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# Ostreococcus tauri Luminescent Reporter Lines as Biosensors for Detecting Pollution From Copper-Mine Tailing Effluents in Coastal Environments

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Phytoplankton cells are excellent biosensors for environmental monitoring and toxicity assessments in different natural systems. Green algae, in particular, appear to be more responsive to copper (Cu) disturbances. This is interesting considering that Cu pollution in coastal environments has increased over the last century, with enormous repercussions to marine ecosystems. Unfortunately, no high-throughput method exists for the environmental monitoring of Cu toxicity in seawater. To assess potential uses as biosensors of Cu pollution, high-throughput screening was performed on five luminescence reporter lines constructed in the green algae *Ostreococcus tauri* RCC745. The reporter line expressing the iron storage ferritin protein fused to luciferase (Fer-Luc) was the most sensitive, responding to Cu concentrations in the  $\mu$ M range. Fer-Luc was also the most sensitive reporter line for detecting toxicity in mining-derived polluted seawater predominantly contaminated by soluble Cu. Nevertheless, the Cyclin-Dependent-Kinase A (CDKA) reporter was most suitable for detecting the toxicity of copper-mine tailing effluents containing other metals (e.g., iron). These results highlight that *Ostreococcus* biosensors can serve as a reliable, inexpensive, and automated, high-throughput laboratory approach for performing seawater analyses of coastal areas subjected to metal disturbances. When challenged with Cu, *O. tauri* not only evidenced a rapid, transcriptional response for the tested genes, but also showed changes in a broad range of genes, especially as related to the stress response. Overall, the obtained results reinforce that a single biosensor is insufficient when dealing with complex mixtures of toxic compounds in natural environments.

**Keywords:** copper pollution, mine tailings, biosensors, *Ostreococcus*, ferritin, CDKA, luciferase reporter

## INTRODUCTION

Biosensors contain biological components (e.g., cells) that react to target substances (e.g., pollutants), and these reactions generate an easily readable signal (e.g., photon emission) proportional to the concentration of the compound of interest or of associated by-products (D'Souza, 2001; Balootaki and Hassanshahian, 2014; Kaur et al., 2015). In recent years, phytoplankton cells have proven to be excellent biosensors for environmental monitoring and toxicity assessments (Gutiérrez et al., 2015; Martín-Betancor et al., 2015). This is because phytoplankton cells quickly respond to disturbances in marine environments (Campanella et al., 2001). In fact, whole-cell biosensors based on phytoplankton cells are used to ecologically screen for pesticides and trace metals (Chouteau et al., 2005; Payne, 2013), and the available evidence reveals a broad tolerance range to trace metals in this group (Davis et al., 2006; Wang et al., 2012).

Green algae, in particular, appear to be more responsive to copper (Cu) disturbances than other phytoplankton taxa (Henríquez-Castillo et al., 2015). Therefore, this group of organisms has potential applications in the design of Cu biosensors. Green algae from the genus *Ostreococcus* (Chlorophyta, Mamiellophyceae) are cosmopolitan marine primary producers that dominate eukaryotic pico-phytoplankton communities (i.e., cells < 3 μm in diameter) in several coastal ecosystems (O'Kelly et al., 2003; Collado-Fabbri et al., 2011; Ottesen et al., 2011). These microalgae include different clades (Monier et al., 2016) that are genetically adapted to specific environmental conditions (Rodríguez et al., 2005; Botebol et al., 2017). In particular, *Ostreococcus tauri* is a model photosynthetic organism used to study biological processes (Corellou et al., 2009; Lozano et al., 2014), including cell division (Moulager et al., 2010), circadian clock architecture (O'Neill et al., 2011), iron metabolism (Botebol et al., 2015; Lelandais et al., 2016), and vitamin assimilation (Paerl et al., 2017). These applications as a model organism arise due to unique characteristics, including a small size, compact sequenced genome, and ease of genetic manipulation. The genetically engineered *O. tauri* RCC745, which expresses the cell cycle gene Cyclin-Dependent-Kinase (CDKA) fused to firefly luciferase (CDKA-Luc), is a very sensitive biosensor in high-throughput screenings for herbicides and antifouling compounds frequently found in the marine environment (Moulager et al., 2010; Sanchez-Ferandin et al., 2013). However, the application of *O. tauri* reporter lines as biosensors for Cu has not yet been tested in the laboratory or in natural seawater conditions. Genomic analyses in *Ostreococcus* spp. suggest that Cu is an essential cofactor in cellular processes, such as in photosynthesis, where cytochrome *b* is replaced by the Cu protein plastocyanin (Piganeau et al., 2011), and in iron acquisition, where multicopper oxidase is the only transporting component in the *O. tauri* genome (Palenik et al., 2007).

While Cu is an essential trace metal for marine organisms, toxicity occurs at high levels, with consequent deleterious effects on ecosystems and for public health (Castilla, 1996; Liu et al., 2011; Zhuang et al., 2013; Vásquez et al., 2015). Over the past century, Cu concentrations have increased in several coastal zones worldwide, with notable cases of Cu pollution from mine

tailings (Paytan et al., 2009). Such cases include the open-cut Ok Tedi mine in Papua New Guinea (Fallon et al., 2002; Kirsch, 2007), the Mole River mine in Australia (Ashley and Lottermoser, 1999), the Dabaoshan mining region in China (Lin et al., 2005), and the El Salvador mine in Chañaral Bay, Chile (Andrade et al., 2006). Current protocols for environmental analyses of Cu are based on spectroscopy, including atomic absorption and inductively coupled plasma-mass instruments. Despite being very sensitive and accurate, these methods are time-consuming, require elaborate sample preparation, and, most importantly, are expensive, thus limiting the number of samples that can be analyzed (Liu et al., 2011). There is, therefore, a need to develop low-cost, highly-sensitive, and high-throughput methods for determining Cu toxicity in coastal environments (Feng et al., 2016).

