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Anthony Montembault, Damien Rioult, Iris Barjhoux, Sandra Villaume, Nathalie Gaveau, et al.. Distinct oxidative and photosynthetic responses to betulin and betulone in the freshwater microalgae Chlamydomonas reinhardtii. Ecotoxicology and Environmental Safety, 2025, 303, pp.119033. ⟨10.1016/j.ecoenv.2025.119033⟩. ⟨hal-05329630⟩

HAL Id: hal-05329630

https://cnrs.hal.science/hal-05329630v1

Submitted on 21 Nov 2025

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Ecotoxicology and Environmental Safety 303 (2025) 119033

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Distinct oxidative and photosynthetic responses to betulin and betulone in the freshwater microalgae *Chlamydomonas reinhardtii*

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ARTICLE INFO

Edited by Laura Bulgariu

Keywords:
Ecotoxicology
Emerging contaminant
Flow cytometry
Photophysiology
Triterpenoid toxicity

ABSTRACT

Natural products are increasingly used in therapeutic, cosmetic, and eco-friendly applications, yet their impacts on aquatic ecosystems remain understudied. We investigated the effects of two birch-derived triterpenes, betulin and betulone, on the freshwater microalga *Chlamydomonas reinhardtii* over 24–72 h at environmentally relevant concentrations (0.01–10 µg/L). Both compounds decreased cell mortality under specific conditions, particularly at 48 h, indicating cytoprotective effects. Betulin increased reactive oxygen species (ROS) at low doses, while betulone consistently reduced ROS. Notably, ROS correlated with mortality only for betulin at 48 h, revealing effects not directly mediated by oxidative stress and pointing to other cellular targets. Betulone, but not betulin, reduced chlorophyll fluorescence, suggesting possible inhibition of chlorophyll synthesis, while photosynthetic efficiency showed transient disruptions followed by recovery, highlighting adaptive cellular responses. These compound-specific, time- and dose-dependent responses suggest that betulin and betulone could interfere with primary producers in freshwater environments, potentially destabilizing photosynthetic function and pigment integrity. Such effects are reminiscent of allelopathic interactions described in terrestrial plants, extending the ecological relevance of triterpenoids to aquatic systems. The observed recovery indicates that *C. reinhardtii* exhibited resilience in our experimental conditions, yet chronic and higher exposures could overwhelm these protective responses, highlighting the importance of monitoring triterpenoid environmental risks. Overall, this study provides the first comparative insight into the differential effects of betulin and betulone in microalgae and underscores the importance of assessing emerging natural compounds for their environmental safety and ecotoxicological implications.

1. Introduction

Natural products are a highly attractive source of various pharmacological substances and therapeutic agents, particularly for the development of new drugs and cosmetics (Tuli et al., 2021). Betulin (lup-20(29)-ene-3β,28-diol, betulinol, betulinic alcohol) and betulone (3β-hydroxy-lup-20(29)-en-28-oic acid) are naturally occurring lupane-type triterpenes predominantly found in the bark of birch trees (genus *Betula*) (Hordyjewska et al., 2019). The concentration of betulin in birch bark can be as high as about 20 % (Šiman et al., 2016; Ferreira et al., 2013), making it a readily accessible natural resource (Jäger et al., 2009). Betulone, though less abundant (1 %), is found within the same bark and can also be synthesized from betulin through oxidation processes (Jäger et al., 2009; Lou et al., 2021). The environmental concentration of betulin and betulone in freshwater varies based on natural and anthropogenic factors. Typical baseline level of these compounds range from 10^{-9} to low 10^{-6} g/L (Coicaud, 2021; Ratia et al., 2013). However, higher concentrations can occur near point sources such as birch processing facilities and in pulp and paper mill effluents. Notably, betulin and betulone have been reported at 10^{-5} g/L in effluents and 10^{-6} to 10^{-5} g/kg in sediments impacted by these discharges (Coicaud, 2021; Ratia et al., 2013).

Both compounds have garnered attention as precursor for synthesizing various bio-based materials, including polymers and

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https://doi.org/10.1016/j.ecoenv.2025.119033
Received 18 July 2025; Received in revised form 6 September 2025; Accepted 7 September 2025
Available online 12 September 2025
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nanomaterials, which are considered eco-friendly alternatives to petroleum-based products (Ma et al., 2022; Zhang et al., 2022). Furthermore, betulin and its derivatives are being explored for their potential for the development of novel pharmaceuticals and therapeutic applications (Lou et al., 2021; Yogeeswari and Sriram, 2005; Rastogi et al., 2015; Denaro et al., 2020). They also share a common lupane-type triterpene backbone (Figure S1). Nontheless, the presence of a ketone group at the C3 position in betulone confers distinct chemical properties and biological activities compared to betulin (Darme et al., 2022a). In the medical domain, betulin exhibits anti-inflammatory, antiviral, antibacterial, and anticancer properties (Lou et al., 2021). Betulone has shown potential in anticancer and antiparasitic research due to its ability to induce apoptosis in cancer cells (Lou et al., 2021), and to fight against *Toxoplasma gondii*, respectively (Darme et al., 2022b). Betulin is also valued in cosmetics for its skin-soothing and anti-aging properties which improve collagen synthesis and skin elasticity, leading to its inclusion in various skincare products such as anti-aging creams and lotions (Drąg-Zalesińska et al., 2019).

