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Fizjologiczno-biochemiczne podstawy
odpowiedzi zielenic planktonowych
Chlamydomonas reinhardtii i *Desmodesmus*
armatus na niesteroidowe leki przeciwzapalne
obecne w środowisku wodnym

Physiological and biochemical basis of the response
of planktonic green algae *Chlamydomonas reinhardtii*
and *Desmodesmus armatus* to non-steroidal
anti-inflammatory drugs present in the aquatic
environment

Praca przedstawiona
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celem uzyskania stopnia doktora
w dziedzinie nauk ścisłych i przyrodniczych
w dyscyplinie nauki biologiczne

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1. Źródła finansowania

Niniejsza rozprawa doktorska powstała przy wsparciu finansowym:

1. Narodowego Centrum Nauki w ramach projektu „Ocena wrażliwości i remediacyjnego potencjału zielenic planktonowych wobec niesteroidowych leków przeciwzapalnych obecnych w środowisku wodnym” o numerze UMO-2019/35/B/NZ9/01567, OPUS 18, kierownik projektu dr hab. Anna Aksmann, prof. UG.
2. Wydziału Biologii w ramach Konkursu Projektów Badań Naukowych Młodych Naukowców: „Analiza toksycznego działania nabumetonu w komórkach zielenicy *Desmodesmus armatus*” o numerze 539-D070-B089-23 (2023); oraz „Zintegrowana analiza molekularna i fizjologiczna działania niskich stężeń kwasu flufenamowego na aparat fotosyntetyczny *Chlamydomonas reinhardtii*” o numerze 539-D230-B223-25 (2025). Kierownik projektu: mgr Dominika Kapuścińska.

2. Streszczenie w języku polskim

Niesteroidowe leki przeciwzapalne (NLPZ) należą do istotnych zanieczyszczeń środowiska wodnego, a ich obecność w wodach powierzchniowych wynika z powszechnego stosowania, rosnącej konsumpcji oraz niewystarczającej skuteczności usuwania tych substancji w procesach oczyszczania ścieków. Zielenice planktonowe są organizmami autotroficznymi należącymi do podstawowych producentów w ekosystemach wodnych, stanowiącymi doskonały model do badań nad wpływem zanieczyszczeń występujących w środowisku. Podjęcie niniejszych badań wynikało z ograniczonej liczby danych dotyczących wpływu NLPZ na organizmy niedocelowe, w tym mikroglony, istotne dla funkcjonowania biocenoz wodnych.

Celem rozprawy było określenie fizjologiczno-biochemicznych podstaw odpowiedzi wybranych zielenic planktonowych na NLPZ obecne w środowisku wodnym. Badania obejmowały dwa gatunki mikroglonów, *Chlamydomonas reinhardtii* oraz *Desmodesmus armatus* i koncentrowały się na analizie parametrów wzrostu populacji, funkcjonowania aparatu fotosyntetycznego, homeostazy redoks i odpowiedzi antyoksydacyjnej.

Analizy wykonane w ramach rozprawy doktorskiej wykazały, że NLPZ indukują odmienne, specyficzne dla poszczególnych substancji profile zmian fizjologicznych i biochemicznych. W badaniach prowadzonych na *C. reinhardtii* stwierdzono, że nabumeton (NBT) wykazywał wyższą toksyczność niż kwas flufenamowy (FFA), natomiast najmniej toksyczne wydawały się być naproksen (NPX) i ibuprofen (IBU). Wiarygodna ocena poziomu stresu oksydacyjnego wywołanego w komórkach zielenic przez NLPZ była możliwa dzięki optymalizacji metody czułego i ilościowego oznaczania nadtlenu wodoru. Analiza wpływu substancji leczniczych na *C. reinhardtii* wykazała, że wszystkie badane związki zwiększały produkcję nadtlenu wodoru i ograniczały wzrost populacji. Mniej toksyczne IBU i NPX aktywowały przede wszystkim skoordynowaną odpowiedź dysmutazy ponadtlenkowej i katalazy, podczas gdy bardziej toksyczne FFA i NBT silniej wpływały na aktywność peroksydazy askorbinianowej, obecnej m. in. w chloroplastach, sugerując większe znaczenie chloroplastowych mechanizmów detoksykacyjnych w ochronie komórki przed skutkami stresu. Badania prowadzone na *D. armatus* wykazały, że substancje zbliżone do siebie budową chemiczną mogą indukować odmienne reakcje organizmu. Taka sytuacja występowała w przypadku NPX i NBT, które wywoływały różne efekty w komórkach glonów: NPX zaburzał

przebieg fotosyntezy, obniżając wydajność PSII, natomiast NBT nie powodował typowej inhibicji, lecz indukował odpowiedź kompensującą skutki stresu w komórkach. W kolejnym etapie badań dowiedziono, że również FFA, IBU i diklofenak (DCF) wywołują odrębne profile zmian, przy czym DCF działa jako silny stresor fotosyntetyczny, FFA indukuje metabolicznie kosztowną aklimatyzację komórek, a IBU powoduje relatywnie łagodną odpowiedź badanej zielenicy. U *D. armatus* odpowiedź na NLPZ obejmowała także reorganizację morfologicznej struktury populacji (względny udział form jedno-, dwu-, cztero- i ośmiokomórkowych w strukturze populacji), co wskazuje na duże znaczenie plastyczności morfologicznej w aklimatyzacji tego gatunku do warunków stresu.