Cu in seawater can be detected in association with suspended particulate material (> 0.45 μm) or as dissolved Cu (< 0.45 μm). Dissolved Cu in seawater can exist in multiple states, including as hydrated ions, in colloidal forms, and complexed with inorganic and organic ligands, the latter of which being the predominant state in aquatic environments (i.e., 99%) (Coale and Bruland, 1988). Free Cu ions and labile complex forms are the most toxic to the biota (Meador, 1991). In contrast, Cu complexation by organic ligands can decrease bioavailability and, hence, potential Cu toxicity (Stauber and Florence, 1985; Gledhill et al., 1997). Discriminating between these Cu forms is analytically complex. Therefore, an appropriate Cu sensor needs to be responsive to the bioavailable fraction of Cu in seawater, which makes any laboratory-based approach difficult because of the inherent difficulties in determining the labile fraction.

In this work, a micro plate-based automated laboratory approach was used to screen five luminescent *O. tauri* RCC745 lines reporting several cellular and physiological processes. The aim of this was to establish potential uses as biosensors of Cu toxicity in the laboratory and in Cu-contaminated seawater samples from the Chañaral area (i.e., south-eastern South Pacific). This coastal area of northern Chile has been the discharge site for thousands of tons of Cu mine-derived waste for the last 60 years (Correa et al., 1999). Available evidence from this highly polluted site indicates that the labile fraction of Cu accounts for most of the toxicity in macro- and microorganisms (Medina et al., 2005; Andrade et al., 2006; Morán et al., 2008; De la Iglesia et al., 2012). After initial biosensor selection, the transcriptional responses of *O. tauri* to a Cu challenge were measured. *O. tauri* evidenced a rapid expression of the tested genes and changes in a broad range of genes related to the stress response. Overall, the obtained results highlight that *Ostreococcus* biosensors can be used as a reliable, inexpensive, and automated high-throughput laboratory approach for analyzing seawater from coastal areas subjected to metal disturbances.

## MATERIALS AND METHODS

### Test Conditions for *Ostreococcus* Growth Inhibition

Wild-type *O. tauri* RCC745 (clade C) was grown in T25 aerated flasks (Sarstedt) in artificial seawater media supplemented

with Keller nutrients (0.88 mM nitrate, 22  $\mu$ M phosphate, and f/2 vitamins) (Keller et al., 1987). Trace metals were added without EDTA (Botebol et al., 2015). Copper was added as a Cu sulfate (20 nM final concentration). Exponential phase cultures were transferred onto 24-well plates at a cell density of 1 million cells mL<sup>-1</sup> and incubated with 20 nM, 40 nM, 0.2  $\mu$ M, 0.4  $\mu$ M, 2  $\mu$ M, 4  $\mu$ M, and 40  $\mu$ M of Cu sulfate. Each concentration was tested in triplicate using independent wells. Subsamples (20  $\mu$ L) were taken, diluted in 180  $\mu$ L of Keller media with glutaraldehyde (0.25% vol/vol final concentration), and stored at -20°C. Cells were counted by flow cytometry (Accuri C6 flow cytometer, BD) using a 488 nm excitation laser with detection through side scatter and red fluorescence, using log-scale amplifiers. After 24 h, Cu toxicity was expressed as the concentration of Cu that induced a 50% reduction in cellular growth, relative to the Cu-free control (50% effective concentration [EC<sub>50</sub>]). For each Cu treatment, cell abundances were normalized against the control samples. The percentages were then plotted against the logarithm of Cu concentrations. Regression analysis was used to determine EC<sub>50</sub> values (Prism 6.0, GraphPad).

### Test Conditions for *Ostreococcus* Luminescence Inhibition

*O. tauri* RCC745 lines engineered with a luciferase gene reporter system were grown until the exponential phase, as described in Sanchez-Ferandin et al. (2013). The following *O. tauri* RCC745 lines were used: (1) pHAPT::Luc, a high-affinity phosphate transporter transcriptional reporter (promoter of the gene fused to luciferase); (2) CCA1-Luc, a circadian clock gene translational reporter (full gene in frame fused to luciferase); (3) CDKA-Luc, the cell cycle controller Cyclin-Dependent Kinase A translational reporter; (4) Fer-RI-Luc, an iron-storage ferritin protein translational reporter (random insertion at the ferritin locus of the Fer-Luc transgene); and (5) Fer-RH-Luc, an iron-storage ferritin protein translational reporter (homologous recombination at the ferritin locus of the Fer-Luc transgene) (Corellou et al., 2009; Moulager et al., 2010; Djouani-Tahri et al., 2011; Lozano et al., 2014; Botebol et al., 2015). Exponential phase cells were transferred into a 96-well microplate at final densities of 5 × 10<sup>6</sup> cells mL<sup>-1</sup>. Luciferin (10  $\mu$ M final concentration) was added to the microplates, and then Cu was added at the following final concentrations: 0.4, 1, 2, 3, 4, 6.5, 10, 20, and 40  $\mu$ M. Each Cu concentration was tested in triplicate using independent wells. Control samples containing 20 nM of Cu (i.e., minimum concentration not affecting growth kinetics) were also included in triplicate. Luminescence linked to the expression of the constructs was monitored every hour for 30 h using a high-throughput luminometer (Berthold Technologies). After 24 h, Cu toxicity was expressed as the Cu concentration that induced a 50% reduction in luminescence relative to the control (i.e., EC<sub>50</sub>). Luminescence curves of the treatments were normalized against control samples. The percentages were then plotted against the logarithm of Cu concentrations. Regression analysis was used to determine EC<sub>50</sub> values (Prism 6.0, GraphPad).