Despite these various promising applications, leading to increased releases into aquatic environments, the environmental risks associated with betulin and betulone remain understudied. As naturally occurring compounds, they have typically received less scrutiny than synthetic chemicals (Denaro et al., 2020; Mathur and Hoskins, 2017; Wainwright et al., 2022). However, with increasing utilization, there is a growing need for comprehensive ecotoxicological assessments of natural products to ensure environmental safety. Regulatory bodies may require data on their toxicity to non-target organisms and their overall environmental impact before their large-scale use.

Understanding the ecotoxicological profile of compounds is crucial for assessing their environmental impact and safety. Initial studies suggest that betulin exhibits low toxicity in biota (Jäger et al., 2008), including in aquatic organisms (Chen et al., 2020). On the other hand, betulin was identified as causing allelopathy in tropical plants by inhibiting seed germination, radicle growth, and the functioning of photosystem II (PSII) (Wang et al., 2014). Recent studies have evidenced mild toxicity in rodents (Mishra et al., 2021) and high cytotoxicity in fish fibroblasts with cellular mitochondria showing a particular vulnerability to these compounds (Małaczewska et al., 2021). Moreover, betulin increased ROS formation and altered energy metabolism in the testis of zebrafish (*Danio rerio)* (de Oliveira et al., 2021). Similarly, the antibacterial activity of betulin is attributed to oxidative stress triggered by this compound in bacterial cells (Oloyede et al., 2017).

To date, few studies have examined the effects of betulin on healthy cells, with most focusing on cytotoxicity assays (Mioc et al., 2018; Coricovac et al., 2021; Surowiak et al., 2009). Moreover, data remain scarce across a broader range of environmentally relevant species. This highlights a critical gap that comprehensive studies must address to better understand the ecological impacts of these compounds (Tuli et al., 2021). As therapeutic and industrial applications of betulin and betulone expand, alongside efforts to improve their solubility (Myz et al., 2019), it becomes increasingly important to evaluate their potential environmental risks and ensure their safe use, thus preventing unforeseen ecological consequences.

Microalgae represent the largest group of primary producers on Earth, contributing approximately 32 % of global photosynthesis and sustaining aquatic food webs by providing essential energy and nutrients (Suresh Kumar et al., 2015). However, anthropogenic pollution threatens their physiological functions, raising concerns about ecosystem health (Almeida et al., 2019). Consequently, the effects of biocides on microalgae have attracted growing scientific interest, as such studies are vital for refining ecological risk assessments.

In this context, our study offers a novel investigation into the effects of environmentally realistic concentrations of betulin and betulone on the green freshwater microalga *Chlamydomonas reinhardtii* over exposure periods ranging from 24 to 72 h. We employed multiparametric flow cytometry (FCM) enabling simultaneous measurement of multiple physiological and biochemical parameters at the single-cell level and providing a comprehensive view of cell health (Almeida et al., 2019). Pulse amplitude fluorometry (PAM) was also used to characterize PSII activity, providing dynamic insights into photosynthetic performance under stress. As such, this study provides original and crucial insights into the differential cellular and photosynthetic responses of *C. reinhardtii* to betulin and betulone, filling a significant gap in the ecotoxicological understanding of these increasingly utilized natural compounds.

2. Materials and methods

2.1. Glassware and material

The material was thoroughly cleaned three times by repeated washing and rinsing with ultrapure water (18.2 MΩ·cm, MilliQ Direct system, Merck Millipore), dried in an oven at 37°C and then autoclaved (1 bar, 121 °C, 20 min) to avoid microbial contamination.

2.2. Microalgae

Chlamydomonas reinhardtii strain CCAP11/32 A (Scottish Association for Marine Science strain) was grown in axenic conditions in 1 % BG-11 media (Sigma-Aldrich) at 28 °C ± 2 °C with a continuous light of 23 $\mu mol \cdot s^{-1} \cdot m^2$, stirring at 115 RPM and a volume ratio of 3.33 (i.e., a culture of 150 mL in a 500 mL Erlenmeyer vial). These conditions were selected based on validated laboratory practices for this strain to ensure optimal growth and reproducibility (Kselíková et al., 2022; Jin et al., 2012; Míguez et al., 2021). The culture was transplanted under a laminar flow hood once a week to keep it in exponential growth phase (Beauvais-Flück et al., 2016). A stock solution of betulin and betulone was prepared in DMSO at a concentration of 0.25 g/L. Working solutions were prepared in 1 % BG-11 media immediately before use in microalgae culture. All exposures were performed in identical culture conditions in triplicate. Two negative controls, culture medium alone and culture medium supplemented with DMSO at the highest concentration used in the assays (0.004 %; Ctl) were included for comparison. As no significant differences were observed between the two controls (Figure S2), only the solvent control is presented in figures and results. Betulin, and betulone were tested at 0.01, 0.1, 1, and 10 µg/L (corresponding to, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} g/L, respectively) covering typical environmental levels and concentrations potentially encountered near point sources (Coicaud, 2021; Ratia et al., 2013). Betulin (442.7 g/mol; CAS: 473–98–3; 98 % purity) and betulone (440.7 g/mol; CAS: 7020–34–0; 90 % purity) were purchased from NTSChemicals (Olaine, Latvia).

