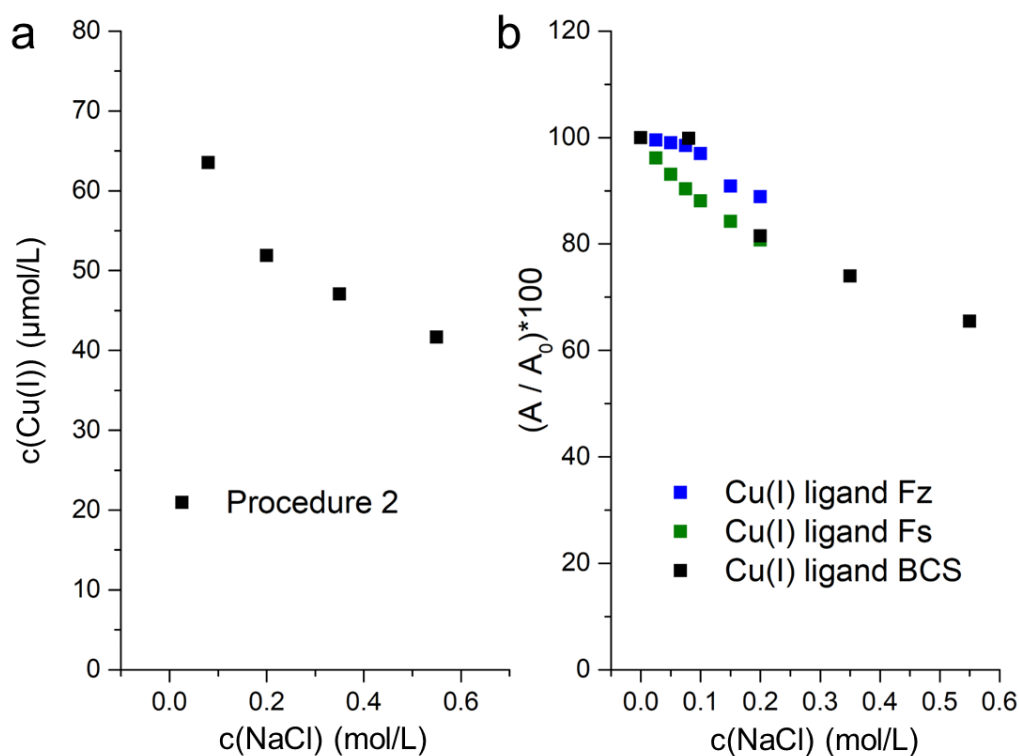


Fig. S2 a) Dependence of Cu(I) concentration in the form of $[\text{CuBCS}_2]^{3-}$ on NaCl concentration. The Cu(I) stock solution was prepared according to procedure 2 (further details on the preparation of Cu(I) stock solutions are provided in the experimental section of the main manuscript). After addition of the Cu(I) ligand (BCS), the Cu(I) concentration was estimated from the measured absorbance (A_{max} at 483 nm) using the Beer–Lambert law. b) Dependence of absorbance (expressed as percentage of A/A_0 at 483 nm) on NaCl concentration for different Cu(I) probes (Fz, Fs and BCS) at a molar ratio of ligands (BCS, Fs and Fz) to Cu(I) of 2:1. The data for the dependence of Cu(I) ligand probes (Fz and Fs) on NaCl concentration are taken from Xiao et al. (2013)

S3. Fitting procedure

S3.1. Fitting of the spectroscopic titration data

The total metal concentration (Cu(I)) in a system containing Cu(I), Cys and BCS can be summarised as follows, assuming negligible contributions from Cu(I) hydrolysis (Xiao et al. 2011; Walsh and Ahner 2013):

$$[\text{Cu(I)}]_{\text{total}} \leftrightarrow [\text{Cu(I)}]_{\text{free}} + [\text{CuCys}] + [\text{CuBCS}_2]^{3-} \quad \text{Eq. 1}$$

Due to the high formation constant of the $[\text{CuBCS}_2]^{3-}$ complex and under conditions where the concentration of free ligand (BCS) exceeds 10^{-5} mol/L , we can assume that the contribution of $[\text{CuBCS}]^-$ is also negligible and that

the concentration of $[\text{Cu(I)}]_{\text{free}}$, is well below 10^{-14} mol/L. Under these conditions, the mass balance given by Eq.1 can be simplified as shown:

$$[\text{Cu(I)}]_{\text{total}} - \text{Cu(I)-Cys} \cong [\text{CuBCS}_2]^{3-} \quad \text{Eq. 2}$$

The competitive reaction and the established equilibrium of the two ligands (BCS versus Cys) for the same metal (Cu(I)) can be summarized as follows:



It is important to note here that the exchange reaction involves two separate reactions, which can be described with the 2 simple equilibria as follows (dissociation of $[\text{CuBCS}_2]^{3-}$ and formation of CuCys):



The summarized equilibrium equation, which describes the equilibria associated with the Eq. 1 is:

$$\frac{K''_{\text{CuL}}}{\beta_{[\text{Cu(I)BCS}_2]^{3-}}} = \frac{[\text{BCS}]^{2-}]^2 \times [\text{CuCys}]}{[\text{CuBCS}_2]^{3-} \times [\text{Cys}]^-} \quad \text{Eq. 6}$$

Based on the experimental data (decrease in absorbance of $[\text{CuBCS}_2]^{3-}$ in UV-Vis spectrophotometry measurements), we can calculate Cu(I)-Cys, free Cys and free BCS using the simple relationship given by Eq. 3. Using Eq. 6, we can determine the K''_{CuL} value. It should be emphasised here that free Cys and free BCS refer to all forms of BCS and Cys not bound to Cu(I). The K''_{CuL} refers to the equilibrium constant, which is conditional with respect to both the Cu and the ligand. The reverse titration data were fitted in Origin software using nonlinear curve fitting and Levenberg-Marquardt iteration algorithm.

S3.2. Fitting of the electrochemical titrations data

For the determination of conditional stability constant by electrochemical titrations of Cu(I)-Cys complex with BCS, Eq.3 is modified as follows:



As in Eq. 4 and 5, it is important to note here that the exchange reaction involves two separate reactions, which can be described with the 2 simple equilibria as follows (dissociation of CuCys and formation of $[\text{CuBCS}_2]^{3-}$):





The summarized equilibrium equation, which describes the equilibria associated with the Eq. 7 is:

$$\frac{\beta_{[\text{Cu(BCS)}_2]^{3-}}}{K''_{\text{CuL}}} = \frac{[[\text{Cu(BCS)}_2]^{3-}] \times [\text{Cys}]^-}{[[\text{BCS}]^{2-}]^2 \times [\text{CuCys}]} \quad \text{Eq. 10}$$

In addition to fitting the spectrophotometric data, based on the experimental data (in this case, the decrease in peak current of Cu(I)-Cys in cathodic stripping voltammetry measurements), we can calculate $[\text{Cu(BCS)}_2]^{3-}$, free Cys, and free BCS using the simple relationship given by Eq. 7. Using Eq. 10, we can determine the K''_{CuL} value. It should be emphasised that free Cys and free BCS refer to all forms of BCS and Cys not bound to the Cu(I). The K''_{CuL} refers to the equilibrium constant which is conditional with respect to both the Cu and the ligand. The reverse titration data were fitted in Origin software using nonlinear curve fitting and Levenberg-Marquardt iteration algorithm.

