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Copper toxicity as stressor in *Chaetoceros calcitrans* under different salinity levels: Growth inhibition, ROS accumulation, and alterations in fatty acid and PUA profiles

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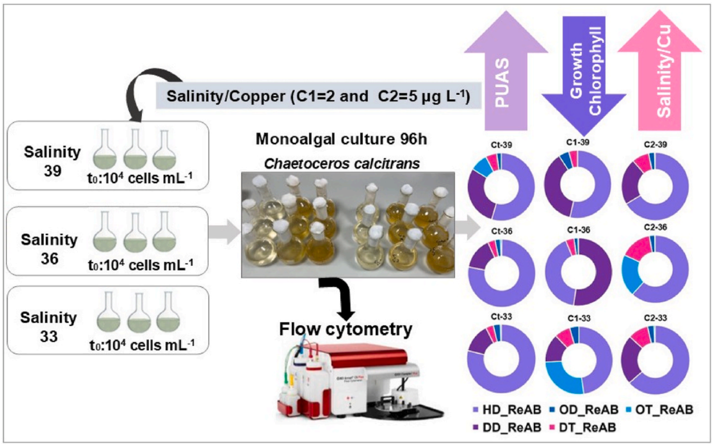
HIGHLIGHTS

- Maximum growth rate of *Chaetoceros calcitrans* occurs at salinity 39 without exposure to Cu.
- Cu at 5 μg L^{-1} inhibited growth and Chl-fluorescence at salinity 33.
- ROS production peaked under combined low salinity 33 and 5 μg L^{-1} Cu exposure.
- Higher salinity partly reduced Cu toxicity, suggesting protection from metal stress.
- *C. calcitrans* exhibited high SFAs, elevated MUFAs and reduced PUFAs under Cu and salinity.

GRAPHICAL ABSTRACT



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ABSTRACT

The sensitivity of marine microalgae to metal contamination, particularly copper (Cu), is well recognized in ecotoxicology, providing key insights into predicting marine pollution impacts. This study examined the combined effects of Cu toxicity and salinity on the diatom *Chaetoceros calcitrans* under controlled laboratory conditions. Toxicity assays were conducted using two environmentally relevant Cu concentrations (2 and 5 μg L^{-1}) and three salinity levels (33, 36, and 39) over 96 h, with cell responses analyzed via flow cytometry. Specific growth rate (SGR), chlorophyll fluorescence, fatty acid methyl ester (FAME) composition, polyunsaturated aldehydes (PUAs), and reactive oxygen species (ROS) production were evaluated. Results showed that SGR increased from 1.7 d^{-1} at salinity 33 to 1.9 d^{-1} at 39, indicating enhanced growth at higher salinity. However, Cu

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exposure significantly reduced SGR, particularly under 5 μg L^{-1} Cu at salinity 33, where SGR dropped to 0.86 d^{-1}. ROS levels were highest under the combined low salinity (33) and 5 μg L^{-1} Cu treatment, suggesting strong oxidative stress. PUA profiles shifted with Cu and salinity, with stress-related compounds such as 2*E*,4*E*/*Z*-decadienal and 2*E*,4*E*/*Z*,7*Z*-decatrienal increasing at low salinity. Fatty acid analysis revealed marked rises in saturated fatty acids, especially palmitic acid (C16:0) and myristic acid (C14:0), under Cu exposure. In the Cu2/39 treatment, C16:0 reached 1.34×10^4 fmol $cell^{-1}$ compared to 1.36×10^3 fmol $cell^{-1}$ in the control. Monounsaturated fatty acids, notably palmitoleic acid (C16:1), also increased, reaching $7.\ 46 \times 10^3$ fmol $cell^{-1}$ versus 1.71×10^3 fmol $cell^{-1}$ in the control. Conversely, polyunsaturated fatty acids, including arachidonic acid (C20:4) and eicosapentaenoic acid (C20:5 n-3), decreased significantly following Cu exposure. Overall, elevated salinity mitigated Cu-induced growth inhibition but intensified oxidative and biochemical stress, underscoring the complex, endpoint-specific responses of *C. calcitrans* to multiple environmental stressors.

1. Introduction

In marine ecosystems, copper (Cu) acts as a vital micronutrient for marine microalgae, serving as an inorganic cofactor in redox proteins that are key to metabolic processes such as photosynthesis and respiration (Kropat et al., 2015). However, the persistence and stability of this metal in the environment can provoke negative effects on marine species and it has implications for ecosystem health (Mu et al., 2018; Du et al., 2024). Like other bioactive metals, free cupric ion Cu^{2+} which is the most toxic and bioavailable form of Cu’ can form inorganic complexes with CO_3^{2-}, OH^-, and Cl^-(Du et al., 2024). Additionally, it can produce organic complexes with organic ligands such as thiols, exopolysaccharides and humic substances (Abualhaija et al., 2015). Their concentration can vary as a function of the marine environment conditions (Benedict et al., 2025), and differ from open ocean to nearshore waters (Leng et al., 2023). Natural concentrations of Cu in coastal seawater generally range between 0.008 and 0.050 Mm (Santiago-Díaz et al., 2023). Nevertheless, human activities that continuously impact marine ecosystems, introduce various pollutants including Cu, especially in coastal areas, increasing these ranges. These activities are derived primarily from nearby mines, harbors, and industrial sites, resulting in local increase in Cu concentrations (Ayu et al., 2016; Lagerström et al., 2018). For example, in the Vigo estuary, Cu levels ranged from 0.7 to 6.9 μg L^{-1} across 18 sampling points from the Cíes Islands to the Rande Strait (Pérez-Cid et al., 2021).

These changes in seawater chemistry have the potential to affect the physiological processes of marine organisms including microalgae. Toxic effects of Cu on marine life have been well documented, even at low concentrations (Debelius et al., 2009; Morin et al., 2017; Kaamoush et al., 2023). Cu concentrations have a negative impact on microalgal growth rate, impairing photosynthesis and respiration, and altering cell and organelles morphology (Cavalletti et al., 2022). For instance, Cu was identified as a highly toxic metal, with an EC_{10} of 3.3 μg L^{-1} for *Phaeocystis antarctica* (Koppel et al., 2021). Any perturbation in marine primary producers can, in turn, affect the quantity and nutritional content of food available to consumers with negative implications for the rest of trophic levels (Gonçalves et al., 2023).

In coastal areas, Cu can reach higher concentrations, such as surface waters of the Pacific and Atlantic Oceans, potentially influenced by various external factors including aerosol deposition and river discharge (Roshan and Wu, 2015; Boiteau et al., 2016). Diatoms, one of the most abundant planktonic primary producers in these regions (Vincent and Bowler, 2020), may be particularly sensitive to Cu fluctuations (Kong and Price, 2020, 2022). Changes in the basal resources provided by diatoms will affect their nutritional quality for consumers, particularly the concentrations of long-chain polyunsaturated fatty acids (PUFAs) (Napolitano, 1999; Taipale et al., 2013) that are synthesized de novo by algae and are essential in animal diet. This can induce the generation of reactive oxygen species (ROS) (Ajitha et al., 2021). Excessive ROS disturb redox homeostasis and damage cell components in microalgae, leading to an induction of cell death and releasing various metabolites (Nowicka, 2022). In fact, over the past decades, diatoms have been demonstrated to release a diverse array of secondary metabolites, derived from PUFA lypoxidation, called polyunsaturated aldehydes (PUAs), which are produced via a wound-activated mechanism when the cell is damaged by grazers (Pohnert, 2000; Jüttner, 2005), lysed by several causes (Brussaard et al., 1995; Casotti et al., 2005) or at senescence conditions (Vidoudez and Georg Pohnert, 2008; Zhu et al., 2023). PUAs are highly reactive molecules that, in most cases, induce a significant decrease in the reproductive success of predators, such as copepods (Caldwell, 2009; Lauritano et al., 2012; Brugnano et al., 2016) but also affect to other primary producers (Ribalet et al., 2007; Franzé et al., 2018). Numerous studies conducted in both controlled cultures and natural environments have demonstrated that the type and amount of PUAs released by diatoms are influenced in part by environmental conditions (Ribalet et al., 2009; Ribalet et al., 2014; Chen et al., 2023).