2.3. Flow cytometry

A BD Accuri C6 flow cytometer (Software version 1.0.264.21) was used to assess intracellular ROS levels via FL1-A detection (488/525 ± 30 nm), using the DHR123 probe (Sigma) at 5 µM after a 30 min incubation in the dark at room temperature. Cells exposed for 1 h to 200 µM tert-butyl hydroperoxide (70 %) (Thermo Fisher Scientific) served as a positive control (Ctl^+). Chlorophyll (Chl) autofluorescence was recorded using the FL3-A channel (488/670LP nm). The median fluorescence intensity (in arbitrary units) on the FL1-A and FL3-A channels was used to reflect ROS and Chl levels, respectively. Cell mortality was evaluated by measuring membrane permeability using propidium iodide (PI; 1 $mg \cdot mL^{-1}$, Sigma-Aldrich), a membrane-impermeant DNA-intercalating dye, applied at 1 % (v/v) final concentration and detected on FL2-A (488/585 ± 30 nm). Boiled cells (5 min) were used as Ctl^+, and a fluorescence threshold was established to distinguish PI-positive (dead) cells.

The corresponding Ctl^+ were verified to validate each endpoint. Analyses were considered valid only if the negative control (Ctl)

confirmed the expected healthy state of untreated cells throughout the assay, and the Ctl^{+} elicited a strong and consistent response. Data were normalized to the mean value of the Ctl, and results are expressed as percentages relative to this reference.

2.4. Photosynthesis activity

Photosynthesis activity was analyzed using a Dual-PAM-100 measurement system (Heinz Walz, Effeltrich, Germany; software version 1.19) with NIR & FR Led Array and Fluo ML Blue AL Led Array modules. For this endpoint analysis, we focused on, cultures treated with 10 µg/L (correspondint to 10^{-5} g/L) of betulin or betulone at 24 and 48 h, representing environmental concentrations potentially encountered near point sources (Ratia et al., 2013). The measurements were performed on 10^{7} cells in 1 mL in quartz tanks at room temperature, under stirring, with a 30 min acclimatization phase in the dark. The minimum fluorescence (F0) and maximum fluorescence (Fm) were determined respectively before and after a saturating light flash (300 ms at 10,000 µmoles photons·m^{-2}·s^{-1}), and the ratio of variable to maximal fluorescence was calculated with Eq. 1. Subsequently, the sample was subjected to actinic light of 250 µmoles photons·m^{-2}·s^{-1} until the sample's fluorescence was stable. Then, a saturating light flash was applied again to measure the maximum fluorescence of the sample adapted to light (Fm'), and the F0' parameter was estimated. Quantum yields were calculated according to Eq. 2 (Kramer et al., 2004) as follows: YII for photochemical energy utilization in PSII, Y(NPQ) for regulated energy dissipation in PSII, Y(NO) for nonregulated energy dissipation in PSII and electron flow through PSII (ETRII). In addition, photochemical quenching (qP), reflecting the fraction of open PSII reaction centers, non-photochemical quenching (qN), indicating protective energy dissipation, and qL, estimating the fraction of open PSII centers accounting for connectivity between units, were determined. Together, these parameters provide an integrated assessment of photosynthetic efficiency and energy allocation in response to betulin and betulone exposure.

$$\frac{Fv}{Fm} = \frac{(Fm - F0)}{Fm} \quad (1)$$

$$YII = \frac{(Fm' - F)}{Fm'} \quad (2)$$

2.5. Statistical analysis

Data were analyzed using R. Correlation analysis was performed with *Hmisc* package utilizing Spearman correlation analysis. For a significant difference against the negative Ctl, the non-parametric multiple comparison procedure of Dunnett-type was used (α=5 %) with the package *nparcomp* on each time point dataset independently (Konietschke et al., 2015).

3. Results and discussion

3.1. ROS abundance

Betulin exposure resulted in a significantly higher ROS abundance compared to the Ctl at 0.1 µg/L after 48 (276 %) and 72 h (421 %), while a slight reduction was observed at 1 µg/L after 24 (89 %) and 48 h (84 %) of exposure (Fig. 1). The highest ROS level (421 %) occurred with betulin exposure at 0.1 µg/L after 72 h. Betulone generally reduced ROS levels compared to the Ctl, notably 66 % of Ctl at 10 µg/L exposure after 24 h. Although no clear concentration-dependent pattern emerged, most differences were statistically significant, except at 0.01 µg/L (24 h) and 1 µg/L (48 h).

Increased ROS if not adequately managed by antioxidant mechanisms, may cause oxidative stress, damaging cellular components. Similarly, betulin increased ROS formation in the testis of zebrafish (*Danio rerio)* (de Oliveira et al., 2021) and in bacterial cells (Oloyede et al., 2017). Another study in human renal proximal tubule epithelial cells reported that 500•µg/L of betulin reduced antioxidant capacity and defenses as well as increased lipid peroxidation (Kruszniewska-Rajs et al., 2022). Other authors also showed that the increased production of ROS by betulin was a consequence of alterations in mitochondrial activity (Dubinin et al., 2020). Conversely, several studies observed that betulin reduced oxidative stress in tumor cell lines (Adepoju et al., 2023). Similarly, both betulone at most time points and betulin at 1 µg/L after 24 h and 48 h reduced ROS levels (66–84 %) compared to Ctl, suggesting potential antioxidant properties or the activation of cellular mechanisms that mitigate ROS production. Betulone has been shown *in vitro* to possess greater antioxidant capacity than betulin, with the ketone group identified as a key structural feature underlying this effect (Mao et al., 2012). Our findings support this observation: betulin trigered higher ROS levels than betulone, particularly at a concentration of 0.1 µg/L.