S4. Formation constants of Cu(I)Cys complex

Table S2 Literature values for the formation constant (formal or conditional) of Cu(I)Cys

method	medium composition	$\log \beta$	$\log K'$	reference
polarography	1 mol/L NH_3 , 1 NH_4Cl , 0.005% gelatin	$\log \beta_{110}$ 19.19		Stricks and Kolthoff 1951
	1 mol/L NaCl	$\log \beta_{110}$ 10.13		Königsberger et al. 2015
	Britton-Robinson buffer	$\log \beta_{110}$ 21.64		Çakir et al. 2000
voltammetry	seawater	$\log \beta_{110}$ 18.50		van den Berg et al. 1988
spectrophotometry	10 mmol/L buffer (MOPS, TRIS, borate)		$\log K'_{\text{Cu(I)-Cys}}$ 11.4	Walsh and Ahner 2013
	Britton-Robinson buffer	$\log \beta_{110}$ 20.81		Çakir et al. 2000

Table S2 presents a literature review of stability constants obtained for the complex between Cu(I) and Cys. However, we note that direct comparison of the derived stability constants is difficult. For example, Stricks and Kolthoff (1951) determined a logarithmic stability constant of 19.19 by polarography in an air-free solution

containing 10^{-3} mol/L Cys in ammoniacal medium after titration with 0.01 mol/L Cu(I) (in their article the dissociation constant is given as 6.5×10^{-20} , but we have converted it to the formation constant for comparison purposes). In the work of van den Berg et al. (1988), the formation constant, with a logarithmic value of 18.5, was calculated from the shift of the Cu-Cys peak in seawater relative to the free Cu(I) (van den Berg et al. 1988). In the cited articles, however, the constants obtained are corrected for ion pairing with NH_3 (Stricks and Kolthoff 1951) or Cl^- (van den Berg et al. 1988). Furthermore, in the work by Cakir et al. (2000), the stoichiometries and stability constants of the complexes between Cu(I) and Cys were determined using the Lingane method (voltammetric) and the Job method (spectrometric). Their logarithmic values were 20.81 and 21.64, respectively, although it is not clear whether, and which side reactions were taken into account, neither is the calculation procedure specified (Çakir et al. 2000). In the work by Vortisch et al. (1976), potentiometric titrations of the Cu(I) and Cys system in 1 mol/L ACN gave $\log K_{\text{CuL}}$ of 14.0. After correction for the side reaction of Cu(I) with ACN ($\text{Cu}(\text{CH}_3\text{CN})_2^+$ with $\log \beta_2$ of 4.34, a logarithmic value of 18.34 was obtained for the Cu(I)-Cys complex, which is in reasonable agreement with the previously reported log values (Stricks and Kolthoff 1951; Vortisch et al. 1976; van den Berg et al. 1988) (Table S2). Königsberger et al. (2015) reported precipitation at $\text{pH} < 5.2$ which prevented the derivation of the stability constant from 4-5 mmol/L Cu(I) solutions containing Cys in the molar ratios of 1:1, 3:2, and 2:1 with respect to the Cu(I). Instead, the complex stability was determined by polarography in 1 mol/L NaCl, yielding a logarithmic value of 13.13, without further information on the side reaction corrections or the calculation procedure (Königsberger et al. 2015). This brief literature review of reported stability constants for the Cu(I)-Cys complex also highlights the need for clearer descriptions of calculation procedures and better definitions of the obtained constants (conditional or formal), i.e. a clear description of any corrections applied to the derived constants for side reactions involving Cu(I) complexation with Cl^- , ACN, NH_3 , as well as corrections for H^+ competition with Cu(I) for the ligand.

S5. Electrochemical investigation of Cys behaviour and cuprous Cys formation on Hg electrode

S5.1. Investigation of Cys behavior on Hg electrode using Square Wave Voltammetry (SWV)

Fig. S3 shows the dependence of the peak potential (E_p) and the peak current (P_i) on the accumulation time (t_a), which increases from 5 s to 600 s for a solution of $0.8 \mu\text{mol/L}$ Cys in $0.55 \text{ mol/L NaCl}/0.01 \text{ mol/L}$ borate buffer as supporting electrolyte. At shorter t_a , the SW voltammograms are characterised by a peak at -0.5 V (denoted C2, representing $\text{Hg}_2(\text{SR})_{2\text{ad}}$ reduction), with the E_p of the C2 peak strongly dependent on the accumulation time used (Heyrovský et al. 1997). Linearity between P_i and t_a exists only for shorter t_a (up to 60 s), which is lost for longer

t_a when another peak (denoted C1) appears, about 400 mV more positive than the C2 peak. These results again agree with the work of Heyrovsky et al. (1997), who observed two anodic processes on the polarographic and voltammetric curves, 400-500 mV apart, and assigned the processes to the oxidation of metallic mercury in two steps in the presence of Cys as follows: $\text{Hg} \rightarrow \text{Hg(I)}$ and $\text{Hg(I)} \rightarrow \text{Hg(II)}$ (Heyrovský et al. 1997). Low Hg electrode surface coverage promotes spontaneous disproportionation of the adsorbed $\text{Hg}_2(\text{RS})_2$ at the potential of $\text{Hg(I)} \rightarrow \text{Hg(II)}$ into Hg(SR)_2 and metallic mercury. At higher Cys concentrations, adsorbed reaction product hinders spontaneous disproportionation and a faradaic reaction occurs instead: $\text{Hg}_2(\text{SR})_{2\text{ad}} + 2\text{RSH} \leftrightarrow 2\text{Hg(SR)}_{2\text{ad}} + 2\text{e}^- + 2\text{H}^+$ at the peak potential C1.

