Copper ecotoxicology of marine algae: a methodological appraisal

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ABSTRACT

The production of accurate and reliable data on copper ecotoxicology of marine algae depends on the use of trace metal clean techniques during experimentation. We reviewed the methodologies used in the literature on copper ecotoxicology of marine macro- and microalgae, specifically the use of trace metal clean procedures such as the labware used (glassware vs. plasticware), methods of cleaning the labware (acid soaking and ultrapure water rinsing), stock solution preparation (copper source and acidification), and measurement and reporting of dissolved copper concentrations. In terms of taxonomic classification, the most studied algal groups were the Phyla Ochrophyta, Bacillariophyta, Rhodophyta, and Chlorophyta. In terms of methodology, ~50% of the articles did not specify the labware, ~25% used glassware, and ~25% plasticware; ~30% of the studies specified cleaning protocols for labware to remove trace metal impurities; the copper form used to prepare the stock solutions was specified in ~80% of studies but acidification to stabilise the dissolved copper was performed in only ~20%; and the dissolved copper concentration was measured in only ~40% of studies. We discuss the importance of following trace metal clean techniques for the comparison and interpretation of data obtained on copper ecotoxicology in algae.

1. Introduction

Algae (both macro- and microalgae) are important components of marine ecosystems. They provide 50% of global primary production, play significant ecological roles as the base of marine food webs, and participate in the cycling of organic and inorganic substances.[1] In coastal environments, macroalgae additionally provide a settling substrate for sessile invertebrates, and provide shelter for nekton from predation.[2–5] Algae are continually exposed to natural (e.g. temperature, UV radiation, and grazing) and anthropogenic stressors.[5] Human activities produce various forms of stressors, including nutrient...
enrichment and discharges of toxic metals. Studies on the toxic effects of metals on different algal groups are needed to provide an effective environmental risk assessment. Because the sensitivity of algae to pollution is species-specific, the same pollutant can have different ecological consequences depending on the algal group affected. For example, negative effects of a toxic compound on the physiology of a microalgal species will have a direct effect on higher trophic levels of the food web, while negative effects on macroalgae do not only affect trophic dynamics but also have severe consequences on the structural (e.g. 3-D habitat) integrity of the coastal ecosystem. Studies on the effects of increased metal concentrations on the biota (e.g. algae) and their environment is termed metal ecotoxicology, and this field has become increasingly important over the past few decades in order to assess the impacts of pollution on marine coastal ecosystems. Metal ecotoxicological studies on algae are important because: (1) adverse impacts of high metal concentrations on algal populations can have flow-on effects that are detrimental to the local coastal environment; (2) metals can be directly accumulated by algae and incorporated into the food web, causing a health risk to other living organisms, including humans; and (3) the information obtained in an ecotoxicological study can serve as a tool for legislators and local governments to determine critical levels of pollutants in coastal areas, allowing them to regulate and/or prevent detrimental ecosystem effects from metal contamination. For example, biomonitoring programmes have been developed to establish spatial and temporal variation in trace metal concentrations in marine environments, some even using macroalgae as biomonitors. Copper is an essential micronutrient required for algal growth and development because it participates in fundamental physiological processes (e.g. photosynthetic electron transport, mitochondrial respiration) and is a cofactor for many enzymes (e.g. superoxide dismutase, cytochrome c oxidase). However, copper in excessive concentrations becomes toxic, causing growth inhibition of algae due to adverse effects on the same cellular processes where it is needed, such as enzyme activity and photosynthetic electron transport. Elevated copper concentrations in marine coastal ecosystems are directly related to human activities involving the production of industrial (e.g. pesticide use and agricultural run-off, mine tailings) and domestic (e.g. urbanisation, automobile exhausts) wastes. The main method used for determining the impacts of a marine pollutant (e.g. copper) on algae is the ecotoxicity test. In general, the algal species is exposed to different metal concentrations. After a predefined exposure time, biological responses such as photosynthesis, growth, spore germination and mortality, and metal biosorption and bioaccumulation are measured. From the resultant dose response curve, an effective concentration at which $x\%$ of the biological response is affected by the metal ($EC_x$) is derived. $EC_x$ is a highly recommended response variable to facilitate ecological comparisons between ecotoxicological studies.