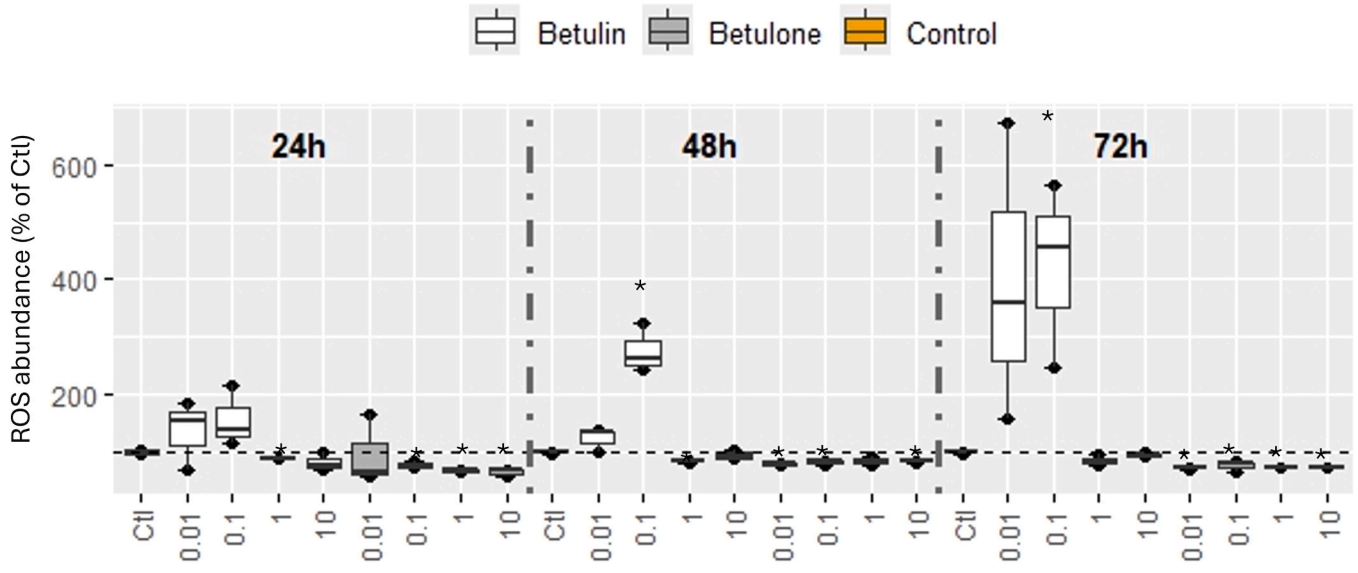


Fig. 1. ROS abundance (% of control) in *C. reinhardtii* exposed to 0.01–10 µg/L betulin and betulone. * indicates significant differences from control.

3.2. Membrane permeability

Membrane permeability used as a proxy for cell mortality was either decreased or unchanged compared to the Ctl, depending on the exposure conditions, without any obvious time or concentration dependence (Fig. 2). A decrease in cell mortality (66 %) was observed after 24 h of exposure to betulin at a concentration of 0.1 μg/L *vs* Ctl. This effect is generalized for all tested concentrations at 48 h (21–67 %), while no significant modulation of this endpoint is observed at 72 h. Betulone also significantly reduced cell mortality at 0.01 (39 %) and 1 μg/L (37 %) at 24 h in comparison with the Ctl. Previous studies have suggested that the cytotoxicity of these compounds is linked to increased ROS production [38; 39]. However, in our study, a significant correlation between ROS abundance and cell mortality was observed only for betulin exposures at 48 h (Table 1). Moreover, betulin at 0.1 μg/L significantly reduced cell mortality at both 24 h (66 %) and 48 h (67 %). These findings suggest that betulin may exert a protective effect against cell death, despite increasing ROS levels, possibly through the modulation of cellular pathways involved in the initiation of cell death. However, this hypothesis would need confirmation in future studies.

3.3. Chlorophyll autofluorescence

Chlorophyll (Chl) fluorescence was unchanged by betulin exposure (Fig. 3). In contrast, betulone caused significant reduction of Chl fluorescence at 48 h in 0.01, 1, 10 μg/L exposures (71–76 %) and at 72 h in 0.01 and 1 μg/L exposure (79–86 %). Since Chl autofluorescence serve as a proxy for pigment abundance and the potential for photosynthesis, the result suggest that betulone compromise Chl synthesis or stability, potentially impairing the photosynthetic capacity in *C. reinhardtii*. Notably, we found no correlation between Chl autofluorescence and ROS levels, indicating that oxidative stress is unlikely to be the main factor driving pigment loss. To date, no studies have directly examined betulone's influence on Chl metabolism in algae nor plants. However, under other stress conditions, decreases in Chl have been attributed to pigment degradation, impaired biosynthesis, and alterations of membrane environments that destabilize pigment-protein complexes (Jan et al., 2025; Beauvais-Fluck et al., 2017; Franklin et al., 2001; Franqueira et al., 2000). Betulone's triterpenoid backbone may disrupt biochemical pathways or structural components required for Chl stability, and could also interact directly with the pigment itself. The synthesis of conjugates between Chl derivatives and triterpenoids, including betulin (Malshakova et al., 2013), demonstrated the feasibility of such direct interactions. This supported the hypothesis that betulone's toxicity could arises from perturbation of Chl integrity, although experimental confirmation is still needed. These results pointed to a novel, ROS-independent mechanism of Chl destabilization by betulone, requiring further investigation into specific targets of this compound.