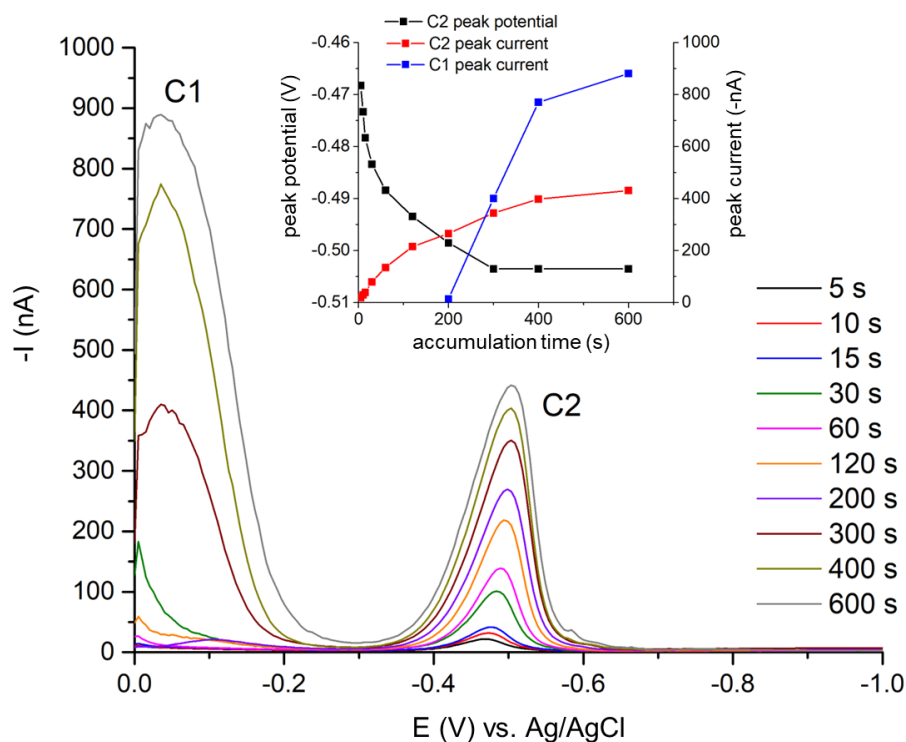
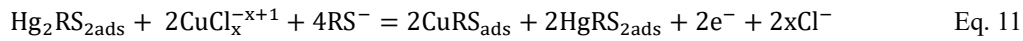


Fig. S3 Square wave (SW) voltammograms of cysteine (Cys) (0.8 μmol/L) in 0.55 mol/L NaCl/0.01 mol/L borate buffer at different accumulation times. Inset: Peak potential (C2 peak) and peak current (C2 peak) as a function of the accumulation time (0.8 μmol/L Cys in 0.55 mol/L NaCl/0.01 M borate buffer)

S5.2. Cuprous Cys formation at the Hg electrode surface

The addition of Cu(II) to the electrolyte containing Cys (0.55 mol/L NaCl/0.01 mol/L borate buffer) led to several observations (Fig. S4). One of these is the appearance of an additional SWV wave (C3), which can be attributed to the reduction of Cu(I)Cys with a stoichiometry of 1:1 for Cu(I):Cys, according to the available literature (Çakır et al. 2000; Mlakar et al. 2017). As expected, the C2 wave decreased with increasing Cu(II) addition, while the C3 wave increased when a fixed t_a was applied. However, two further observations can be derived from the results shown in the Figs. S4 and S5. The P_i of the C1 wave increases with the addition of Cu(II) although C2 decreases, which is counterintuitive, i.e. in agreement with the investigation of electrochemical behavior of Cys described in previous section- C1 should decrease as C2 decrease. As shown in Fig. S5 depending on the t_a applied under conditions of fixed Cys concentration (0.8 $\mu\text{mol/L}$), increasing Cu(II) concentration causes a shift of the C2 E_p towards more positive potential values. The phenomena is more pronounced with increasing Cu(II) concentration. The explanation which can reconcile observation 1) and 2) is that adsorbed $\text{Hg}_2(\text{RS})_2$ is reacting with the Cu(I)Cl_x^{x+1} as follows:



The Cu(I)Cl_x^{x+1} complex is generated electrochemically due to the stabilization of Cu(I) by Cl^- at the Hg electrode surface (Nekrasov and Berezina 1962; Pinchin and Newham 1977; Nelson and Mantoura 1984). As the Cu(II) concentration increases, the exchange reaction between $\text{Hg}_2\text{RS}_{2\text{ads}}$ and CuCl_x^{x+1} progresses, resulting in the formation of more $\text{HgRS}_{2\text{ads}}$ (increase in the C1 peak). As t_a increases, the Hg electrode becomes increasingly saturated with the adsorbed reaction product, resulting in the Hg^+ reduction. This phenomenon is already known from the literature, which shows that metal ions (Co, Ni, Zn, Fe, etc.) exchange with Hg^{2+} in the HgS surface layer, where the HgS peak potential can be completely shifted towards more positive potentials at a high metal/ $\text{HgS}_{(\text{ads})}$ ratio (Spataru and Banica 2001; Bănică et al. 2009).

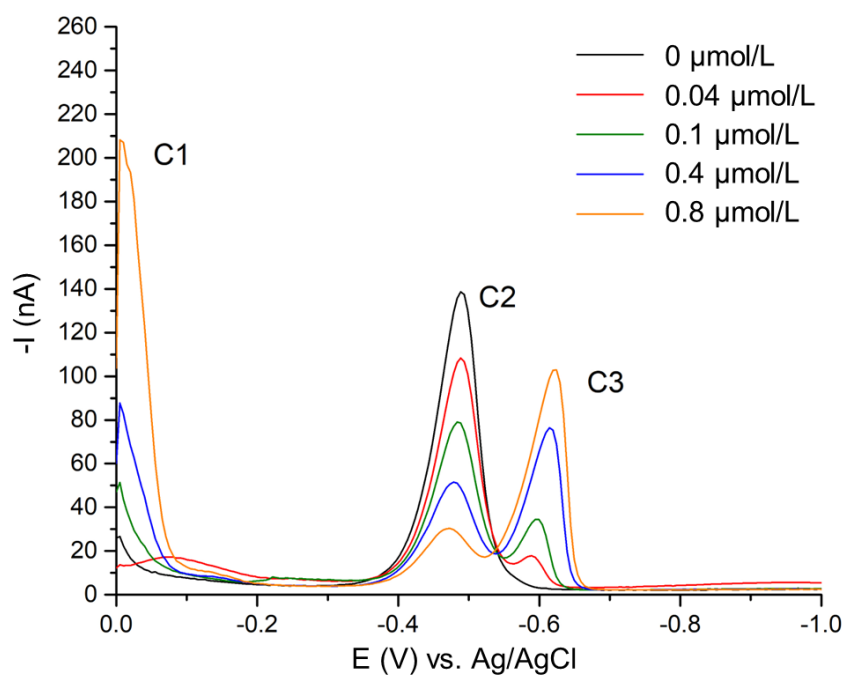


Fig. S4 Square wave (SW) voltammograms of cysteine (Cys) (0.8 $\mu\text{mol/L}$) in 0.55 mol/L NaCl/0.01 mol/L borate buffer with the Cu(II) addition. Accumulation potential 0.0 V; accumulation time 30 s (amplitude (a) and frequency (f) used are given in the experimental section of the main manuscript)