Very important for acquiring accurate results for ecotoxicity tests is the use of trace metal clean techniques for (1) cleaning and storage of labware for sampling and experiments, (2) experimental and sampling handling, (3) stock solution preparation, and (4) samples storage. The use of rigorous trace metal clean techniques can considerably reduce sampling contamination, subsequent procedural contamination or the loss of the analysed metal. In 1976, Maienthal and Becker reported that most
published studies on trace metal analysis, especially in the field of marine biology, are inaccurate and unreliable as a result of not using trace metal clean techniques. However, these early recommendations have largely been ignored in subsequent trace metal studies.[34]

Another potentially confounding problem in metal ecotoxicology is the choice of material for the labware. For example, while some metals such as copper adsorb onto glass, others (e.g. iron, aluminium, lead) continuously leach from it.[35] The use of glassware to prepare, store, and/or to incubate the experimental organism can decrease the actual dissolved (i.e. measured) metal concentration by up to 50% when compared to the nominal (i.e. added) concentrations.[36] Electrochemical analysis of total copper concentrations in seawater with additions of 100, 200, 300, and 400 µg Cu L\(^{-1}\), after 9 days incubation showed only 54, 91, 131, and 171 µg Cu L\(^{-1}\), respectively, left in solution.[36] Therefore, rather than being dependent on the prepared nominal metal concentrations in stock solutions and culture media, a routine measurement of the actual dissolved metal concentration is needed.[23,37] In addition, algae can also bioadsorb and bioaccumulate metals in solution, causing variation of the metal concentrations in the culture medium.[12] For example, the cell wall polysaccharide of brown macroalgae, alginate, has a high affinity for binding metals, which leads to their bioaccumulation.[15]

Here, we conduct an analysis of the literature published on copper ecotoxicology of marine macro- and microalgae since 1977 to assess the rigour of trace metal clean protocols used. The goals of this study were to assess (1) whether or not the recommendations of Maienthal and Becker [33] for trace metal clean procedures have been incorporated into subsequent copper ecotoxicological studies; (2) the proportion of studies using glassware and plasticware and the proper cleaning procedures of all labware; (3) methods of preparation of stock solution, for example, acidification and storage; (4) the measurement of actual dissolved copper concentration in the test solutions; and (5) to compile a comprehensive list of algal species (taxonomic classification) used in studies on copper ecotoxicology.

2. Material and methods

2.1. Literature search

The search for English peer-reviewed literature was conducted with the Web of Science™ database.[38] The primary keywords used were algae and copper, and the initial number of articles provided by the search engine was >5000. An advanced search was performed using one or both of the primary keywords in combination with the following keywords: seaweed, microalgae, heavy metal, trace metal, toxicity, ecotoxicity, ecotoxicology, bioaccumulation, biomonitoring, bioremediation, biosorption, copper uptake, and effective concentration.

To refine the number of articles displayed by the search engine, the main criteria for the inclusion of articles in this review was that the study performed copper dose–response experiments on marine macro- and/or microalgae or biosorption and/or bioaccumulation experiments.

2.2. Experimental protocols

The materials and methods of each article were scrutinised and the following information was recorded and analysed: (1) type (i.e. glass or plastic) of the labware used; (2) labware
cleaning protocol, that is, acid washing and ultrapure water rinsing; (3) copper stock solution preparation, that is, copper forms, acidification and the addition of chelating agent; (4) reported copper concentrations (i.e. nominal and/or dissolved); and (5) analytical technique for dissolved copper concentrations.