The interaction between metal toxicity and environmental factors, such as salinity, is crucial for understanding the ecological impacts on marine microalgae. The toxicity of metals to microalgae is influenced not only by their concentration but also by their speciation, which is affected by environmental conditions and the presence of co-existing substances (Hu et al., 2016; Schuler and Relyea, 2018). In particular, salinity affects the chemical speciation of metals, which in turn, affects their bioavailability and change metal uptake and cellular response (Wang et al., 2021; Amaro et al., 2023). For example, changes in salinity affect organism physiology by influencing cellular membrane potentials and modifying major ion influx and efflux mechanisms, which are critical for maintaining homeostasis (Glover et al., 2016). Toxicity of Cu to marine microalgae depends not only on ion concentration but also on variations in environmental factors (Schuler and Relyea, 2018). Thereby, interaction between Cu and salinity could alter their toxicity (Gensemer et al., 2002) and accumulation capacity. Moreover, Hassan et al. (2021) found that an increase of salinity adversely affects the uptake rate of some metals by the halophilic alga *Dunaliella salina.* Thus, it is important to understand how variations in salinity might jointly influence the toxicity of Cu to marine microalgae. Such variations are expected to occur in the context of climate change, making it vital to study and understand how these populations respond under such conditions, especially when considering the potential presence of contaminants in their habitat.

The combined effects of Cu exposure on monoalgal cultures under varying environmental conditions have not been studied comprehensively. To address this knowledge gap, we hypothesized that the interaction between Cu and salinity induces structural and functional alterations in microalgae during short-term exposure, which may lead to the release of metabolites and modification of their nutritional value for consumers. We investigated the effects of salinity and Cu toxicity on the coastal diatom *Chaetoceros calcitrans* by analyzing its polyunsaturated fatty acids (PUFA) and polyunsaturated aldehyde (PUAs) profiles, along with various physiological parameters, including specific growth rate, chlorophyll fluorescence, and oxidative stress, as indicated by reactive oxygen species (ROS) production.

2. Material and methods

2.1. Microalgae culture

The microalga *Chaetoceros calcitrans* (Paulsen) H. Takano (Bacillariophyta) (strain CCMM 07/0101) was selected from the Marine Microalgae Culture Collection at the Institute of Marine Sciences of Andalusia (CSIC-ICMAN) in Puerto Real, Spain. Monoalgal cultures were maintained in sterile f/2 plus silica (50 mg L^{-1}) medium at 20 °C within a climatic chamber under continuous white light (approximately 30 μmol photons m^{-2} s^{-1}). Cells were handled under sterile conditions in laminar flow chambers (Telsar AV-100). Cultivation occurred in 200 mL glass Erlenmeyer flasks, using 0.20 μm filtered and autoclaved seawater sourced from the Bay of Cádiz (36°32′33″N, 006°16′46″W). The medium was prepared without EDTA or trace metals to prevent the presence of chelating molecules. Seawater salinity was adjusted by dilution with Milli-Q water or evaporation through heating and monitored using an electronic refractometer (HI96822 Refracto-Meter, Hanna). The selected species were incubated at a salinity of 36, in accordance with laboratory culture standards, for two weeks before the experiment. Salinity is reported as a dimensionless value, according to the Practical Salinity Scale (PSS-78), and therefore no unit is given.

2.2. Chemical preparation

Copper (II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$, Sigma) was used to prepare initial stock solutions at a concentration of 5 mg L^{-1}. Two concentrations were tested (2 and 5 μg L^{-1}) to assess the toxic effects of Cu on *C. calcitrans*. These concentrations were chosen based on previous literature (Joonas et al., 2021), and prepared from stock solutions with ultra-pure Milli-Q water. Actual concentration of the stocks solutions is routinely checked in the lab by ICP-MS (Thermo Scientific, model iCAP Q). Background levels of Cu in water sampled in the same zone of the sampling for this experiment is around 14 μg L^{-1}.

2.3. Experimental design

Acclimated monoalgal cultures of *C. calcitrans* in the exponential growth phase were used for the experiments. The initial concentration of *C. calcitrans* was set at 10^4 cells mL^{-1}, following established standard toxicity test procedures (APHA-AWWA-WPCF,; ISO, and OECD). Cultures were subjected to a two-factor experimental design involving salinity and Cu concentration for 96 h. The experimental conditions adhered to the cited guidelines (50 mL of experimental volume, 20 ± 1 °C, continuous light), with modifications, as only two pollutant concentrations were assayed. Three salinity levels (33, 36, and 39) were chosen to simulate potential scenarios of climate change, considering both rising and falling salinity levels in various regions due to factors such as evaporation and melting of inland ice layers (Kholssi et al., 2023). Seawater adjusted to different salinities were prepared prior to the experiments through dilution with ultra-pure water (Milli-Q, Millipore), with a resistivity of 18.2 MΩ cm at 25 °C and <5 μg L^{-1} Total Organic Carbon. The experiment involved exposing treatments to two concentrations of Cu at 20 °C. Each treatment was prepared in three independent biological replicates (three separate 200 mL Erlenmeyer flasks) each containing 50 mL of medium without EDTA nor trace metals, with a pH value 8 (±0.1). Control groups without the pollutant were also included Ct/33, Ct/36, and Ct/39 (Table 1). These conditions maintained consistent light and temperature settings throughout the experiment. All incubated flasks were shaken twice daily by hand during the test period. Continuous orbital shaking (suggested by some guidelines) was not performed as some of the used species can stick to the surface layer, adhere to the glass and dry there, providing an additional source of carbon to the culture.

Table 1
Description of the treatments used in the experimental design (n = 3 each treatment).

Treatments	Treatments code	Salinity	Cu concentration (μg L^{-1})
1	Ct/33	33	No Cu
2	Ct/36	36	No Cu
3	Ct/39	39	No Cu
4	Cu1/33	33	2
5	Cu1/36	36	2
6	Cu1/39	39	2
7	Cu2/33	33	5
8	Cu2/36	36	5
9	Cu2/39	39	5

2.4. Flow cytometry analysis

Flow cytometry was employed to examine the toxicological effects of Cu at exposure intervals of 0, 48, 72, and 96 h. Cell density and chlorophyll fluorescence were assessed using a BD Accuri C6 flow cytometer (Becton Dickinson) equipped with a blue excitation laser (488 nm), two optical detectors for light scatter signals forward scatter (FSC) and side scatter (SSC) and four fluorescence detectors (FL). The FL3 channel (670 nm) for chlorophyll fluorescence versus FSC cytograms were used for both endpoints. The FSC detector provided data on the cell size. Prior to flow cytometry analysis, all treatments were manually agitated for 15 s. Typical FCM parameter settings were: flow rate 66 μL min^{-1}, sample volume of 25 μL, and washing volume 100 μL. The specific growth rate (SGR) was calculated as the slope of the natural logarithm of cell density versus time (96 h).

$$\mu = \ln\left(\frac{N2}{N1}\right)(t2 - t1)$$

where, μ (d^{-1}) represents the SGR, while N1 and N2 are the cell concentrations at times t1 and t2, respectively.

2.5. Oxidative stress measurement: determination of intracellular levels of ROS

Reactive oxygen species (ROS) levels were assessed at the end of the experiment to evaluate oxidative stress. The method of (Stachowski-Haberkorn et al., 2013) was employed to quantify ROS production using the 2′,7′-dichlorofluorescein di-acetate (DCFH-DA) (Invitrogen Molecular Probe, USA). H_2DCFDA, a non-polar compound, permeates algal cells, where it undergoes deacetylation by esterases and H_2O_2, resulting in the formation of fluorescent 2′,7′-dichlorofluorescein, a polar compound that exhibits maximum emission between 517 and 527 nm (Perreault et al., 2012). A stock solution of 10 mM was prepared in phosphate buffered saline medium (PBS) in the dark with 137 mM NaCl and 2.7 mM KCl at pH 7.4 to make a 2 mM working solution (20 %). H_2DCFDA was used at a final concentration of 80 μM (0.8 % DMSO) in each 500 μL sample. All treated samples were incubated for 30 min in the dark at room temperature (about 22–25 °C) before analysis by FCM at 530 nm (FL1). A positive control with 100 μM of H_2O_2 (with addition of DCFH-DA) and a negative control with fresh algal co-culture were employed (without addition of DCFH-DA). Ultimately, the degree of oxidative stress was calculated as the percentage of positive cells that stained with DCFH-DA with respect to the total microalgae population (Fig. S1).