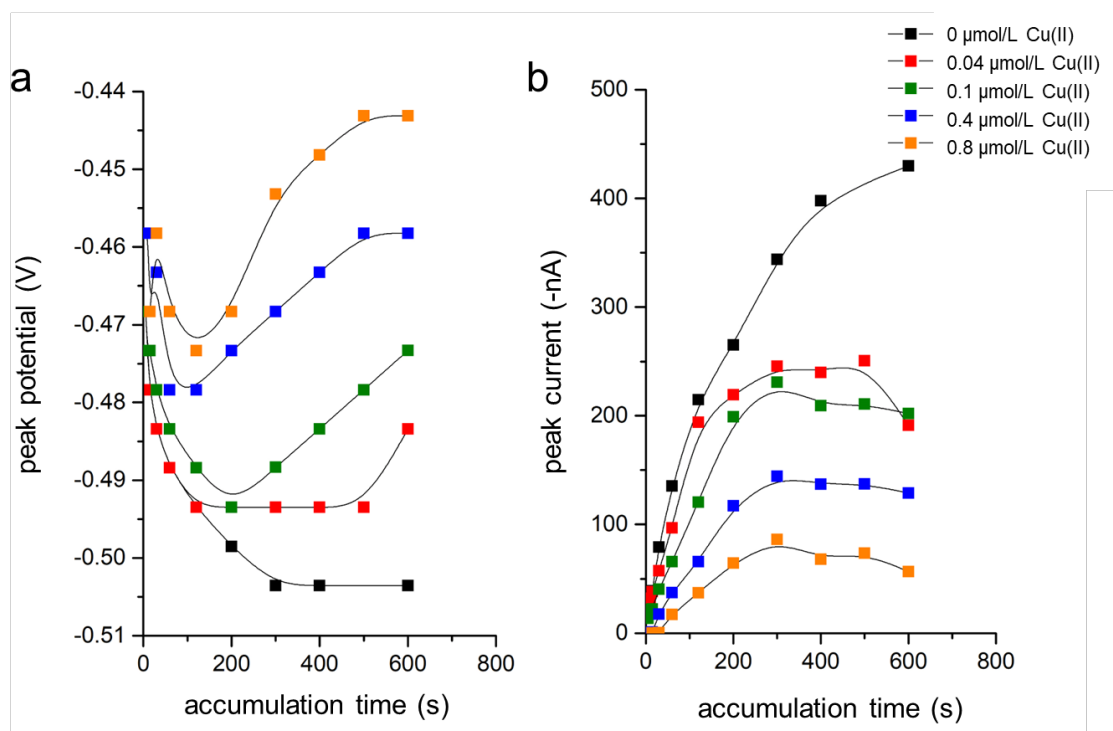


Fig. S5 a) C2 peak potential vs. accumulation time and b) C2 peak current vs. accumulation time. The accumulation potential is 0.0 V, while amplitude (*a*) and frequency (*f*) are given in the experimental section of the main manuscript. Composition of the solution: cysteine (Cys) (0.8 $\mu\text{mol/L}$) in 0.55 mol/L NaCl/0.01 mol/L borate buffer after increasing addition of Cu(II)

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A.4. Effect of chloride concentration on copper(I) stability

Stock solutions of Cu(I) were prepared by dissolving CuCl in deoxygenated media containing different concentrations of NaCl (0.1 M, 0.55 M, 1 M and 4 M), and the stability of Cu(I) was monitored by UV–Vis spectrophotometry using BCS and EDTA as Cu(I)- and Cu(II)-binding ligands, following the procedure described in Research Paper I.

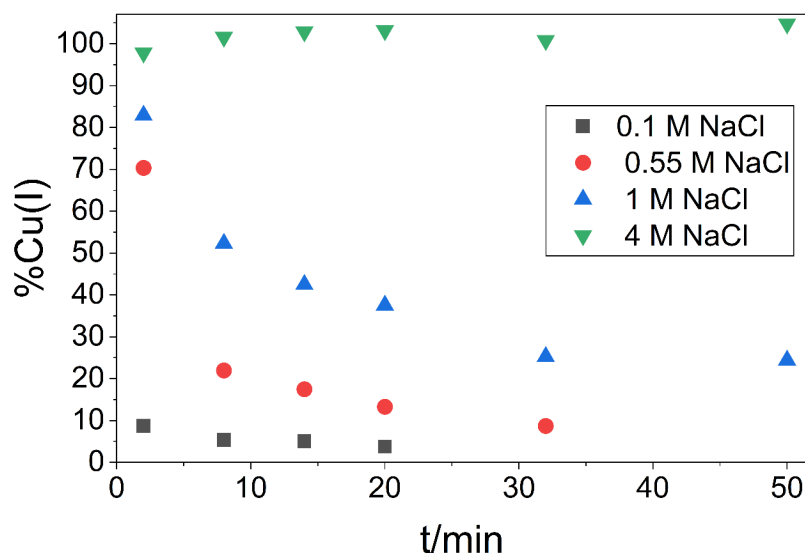


Figure A1. Stability of Cu(I) over time in media containing different concentrations of NaCl.

%Cu(I) denotes the measured concentration of Cu(I) with respect to the concentration of Cu(I) measured in the starting Cu(I) stock solution prepared in 4 M NaCl.

A.5. Additional data on sampling campaigns in the Krka River estuary

A.5.1. Sampling campaign: spring 2024 (E1 and E2)

Sampling and sampling site

Sampling was conducted on sampling sites E1 on April 23 2024 at 10:00 h, and on E2 on May 10 2024 at 9:30 h, following the same procedures as described in Research Papers II and III. In brief, samples were collected from a boat at seven depths (0.2 m, 1 m, 2 m, 4 m, 10 m, 20 m, and 35 m), covering the surface brackish layer, halocline, and seawater layer. Vertical profiles of salinity and Chl *a*) were measured using an EXO2 multiparameter probe (Xylem).

Chemicals and preparation of stock solutions

All sample collection and storage bottles and tubes (FEP or polycarbonate; Nalgene) were pre-cleaned by soaking in 10% HNO₃ for ≥ 24 h and rinsing thoroughly with UP water (18.2 M Ω cm; Sartorius Arium® Mini). Analytical-grade or higher-purity chemicals were used throughout. BCS and EDTA stock solutions, as well as HCl, methanol and borate buffer used for SPE column pre-cleaning, were prepared as described in the Experimental sections of Research Papers II and III.

Sample handling and analyses

Samples were filtered within 1 h of collection through 0.45 μ m cellulose-nitrate filters (Sartorius) under N₂ pressure. Measurements of DOC, absorbance and fluorescence are described in detail in Marcinek et al. (2020), and briefly described in Research Paper II. For Cu redox speciation, samples were divided into three 100 mL aliquots immediately after filtration, followed by addition of BCS and EDTA, followed by SPE and DP-ASV determination of Cu(I), Cu(II), and Cu_T using the standard addition method, as described in Research Paper II.