2.3. Taxonomic classification of test organisms

The marine macro- and microalgae used in published studies on copper ecotoxicity tests were grouped according to taxa into Phyla and Order. Species names were according to the current nomenclature on AlgaeBase.[39]

3. Results

3.1. Ecotoxicological studies: 1977–2015

A total of 93 articles (Supplementary Table 1) which satisfied our criteria were examined thoroughly. These articles on copper toxicity of algae cover the 39-year period between Maienlthal and Becker,[33] and March 2016. When grouped into 10-year periods, the number of studies has increased from six articles (five on macro- and one on microalgae) during the first decade (1976–1985) to 46 (36 on macro- and 11 on microalgae) in the decade corresponding to 2006–2015 (Figure 1). During the whole period (1976–2015), the total numbers of articles that focused on marine micro- and macroalgae were 19 and 74, respectively (Figure 1).

The 93 reviewed articles were grouped according to their study focus. Out of these, 77 studies were focused on copper ecotoxicity, whereas 16 were on copper bioremediation of which 10 focused on copper bioaccumulation and 6 on copper biosorption (Figure 2).

3.2. Review of trace metal clean techniques

The labware material used in toxicity tests was detailed in 50 of the 93 articles (Figure 3a; Supplementary Table 1). Glassware was used in 24 studies (borosilicate glass in three studies) and plasticware in 26 studies, (fibreglass in 1, polycarbonate in 11, polyethylene

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Figure 1. Number of articles (93 in total) on micro- and macroalgal copper ecotoxicology, grouped into 10–year periods. Details of the articles and algal classification are listed in Supplementary Tables 1 and 2. Note: Ross and Bidwell [62] studied macro- and microalgae; Leal et al. [36] was not included in this figure.
in 2, polypropylene in 1 and polystyrene in 1), while 43 studies (~50%) did not specify the material of the labware used (Figure 3a; Supplementary Table 1).

The labware cleaning techniques were detailed in only ~70% of articles (Figure 3b–c; Supplementary Table 1). Acid soaking was performed in 29 studies: 14 used HNO₃, 7 used HCl, 8 an unspecified acid and 1 a cleaning detergent (Figure 3b, Supplementary Table 1). Acid concentrations varied from 2% to 10% or 1 to 2 N (~1 M), and the washing period varied from 12 h to several days (Supplementary Table 1). The labware water rinsing protocol was reported in 22 articles: the utilisation of ultrapure water was specified in 18 studies and distilled water in 4 studies (Figure 3c, Supplementary Table 1) and the number of rinses varied from 1 to 6 times. Around 70% of the articles did not specify any acid cleaning (63 articles) and/or use of ultrapure water (71 articles) for rinsing the copper toxicity test labware (Figure 3b–c, Supplementary Table 1).

The inorganic copper form used in toxicity tests varied between studies (Figure 3d, Supplementary Table 1). Five different inorganic copper compounds were specified: CuSO₄ (37 articles) and CuCl₂ (32 articles) being the most preferred copper forms, including their hydrated forms (CuSO₄·H₂O, CuSO₄·2H₂O, CuSO₄·5H₂O, CuCl₂·2H₂O). In 15 studies the chemical formula of the copper source was not detailed (Supplementary Table 1).