### Ecotoxicology Test Using Natural Seawater From a Mine-Tailing Polluted Area

Seawater samples were collected in March 2013 from the Chañaral area (Figure S1) using metal-free Kemmerer bottles placed at a 1 m depth. The Chañaral area is heavily disturbed due to Cu mine tailings, with Cu levels being well-characterized (Andrade et al., 2006). Six sites were sampled, including one directly affected by riverine Cu discharge (i.e., Chañaral, Palito 200, Playa Palito, Canal Palito, and La Lancha) (Figure S1). Due to low Cu levels, the Playa Blanca site (15 km north of the Canal Palito site) was used as a reference site (Andrade et al., 2006; Henríquez-Castillo et al., 2015).

For ecotoxicology tests, the *O. tauri* CDKA-Luc and Fer-RI-Luc luminescent reporter lines were pre-acclimated in seawater supplemented with Keller nutrients. Late exponential phase cells were transferred onto 96-well microplates containing seawater dilutions for the six sample sites (5 × 10<sup>6</sup> cells mL<sup>-1</sup> final densities). For this, seawater from each polluted site was diluted using seawater from the reference site (i.e., Playa Blanca). The following dilutions were used: 25 mL of polluted seawater with 150 mL of reference seawater (25/100), 50/125, 75/100, 87.5/87.5, 100/75, 125/50, 150/25, and 175/0. In the case of the seawater from Canal Palito, salinity was previously adjusted down using MiliQ water to values for the reference seawater (Playa Blanca). All seawater dilutions used in treatments were enriched with Keller nutrients plus Cu sulfate (20 nM) and iron-citrate (0.1  $\mu$ M). Luciferin (10  $\mu$ M final concentration) was added directly to the plates. Luminescence of the reporter lines was measured every hour for 60 h with a high-throughput luminometer (Berthold Technologies). After 48 h, seawater toxicity was expressed as the dilution of polluted seawater in reference seawater that induced a 50% reduction in luminescence relative to the control (i.e., with no polluted seawater). Luminescence curves of the treatments were normalized against control samples. Percentages were then plotted against the logarithm of the dilution.

### Cu Concentration in Seawater From Polluted Sites

For determinations of total dissolved metals, seawater samples were acidified to pH < 2 with bidistilled ultra-pure grade HNO<sub>3</sub> in a class-100 HEPA laminar flowhood. Concentrations of metals (i.e., Ag, Cd, Co, Cu, Fe, Mo, Ni, Pb, V, and Zn) were determined by inductively coupled plasma mass spectrometry (XSERIES 2 spectrophotometer, Thermo Fisher Scientific) after pre-concentration using the APDC/DDDC organic extraction method (Bruland et al., 1979; Tovar-Sánchez, 2012). Furthermore, labile Cu concentrations (Cu<sub>ASV</sub>) were determined by square wave anodic stripping voltammetry (SW-ASV) as described in Andrade et al. (2006).

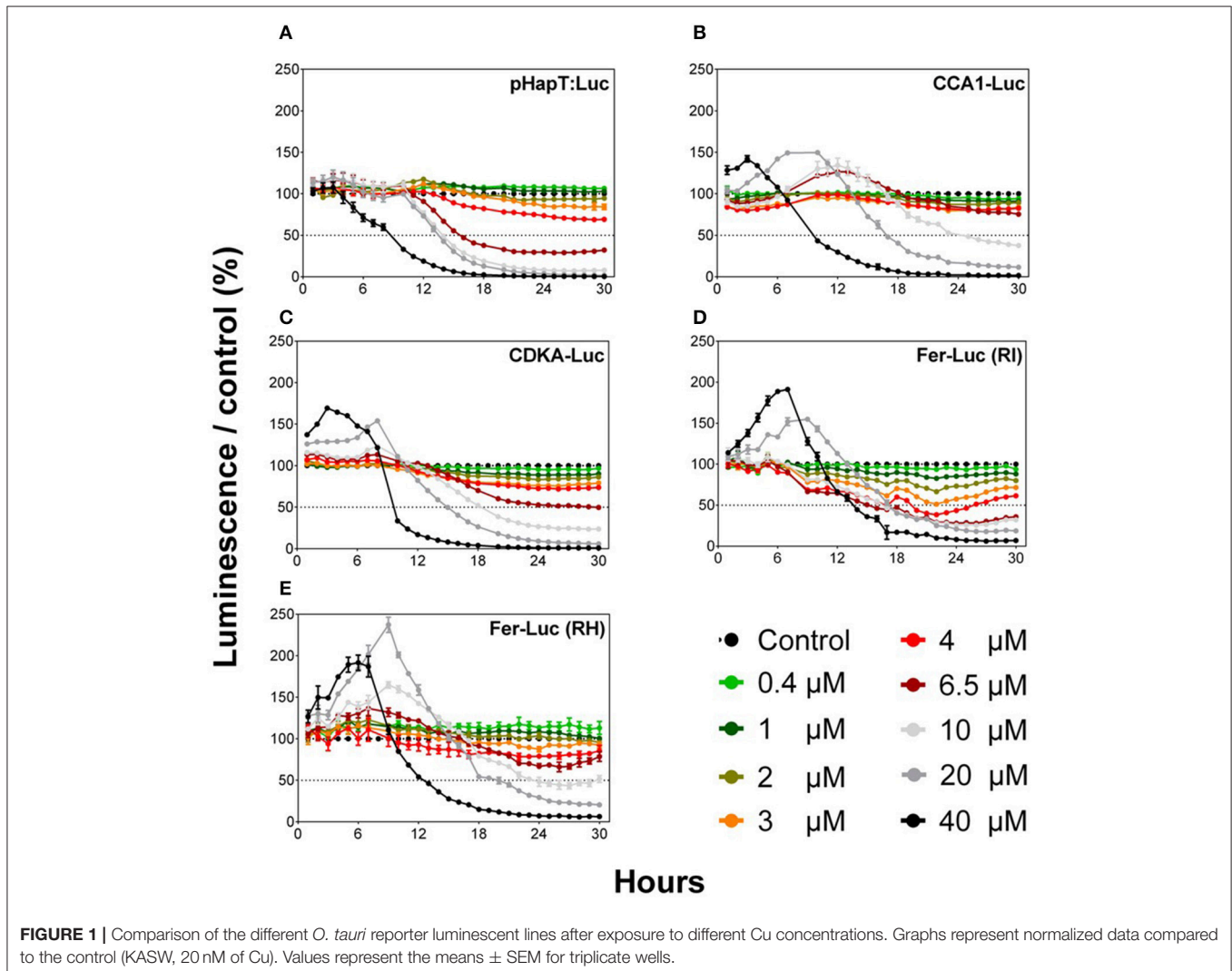
### Transcriptomic Analyses of *Ostreococcus*