2.6. Analysis of fatty acids

For the analysis of fatty acids, cells from 200 mL cultures at different conditions were collected by centrifugation and the pellets were extracted 5 times with a solution of acetone: methanol (1:1) and sonicated (ultrasound bath, 200 W–50Hz for 5 min). The combined extracts were filtered, evaporated under reduced pressure, and frozen until the analysis of fatty acid methyl esters (FAME) (Moser et al., 2022). The transmethylation of fatty acids was carried out by treating the extracts with 1 mL of MeOH/HCl (10:1) and heating under reflux for 1 h. After cooling, the reaction was extracted with hexane (3 × 3 mL), and the organic layers were combined, dried over anhydrous $MgSO_4$, and taken to dryness by rotary evaporation. Fatty acid methyl esters were analyzed by GC-MS on an Agilent Technologies 7890A GC gas chromatography instrument (Agilent Technologies Inc., Santa Clara, CA, USA) coupled to a triple quadrupole spectrometer with an electron impact ionization (EI) source at 70 eV and scanning the mass range *m/z* 50–550. Chromatographic separation of FAME was carried out using an HP-5MS column (30 m × 0.25 mm (i.d.) × 0.25 mm, 5 % phenyl and 95 % polydimethylsiloxane), at flow rate 1 mL min^{-1} and injection temperature of 280 °C. Fatty acid identification and quantification (validation data in Table S1) were established by comparing their retention time and mass spectrum with MS spectra of the commercial FAME standards Supelco 37 Component FAME Mix (ref. 47885-U, Sigma-Aldrich, Darmstadt, Germany) analyzed by GC-MS under the same conditions of FAME samples and using C17:0 as internal standard and their molecular formula obtained by high resolution mass spectrometry using a high-resolution SYNAPT G2 (Waters, Milford, MA, USA) instrument equipped with a quadrupole-time-of-flight (QTOF) analyzer using atmospheric pressure ionization (API) in positive ionization mode. The chromatograms were processed with MassLynx software (version 4.1, Waters, Milford, MA, USA).

2.7. *Polyunsaturated aldehydes: sampling, extraction, and quantification*

For analysis of the PUAs fraction, 200 mL samples were collected from the different cultures and the algal biomass was concentrated by centrifugation. Then, 2 mL of derivatization reagent (25 mM solution of *O*-(2,3,4,5,6-pentafluorobenzylhydroxylamine) hydrochloride; PFBHA, Fluka, Basel, Switzerland) in 100 mM Tris-HCl, pH 7, Trizma, Sigma, Steinheim, Germany) and benzaldehyde as internal standard were added to the resulting pellet. For mechanical disruption of the cells, the samples were sonicated by ultrasound (Bandelin Sonoplus, HD2070, 97 %). The extraction was performed according to the protocol described in (Morillo-García et al., 2014)

The obtained extracts of pPUA were analyzed by GC-MS using an Agilent 7890A GC gas chromatography instrument (Agilent Technologies Inc., Santa Clara, CA, USA) coupled to either a Synapt G2 Q-TOF high-resolution mass spectrometer (Milford, MA, USA) with an atmospheric pressure ionization source (atmospheric pressure gas chromatography, APGC) in positive mode, or to a triple quadrupole spectrometer with an electron impact ionization (EI) source in multiple reaction monitoring (MRM) mode. Chromatographic separation of PUA was carried out using an HP-5MS column (30 m × 0.25 mm (i.d.) × 0.25 mm, 5 % phenyl and 95 % polydimethylsiloxane), at flow rate 1 mL min^{-1} and injection temperature of 280 °C. The temperature ramp used was: 70 °C for 1 min, incrementing at 35 °C min^{-1} up to 180 °C, and 4.50 °C min^{-1} up to 290 °C, and maintaining 290 °C for 8 min. TOF-MS analyses in API mode were performed in a range $m/z = 50–1200$, with a corona voltage of 2 kV, chamber temperature of 130 °C and a corona voltage between 10 and 40 V. PUA were identified comparing the retention times and exact molecular mass measurement (error less than 5 ppm) with those obtained from commercially available standard samples, 2*E*,4*E*-heptadienal (90 %, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 2*E*,4*E*-octadienal (>96 %, Sigma-Aldrich Chemie GmbH), and 2*E*,4*E*-decadienal (85 %, Sigma-Aldrich Chemie GmbH, St. Louis, MO, USA) as in (Bartual and Ortega, 2013). For the correct quantification of PUA, calibration lines were performed (1–7000 nM in hexane-2 mL) by comparing the intensity of the signals of the molecular ions of the standard samples. Different synthetic standard solutions of commercial HD, OD, and DD were used to obtain calibration curves to cover the wide range of molarities in the analysis of pPUA.

The results were obtained by plotting the peak area of each aldehyde against the area of the internal standard (benzaldehyde). Reproducibility and repeatability of this methodology were evaluated by reanalysis of the standard samples two weeks after the first analysis (validation data in Table S2). The chromatograms were processed with MassLynx software (version 4.1, Waters, Milford, MA, USA).

2.8. *Statistical analysis*

Data were analyzed using IBM SPSS Statistics. Normality and homogeneity of variances were assessed using the Kolmogorov–Smirnov and Levene tests, respectively. Two-way ANOVA, with significance defined at $p \leq 0.05$, followed by Tukey's post-hoc test, was applied to compare means between treatment groups. When the assumptions of normality and homogeneity were not met and data could not be transformed, a non-parametric test was used ($p < 0.05$). Additionally, a PERMANOVA analysis was performed to assess multivariate differences among groups. PCA and PERMANOVA analyses were conducted using R. Results are presented as means ± standard deviation.

3. Results and discussion

3.1. *Structural changes and population growth*

Results of SGR (μ, day^{-1}) of *C. calcitrans* exposed to two Cu concentrations under three salinity levels (33, 36, 39) over 96 h are presented in Fig. 1a. The Tukey HSD post-hoc test ($\alpha = 0.05$) revealed no statistically significant differences ($p < 0.05$) in SGR among the treatments exposed solely to varying salinity levels. Nevertheless, the control treatments (Ct/33, Ct/36, Ct/39) consistently demonstrated elevated growth rates, with Ct/39 (1.9 μ day^{-1}) surpassing all other group.

However, exposure to Cu reduced SGR, with the strongest inhibition observed at 5 μg L^{-1} combined with salinity 33 (0.86 μ day^{-1}). In both Cu treatments, higher salinity (39) consistently alleviated Cu-induced inhibition, indicating a potential protective role of elevated salinity against Cu toxicity. These data provided evidence that salinity plays a significant role in modulating the toxic effects of Cu on *C. calcitrans*, particularly with regard to SGR Fig. 1a). Although Cu exposure led to a reduction in both cell density and SGR, the attenuating effect of elevated salinity was more apparent in SGR than in cell density parameter. The variation in cell densities of *C. calcitrans* cultures during the experimental period are depicted in Fig. 1b. A significant increase (Tukey's post-hoc test $p < 0.05$) in cell density was observed in the control treatments, particularly Ct/36 with value of cell density $17.21 \pm 0.22 \times 10^4$ cells mL^{-1}, compared to treatment Ct/39. Exposure to 2 μg L^{-1} Cu caused a marked reduction in cell density at salinity 36 ($7.10 \pm 0.56 \times 10^4$ cells mL^{-1}), while the effect was less pronounced at 39 (Cu1/39). In contrast, treatments exposed to 5 μg L^{-1} Cu (Cu2/33, Cu2/36, and Cu2/39) exhibited a significant reduced algal density, decreasing to approximately $2.02 \pm 0.05 \times 10^4$ cells mL^{-1} compared to all others treatments tested in this experiment, corresponding to inhibition rates up to 90 %.