3.4. Photosynthesis activity

To further assess the potential effect of betulin and betulone on photosynthetic efficiency, we used PAM at a concentration of 10 μg/L, with exposures at 24 h and 48 h. Exposures resulted in different alterations of photosynthesis activity in microalgae exposed 24 h to a concentration of 10 μg/L of betulin and betulone, while more similar alterations of photosynthesis activity were observed at 48 h (Table 2). At 24 h, betulone significantly increased Y(NO) (115 %). At the same time, it reduced photochemical quenching (qP) (59 %) and the maximum efficiency of PSII photochemistry after dark adaptation (Fv/Fm) (55 %), suggesting a higher energy loss as fluorescence or heat, not regulated by protective mechanisms, fewer open PSII reaction centers available for photochemistry and a reduced photosynthesis activity. Betulin at 24 h reduced YII (91 %), indicating that a lower proportion of absorbed light energy is used for photochemical reactions. At 24 h, data globally suggest that the photosynthetic machinery is less efficient, with fewer active PSII centers participating in photochemistry and fewer protective mechanisms, potentially leading to increased photodamage or malfunction of the photosynthetic apparatus. These data suggest an early impairment of the photosynthetic machinery, reflected by reduced efficiency and protective capacity. This interpretation is partly consistent with FCM observations, as changes in Chl fluorescence were not always statistically significant due to high variability. Other authors have identified betulin as reducing the electron transport rate (ETR), the rate at which electrons are transported through the PSII during the light reactions of photosynthesis, and ETRmax in cucumber seedlings (Wang et al., 2014), indicating a marked inhibition of PSII activity and consequently reduced production of ATP and NADPH. While the experimental context differs, higher triterpenoid concentrations (≈10^6 μg/L) applied to terrestrial plants *vs* trace-level exposures (10 μg/L) in microalgae, the findings converge on the ability of triterpenoid structures to disrupt photosynthetic function. Such reduction may reflect damage to the photosynthetic apparatus, affecting the Chl content, thylakoid membrane integrity, or other critical components of PSII. Our findings in

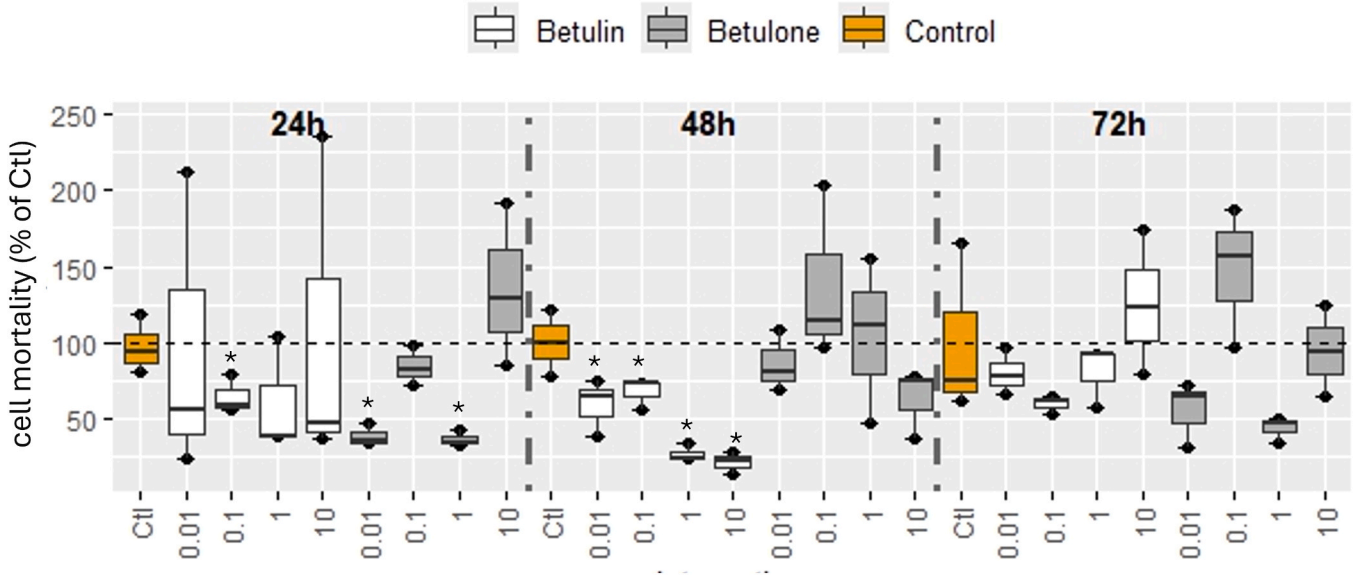


Fig. 2. Membrane permeability (% of control) in *C. reinhardtii* exposed to 0.01–10 μg/L betulin and betulone. * indicates significant differences from control.