*O. tauri* RCC745 cultures were grown until the exponential phase (15 × 10<sup>6</sup> cells/mL) in T175 aerated flasks under a 12:12 light-dark cycle (20  $\mu$ mol photons cm<sup>-2</sup> s), transferred on 27 T75

flasks (80 mL each), and incubated with a final Cu concentration of 0.4 or 2  $\mu\text{M}$ . Control samples containing 20 nM of Cu were also included in triplicate. To obtain cell counts, 20  $\mu\text{L}$  triplicate samples were collected 1, 4, and 12 h after the addition of Cu. These samples were diluted in 180  $\mu\text{L}$  of Keller medium with glutaraldehyde (0.25% vol/vol final concentration) and stored at  $-20^\circ\text{C}$ . Cells were counted by flow cytometry (Accuri C6 flow cytometer, BD) using a 488 nm excitation laser with detection through side scatter and red fluorescence, using log-scale amplifiers. For RNA extraction, the remnant culture volume was split into two 50 mL polypropylene tubes. Pluronic F68 was added (0.01% vol/vol final concentration) and centrifuged for 30 min at  $12,000 \times g$ . Cell pellets were frozen in liquid nitrogen and stored at  $-20^\circ\text{C}$  until RNA extraction.

Total RNA was extracted from frozen cell pellets using the TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA concentrations were determined by absorption at 260 nm, and purity was evaluated by the 260/280 nm absorption ratio. Total RNA integrity was assessed using an Agilent 2100 Bioanalyzer. RNAseq libraries were

constructed using the TruSeq Stranded mRNA Library Preparation Kit (Illumina) by the NGS Service FASTERIS (Switzerland). Libraries were multiplexed in one channel of  $1 \times 50$  bp run on a MiSeq run for titration and quality control. Finally, the libraries were sequenced using an Illumina HiSeq 2000 sequencer. Raw image data were converted into sequence data by base-calling, defined as raw reads. Pandaseq was used to assemble the paired-end sequences with default values. All sequences were searched for homologies against the KEGG database and against the most recent *O. tauri* genome annotation (Blanc-Mathieu et al., 2014) using the DIAMOND protein aligner (Buchfink et al., 2014). The DEseq2 routine (Love et al., 2014) was applied for comparisons between treatments. Genes with absolute  $\text{Log}_2\text{FC}$  values over 0.3 and p-adjusted values below 0.1 were selected for further analysis. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE114058 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114058>).



## Statistical Analyses

All statistical analyses were performed in R v.3.4.1. Dissimilarity matrices were created using Euclidian distances. Non-metric multidimensional scaling was performed using the isoMDS function in the MASS package. Heatmaps were generated using the Heatmap, Vegan, and gplots packages.

## RESULTS

### Responses of Different *Ostreococcus* Luminescent Reporters to Cu Amendment

Five *O. tauri* luciferase reporter lines were tested using an automated microplate approach to determine potential applications as biosensors for Cu toxicity. These lines were pHAPT::Luc, CCA1-Luc, CDKA-Luc, Fer-RI-Luc, and Fer-RH-Luc. The addition of Cu resulted in a differential response of the reporter lines, with an initial increase in luminescence for first 6 h post-exposure, followed by dose-dependent decreases (Figure 1). For all lines, excepting pHAPT::Luc, this initial luminescence increase occurred at the highest Cu concentration tested. Clear dose-dependent kinetic responses were observed, with a maximal decrease in luminescence after 24 h of Cu exposure (Figure 1). EC<sub>50</sub> values for Cu were calculated for each line 24 h after Cu addition (Table S1). EC<sub>50</sub> values varied among the different lines, but ranged between 4 and 10 μM. Fer-RI-Luc was the most Cu-sensitive luminescent line, with an EC<sub>50</sub> of 3.83 μM after 24 h.

### Response of Fer-RI-Luc and CDKA-Luc Lines to Cu-Polluted Seawater

The sensitivities of Fer-RI-Luc and CDKA-Luc were tested using natural seawater collected from five Cu-mining polluted sites in the Chañaral area (i.e., Chañaral, Palito 200, Playa Palito, Canal Palito, and La Lancha), as well as from an unpolluted reference site (Playa Blanca) (Figure S1). The seawater from all polluted sites showed high levels of both total dissolved Cu (> 0.1 μM) and labile Cu (> 0.01 μM) (Table S2). Variable concentrations of Cu, Fe, Zn, Pb, V, Ni, and Co were found among sites (Figure 2A), with a clear difference between polluted sites and the reference site, as revealed by non-metric multidimensional scaling analysis of metal concentrations (Figure 2B). Similar concentrations of trace metals, other than the two Cu fractions, were found between La Lancha and the reference site (paired *t*-test, Cu, labile Cu; *P* < 0.001).