Table A1. Values of optical parameters at stations E1 in spring 2024.

stati on	dept h (m)	BIX	HIX	peak A (R.U.)	peak M (R.U.)	peak C (R.U.)	peak T (R.U.)	<i>S</i> _{275- 295} (μ m ⁻¹)	<i>a</i> ₂₅₄ (m ⁻¹)	SUV A (m ² g ⁻¹)
E1	0.2	0.9015	3.49 60	0.0940	0.0532	0.040 9	0.03 77	25.4 9	3.0 9	1.59

E1	1	0.8898	3.58 34	0.0920	0.0530	0.039 5	0.03 90	26.6 7	3.0 6	1.66
E1	2	0.9521	2.48 99	0.0577	0.0304	0.023 7	0.03 91	29.9 1	2.5 0	1.10
E1	4	1.0344	1.74 96	0.0310	0.0188	0.011 8	0.02 24	35.6 2	1.9 7	0.98
E1	10	0.9972	1.80 85	0.0270	0.0158	0.011 4	0.02 05	36.3 9	1.9 1	0.95
E1	20	0.9978	2.00 43	0.0334	0.0190	0.014 6	0.02 22	35.7 2	1.9 3	1.00
E1	35	1.0484	1.62 56	0.0268	0.0146	0.011 0	0.02 25	40.4 1	1.8 7	0.96

A.5.2. Sampling campaign: summer 2024 (E1 and E2)

Table A2. Values of optical parameters at stations E1 and E2 in summer 2024.

stat ion	dept h (m)	BI X	HI X	peak A (R.U.)	peak M (R.U.)	peak C (R.U.)	peak T (R.U.)	$S_{275-295}$ (μm^{-1})	a_{254} (m^{-1})	SU VA (m^2 g^{-1})
E1	0.2	1.0 340	1.1 360	0.0597	0.0334	0.0236	0.0759	33.48	3.43	1.16
E1	1.5	1.0 323	1.4 162	0.0566	0.0318	0.0224	0.0586	32.89	3.41	1.15
E1	2	1.0 507	1.3 526	0.0506	0.0278	0.0199	0.0546	31.01	2.78	0.99
E1	3.5	1.0 590	0.9 406	0.0302	0.0168	0.0115	0.0445	34.52	2.26	0.89
E1	10	1.0 531	0.8 799	0.0316	0.0182	0.0122	0.0497	34.51	2.14	0.87
E1	20	1.0 203	0.6 448	0.0395	0.0226	0.0161	0.0866	34.59	2.17	0.93
E1	35	0.9 724	1.1 507	0.0349	0.0198	0.0153	0.0439	33.22	2.02	0.92
E2	0.2	1.1 625	1.3 008	0.0686	0.0398	0.0274	0.0758	30.29	3.47	1.19
E2	2	1.0 052	1.3 306	0.0596	0.0329	0.0242	0.0654	29.88	3.27	1.12
E2	9	0.9 466	1.9 891	0.0658	0.0376	0.0301	0.0505	27.63	2.92	1.20

A.5.3. Seasonal and vertical distribution of total dissolved Cu and Cu(I) fraction at E1 and E2 over 2023 and 2024 sampling campaigns.

Table A3. Seasonal and vertical distribution of Cu_T and Cu(I) fraction at sampling sites E1 and E2 in 2023 and 2024, with seasonal median values.

Season	Sampling site	Depth (m)	Cu _T (nM)	Cu(I)/Cu _T (%)
Spring 2023	E1	0.2	11.66	88.7
		1.5	7.39	86.6
		2.0	8.37	68.6
		2.5	7.10	66.5
		10	3.55	36.1
		20	3.02	43.7
		38	2.54	44.1
Spring 2024	E1	0.2	7.22	72.4
		1.0	7.79	65.6
		2.0	16.42	64.9
		4.0	8.33	49.3
		10	4.41	43.3
		20	6.42	56.2
		35	4.41	47.6
Spring 2023 and 2024 median	E1		60.6	
Spring 2023	E2	0.2	27.47	73.8
		2.0	14.04	72.8
		10	10.29	63.2
Spring 2024	E2	0.2	63.47	79.2
		2.2	20.18	64.4
		9.0	11.32	47.4
Spring 2023 and 2024 median	E2		68.6	
Summer 2023	E1	0.2	8.02	50.9
		2.2	9.62	78.6
		2.5	9.31	83.4
		2.8	6.23	71.9
		20	5.18	69.9
		38	3.04	72.4
Summer 2024	E1	0.2	13.6	82.5
		1.5	14.8	72.6
		2.0	23.2	71.6

		3.5	7.48	69.9
		10	6.40	67.5
		20	5.04	50.2
		35	3.18	51.9
Summer 2023 and 2024 median	E1		71.6	
Summer 2023	E2	0.2	89.58	35.3
		2.0	33.55	59.3
		10	11.60	20.7
Summer 2024	E2	0.2	91.13	47.5
		2.0	67.19	63.9
		9.0	10.25	37.9
Summer 2023 and 2024 median	E2		42.7	

A.5.4. Rainwater sampling and seasonal sampling of the estuarine surface layer (E3)

Sampling and sampling site

Rainwater samples were collected on an event basis at the station E3, in the proximity of previously investigated stations E1 and E2 in the Krka River estuary, near the city of Šibenik . In parallel, samples of surface estuarine layer were collected.

All the bottles used for trace metal analysis were made either of fluorinated polypropylene or polycarbonate. The bottles, syringes and tubes used for sample collection, filtration, storage and analysis were pre-cleaned by soaking in 10 % v/v HNO₃ or HCl, depending on analysis, for 24 h and rinsed several times with UP water. Bulk rainwater samples (n=11) were collected on an event basis between October 2023 and September 2024. Some events were discarded as the volume of collected rainwater was insufficient for analysis and/or 2 or more days passed between precipitation event and collection of the samples. Rainwater was collected in polycarbonate carboy (Nalgene) inside the rainwater sampler. Rainwater sampler was home-made automatic rainwater sampler with precipitation-controlled funnel lid and high density polypropylene beaker with 27 cm diameter. The rainwater sampler was located in proximity of

laboratory which allowed us immediate collection and analysis of the samples. Estuarine samples (n=16) were collected as described in Research Papers II and III.



Figure A2. Rainwater sampler at the E3 sampling site.

Sample handling and analyses

Within 1 h of sample collection (both estuarine and rainwater), filtration and reagent (BCS and EDTA) addition were carried out, and Cu redox speciation was performed 1 h after reagent addition, as described in detail in Research Paper II. Organic matter characterisation and organic Cu speciation in estuarine samples, as well as DOC measurements in both estuarine and rainwater samples, are also described in detail in Research Paper II. pH of rainwater samples was measured using 913 pH meter (Metrohm). Concentrations of anions (chloride, nitrite, nitrate and sulphate) in rainwater samples were determined by an ECO-IC ion chromatograph (Metrohm) with a Metrosep A Supp 7 anion separation column (150 mm length, 4.0 mm inner diameter) in combination with the conductivity detector 945 Professional Detector Vario and chemical suppression with the Metrohm Suppressor Module. The instrument was operated by MagIC Net software at flow rate of 0.70 mL/min and sample injection volume of 20 μ L at standard room temperature of 23 °C. The concentrations of dissolved elements in samples were measured by Inductively Coupled Plasma Mass Spectrometer (Agilent 8900 QQQ) (ICP-MS) in He mode. All samples were spiked with an Internal Standard (indium) at concentration of 10 μ g/L. External calibration was used for concentration quantification. A certified reference material (SLRS-6, River Water Certified Reference Material for Trace

Metals and other Constituents, National Research Council Canada) was used as a quality control (QC). Concentrations of metals in QC samples were within 10% of the assigned values. Relationships between Cu concentrations, Cu redox speciation and accompanying physicochemical and chemical parameters were evaluated using Spearman's rank correlation coefficient (ρ). Statistical significance was assessed at $p < 0.05$.