In diatoms, certain salinity levels can trigger protective responses, and one of the most likely mechanisms is the accumulation of organic osmolytes that help the cells maintain osmotic balance (Masmoudi et al., 2013). Additionally, in natural (Verslycke et al., 2003) and in artificial seawater, toxicity of Cu decreases with increasing salinity which it probably be linked to enhanced competition between Cu and major cations such as Ca^{2+} and Mg^{2+} for binding sites on algal surfaces (Ytreberg et al., 2011). Our findings align also with previous studies involving the same species tested in our experiment; though those studies used much higher Cu concentrations; they found that Cu inhibited the growth of *C. calcitrans*, with growth rates at 96 h of 1.23, 1.07, 0.42, and 0.14 divisions per day at 240, 320, 530, and 840 μg Cu L^{-1}, respectively. The corresponding growth inhibitions were 9.2, 21.2, 68.8, and 90 % (Anu et al., 2016). Previous work by Biswas et al. (2013) reported that concentrations between 150 and 500 nM had significant

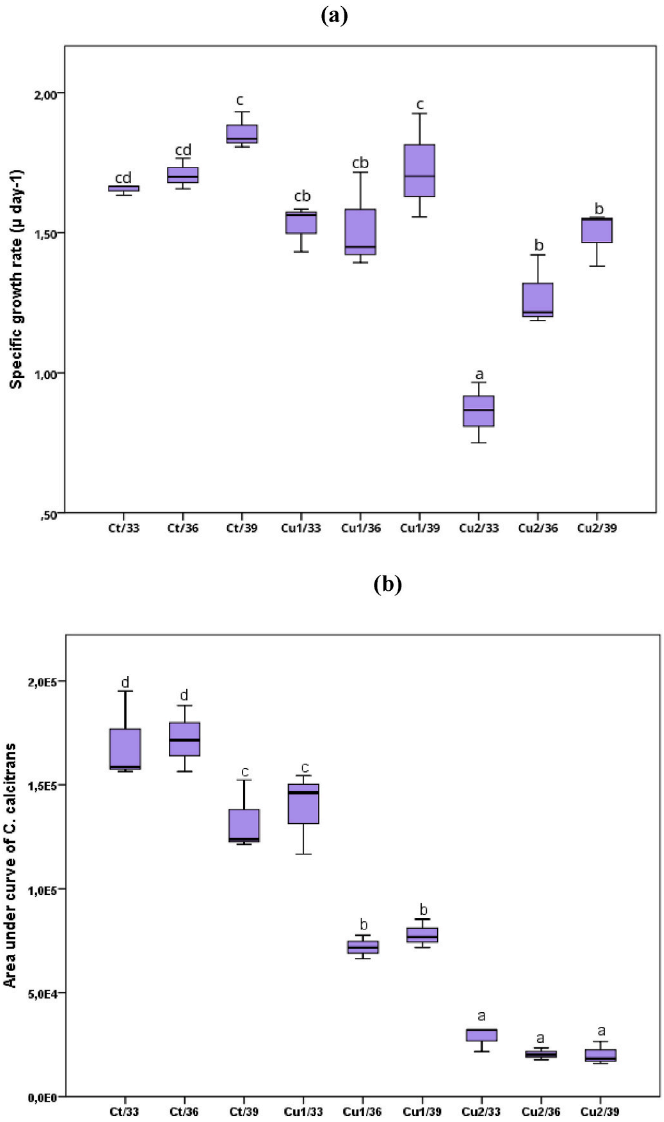

Fig. 1. Effect of three salinity levels (33, 36, and 39) on the toxicity of Cu (Cu1 = 2 µg L^{-1} and Cu2 = 5 µg L^{-1}) on (a) specific growth rate (µ), and (b) Area Under the Curve (AUC) of *C. calcitrans* over 96 h. Error bars represent mean ± SD ($n = 3$). Different letters above bars indicate statistically significant differences among treatments (Tukey's post-hoc test, $p \leq 0.05$).

inhibitory effects on the growth of *Chaetoceros gracilis,* which is considered growth-inhibitory. Based on these findings, Moreno-Garrido et al. (2000) demonstrated that sensitivity to Cu (72-h IC_{50}, µg L^{-1}) decreased in the following order: *Isochrysis galbana* (prymnesiophyte; 0.4 µg L^{-1}) $\gg$ *Chlorella autotrophica* (green alga; 9.6 µg L^{-1}) $\approx$ *Phaeodactylum tricornutum* (diatom; 9.8 µg L^{-1}) $>$ *Nannochloris atomus* (green alga; 16.7 µg L^{-1}), indicating that also other taxonomic groups of microalgae. In another study examining the sensitivity of 11 marine species to Cu (with 72-h IC50 values of <5 µg L^{-1}), the small centric diatom *Minutocellus polymorphus* was identified as the most sensitive (Levy et al., 2007). Stauber and Florence (1987) investigated the disruption of algal growth by Cu, and they found that Cu in cytoplasm can decrease the ratio of reduced to oxidized glutathione, leading to impairment of spindle formation and interferes with normal cell division.

It has already been established that toxicity effects induced by Cu varies between species, and it also depends on others factors such as initial cell density, temperature, light illumination, media used and exposure time (Fawaz et al., 2018; Joonas et al., 2021; Manimaran et al., 2012; Moreno-Garrido et al., 2000). Furthermore, the toxicity of this element could be reduced through algal excretion of metal-binding compounds or the production of intracellular metal-binding peptides (Bossuyt and Janssen, 2004; Monteiro et al., 2012; Wang et al., 2017). Such modified physical and chemical conditions inevitably impacts marine microalgae as primary producers, potentially jeopardizing their survival, distribution, and nutritional quality.

The autofluorescence patterns of chlorophyll-*a* in *C. calcitrans* culture exposed to three levels of salinity (33, 36, and 39) and under two concentrations of Cu (2 and 5 µg L^{-1}) are presented in Fig. 2. The effects of salinity, when tested as single factor, induced a significant (Non-parametric test, Kruskal Wallis test, df = 8; p = 0.02) decrease in chl-fluorescence by 30 % at salinity of 39 compared to treatment of 36. However, the presence of Cu intensified the toxicity of salinity, leading to a sharp decline of 76 % in chl-fluorescence, particularly at the lowest salinity level examined 33 at 5 µg L^{-1}; the lowest across all treatment.

Chlorophyll plays a vital role in photosynthesis, facilitating the conversion of light energy absorbed by microalgae into useable energy (Sun et al., 2024). The values of chl-fluorescence were highest in *C. calcitrans* grown under 33, and exhibited a decreasing trend with the increase of salinity till 39. These results indicated that chl-fluorescence was negatively impacted with the variation of salinity. However, at higher salinities (36 and 39), a slight recovery in fluorescence was observed under 5 µg L^{-1} Cu exposure, suggesting that salinity may partially alleviate copper-induced stress.

Photosynthesis inhibition by exposure to variation of salinity has been observed previously in the study of (Liang et al., 2014) who observed a decline in the relative chlorophyll content of *P. tricornutum* and *C. gracilis*, indicating that extreme salinity (>50) negatively influenced the chlorophyll content. Results obtained in the present work showed that chl-fluorescence of *C. calcitrans* cultures decreased when Cu concentration increased from 2 to 5 µg L^{-1} at the three salinities tested. Similar results were obtained for Neethu et al. (2021), where different concentration of Cu caused reduction of Chl-a and c on marine diatoms *C. calcitrans* and *Nitzschia closterium*. Our data corroborate the study of (Perales-Vela et al., 2007) who investigated growth, photosynthesis and respiratory responses at sub-lethal Cu concentrations in *Scenedesmus incrassatulus* and observed that, at Cu concentrations below the EC_{50}, the level of chlorophylls and carotenoids rise when compared with control algae.