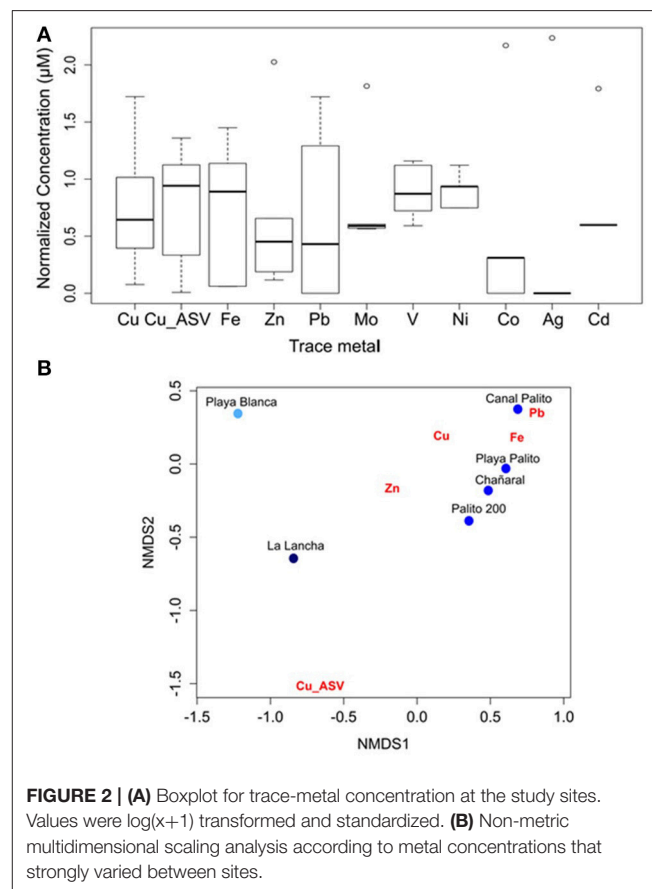
Cu-polluted seawater had negative, dose-dependent effects on the Fer-RI-Luc and CDKA-Luc reporter lines (Figure 3). The Fer-RI-Luc line showed a dual response to Cu additions, with an initial increase in luminescence followed by a dose-dependent decline for almost all treatments (Figures 3A,E). In contrast, CDKA-Luc luminescence decreased immediately after the addition of Cu amendments (Figures 3B,D,F).

### Transcriptional Response of *Ostreococcus tauri* to Cu Challenge

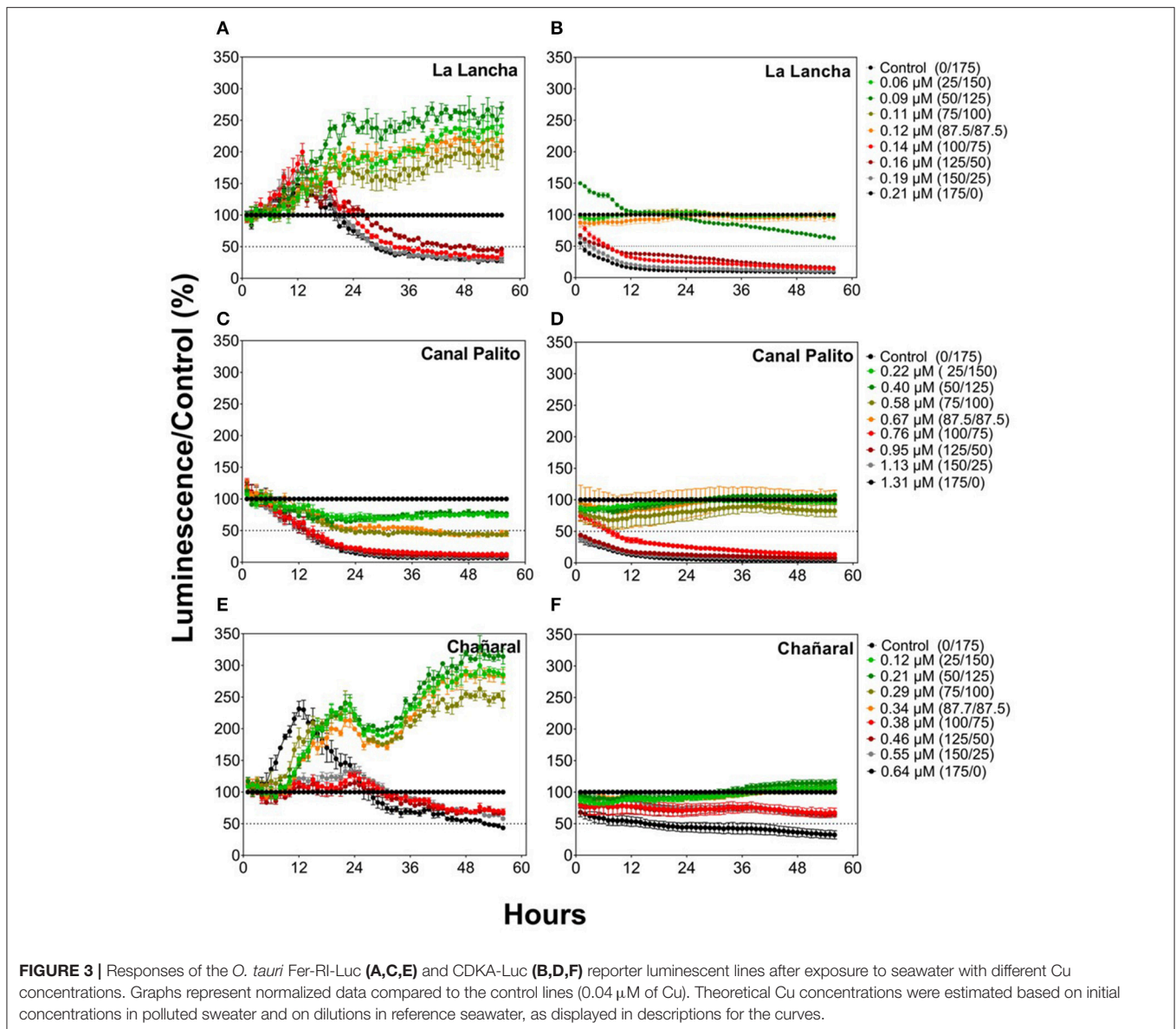
To study the effect of increased Cu concentrations on gene expressions in the luminescent reporter lines, transcriptional