Table 1
correlation analysis of FCM endpoints considering molecule and time points. Spearman correlation coefficient and respective *p*-value are shown. Significant coefficients ($p < 0.05$) are in bold.

	Betulin 24 h	Betulone 24 h	Betulin 48 h	Betulone 48 h	Betulin 72 h	Betulone 72 h
ROS/mortality *R*	0.24	-0.14	**0.62**	-0.34	-0.41	0. 12
p-value	0.45	0.66	*0.03*	0.27	0.18	0.72
ROS/Chlorophyll abundance *R*	-0.28	0.11	-0.09	0.27	-0.02	**0.62**
p-value	0.38	0.74	0.77	0.40	0.94	*0.03*
Chlorophyll abundance/mortality R	-0.14	0.01	-0.35	0.40	0.27	0.00
p-value	0.66	0.98	0.27	0.19	0.40	0.99

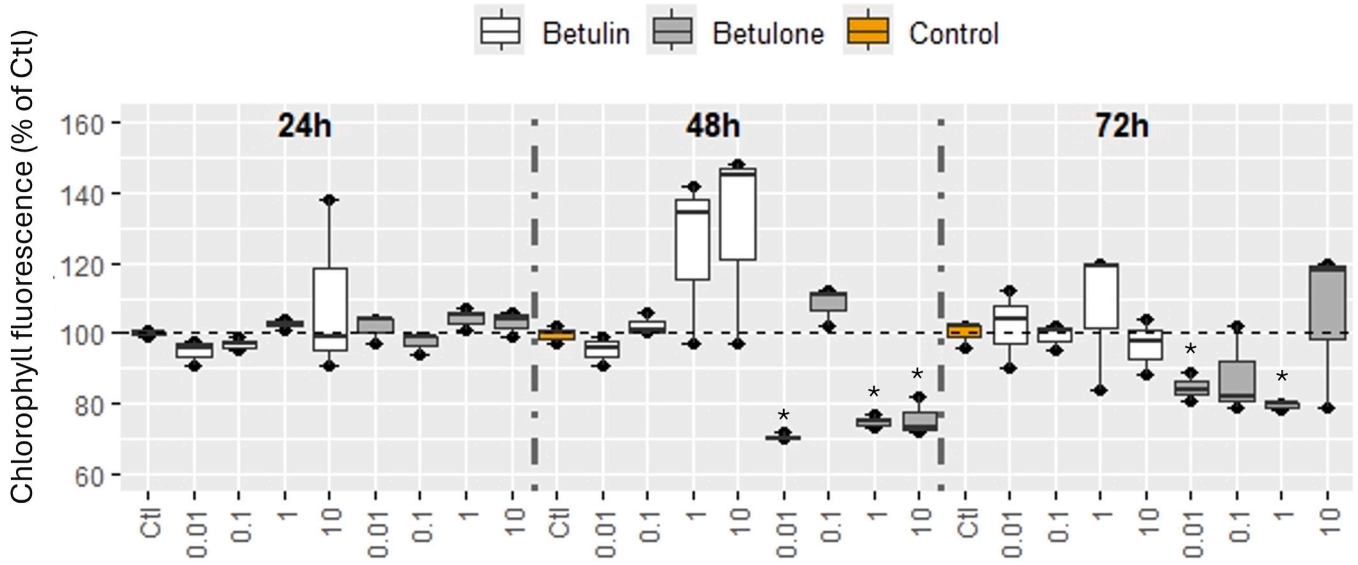


Fig. 3. Chlorophyll autofluorescence (% of control) in *C. reinhardtii* exposed to 0.01–10 μg/L betulin and betulone. * indicates significant differences from control.

Table 2
Effects of 10 μg/L betulin and betulone on physiological endpoints in *C. reinhardtii* after 24 and 48h-long exposure. Quantum yields, designated by YII [YII = (Fm' – F)/Fm'] for photochemical energy utilization in PSII, Y(NPQ) for regulated energy dissipation in PSII, Y(NO) for nonregulated energy dissipation in PSII and electron flow through PSII (ETRII) were calculated according to (Kramer et al., 2004). Photochemical quenching (qP), reflecting the fraction of open PSII reaction centers, non-photochemical quenching (qN), indicating protective energy dissipation, and qL, estimating the fraction of open PSII centers accounting for connectivity between units, were determined. Values in bold are significantly different from the control (Ctl; $p < 0.05$).