**Table 1.** Suggested trace metal clean procedures to perform in a copper ecotoxicity test.

| Trace metal clean procedure                  | Description                                                                 |
|---------------------------------------------|-----------------------------------------------------------------------------|
| 1 Trace metal clean area                    | Performing labware cleaning and toxicity tests in a laminar flow hood.      |
| 2 Plastic labware                           | All labware used during sampling and the toxicity test should be made of    |
|                                             | chemically inert synthetic polymers such as Teflon or polyethylene.         |
| 3 Trace metal cleaning                      | Soak labware in 2 M HCl or HNO₃ (1 week minimum); then rinse labware with   |
|                                             | ultrapure water (3 times minimum).                                          |
| 4 Stock solution acidification              | Stabilise metal in solution with acid (ultrapure HCl or HNO₃; pH 2) to avoid |
|                                             | adsorption into container walls.                                            |
| 5 Metal-chelating agent addition            | The addition of metal-chelating agent (e.g. EDTA) to the stock solution/culture |
|                                             | medium should be noted.                                                     |
| 6 Dissolved metal concentrations            | Measure dissolved metal concentrations during experimentation to monitor     |
|                                             | potential changes in metal concentrations due to contamination and/or       |
|                                             | adsorption.                                                                |
| 7 Wear gloves and proper laboratory         | Wear gloves (polyvinyl chloride or polyethylene, powder-free) and proper    |
| garments                                     | laboratory coat, head and shoe covers (nylon or polyester) during the       |
|                                             | performance of labware cleaning and toxicity tests.                        |
Figure 3. Quantitative analysis of the trace metal clean techniques used in the 93 copper ecotoxicology studies of micro- and macroalgae: (a) labware material, (b) type of acid used in the acid washing procedure and (c) type of water used to rinse labware, (d) form of inorganic copper used for the stock solution, (e) type of acid added into the stock solution, (f) chelating agent added to the stock solution and/or culture medium, (g) reporting of nominal or dissolved copper concentrations, and (h) analytical techniques for measuring dissolved copper concentrations. Details of the articles and technical procedures are listed in Supplementary Table 1. Note: Guo et al. [63] used CuSO₄ and CuCl₂.
The addition of acid to reduce the pH of the copper stock solution also varied between studies (Figure 3e, Supplementary Table 1). The copper stock solution was noted as acidified in 17 studies, using HCl (six studies), HNO₃ (three studies) and H₂SO₄ (two studies) at different concentrations (listed in Supplementary Table 1). Only three studies used pH <2 for the copper stock solution. In six studies, the acid form added to the stock solution was not specified. Acid addition in the stock solution was not specified in 76 articles (Figure 3e; Supplementary Table 1).

The addition of chelating agents to the stock solution varied between studies (Figure 3g; Supplementary Table 1). The addition of ethylenediaminetetraacetic acid (EDTA) was reported in 6 articles whereas chelating agents were intentionally avoided in 19 articles. The presence of chelating agents in the copper stock solution was not detailed in 68 articles (Figure 3f; Supplementary Table 1).

Reported concentrations of copper used in the toxicity tests varied between studies (Figure 3g, Supplementary Table 1). Nominal (i.e. added) concentrations for copper treatment were mentioned in 69 articles but dissolved (i.e. measured) concentrations were only specified in 24 articles (Figure 3g, Supplementary Table 1).

The 24 studies that reported dissolved copper concentrations (Figure 3f; Supplementary Table 1) used different analytical techniques to measure dissolved copper (Figure 3h; Supplementary Table 1). Three different analytical techniques were specified: inductively coupled plasma methods (ICP; 11 articles); voltametric analytical techniques (6 articles) that included anodic stripping voltammetry (ASV), cathodic stripping voltammetry (CSV) and potentiometric stripping analysis (PSA); and atomic absorption spectroscopy (AAS; 5 articles) (Figure 3h; Supplementary Table 1).

### 3.3. Taxonomic classification of algal species

From the 93 articles, 99 distinct marine algal species (46 microalgae, 53 macroalgae) were experimentally exposed to copper (Figure 4, Supplementary Table 2). The number of species subjected to ecotoxicity tests, when grouped into Phyla was: Bacillariophyta = 20, Chlorophyta = 14, Cryptophyta = 1, Cyanobacteria = 2, Dinophyta = 12, Haptophyta = 7, Ochrophyta = 28, and Rhodophyta = 15 (Figure 4a).