responses were studied through an RNAseq-based approach. Growth-curve analysis showed that the addition of 0.4 and 2 μM of Cu inhibited growth by 30 and 60%, respectively, at 24 h post-Cu amendment addition (Figure S2). Based on this, high-throughput transcriptomic analysis was performed at 1, 4, and 12 h post-Cu addition. General patterns of gene expression were found (Figure 4; Table S3). Significant changes in gene expression were observed 4 h post-Cu exposure, especially for the 2 μM treatment (Figure 4, cluster 2).

Expressional changes were evaluated for genes used in the ecotoxicology tests and for genes involved in the regulation of Cu homeostasis (Figure 5). HapT (OT\_ostta02g02460) and CCA1 (OT\_ostta02g04817) were upregulated in all conditions in response to Cu, especially in the 0.2 μM Cu treatment. While ferritin transcripts (OT\_ostta02g03400) were significantly upregulated at 4 and 12 h after the addition of the 2 μM Cu treatment, CDKA transcripts (OT\_ostta15g00670) were upregulated only at 0.2 μM (Figure 5). Overall, CDK transcript levels decreased in response to the addition of Cu. Genes related to Cu homeostasis, such as Superoxide Dismutase Cu/Zn-dependent (SOD1), the respective SOD1 Chaperone (CCS), and Cu-exporting ATPase (copB), displayed various expression patterns. Some were downregulated (e.g., copA), while others appeared upregulated (e.g., SOD1).



**FIGURE 2 | (A)** Boxplot for trace-metal concentration at the study sites. Values were  $\log(x+1)$  transformed and standardized. **(B)** Non-metric multidimensional scaling analysis according to metal concentrations that strongly varied between sites.