	Fv/Fm (% of Ctl)	YII	Y(NPQ)	Y(NO)	ETR(II) (% of Ctl)	qP	qN	qL
24 h								
Ctl	1.000 ± 0.368	0.717 ± 0.037	0.055 ± 0.031	0.661 ± 0.075	1.000 ± 0.368	0.407 ± 0.123	0.095 ± 0.047	0.176 ± 0.056
Betulin	0.744 ± 0.176	**0.656 ± 0.014**	0.057 ± 0.030	0.731 ± 0.022	0.745 ± 0.177	0.336 ± 0.070	0.096 ± 0.045	0.158 ± 0.035
Betulone	**0.549 ± 0.124**	0.681 ± 0.025	0.083 ± 0.017	**0.761 ± 0.018**	0.548 ± 0.122	**0.241 ± 0.062**	0.128 ± 0.020	0.102 ± 0.037
48 h								
Ctl	1.000 ± 2.236	0.558 ± 0.126	0.075 ± 0.028	0.890 ± 0.072	1.000 ± 0.535	0.205 ± 0.098	0.011 ± 0.025	0.112 ± 0.073
Betulin	**6.228 ± 1.393**	**0.707 ± 0.031**	**0.045 ± 0.003**	0.801 ± 0.151	1.459 ± 1.413	0.229 ± 0.202	**0.071 ± 0.016**	0.086 ± 0.086
Betulone	6.023 ± 3.226	**0.713 ± 0.010**	**0.042 ± 0.017**	0.787 ± 0.132	1.615 ± 1.090	0.247 ± 0.167	**0.069 ± 0.037**	0.100 ± 0.083

C. reinhardtii at 24 h were consistent with this mode of action, underscoring the ability of triterpenoid structures to interfere with photosynthesis. However, by 48 h both compounds induced patterns indicative of recovery: effective PSII quantum yield (YII) were increased compared to the Ctl (109 and 128 %), while Y(NPQ) decreased (56 and 60 %). Most strikingly, betulin strongly enhanced qN (645 %) and the maximum efficiency of PSII photochemistry after dark adaptation (Fv/Fm) (623 %), suggesting not only restoration of PSII efficiency but also an activation of overprotective mechanisms that safeguard the photosynthetic apparatus from excess energy and potential photodamage. These adjustments indicate a rapid and robust compensation, allowing the photosynthetic system to regain optimal functionality despite the initial stress, suggesting a hormesis effect (Calabrese and Mattson, 2017). Our results therefore extend the evidence of triterpenoid phytotoxicity to aquatic primary producers and highlight the duality of their effects: an initial destabilization of PSII followed by compensatory restructuring that enhances resilience and protective capacity (Wang et al., 2014). Notably, our study is, to our knowledge, the first to examine the effects of betulin and betulone on microalgal photosynthesis. This originality highlighted a significant gap in the literature and underscored the need for further investigations into the ecotoxicological consequences of these triterpenoids in aquatic primary producers.

From an ecotoxicological perspective, these findings are particularly relevant. Betulin and betulone are increasingly released into aquatic environments through industrial processes, potentially exposing microalgal communities to biologically active triterpenoid concentrations (Ratia et al., 2013; de Oliveira et al., 2021). Impairment of Chl

integrity and PSII efficiency, even transiently, could reduce primary productivity, alter energy transfer within the food web, and impact ecosystem functioning (Field et al., 1998). The observed recovery suggests resilience in *C. reinhardtii*, but chronic or higher-level exposures may overwhelm protective mechanisms, emphasizing the need for environmental monitoring and risk assessment of triterpenoid contaminants.

3.5. Toxicity mechanisms and ecotoxicological implications

By identifying early and sublethal effects on microalgal physiology, our results offer critical parameters for environmental monitoring and risk assessment, especially in regions where triterpenoid discharge is expected to increase. This study provided the first mechanistic evidence of how betulin and betulone affected primary producers at environmentally relevant concentrations, bridging a critical knowledge gap in the ecotoxicological assessment of naturally occurring triterpenoids in aquatic ecosystems. The constrating effects of betulin and betulone on *C. reinhardtii* across various endpoints, ROS production, membrane permeability, Chl autofluorescence, and photosynthesis activity, revealed a complex interplay between oxidative stress, cell viability, and photosynthesis. Increased ROS production following betulin exposure does not correlate with variable impacts on cell mortality. Similarly, ROS production is expected to contribute to the reductions in Chl autofluorescence, suggesting impaired Chl synthesis or degradation, which directly affects photosynthetic capacity. However, the absence of correlation between endpoints suggests that ROS are not directly linked to the reduction of Chl observed with betulin and betulone exposure at environmental concentrations, indicating that other mechanisms may be at play. Future research should investigate the toxicity pathways triggered by betulone to identify its precise targets. The compromised photosynthetic efficiency at 24 h could illustrate the initial detrimental impact on the photosynthetic apparatus. These disruptions could be critical as they might affect the microalgae's overall energy balance and metabolic functioning. Therefore, it is an interesting endpoint in ecotoxicology because its regulation mechanism under stress can shed light on microalgae physiology (White et al., 2011). Photosynthesis plays a central role in converting matter into energy, forming the foundation of aquatic food webs. As a model primary producer, *C. reinhardtii* offers valuable insights into the potential early effects of betulin and betulone exposure. While extrapolation to ecosystem-level impacts requires caution, the observed cellular responses may suggest possible implications for the functioning of aquatic trophic networks. Notably, the partial recovery of photosynthetic parameters by 48 h points to an adaptive response that may help restore photosynthetic function and activate protective mechanisms, consistent with the increased Chl abundance observed at 72 h. This recovery highlights the complex and time-dependent nature of the cellular response, underscoring the importance of considering both stress dynamics and toxicokinetics when evaluating the ecological relevance of such compounds. Beyond photosynthetic impairment, triterpenoids are recognized as phytotoxic compounds in terrestrial ecosystems, where they inhibit seed germination, root elongation, and growth, often acting as allelochemicals that suppress competitors. Betulinic and ursolic acids, for example, have been implicated in allelopathic interactions that shape plant–plant dynamics (Wang et al., 2014). Our results extend this ecological role to aquatic systems, suggesting that betulin and betulone released into freshwater environments could interfere with primary producers not only through growth inhibition but also by destabilizing pigment integrity and photosynthetic performance. This dual action, direct effects on chlorophyll stability and functional impairment of PSII, highlighted their potential ecotoxicological significance as allelochemicals beyond terrestrial habitats. Indeed, this study provided novel insights into the cellular and photosynthetic responses of *C. reinhardtii* to environmentally relevant concentrations of betulin and betulone, using cellular-level effects to highlight potential consequences for aquatic ecosystems.