Data regarding rainwater and surface estuarine samples

Table A4. Concentration of dissolved elements in seawater measured by ICP-MS. All the concentrations are in nM.

Date	Li	Na	Mg	Al	K	Ca	Ti	V	Cr	Mn	Fe	Co	Ni	Cu	Zn	As	Rb	Sr	Mo	Cd	Sn	Sb	Cs	Ba	Tl	Pb	U
22/10/2023	20.3	1.911 e+05	2.728 e+04	265.3	7440	1.848 e+05	0.585	9.01	0.731	3.08	25.9	0.119	0.596	3.95	6.21	1.43	3.57	310.1	0.334	0.667	0.118	0.485	0.007 5	61.4	0.019 6	0.043 7	0.012 6
18/11/2023	5.19	2.042 e+05	6091	179.2	1727	1.501 e+04	0.762	1.47	0.827	57.5	109.7	0.899	5.76	5.11	201.8	0.32	0.831	19	0.292	2	2.59	0.386	0.007 5	3.72	0.004 9	0.912	0.004 2
22/11/2023	54.6	2.199 e+04	4634	104.2	1.533 e+05	4.499 e+04	1.44	2.89	0.404	37.2	33.7	1.71	2.42	6.86	189.6	0.28	5.39	23.5	0.49	0.035 6	1.23	0.205	0.007 5	5.99	0.004 9	0.092 2	0.004 2
06/12/2023	3.17	5.61e +04	5978	32.1	1355	4902	0.543	0.982	0.115	8.12	9.28	0.322	3.66	48.3	128.3	0.133	0.527	13.6	0.156	0.044 5	0.118	0.181	0	2.86	0.004 9	0.097	0
18/01/2024	9.8	8.763 e+04	9886	171.3	4992	1.222 e+04	0.501	1.83	0.596	49.9	24.2	1.34	3.49	227.4	650.1	0.547	4.22	25.5	0.511	0.116	0.152	0.6	0.022 6	8.63	0.048 9	0.238	0.008 4
11/02/2024	23.6	3.548 e+05	3.942 e+04	130.8	8676	3.702 e+04	0.815	2.16	0.558	41.2	22.6	0.916	2.4	211.8	755.4	0.387	3.23	94.1	0.5	0.116	0.514	0.337	0.007 5	14.9	0.014 7	0.267	0.008 4
24/02/2024	28.2	4.271 e+05	4.597 e+04	176.8	9436	4.207 e+04	1.67	4.36	0.539	7.21	11	0.255	3.83	200.2	1571	1.03	3.86	101.1	0.657	0.107	0.177	1.01	0.015	19.2	0.034 2	0.116	0.004 2
05/03/2024	14.1	1.033 e+05	1.984 e+04	146	3661	7.027 e+04	1.53	4	0.327	42.2	15.5	0.492	1.24	112.5	879.6	0.841	1.94	131.1	0.438	0.080 1	0.135	0.682	0.007 5	18.1	0.024 5	0.048 5	0.004 2
11/03/2024	26.7	2.39e +05	2.424 e+04	74.7	5694	2.396 e+04	1.23	2.16	0.231	25.9	7.93	0.407	4.24	117.2	833.5	0.214	1.29	64.8	0.188	0.080 1	0.168	0.353	0	11.2	0.009 8	0.189	0.004 2
22/05/2024	13	5.909 e+04	1.49e +04	1398	2198	7.083 e+05	2.88	4.59	0.981	2.68	29.5	0.238	6.44	19	84.1	0.934	1.32	155.7	0.334	0.008 9	1.07	0.591	0.015	68.5	0.004 9	0.048 5	0.096 6
06/09/2024	6.77	4.517 e+04	5943	313.8	2961	2.329 e+04	3.47	3.18	0.923	78.7	89.5	0.865	2.66	33.1	154.5	0.681	1.66	28.6	0.167	0.071 2	0.396	0.443	0.015	7.09	0.014 7	0.257	0.008 4

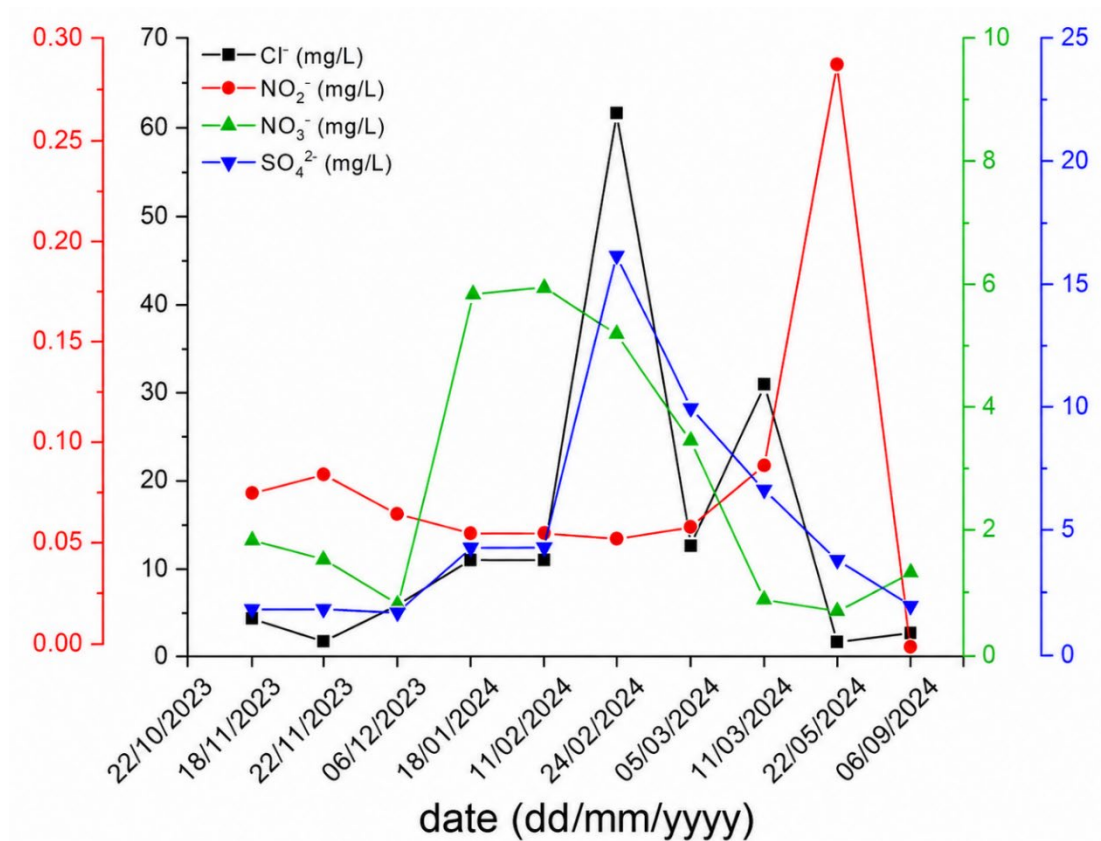


Figure A3. Temporal variation in chloride (Cl^-), nitrite (NO_2^-), nitrate (NO_3^-), and sulphate (SO_4^{2-}) concentrations in rainwater samples collected between October 2023 and September 2024 at E3.