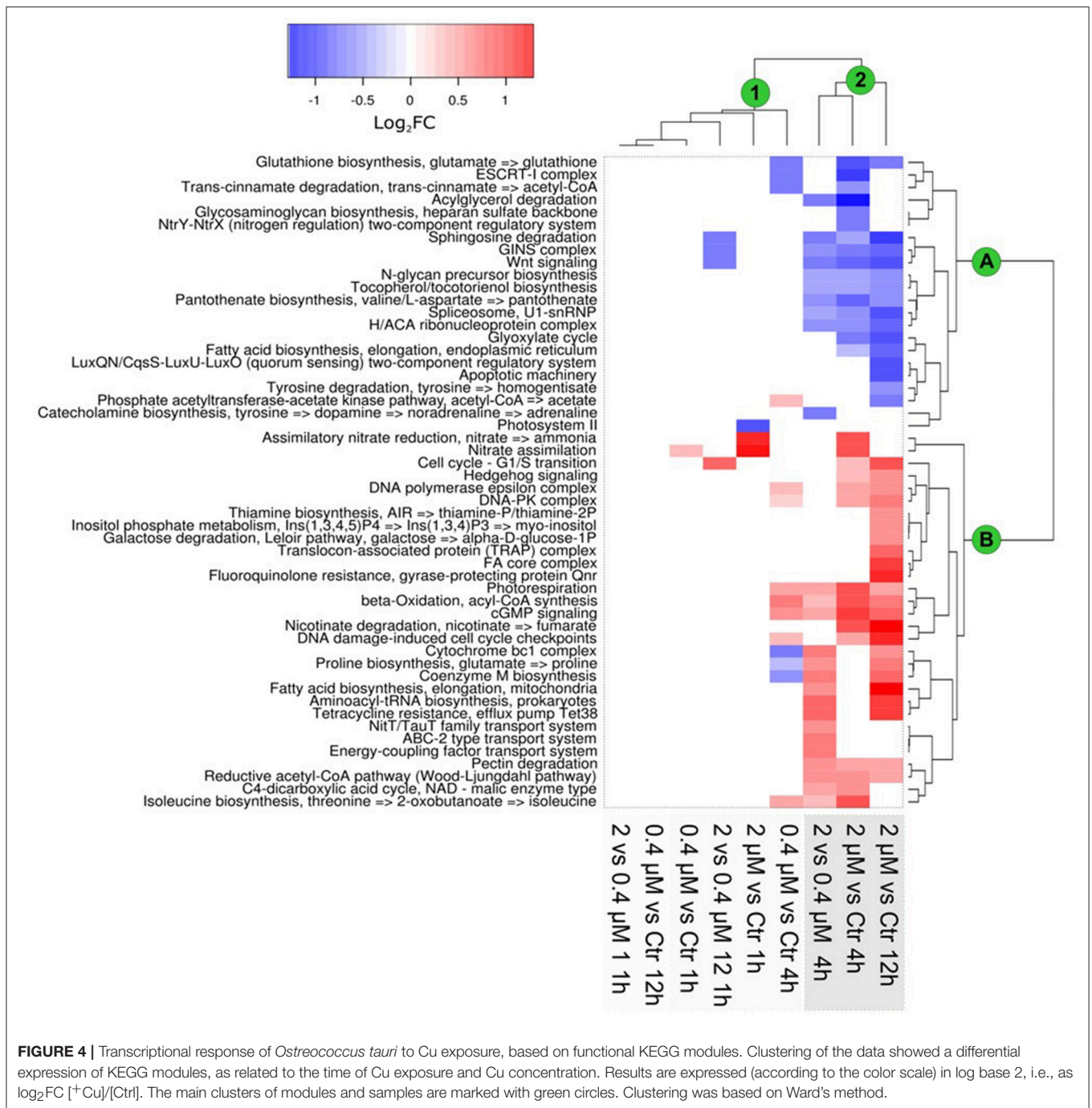


## DISCUSSION

Five different *O. tauri* luminescent lines were analyzed to determine applicability as a high-throughput system for Cu monitoring in coastal environments. Luminescent lines included a broad spectrum of genes and genetic constructions involved in different biological processes, including the circadian clock (CCA1), cell division (CDKA), metal homeostasis regulation (Fer), and phosphate transport (*HapT*) (Moulaguer et al., 2010; Sanchez-Ferandin et al., 2013). The obtained results indicate that increases in Cu exert global effects on gene expression, as reflected by the physiology of *O. tauri*. Impacts to gene expression were particularly notable for the Fer-RI-Luc iron-related luminescent line, which was the most effective in sensing toxicity in natural seawater samples predominantly contaminated by labile Cu. The differences in sensitivity between

the Fer-RI-Luc and the CDKA-Luc lines highlight the need to use different biosensors when environmental pollutants are mixed, as usually occurs. Importantly, the conducted assays required small amounts of polluted seawater and non-complex sample preparation, thus facilitating the combination of multiple luminescence reporter lines to accurately detect metal pollution. The feasibility of assays, in terms of cost and time, represents a valuable contribution to the environmental monitoring of coastal areas. The presently tested luminescent biosensors showed major advantages compared to traditional assessment tests based on cell-growth inhibition. Specifically, the monitoring process is fully automated and can provide rapid, low-cost responses while using a high-throughput approach applicable to routine coastal pollution studies.

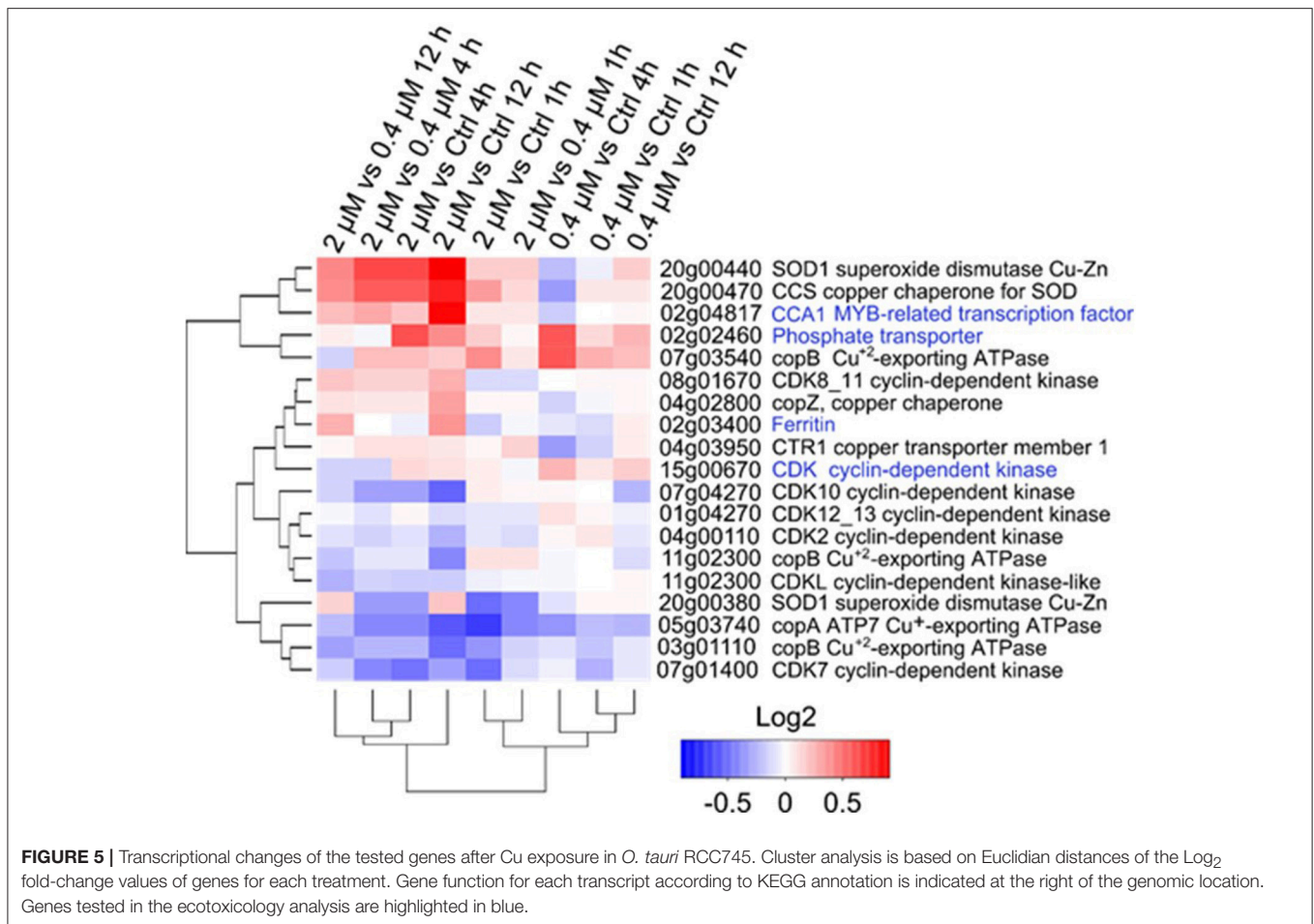
All luciferase reporter lines displayed dose-dependent growth inhibition in response to Cu amendments. These results suggest