Significant pH variation in all treatments assayed in this experiment

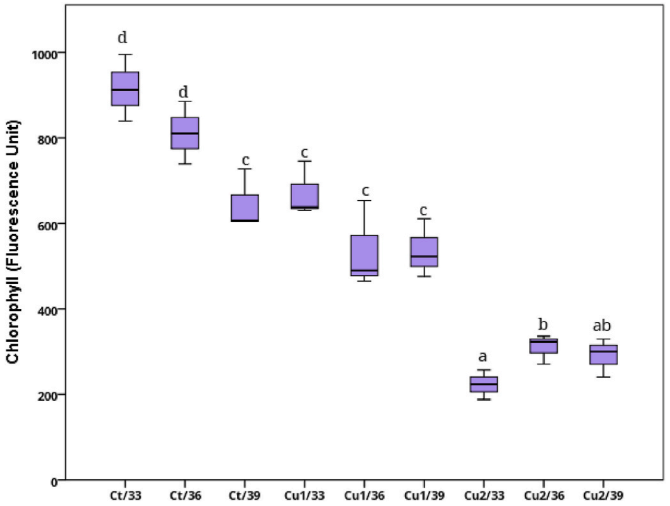

Fig. 2. Chlorophyll autofluorescence in *C. calcitrans* after 96 h of exposure to three salinity levels (33, 36, and 39) and two Cu concentrations (Cu1 = 2 µg L^{-1}, Cu2 = 5 µg L^{-1}). Error bars represent mean ± SD ($n = 3$). Different letters denote homogeneous groups from post-hoc comparisons (Kruskal–Wallis test with pairwise comparisons, $p \leq 0.05$).

related to both salinity and Cu concentration factors was observed (Fig. 3). Generally, the highest value of pH was recorded in the control at salinity 33 (Ct/33, 9.48), followed by Cu1/33 (9.44) and Cu1/36 (9.35). In contrast, values of pH decreased under exposure with Cu (Cu2 treatments), and the lowest value observed in Cu2/39 (8.43). Previous studies have shown that the toxicity of metals such as Cu and Zn to *Chlorella* sp. increases at higher pH levels (Wilde et al., 2006). This variation is likely due to changes in metal speciation, which can affect the bioavailability of the metals (Amaro et al., 2023). Speciation of dissolved Cu in seawater is a complex process. Although the dominant dissolved Cu species in marine environments is expected to be Cu^{2+}, as it is the most stable form, this metal readily complexes with organic matter, including compounds excreted by microalgal cells (Monteiro et al., 2012; Wang et al., 2017). The negatively charged surfaces of microalgal cells also act as binding sites for Cu, enabling a fast, non–metabolic-dependent adsorption process. Following this initial adsorption, a second, slower, metabolic-dependent absorption step into the cytoplasm has been described, and both adsorption and absorption capacities can vary among taxonomic groups or even be species-specific (Maeda and Sakaguchi, 1990). In addition, the high-pH microenvironment surrounding actively photosynthesizing cells can decrease the solubility of Cu. Therefore, the speciation of Cu in seawater is influenced by multiple factors, including Cu concentration, salinity, temperature, dissolved organic matter (which may also change in response to pollutant exposure), pH, cell density, and the taxonomy of the species involved, among others.

3.2. ROS production

Fig. 4 shows the production of ROS (%) in *C. calcitrans* exposed to three levels of salinity (33, 36, and 39) and two Cu concentrations (Cu1 = 2 μg L^{-1} and Cu2 = 5 μg L^{-1}). The control groups (Ct/33, Ct/36, Ct/39) exhibited no significant ROS production, with all values remaining below 10 %. However, in the presence of Cu, ROS levels increased significantly (p-value = 0.02) across all salinities, reaching approximately 77 % at 5 μg L^{-1} Cu and 36 compared to their control groups (Ct/33, Ct/36, Ct/39). A significant increase was observed at 39, where ROS production reached 99 % under both Cu concentrations (Cu1 and Cu2) compared to treatment without Cu at the same salinity.

The oxidative stress response is an important biomarker that is often used to evaluate heavy metal exposure in marine organisms. Cu induce ROS production in *C. calcitrans*, with the percentage of ROS showing different trends depending on salinity and Cu levels used in this study. These results demonstrate that while salinity alone had minimal effect on ROS accumulation, the combination of Cu stress at higher salinities enhanced oxidative stress response, suggesting a synergistic effect between salinity and Cu on ROS production. The exposure history of microalgal population communities plays a fundamental role in determining their response to a given dose of Cu (Bossuyt and Janssen, 2004; Neethu et al., 2021). Previous research by (Sendra et al., 2023) has examined the toxicity of bisphenol (BPA) at concentrations ranging from ng L^{-1} to μg L^{-1} in diatom and other taxa of marine microalgae. These findings have shown that the diatom *P. tricornutum* was more sensitive to BPA exposure, compared with *Tetraselmis suecica* and *Nannochloropsis gaditana*, showing significantly higher levels of percentage of ROS compared to the other species, indicating a stronger oxidative stress response. Our findings further confirm that the experimental Cu concentrations triggered ROS production, aligning with recent studies on Cu-toxicity in microalgae (Akcha et al., 2022; Parkavi et al., 2025). In general, the toxicity of Cu has been closely associated with the induction of oxidative stress and the resulting disruption of cellular redox balance (Pinto et al., 2003).

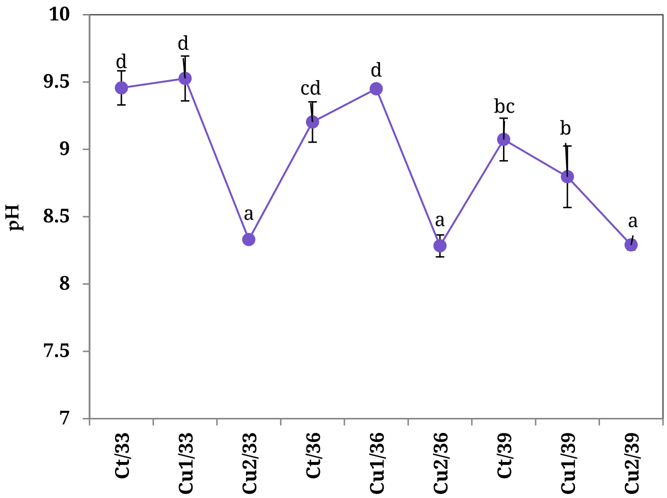

Fig. 3. Effect of three different salinity levels (33, 36, and 39) on pH changes in *C. calcitrans* over a 96-h period under two Cu toxicity concentrations (Cu1 = 2 μg L^{-1} and Cu2 = 5 μg L^{-1}). Significant differences relative to the control within each treatment were tested at $\alpha = 0.05$. Error bars represent mean ± SD ($n = 3$). Different letters above bars indicate statistically significant differences among treatments (Kruskal Wallis test, $p \leq 0.05$).

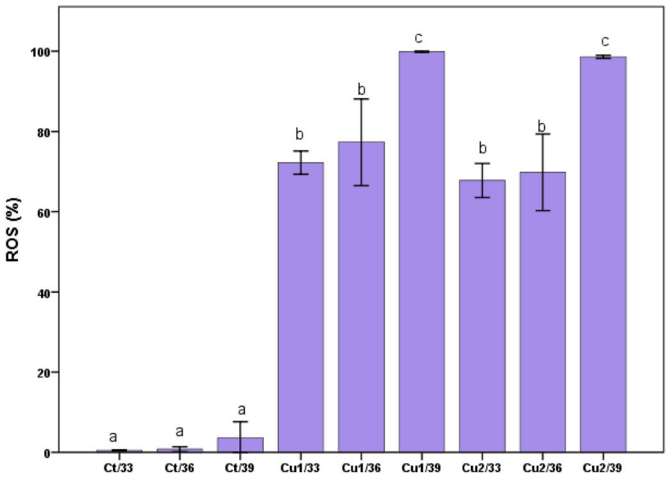

Fig. 4. Reactive oxygen species (ROS) production (%) in *C. calcitrans* under exposure to three different salinity levels (33, 36, and 39) and two Cu concentrations (Cu1 = 2 μg L^{-1} and Cu2 = 5 μg L^{-1}). Error bars indicate mean ± SD ($n = 3$). Letters above bars represent homogeneous groups from Tukey's post-hoc test ($p \leq 0.05$).

3.3. Fatty acid composition

The fatty acid methyl ester (FAME) profiles of *C. calcitrans* cultivated under varying salinities (33, 36, and 39) and copper concentrations (2 and 5 μg L^{-1}) revealed the presence of 31 fatty acids, including saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) (Table 2). Significant changes in lipid composition were observed across treatments ($P < 0.05$), highlighting a strong influence of Cu and salinity stress.

SFAs, particularly palmitic acid (C16:0) and myristic acid (C14:0), increased markedly in Cu-exposed cultures. For instance, C16:0 peaked in Cu2/36 and Cu2/39, reaching values over 13,000 fmol·$cell^{-1}$, compared to much lower levels in the respective controls. MUFAs also rose under Cu stress, notably palmitoleic acid (C16:1) and oleic acid (C18:1), which showed 5–10-fold increases under Cu2 conditions at 36 and 39 salinities.