The 20 diatom species studied were in 11 Orders of the Phylum Bacillariophyta with both pennate and centric species being examined (Figure 4b). Among the green algae (Phylum Chlorophyta), species examined belonged to six different Orders: Ulvales (six species) and Cladophorales (four species) were the most studied (Figure 4c). The two species of the Cyanobacteria examined belonged to the Order Synechococcales (Supplementary Table 2). The only species in the Cryptophyta investigated was the microalga, *Proteomonas sulcata* in the Order Pyrenomonadales (Supplementary Table 2). Among the Dinophyta, species investigated belonged to five Orders and the most studied was the Peridiniales with four species (Figure 4d). For the Haptophyta, species investigated belonged to three Orders, with the Order Coccolithales and Isochrysidales being the most studied with three species each (Figure 4e). Among the brown macroalgae (Phylum Ochrophyta), species belonging to six Orders were studied and the most studied taxa were the Fucales (11 species) and Laminariales (8 species) (Figure 4f). For the red algae (Phylum Rhodophyta), the species tested belonged to six Orders, with the Orders Gracilariales and Ceramiales being the most studied with five species each (Figure 4g).
Figure 4. Quantitative analysis of species used for copper toxicity experiments found in the review of 93 articles. Species (99 in total) were grouped into (a) Phyla and (b–g) Orders within each Phylum: (b) Bacillariophyta, (c) Chlorophyta, (d) Dinophyta, (e) Haptophyta, (f) Ochrophyta, and (g) Rhodophyta. The Order Synechococcales for the Phylum Cyanobacteria and Order Pyrenomonadales for the Phylum Cryptophyta are not detailed because there were too few studies. Details of the articles and algal classification are listed in Supplementary Tables 1 and 2.
From the 93 articles examined, the most studied species were the brown macroalgae *Fucus serratus* Linnaeus (8 studies) and *F. vesiculosus* Linnaeus (12 studies) (Supplementary Table 2).

### 4. Discussion

Our critical evaluation of the literature on copper ecotoxicology of marine algae, since Maienthal and Becker [33] highlighted the importance of using trace metal clean methods, and reveals a number of deficiencies in both the reporting of methods and trace metal clean protocols. In particular, there was under-reporting of important experimental procedures, including labware material (~45% not reported), acid washing and water rinsing (~70% not reported), the chemical form of the copper source (~15% not reported), stock solution acidification (~80% not reported), and the addition of artificial inorganic chelators (~70% not reported). Furthermore, only ~25% of studies reported the dissolved copper concentrations in the culture medium during the toxicity test, and these concentrations were measured using a variety of analytical techniques. Given the importance of ecotoxicological research in setting ecological standards for policymakers to protect and conserve ecosystems,[10] these findings indicate that more rigorous experimental protocols, and more extensive data reporting, would greatly benefit ecotoxicological research as it will allow scientists and managers to better compare the results of different studies.

In addition to the biological criteria (i.e. ecologically relevant biological responses) and data reporting (e.g. EC₅₀), an ecotoxicology test must consider technical procedures to avoid or reduce metal contamination and/or losses. Based on our review and Maienthal and Becker’s [33] methodological recommendations, we suggest that the following seven procedural steps must be included in an ecotoxicity test (Table 1):

1. **Perform the toxicity test and trace metal analysis in a trace metal clean area.**[30,33–35] Airborne dust is a major source of external contamination during experimentation. For example, dust particles are mainly composed of metals such as copper, magnesium, and iron.[35] The best approach to control and avoid airborne contamination is the use of a laminar flow hood during the entire toxicity test, because they filter out the airborne dust, providing filtered air areas for sample handling, analysis, and storage.[32–35,40] Only one study [41] described the use of a laminar flow hood during the experimentation. If the entire experiment cannot be performed in a clean air environment, measures should be taken to minimise airborne particles contaminating the solutions. This could be achieved by covering the containers, for example.

2. **Selection of the proper container for toxicity testing.** The most recommended container for trace metal analysis is plastic material made of synthetic polymers such as Teflon, polyethylene, polycarbonate, and polypropylene [33,35] because of their low trace element content and resistance to chemicals.[31,42] These features make them ideal for toxicity testing. In contrast, borosilicate glass is not a suitable material for trace metal analysis [33] although it is the most common material used for laboratory containers. Besides adsorbing metals in solution,[42,43] borosilicate glass containers may release metal contaminants that can modify the form of trace metals in...
Only ∼25% of studies used containers made of synthetic polymers, and some of these [45–49] used glass cover slips as a substratum for the algae, and this glass is also likely to adsorb metals.