Table A5. Precipitation, insolation and air temperature on the days of investigated rain events; DOC and pH in rainwater. Data for precipitation, duration of insolation and air temperature was taken from (Croatian Meteorological and Hydrological Service, 2023/2024).

sampling date	precipitation/mm	insolation/h	T/°C	DOC (mg/L)	pH
22/10/2023	16.9	8.4	20.1	0.652	7.02
18/11/2023	6.7	7.9	12.0	0.604	6.01
22/11/2023	42.6	0.2	11.1	1.063	6.2
06/12/2023	41.8	7.3	7.2	0.402	5.88
18/01/2024	5.7	1.0	15.9	1.450	5.51
11/02/2024	16.0	2.4	13.2	1.717	6.45
24/02/2024	11.9	2.1	11.6	1.716	6.21
05/03/2024	8.6	8.5	12.8	1.444	5.81
11/03/2024	38.1	1.5	11.2	0.550	5.50
22/05/2024	12.5	11.9	19.9	1.362	6.79
06/09/2024	22.3	7.0	24.2	1.781	6.51

Table A6. DOC and salinity in the estuarine samples.

sampling date	DOC (mg/L)	salinity
18/10/2023	1.452	22
23/10/2023	1.341	26
07/11/2023	0.797	16
21/11/2023	0.875	6
06/12/2023	0.402	6
10/01/2024	0.881	17
18/01/2024	0.87	8
26/01/2024	0.849	10
06/02/2024	0.858	5
12/02/2024	0.882	10
26/02/2024	0.913	7
05/03/2024	0.938	5
11/03/2024	0.891	9
05/04/2024	0.795	15
19/04/2024	0.851	16
06/09/2024	1.413	23
18/10/2023	1.452	22
23/10/2023	1.341	26
07/11/2023	0.797	16
21/11/2023	0.875	6

Table A7. Copper organic speciation and complexing parameters at E3 (autumun 2023-spring 2024).

Sampling date	[Cu _T] (nM)	[Cu ²⁺] (pM)	[L ₁] (nM)	log K'_{CuL1}	[L ₂] (nM)	log K'_{CuL2}
21/11/2023	6.83	0.143	3.04	13.60	13.91	11.33
10/01/2024	4.11	0.159	3.67	14.08	10.62	11.65
6/2/2024	7.10	0.0637	2.67	13.70	19.20	11.76
26/2/2024	3.27	0.065	3.34	13.75	16.00	11.74
11/3/2024	4.46	0.622	2.02	14.13	12.10	11.62
5/4/2024	3.16	0.132	2.70	13.43	12.60	11.49
19/4/2024	7.52	0.487	6.40	13.34	17.41	11.46

Table A8. Values of optical parameters of CDOM at station E3 (autumn 2023-spring 2024).

sampling date	BIX	HIX	peak A (R.U.)	peak M (R.U.)	peak C (R.U.)	peak T (R.U.)
21/11/2023	0.8797	4.2652	0.1323	0.0831	0.0649	0.0498
6/2/2024	0.8793	3.1518	0.1002	0.0574	0.0439	0.0415
26/2/2024	0.9936	2.5436	0.0952	0.0552	00.0415	0.0467
11/3/2024	0.8934	3.1851	0.0758	0.0437	0.0339	0.0337
5/4/2024	0.8510	3.8621	0.0874	0.0489	0.0375	0.0338
19/4/2024	0.8878	2.7404	0.0650	0.0335	0.0260	0.0344

A.5.5. Sampling campaign: summer 2025 (E1)

Sampling

Sampling was conducted on 30 July 2025 at 10:00 h at the E1 sampling site in the Krka River estuary, as described in Research Papers II and III. Samples were collected from a boat at three depths: 0.2 m (surface brackish layer), 3 m (halocline), 5 m (seawater layer), using a home-built 4.5 L “butterfly”-type water sampler. Vertical profiles of salinity and Chl *a* were measured using an EXO2 multiparameter probe (Xylem).



Figure A4. Sampling in the Krka River estuary (summer 2025).

Chemicals and preparation of stock solutions

All bottles and tubes used for sample collection and storage were made of fluorinated ethylene propylene (FEP; Nalgene) or polycarbonate (Nalgene) and were previously cleaned by soaking in 10% HNO_3 for at least 24 h, followed by multiple rinses with UP water. All chemicals were of analytical grade or higher. Stock solutions of BCS and EDTA, as well as the solutions used for SPE column pre-cleaning (HCl, methanol and borate buffer), were prepared as described in the Experimental section of Research Papers II and III.

Sample handling and analyses

Samples were filtered within 1 h of sampling using 0.45 μm cellulose-nitrate membrane filters (Sartorius) under nitrogen pressure. Aliquots of each sample were acidified with Suprapur® HCl and stored in a refrigerator for 1 week prior to DOC determination by high-temperature catalytic oxidation using a Shimadzu TOC-L Online carbon analyser. The remaining volume of each sample was divided into three 100 mL aliquots. For the first set, Cu redox speciation was performed immediately after filtration by adding BCS and EDTA to the filtered samples,

followed by SPE approximately 1 h after ligand addition, as described in detail in Research Paper II. The second set of aliquots was stored on a balcony under ambient day/night conditions and temperature (22-35°C), and Cu redox speciation was performed after 48 h. The third set of aliquots was stored in the dark at room temperature (23 °C), and Cu redox speciation was performed after 48 h. Concentrations of Cu(I), Cu(II) and Cu_T were determined by DP-ASV using the standard addition method and an Autolab PGSTAT204 instrument controlled by NOVA 2.1 software. Measurements were performed in a three-electrode cell with a hanging mercury drop electrode (HMDE) as the working electrode, as described in detail in Research Paper II.

Table A9. Concentrations of Cu(I) and Cu(II) in estuarine samples (E1) measured in situ and after different light exposure.

Depth (m)	Cu(I) in situ (nM)	Cu(II) in situ (nM)	Cu(I) after 48 h in dark (nM)	Cu(II) after 48 h in dark (nM)	Cu(I) after 48 h under ambient light/dark conditions (nM)	Cu(II) after 48 h under ambient light/dark conditions (nM)
0.2	6.85±0.71	2.95±0.21	2.25±0.12	7.59±0.82	5.36±0.44	4.43±0.51
3	6.86±0.63	5.51±0.45	8.58±0.59	3.92±0.22	7.79±0.91	4.12±0.27
5	2.30±0.36	2.28±0.41	3.51±0.21	2.61±0.12	3.11±0.38	2.84±0.19

A.6. Investigation of possible mechanisms of Cu(II) photochemical reduction and Cu(I) stabilisation in model solutions