PUFAs displayed a more complex response pattern. EPA (C20:5n3) increased under high Cu concentration combined with elevated salinity (Cu2/39), suggesting a potential stress-induced stimulation of its

Table 2

Fatty acid profiles of *C. calcitrans* after exposure to salinity treatments (33, 36 and 39) at 20 °C. Means and standard deviation are shown, expressed in fmol cell^{-1} (n = 3). Trace concentrations are not included. Sample key: SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; EPA = (C20:5n3) *cis*-5,8,11,14,17-eicosapentaenoic acid; - = non detected. SFA: capric acid (C10:0), undecanoic acid (C11:0), lauric acid (C12:0), tridecanoic acid (C13:0), myristic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), stearic acid (C18:0), arachidic acid (C20:0), henicosanoic acid (C21:0), behenic acid (C22:0), lignoceric acid (C24:0); MUFA: myristoleic acid (C14:1), *cis*-10-pentadecenoic acid (C15:1), palmitoleic acid (C16:1), oleic acid (C18:1n9c), *cis*-11-eicosenoic acid (C20:1n9), erucic acid (C22:1n9), and PUFA: linoleic acid (C18:2n6c), linolelaidic acid (C18:2n6t), γ-linolenic acid (C18:3n6), α-linolenic acid (C18:3n3), (C18:4), *cis*-11,14-eicosadienoic acid (C20:2), *cis*-8,11,14-eicosatrienoic acid (C20:3n6), *cis*-11,14,17-eicosatrienoic acid (C20:3n3), arachidonic acid (C20:4n6).

	Component	Ct/33		C1/33		C2/33	
FAME (fmol cell^{-1})		Mean	SD	Mean	SD	Mean	SD
SFA	C10:0	0.57	0.08	0.09	0.01	8.27	10.50
	C11:0	0.37	0.07	0.10	0.01	4.11	0.75
	C12:0	10.07	0.12	7.61	0.50	31.89	4.66
	C13:0	3.08	0.35	2.44	0.39	10.49	0.86
	C14:0	196.03	46.26	263.85	26.40	10269.30	1064.16
	C15:0	21.35	4.44	97.81	9.32	566.60	123.23
	C16:0	369.20	37.90	218.80	33.90	19478.50	1045.00
	C18:0	59.7	10.00	148.80	20.80	1050.40	179.50
	C20:0	1.78	0.70	79.70	13.10	11.20	1.00
	C21:0	0.10	0.03	23.90	3.40	–	–
	C22:0	1.30	0.50	67.30	7.20	–	–
MUFA	C14:1	10.10	1.20	22.10	2.10	–	–
	C15:1	0.60	0.30	84.70	7.30	–	–
	C16:1	636.00	74.80	90.60	2.00	5528.40	568.70
	C18:1n9c	22.90	3.20	101.20	11.30	419.00	31.80
	C20:1n9	–	–	26.60	5.60	–	–
	C22:1n9	–	–	14.60	1.20	–	–
PUFA	C16:3	54.70	6.70	–	–	33.10	3.10
	C16:4	0.20	0.04	1.09	0.20	2.80	0.30
	C18:2n6c	2.10	1.00	40.60	9.00	–	–
	C18:2n6t	–	–	–	–	–	–
	C18:3n6	47.57	8.74	52.80	4.22	48.77	1.90
	C18:3n3	–	–	42.70	2.92	47.55	6.70
	C20:2	–	–	10.90	2.60	–	–
	C20:3n6	–	–	27.80	2.40	–	–
	C20:3n3	–	–	26.0	0.80	–	–
	C20:4n6	199.80	22.20	26.30	1.10	–	–
EPA	C20:5n3	404.70	7.60	48.62	4.03	503.10	28.00
FAME (fmol cell^{-1})		Ct/36		C1/36		C2/36	
		Mean	SD	Mean	SD	Mean	SD
SFA	C10:0	–	–	–	–	–	–
	C11:0	–	–	–	–	–	–
	C12:0	3.90	0.70	2.00	0.03	7.30	0.40
	C13:0	–	–	1.30	0.24	4.10	0.16
	C14:0	515.20	39.60	1694.50	207.40	13083.50	1083.70
	C15:0	30.40	6.83	109.50	1.62	1097.40	177.30
	C16:0	416.80	32.30	2079.70	166.70	1323.00	700.00
	C18:0	91.20	4.80	259.00	17.30	906.40	44.00
	C20:0	2.00	0.70	5.10	0.30	13.00	0.60
	C21:0	–	–	–	–	–	–
	C22:0	–	–	5.10	1.20	–	–
	C24:0	–	–	27.86	0.70	–	–
MUFA	C14:1	11.99	1.00	17.20	2.00	27.30	2.40
	C15:1	0.94	0.30	1.90	0.30	–	–
	C16:1	737.90	49.00	1972.00	118.00	5897.30	216.00
	C18:1n9c	63.20	7.60	108.60	14.50	572.60	20.70
PUFA	C16:2	4.02	0.10	9.30	0.90	–	–
	C16:3	92.20	7.80	170.80	28.10	399.00	13.20
	C16:4	0.10	0.03	3.20	0.40	19.70	1.10
	C18:2n6c	–	–	11.23	1.06	–	–
	C18:2n6t	–	–	–	–	–	–
	C18:3n6	75.85	1.68	131.24	25.47	20.39	4.50
	C18:4	11.05	0.90	18.00	2.26	–	–
	C20:4n6	375.00	26.40	429.00	26.00	106.60	10.20
EPA	C20:5n3	952.00	61.60	1486.10	192.10	482.00	32.50
FAME (fmol cell^{-1})		Ct/39		C1/39		C2/39	
		Mean	SD	Mean	SD	Mean	SD
SFA	C10:0	0.20	0.04	–	–	–	–
	C11:0	–	–	–	–	–	–
	C12:0	41.70	2.50	10.10	2.40	2.30	0.20
	C13:0	6.60	0.70	2.70	0.30	7.30	0.70
	C14:0	1954.40	143.10	1763.10	238.20	13564.00	390.63
	C15:0	77.00	3.12	105.30	8.00	342.86	46.30

(*continued on next page*)

Table 2 (*continued*)

	Component	Ct/33		C1/33		C2/33	
FAME (fmol cell^{-1})		Mean	SD	Mean	SD	Mean	SD
	C16:0	1356.10	168.00	1561.10	54.10	13383.00	1394.41
	C18:0	156.60	5.30	154.00	12.30	814.22	22.40
	C20:0	2.60	0.50	2.80	0.50	20.48	2.41
	C21:0	–	–	–	–	–	–
	C22:0	3.80	0.50	4.00	0.40	8.30	1.30
	C24:0	14.90	1.30	15.00	0.90	12.70	1.74
MUFA	C14:1	74.00	4.00	44.60	5.04	22.10	3.40
	C15:1	2.80	0.40	5.10	0.20	–	–
	C16:1	1711.00	–	2054.10	–	7458.50	–
	C18:1n9c	104.10	6.90	102.70	5.50	486.00	17.00
PUFA	C16:2	9.80	1.10	12.10	2.10	267.70	29.20
	C16:3	219.20	28.40	157.40	12.60	144.7	18.04
	C16:4	14.20	1.60	10.00	2.30	16.30	1.80
	C18:2n6c	13.58	2.27	9.73	2.00	–	–
	C18:2n6t	–	–	10.29	0.31	–	–
	C18:3n6	160.80	21.70	109.00	15.50	94.46	5.10
	C18:4	20.94	0.70	13.40	4.00	–	–
	C20:4n6	841.10	69.00	498.20	25.00	279.00	20.30
EPA	C20:5n3	2968.10	171.00	1380.00	117.20	3630.67	357.56