(3) Labware cleaning before performing the toxicity test. Trace metal impurities can be leached from the container walls and the adoption of a strict cleaning procedure to reduce contamination is encouraged.[40,50–52] The ideal labware cleaning method consists of: (1) an acid (2–6 M HCl or HNO₃ depending on the type of plastic) soaking regimen, for a week or more, to remove metal and other impurities from the container wall surface, and (2) repeated soaks and rinsing (three or more) in ultrapure distilled water to remove trace metals and acid residues.[30,31,33] All cleaning and sample treatment should be done in a laminar flow chamber or a trace metal clean room.[30,33]

(4) Stock solution preparation. Around 80% of the studies described the chemical form of the copper source used to prepare the stock solution but the stock acidification was not specified in ∼80% of studies. Metals in solution may be adsorbed by container walls,[42,43] decreasing the concentration that test organisms are exposed to and leading to an over-reporting of the actual metal concentration and thereby the effect on the algal physiology. However, the acidification (pH 2 with 2 M HCl or HNO₃) of the stock solution and culture medium samples taken during the toxicity test can stabilise the metal concentration in solution, avoiding the loss of metal by container walls adsorption.[30] To avoid a pH decrease in the culture medium used in the ecotoxicity test by the addition of the acidified copper solution, the stock solution should be prepared sufficiently concentrated to use only small volumes (<1 mL). The final pH of the culture medium should be measured following the addition of the acidified copper solution.

(5) The use of synthetic metal-chelating agents (i.e. EDTA) in the stock solution and/or culture media was reported in only ∼6% of studies. This is important to report because the toxicity of copper depends of the concentration of its ionic form (i.e. Cu²⁺).[37] The use of synthetic metal-chelating agents in the copper stock can change the form of copper in solution by reacting with Cu²⁺ to form organic complexes (e.g. CuEDTA), which changes the copper bioavailability in the media.[53,54] For instance, the resulting CuEDTA complexes are not directly bioavailable for algal cellular uptake while the non-complexed copper (e.g. Cu²⁺) and inorganic metal complexes (e.g. CuCl₂) are bioavailable for algal uptake.[53] Because the free Cu²⁺ concentration is controlled by the presence or absence of metal-chelating agents in the medium, EDTA (or other complexing reagents) can be used to better dose the bioavailability of copper in studies spanning a very large concentration range of Cu²⁺. The Cu²⁺ concentrations can be calculated using speciation programs such as MINEQL and WHAM.[53]

(6) Analytical measurement of dissolved metal concentrations. Periodic measurements of the actual dissolved metal concentrations during the toxicity test are needed in order to monitor changes due to metal adsorption by container walls and/or target organisms. For example, analytical measurements during an ecotoxicity test showed that copper concentrations decreased by 50% compared to the nominal concentrations due to adsorption onto the container (glassware and plasticware) walls, when the stock solution was not acidified.[36] Determination of dissolved metal concentrations
during experiments can help avoid misinterpretation of the ecotoxicity test results due to overestimation of the actual copper concentration. (7) Wearing gloves and proper protective garments. In order to control contamination by touching or handling samples with bare hands (dried skin may contain metals such as copper and zinc), the analyst must wear polyvinyl chloride or polyethylene powder-free gloves.[30,35] The analysts also should use appropriate garments such as laboratory coat and head and shoe covers made of nylon or polyester that are resistant to acid and will not shed fibres.[35] Contamination may also be induced by material leached from the sampling apparatus, which may contain metallic components, rubber washers, stoppers, and tubing.[33,55] In addition, measuring equipment (e.g. micropipettes) must be regularly checked and calibrated against certified reference materials (CRMs) to guarantee accuracy and precision. This was not considered in the present review because descriptions of the sampling apparatus such as micropipettes (with plastic and metallic components) and conventional pipettes (made of plastic or glass), the use of CRMs and the wearing of gloves and garments are usually omitted in the literature.