The identification of concentration-dependent and reversible effects further suggested that *C. reinhardtii* may serve as a sensitive model for monitoring triterpenoid contamination in freshwater systems. The data suggest that betulin and betulone exert significant sublethal effects on *C. reinhardtii* under our experimental conditions. Both molecules exhibited complex and distinct impacts, varying according to concentration and exposure time, which influence ROS production, membrane permeability, Chl content, and PSII efficiency. Previous studies have documented the cytotoxic potential of these molecules, particularly their ability to induce oxidative stress and impair cell viability across various organisms (Małaczewska et al., 2021; de Oliveira et al., 2021; Oloyede et al., 2017). The observed increase in ROS production, reduced Chl autofluorescence, and decreased PSII activity at specific time points align with earlier findings showing that betulin and betulone can disrupt cellular redox balance and compromise photosynthetic function. Betulin generally appears to have both the capacity to increase ROS abundance and a more protective role, particularly evident in its ability to reduce mortality and enhance photosynthetic recovery after initial stress. In contrast, betulone consistently reduce ROS levels but other effects are more variable and sometimes appear detrimental, as shown by decreased Chl content and PSII activity. These findings highlight for the first time distinct, time-dependent effects of both compounds on *C. reinhardtii*, revealing not only oxidative stress but also potential adaptive responses in photosynthetic efficiency, along with disruptions in Chl synthesis and the photosynthetic machinery. The data further suggest involvement of additional, yet unidentified, toxicity targets. Overall, these results underscored the importance of considering species-specific dynamics, temporal variation, and recovery mechanisms in ecotoxicological assessments. By detailing the complex interactions between ROS, Chl content, and PSII function across concentrations and time points, this study advances the current understanding of natural product toxicity. These findings highlight the necessity to include triterpenoids such as betulin and betulone in future ecotoxicological guidelines and monitoring programs, as their sublethal impacts may propagate through aquatic food webs. Future work should focus on elucidating the molecular pathways underlying these effects to better inform environmental risk assessment and management in aquatic ecosystems.

4. Conclusion

Our results revealed compound-specific effects: betulin increased ROS at low concentrations, while betulone reduced it consistently. Both compounds decreased cell mortality under certain conditions, particularly at 48 h, indicating a cytoprotective effect. Betulin did not affect Chl fluorescence, whereas betulone reduced it, suggesting possible inhibition of Chl synthesis. Changes in photosynthetic efficiency were transient, with initial disruption followed by recovery. Furthermore, the compound-specific, time- and dose-dependent responses observed here highlighted the complexity of algal sensitivity to natural bioactive molecules. Notably, ROS levels correlated with mortality only at 48 h for betulin, pointing effects not directly linked to ROS levels and the need to identify the underlying target. These results expand upon current scientific knowledge, as no prior study has evaluated the effects of betulin and betulone on microalgae. This study provides the first comparative insights into their differential impacts on *C. reinhardtii*, a widely used model organism in ecotoxicology. Beyond characterizing toxicity, this work also introduces an innovative multiparametric approach combining FCM and PAM fluorometry to uncover subtle and dynamic cellular processes in *C. reinhardtii*. This integrative strategy represents a novel framework for investigating the ecotoxicological effects of emerging natural compounds in primary producers.

CRediT authorship contribution statement

Anthony Montembault: Investigation, Formal analysis. **Damien Rioult:** Writing – review & editing, Validation, Supervision, Resources, Methodology. **Nicolas Borie:** Validation, Supervision, Methodology. **Simon Remy:** Writing – review & editing, Methodology. **Jean-Hugues Renault:** Writing – review & editing, Validation, Resources, Methodology. **Cosio Claudia:** Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Iris Barjhoux:** Writing – review & editing, Validation, Supervision, Methodology. **Sandra Villaume:** Visualization, Investigation, Formal analysis. **Nathalie Gaveau:** Writing – review & editing, Validation, Resources, Methodology.

Funding

This research was funded by SFR Condorcet to POLEMIC program and Grand Reims to chaire BASE.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Claudia Cosio reports financial support was provided by Greater Reims Urban Community. Claudia Cosio reports financial support was provided by SFR Condorcet. Jean-Hugues Renault reports financial support was provided by Horizon Europe. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We are grateful to the URCATech-Mobicyte core facility at URCA for access to FCM facilities and technical support. Authors also benefited from the French GDR “Aquatic Ecotoxicology” framework, and the HORIZON-MSCA-2022-SE-01 “GreenCosmIn” project (code 101131346) aiming at fostering stimulating scientific discussions and collaborations for more integrative approaches.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.119033.

Data availability

Data will be made available on request.

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