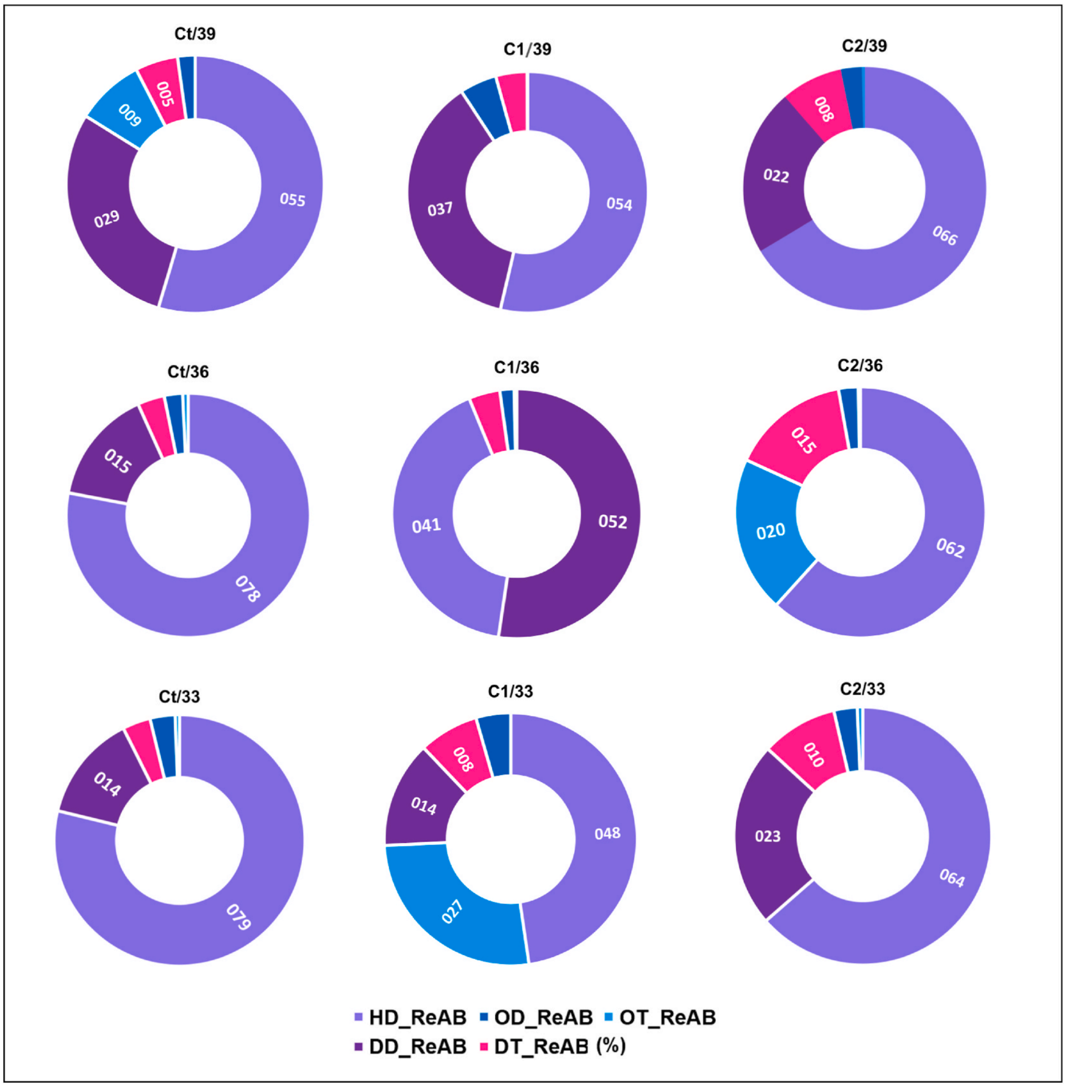

Fig. 5. Sector diagram of the relative abundance (ReAb) of different pPUA types (%) detected in *C. calcitrans* cultures quantified in late exponential growth phase at the two concentrations of Cu assayed (Cu1 = 2 μg L^{-1} and Cu2 = 5 μg L^{-1} at 33, 36 and 39 of salinities, (n = 3); 2*E*,4*E*/*Z*-heptadienal (HD), 2*E*,4*E*/*Z* -octadienal (OD), 2*E*,4*E*/*Z*-decadienal (DD), 2*E*,4*E*/*Z*,7-octatrienal (OT), and 2*E*,4*E*/*Z*,7*Z*-decatrienal (DT). Error bars show mean ± SD (n = 3). Different letters indicate statistically significant differences among treatments (Kruskal–Wallis's test, p ≤ 0.05).

biosynthesis. In contrast, arachidonic acid (ARA, C20:4n6) increased with salinity in control treatments (from 199 to 841 fmol·cell^{-1}) but showed a sharp decline across all Cu2 treatments. This indicates that elevated Cu levels override the salinity-driven accumulation of ARA in *C. calcitrans*.

These findings underscore that Cu exposure, especially in combination with high salinity, shifts FAME profiles towards greater SFA and MUFA content, while generally reducing PUFA levels. This trend aligns with previous observations in other diatoms and microalgae species under environmental stress (Liang & Kangsen, 2005; Griffiths et al., 2012; Chen et al., 2023; Parkavi et al., 2025). Elevated SFA and MUFA levels under stress may reflect a physiological adaptation to oxidative stress, potentially linked to photosynthetic inhibition (Akcha et al., 2022). Notably, despite the reduction in total PUFA content, *C. calcitrans* still produced key PUFAs such as EPA and ARA, known precursors for bioactive polyunsaturated aldehydes (PUAs) like heptadienal, decatrienal and decadienal (D'Ippolito et al., 2004; Pohnert, 2000). These biochemical shifts may serve as an adaptive mechanism to mitigate ROS-induced stress, a hypothesis supported by elevated ROS levels detected via flow cytometry in Cu-exposed cultures.

3.4. *Polyunsaturated aldehydes*

PUAs produced by *C. calcitrans* culture grown under different salinities (33, 36 and 39) individually and in combination with two Cu concentrations (2 and 5 μg L^{-1}), were analyzed after 96 h (Fig. 5). According to One-way ANOVA, there was a significant difference in PUA content among all treatments ($P < 0.05$), including 2*E*,4*E*/*Z*-heptadienal (HD), 2*E*,4*E*/*Z* -octadienal (OD), 2*E*,4*E*/*Z* -decadienal (DD), 2*E*,4*E*/*Z*,7-octatrienal (OT), and 2*E*,4*E*/*Z*,7*Z*-decatrienal (DT). In the control treatments (Ct/33, Ct/36, Ct/39), HD was the dominant PUA, with particularly high relative abundances at 33 and 36, picked to 79 and 78 %, respectively. However, when treatments were exposed to both Cu concentrations (2 and 5 μg L^{-1} Cu), a marked decrease in the percentage of HD was observed, especially at the lowest concentration of Cu tested combined with salinity of 36. A Kruskal-Wallis's test confirmed significant differences in HD levels among treatments ($p = 0.002$). Conversely, OD levels appeared at low levels in all control treatments (Ct/33, Ct/36, Ct/39), but a significant increase ($p = 0.004$) was elicited under Cu exposure, with the highest relative abundance recorded in Cu1/39.

The relative abundance of OT was minimal in all control treatments, but exhibited a significant increase ($p = 0.002$) in Cu1/33 (27 %) and Cu2/36 (20 %) compared to their controls. DD levels increased significantly in all Cu-exposed treatments ($p = 0.002$), especially at Cu1/36 with 52.3 % compared to the corresponding control Ct/36 (15.3 %). Finally, DT was the least abundant in controls and reached significantly its highest level ($p = 0.003$) at 5 μg L^{-1} Cu and salinity 36 (Cu2/36, 15.34 %).

PUAs have been proposed to play a crucial role as infochemicals that facilitate the transmission of information within diatom population (Pohnert, 2005; Vardi et al., 2006; Leflaive and Ten-Hage, 2009). In the past, the production of bioactive PUAs such as HD-heptadienal; OD-octadienal; DD-decadienal; OT-octatrienal; DT-decatrienal have been well documented as the dominant bioactive PUAs released by diatoms (Pohnert et al., 2004; Bartual et al., 2014; Morillo-García et al., 2014; Hernanz-Torrijos et al., 2023). Quantitative analyses have revealed the variability in the amounts of aldehydes produced by different diatom species, ranging from 30 to 869 fg per cell (Pohnert et al., 2004; Ribalet et al., 2007). These differences could be attributed to several factors (e.g; nutrient concentration, cell concentration, type of light use) which may collectively contribute to increased PUAs concentrations.