that Cu has a global toxic effect on the physiology and growth of *O. tauri*, a finding similar to prior reports for other phytoplankton species (Davis et al., 2006; Stuart et al., 2009; Jordi et al., 2012). Of the lines tested, Fer-RI-Luc was the most sensitive to Cu. This is in line with the nutritional coupling between Cu and Fe reported for other microbial species (Crichton and Pierre, 2001; Peers et al., 2005; Maldonado et al., 2006; Sunda, 2012), a coupling that can lead to nutritional imbalance at high Cu concentrations. Fer-RI-Luc construction was also highly sensitive when *O. tauri* was challenged with Cu-polluted seawater. Indeed,

the minimum detection level was  $0.06 \mu M Cu L^{-1}$ , one of the lowest detection limits reported to date for a Cu whole-cell biosensor (Tag et al., 2007; Jarque et al., 2016). For the La Lancha site, where Cu was the only metal to evidence significant differences with the reference site, soluble copper, in a dose-dependent manner, accounted for seawater toxicity. The Fer-RI-Luc line could, therefore, be a sensitive biosensor for Cu toxicity when Cu is the sole source of pollution. However, when this luminescent line was tested with the riverine discharge water, luminescence inhibition was observed for all of the tested





concentrations. Therefore, the recorded luminescence decrease cannot be attributed to only dose-dependent Cu effects. Water coming from the riverine discharge point contained a complex mixture of metals, including Mo, Ni, Co, Ag, Zn, Cd, and Pb. Metal interactions may account for the increased water toxicity at the riverine discharge site (Franklin et al., 2002). The CDKA-Luc reporter showed decreased luminescence for all tested polluted seawater samples, with dose-dependent decreases aligning with earlier data showing that the CDKA-Luc line is a sensitive biosensor of general toxicity (Sanchez-Ferandin et al., 2013). These results indicate that, when dealing with complex metal complexes such as those near the riverine discharge site, the CDKA-Luc line is more suitable to detect seawater toxicity. Overall, the obtained results reinforce the idea of combining several reporter lines, such as CDKA-Luc and Fer-RI-Luc, to discriminate between general and specific Cu toxicity in polluted seawater.

*O. tauri* showed a strong transcriptional response to Cu amendments, as inferred from RNAseq data. Prior analyses on the main KEGG modules during the initial response to Cu amendments revealed a transcriptional inhibition of genes involved Photosystem II and nitrogen metabolism (Harrison et al., 1977; Harismendy et al., 2009). Later responses to Cu

amendments included a well-defined group of overexpressed genes, some of which were related to the general stress response, such as the DNA damage-induced cell cycle checkpoint. Gene groups inhibited at the end of the experiments included genes related to cellular growth and biosynthesis. RNA-seq analysis confirmed the luminescence patterns for ferritin, which was induced soon after Cu addition before being downregulated later. For other genes, such as CCA1, hapT, and CDK, luminescence responses and expression patterns were quite different, and no clear dose-dependent response was observed. These differences may be due to the nature of the CDKA-Luc line, which reports CDKA protein levels vs. transcript levels in RNAseq data. Unlike RNAseq data, the luminescence curves established clear Cu dose-dependent responses of luminescence inhibition at 24 h. In addition to being low-cost, luminescent biosensors appear more sensitive, being able to detect toxicity in a dose-dependent manner.

Coastal pollution is increasing in step with expanding human activities. Despite the high impact of metallic pollutants on the environment, current tools for determining metal toxicity in coastal areas are scarce, expensive, and far from being routinely applied. The Chañaral area in northern Chile provides an excellent natural laboratory for understanding the complex

repercussions of Cu pollution on aquatic and coastal ecosystems (Medina et al., 2005; Andrade et al., 2006; De la Iglesia et al., 2012). The northward current in this area generates a gradient in Cu concentrations, being highest at the riverine discharge site and decreasing in proximity to the northern reference site. A decrease in the complexity of the metal mixtures was also observed in a gradient from the discharge point to the reference site, thus permitting evaluations on the response of biosensors to different metal mixtures in natural marine samples. In this work, seawater from the most polluted site (i.e., La Lancha) was used to determine the effect of total dissolved Cu and labile dissolved Cu on two luciferase reporter lines, without interference from other metals present in seawater from the polluted sites located in the vicinity of the riverine discharge site.

## CONCLUDING REMARKS

In this study, *O. tauri* biosensors were useful for studying Cu toxicity in seawater matrices. The presented data suggest that, when dealing with complex environmental samples, a combination of several reporter lines is required to assess seawater toxicity. Further interference and selectivity investigations are needed to fully understand responses to Cu by genetically engineered *O. tauri* biosensors and to develop

more specific, sensitive biosensors based on the identification of Cu-responding genes, e.g., as derived from RNAseq approaches.

## AUTHOR CONTRIBUTIONS

RD, F-YB, and CH-C design the study. CH-C, RD, NT, and SA collected the samples. CH-C, AM, HB, J-CL, GL, and SA performed the experiments and data analysis. CH-C, RD, NT, SR-F, and GL perform bioinformatic analyses. All authors participate in writing the paper.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fenvs.2018.00022/full#supplementary-material>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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