Our literature review also indicates that there is a clear increase in the number of studies on copper ecotoxicology of algae since the mid-1970s. In general, microalgae are more often used to perform ecotoxicological research because they are easier to culture under laboratory conditions than macroalgae.[13] For copper ecotoxicology, however, the results obtained in this review indicate that macroalgae were used in preference to microalgae. Of the three Phyla that comprise macroalgal species, Ochrophyta was the main group selected for copper ecotoxicology, probably because this macroalgal group includes the Orders Fucales and Laminariales, which are considered to have a high ability to bind metal ions.[15] The cell wall of members of Ochrophyta are mainly formed by alginic acid (as alginate) and sulphated polysaccharides such as fucoidan.[15,56] These two cell wall components have negatively charged hydroxyl, sulphate and carboxyl groups that are strong metal ion (e.g. Cu^{2+}) complexing sites.[57,58] These characteristics have increased the interest in studying the bioremediation properties of members of the Phylum Ochrophyta.[15,56]

Copper toxicity tests are typically short-term (<5 days), involving the exposure of algae to different copper concentrations.[8,13] The main biological responses measured in copper ecotoxicity tests are growth, photosynthesis, bioadsorption, and bioaccumulation. However, the necessity to standardise toxicity testing, especially for macroalgae, is still in discussion.[8,13,23] The main issues are: (1) definition of biological response (e.g. photosynthesis, development of early life stages) other than growth for long-lived and slow-growing macroalgae and the life stage of the complex life history of the seaweed under investigation[36,59]; (2) standardisation of data reporting (e.g. EC_{50}) for result comparison and interpretation[10,23] because long-term exposure may give a different EC_{50} value compared to short-term exposure[36]; and (3) exposure time of algae to metal during toxicity tests.[36] For example, comparison between meiospores of two kelp species showed a higher EC_{50} for germination of Undaria pinnatifida (Harvey) Suringar compared to Macrocystis pyrifera (Linnaeus) C. Agardh. However, the subsequent development of gametophytes of both species were equally inhibited by all copper concentrations.[36]
Therefore, the use of different biological criteria and exposure times could lead to a misinterpretation of ecotoxicological results.

Most ecotoxicological studies use concentrations higher than those found in the environment because such a wide range of copper concentrations may allow an understanding of the tolerance limit of algae to copper. For example, Leal et al. [36] used concentrations ranging from 54 to 171 µg L\(^{-1}\) \(Cu_T\) to determine ecotoxicological effects of copper on kelp physiology, even though in the Otago Harbour study site copper concentrations were substantially lower (9.5 µg L\(^{-1}\) \(Cu_T\)). Moreover, some algal species have been reported to display increased tolerance to copper due to a constant exposure to elevated copper concentrations.[48,60,61] Therefore, the use of unrealistically high copper concentrations in ecotoxicological studies can also have physiological relevance for the toxicity threshold of copper-tolerant marine algal species.[36]

5. Conclusions

Our review indicates that the knowledge of copper ecotoxicity on algae has increased in the last 39 years. However, the recommended use of trace metal clean techniques during ecotoxicity testing was not followed in \(\sim 70\%\) of the literature reviewed, even though these clean procedures have been available for more than 40 years. Reliable data can only be obtained if the toxicity test is performed using the appropriate labware (i.e. plastic), cleaning procedure (i.e. acid and ultrapure water rinsing), and in a trace metal clean area (e.g. laminar flow hood). Failure to follow these steps and report dissolved metal concentrations bring into question any ecotoxicity test results because the negative effects of copper may be underestimated due to metal contamination and overestimated due to metal loss by adsorption.

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