Laboratory experiments were conducted in model solutions containing Cu(II) with different combinations of BCS and His. The pH was adjusted to 8.2 using 0.1 M borate buffer. The final concentrations were 0.3 mM Cu(II), 0.9 mM BCS, and 10 mM His, where applicable. Three systems were investigated: (1) Cu(II)–His, (2) Cu(II)–BCS, and (3) Cu(II)–His–BCS. For the Cu(II)–His system, Cu(II) reduction was assessed using the same approach described for Cu(II) and thiols in Research Paper I, i.e. by mixing aliquots of model solutions with an excess of BCS and EDTA and recording the absorbance of the Cu(I)–BCS complex. For the Cu(II)–BCS and Cu(II)–His–BCS systems, Cu(I) formation was monitored directly in the reaction solutions

by UV–Vis spectrophotometry. The experiments were conducted under two light regimes: dark and UV irradiation. For the dark treatment, the cuvette containing the reaction mixture was kept inside a closed dark box to prevent exposure to ambient light. For the light treatment, the cuvette containing the reaction mixture was irradiated using a UV lamp at 365 nm (Handheld UV Lamp 254nm/365nm, XEPU). At predetermined time intervals, UV–Vis spectra of the reaction mixture were recorded, and the A_{483} , corresponding to the Cu(I)–BCS₂ complex, was used to monitor Cu(I) formation over time. UV–Vis measurements were performed using a Cary 50 Bio UV–Visible spectrophotometer (Agilent Technologies). The concentration of Cu(I) formed was calculated from the measured A_{483} using the ϵ of the Cu(I)–BCS₂ complex reported in the literature (Xiao et al., 2004) and expressed as a percentage of the total Cu concentration in the solution.

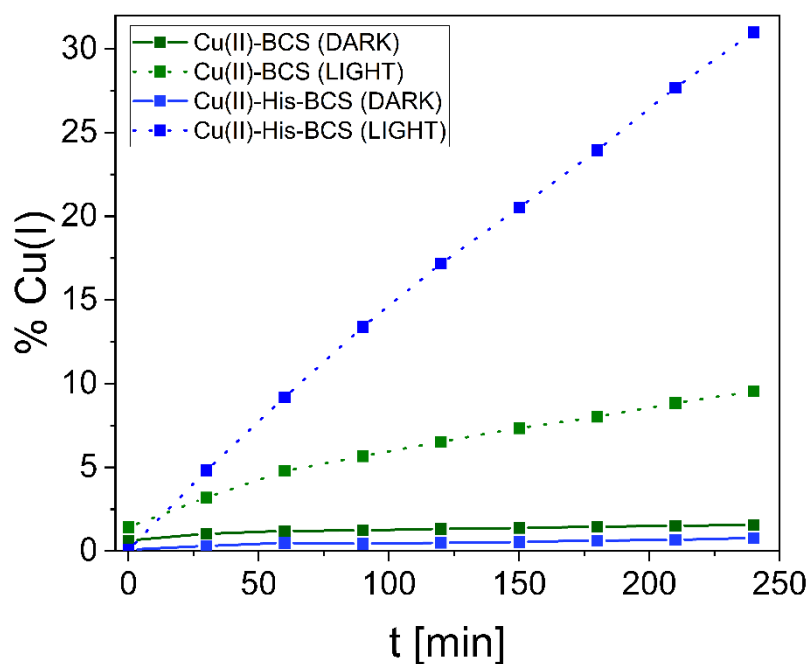


Figure A5. Formation of Cu(I) in Cu(II)–BCS (green) and Cu(II)–His–BCS (green) model solutions under dark (solid line) and light (UV irradiation; dotted line) conditions.

Author Resume

Dora Crmarić was born in Split, Croatia, where she completed her primary and secondary education. She obtained a Bachelor's degree in Chemical Engineering from the Faculty of Chemical Engineering and Technology, University of Zagreb, in 2018, and a Master's degree in Chemical Engineering in 2020. Her Master's thesis, "Photocatalytic removal of organic micropollutants under simulated solar irradiation", resulted in a peer-reviewed publication. In December 2021, she enrolled in the Interdisciplinary Doctoral Programme in Oceanology at the Faculty of Science, University of Zagreb, where she is currently a PhD candidate. Her research focuses on the biogeochemistry of trace metals in marine and estuarine environments, with particular emphasis on the redox speciation of copper and its interactions with natural organic ligands. In October 2021, she was employed as a Professional Associate in Science and Higher Education at the Ruđer Bošković Institute, Division for Marine and Environmental Research, where she worked until December 2023 on the project "Application of artificial intelligence in advanced predictive technologies for online water quality control" (IOTSIEMENS, KK.01.2.1.02.0242). From January to July 2024, she was employed as a substitute Research Assistant at the Ruđer Bošković Institute, Division for Marine and Environmental Research. From July 2024 to January 2025, she worked as a Project Associate at the Institute of Oceanography and Fisheries (Croatia) on the project "Systematic Monitoring and Assessment of Transitional and Coastal Waters and Eutrophic Areas in the Central and Southern Adriatic Sea". Since January 2025, she has been employed as a Project Associate at the Ruđer Bošković Institute on the project "Developing an innovation network for the uptake of solutions in robotics and sensors to improve monitoring and prevention of pollution of freshwater and marine ecosystems" (ROBONETCBC, HR-BA-ME00269). During her doctoral studies, she has been involved as a project associate on the Croatian Science Foundation project "Understanding copper speciation and redox transformations in seawater" (SeCuTrans, IPCH-2020-10-4965), which lasted from December 2021 to March 2025. Through her various research and professional positions, she has gained extensive experience in fieldwork, including trace metal clean sampling of natural waters, as well as laboratory analysis using voltammetry, UV-Vis and EPR spectroscopy, ion chromatography, and solid-phase extraction, together with scientific writing. She is the first author of three scientific papers and a co-author of three additional publications. She has presented her research at international and national scientific conferences, with a total of sixteen contributions. During her PhD, she was awarded four scholarships that enabled her to participate in the GEOTRACES Summer School (Alfred Wegener Institute, Bremerhaven, Germany, July 2022; Volkswagen Stiftung grant), as well as three research stays at the Institut des Sciences Moléculaires de Marseille (France) in 2024 (NECTAR COST Action Short-Term Scientific Mission grant; Financial support from the City of Zagreb for doctoral students' education; Scholarship of the Government of the French Republic for a research stay). Furthermore, she was awarded three grants for participation in scientific conferences (Support for the participation of young scientists from the Division for Marine and Environmental Research in scientific conferences; Award of financial grants for the participation of Independent Union of Research and Higher Education Employees of Croatia members in scientific conferences; Financial support from the City of Zagreb for doctoral students' education). In 2025, her project "Investigation of the influence of humic acid and solar radiation on copper redox speciation in estuarine waters" was financially awarded by the Croatian Academy of Sciences and Arts Foundation. Her engagement in science communication and public outreach includes leading workshops for primary school children at the Faust Vrančić Creative Days Festival (Prvić Luka, Croatia; October 2021, 2023, 2025) and at the International Children's Festival (Šibenik, Croatia; July 2023). She is a member of the International Society of Electrochemistry, the Association of

Chemistry and the Environment, and the Chem2Change early-career research network, where she serves as a member of the organisational board. Her native language is Croatian, and she holds language diplomas in English (C1 level) and Swedish (B1 level).

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