In this study, HD was the dominant aldehyde released in controls treatments especially in Ct/33 and Ct/36, but decreases under exposure to Cu at the same salinities, indicating it may be metabolized or replaced as other aldehydes. Despite the availability of EPA and ARA as precursors, exposure to Cu led to a decline in both HD and OD, while DT production increased, suggesting altered enzymatic activity and physiological disfunctions under stress. These trends were also observed by Ribalet et al. (2009), who reported that the production of PUAs (HD and OD) in *Skeletonema marinoi* was limited by enzymatic activity under N-limitation and also by substrate availability. In other side, a significant increase in DD percentage was observed under low Cu-exposure in Cu1/36 (52.35 %). Previous works have been reported that DD triggered a degenerative process, through change in cell membrane, leading to cell death (Casotti et al., 2005). In addition, previous studies have proposed that DD, may play an important role as infochemicals, functioning as a surveillance system to assess stress within diatom assemblages during blooms (Vardi et al., 2006). The result demonstrated a minimal production of OT level, probably due to lower lipase and lipoxygenase activity (Ribalet et al., 2009). Furthermore, a comparison of the major PUA isolated from *Thalassiosira rotula* and *Skeletonema costatum* showed that both OD and DD exhibit antiproliferative activity and inhibit cleavage of sea urchin eggs (Pohnert, 2005). Altogether, the variation in the production of a wide range of PUAs may indicate a balancing mechanism in response to environmental stress. Therefore, PUAs can reach high concentration in *C. calcitrans* and may work together to produce combined effects that reactive aldehydes can reach high concentrations and may work together to produce combined effects that has not yet been thoroughly studied.

Principal Component Analysis (PCA) revealed clear patterns in the biological responses of *C. calcitrans* to Cu and salinity exposure (Fig. 6). The first two principal components (PC1 and PC2) explained 77.2 % of the variance, i.e., PC1 60.2 % and PC2 17.2 %. PC1 (60.2 % of variance) separated the samples mainly according to Chl (−0.40), pH (−0.39), HD (0.40), OD (0.40), with high positive loadings of HD and OD and negative loadings of Chl and pH. The control groups without exposure to Cu (Ct/33, Ct/36, Ct/39) clustered on the negative side of PC1 and were closely associated with higher SGR, chl-fluorescence, and pH, indicating favorable physiological conditions in the absence of Cu. In contrast, according to statistical analyses, the correlations between variables on first two principal components (PCs) indicated that treatments with the highest Cu concentration (5 μg L^{-1}; Cu2/33 and Cu2/36) were strongly associated with elevated ROS and PUAs, particularly OD, HD, and DD, reflecting significant oxidative and biochemical stress (Supplementary Material Table S3). Interestingly, treatment Cu2/39 grouped closer to the center of the PCA plot and away from the stress markers, indicating that high salinity may buffer or mitigate Cu-induced stress.

To assess these multivariate patterns, a PERMANOVA analysis was performed after confirming homogeneity of multivariate dispersion was not significant ($F = 2.81$, $p = 0.08$), indicating that dispersions were reasonably homogeneous across groups. The results were consistent with the PCA interpretation. The analysis showed that Cu was the dominant factor, exerting a highly significant effect ($F = 140.82$, $p < 0.001$) and explaining 83.7 % of the multivariate variation. Salinity also had a significant effect ($F = 6.68$, $p = 0.012$; 4.0 % of variation). Furthermore, a significant Cu × Salinity interaction was detected ($F = 5.83$, $p = 0.003$; 6.9 % of variation), indicating that the multivariate response depended on the combined effects of both factors, although Cu clearly remained by far the major contributor to the overall variation. Residual variation accounted for approximately 5.4 % of the total (Supplementary Material Table S4).

4. Conclusions

In the present paper, a complex experimental approach was employed to study the effects of salinity on the toxicity of Cu in culture of *C. calcitrans* in short-term acute toxicity tests.

A key finding was that salinity 39 partly alleviated Cu-induced inhibition of growth rate and chlorophyll fluorescence, while oxidative stress increased under copper exposure and was further amplified at higher salinity, as shown by ROS production.

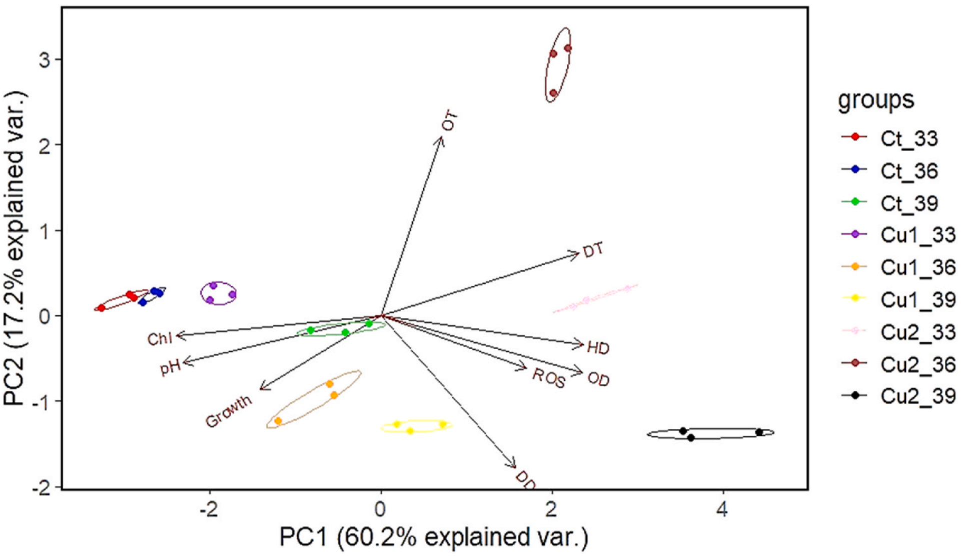

Fig. 6. Principal Component Analysis (PCA) displaying the relationships among physiological variables in *C. calcitrans* after 96 h of exposure to combined salinity and Cu stress. The data were scaled and centered as a part of the preprocessing step. The percentage of variance explained by PC1 was 60.2 % and by PC2 17.2 %. The PCA includes chlorophyll fluorescence (Chl), ROS, PUAs (OT, DT, HD, OD, DD), pH and growth rate The analysis included 27 observations and 9 variables, giving a sample-to- variable ratio of 3. We considered loadings $\geq |0.4|$ as meaningful. Under this criterion, PC1 was mainly associated with Chl (−0.40), pH (−0.39), HD (0.40), OD (0.40), and DT (0.39). PC2 was mainly driven by OT (0.66) and DD (−0.56).

This study aimed to ascertain the impact of Cu and salinity exposure on the fatty acid composition of *C. calcitrans*, confirming that Cu contamination is a determinant of lipid metabolism in diatoms.

Fatty acid composition shifted toward greater SFA (15:0) and (20:0), and MUFA (16:1n-5), with reduced PUFA, and PUA profiles were significantly altered under combined factors Cu-salinity, indicating stress-related biochemical changes rather than mitigation. Fatty acid composition is commonly employed as a trophic marker, and the impact of metals illustrates their potential to affect trophic ecology and, consequently, the diversity and function of ecosystems. Based on our findings, further experiments are necessary to comprehend the complex roles of PUAs and PUFAs in phytoplankton signaling.

Additionally, a long-term experimental design would allow a deeper insight into how metals affect diatoms and their interactions with other marine organisms in more natural, mesocosm-based settings. In the context of global climate change, it is also important to provide investigation on how environmental factors such as temperature and pH variations influence both the direct effects of metal exposure and the indirect effects through trophic transfer on marine ecosystems health.

CRediT authorship contribution statement

Rajaa Kholssi: Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Juan Rodríguez-Márquez:** Writing – review & editing, Software, Methodology, Investigation. **María Úbeda-Manzanaro:** Writing – review & editing, Visualization, Investigation. **Marta Sendra:** Writing – review & editing, Methodology, Investigation. **Ignacio Moreno-Garrido:** Writing – review & editing, Visualization, Validation, Supervision, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Ana Bartual:** Writing – review & editing, Visualization, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **María J. Ortega:** Writing – review & editing, Visualization, Supervision, Methodology, Investigation, Conceptualization.

Declaration of interest's statement

The authors declare no conflict of interest.

Data availability statement

No external data was used for the research described in the article.

Additional information

No additional information is available for this paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2025.144790.

Data availability

Data will be made available on request.

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