Uzyskane wyniki wskazują, że wrażliwość zielenic na NLPZ zależy od właściwości chemicznych związku oraz cech biologicznych organizmu. Sama inhibicja wzrostu i wyznaczenie wartości EC (stężenie wywołujące określony efekt) nie są wystarczające do oceny toksyczności danej substancji, ponieważ podobne efekty końcowe mogą mieć różne podłoża fizjologiczno-biochemiczne. Niniejsza dysertacja pokazuje, że wiarygodna ocena ekotoksyczności substancji leczniczych obecnych w środowisku wodnym wymaga wielopoziomowego podejścia, integrującego wskaźniki fizjologiczne, biochemiczne, a w wybranych przypadkach także molekularne i morfologiczne.

3. Summary in English

Non-steroidal anti-inflammatory drugs (NSAIDs) are among significant contaminants of the aquatic environment, and their presence in surface waters results from their widespread use, increasing consumption, and the insufficient efficiency of their removal during wastewater treatment processes. Planktonic green algae are autotrophic organisms and primary producers in aquatic ecosystems, and at the same time they constitute an excellent model for studying the toxicity of environmental contaminants. The undertaken research was motivated by the limited amount of available data on the effects of NSAIDs on non-target organisms, including microalgae.

The aim of the dissertation was to determine the physiological and biochemical basis of the response of selected planktonic green algae to NSAIDs present in the aquatic environment. The study focused on two microalgal species, *Chlamydomonas reinhardtii* and *Desmodesmus armatus*, and examined changes in population growth, the functioning of the photosynthetic apparatus, redox homeostasis, and the antioxidant response.

The results demonstrated that NSAIDs induce distinct, compound-specific profiles of physiological and biochemical changes. In *C. reinhardtii*, nabumetone (NBT) was found to be more toxic than flufenamic acid (FFA), whereas naproxen (NPX) and ibuprofen (IBU) appeared to be the least toxic. A reliable assessment of oxidative stress induced in green algal cells by NSAIDs was made possible by the optimization of a sensitive and quantitative method for hydrogen peroxide determination. All tested compounds increased hydrogen peroxide production and inhibited population growth; however, IBU and NPX primarily induced a coordinated superoxide dismutase and catalase response, while FFA and NBT more strongly activated ascorbate peroxidase, which is present, among others, in chloroplasts, suggesting a greater importance of chloroplast detoxification mechanisms in protecting the cell against the effects of stress. Studies conducted on *D. armatus* revealed that chemically similar compounds may induce different responses in the organism. NPX disrupted photosynthetic performance by reducing PSII efficiency, whereas NBT did not cause typical inhibition but rather induced a compensatory response to stress in the cells. Furthermore, FFA, IBU, and diclofenac (DCF) produced distinct response profiles: DCF acted as a strong photosynthetic stressor, FFA triggered metabolically costly acclimation processes, and IBU caused a relatively mild response. In *D. armatus*, the response to NSAIDs also involved the reorganization

of morphological structure of population (the relative abundance of single cells, 2-, 4-, and 8-celled coenobia in population structure), highlighting the role of morphological plasticity in the acclimation of this species to stress conditions.

Overall, the obtained results indicate that the sensitivity of green algae to NSAIDs depends on both the chemical properties of the compounds and the biological characteristics of the organisms. Growth inhibition and EC values alone are insufficient to assess toxicity, as similar endpoints may arise from different physiological and biochemical mechanisms. The study demonstrates that a reliable assessment of the ecotoxicity of pharmaceuticals present in the aquatic environment requires a multi-level approach integrating physiological, biochemical, and, in selected cases, also molecular and morphological indicators.

4. Wykaz prac wchodzących w skład rozprawy doktorskiej

Praca nr 1

Kapuścińska D., Narajczyk M., Liakh I., Wielgomas B., Aksmann A. Nabumetone and flufenamic acid pose a serious risk to aquatic plants: A study with *Chlamydomonas reinhardtii* as a model organism. *Chemosphere* 2024;349:140853. <https://doi.org/10.1016/J.CHEMOSPHERE.2023.140853>

Impact Factor = 8,1 Punktacja MNISW = 140 pkt Kwartyl = Q1

Praca nr 2

Hejna M., **Kapuścińska D.**, Aksmann A. A sensitive and reliable method for the quantitative determination of hydrogen peroxide produced by microalgae cells. *Journal of Phycology*, 2024, 60(6), 1356–1370. <https://doi.org/10.1111/JPY.13524>

Impact Factor = 3,4 Punktacja MNISW = 100 pkt Kwartyl = Q1

Praca nr 3

Kapuścińska D., Hejna M., Pokora W., Aksmann A. Differential antioxidant pathways displayed by *Chlamydomonas reinhardtii* exposed to selected NSAIDs. *Scientific Reports*, 2026, 16, 27289, <https://doi.org/10.1038/s41598-026-57688-8>

Impact Factor = 4,3 Punktacja MNISW = 140 pkt Kwartyl = Q1

Praca nr 4

Kapuścińska D., Harshkova D., Shams M., Liakh I., Wielgomas B., Aksmann A. Distinct biochemical effects of naproxen and nabumetone drive their differential toxicity in *Desmodemus armatus*. (2026 - manuskrypt)

Praca nr 5

Kapuścińska D., Harshkova D., Shams M., Liakh I., Wielgomas B., Aksmann A. NSAID-induced stress triggers physiological and morphological plasticity in *Desmodemus armatus*: a multi-biomarker approach. (2026 - manuskrypt)

5. Wprowadzenie

Niesteroidowe leki przeciwzapalne (NLPZ) stanowią jedną z najbardziej różnorodnych pod względem budowy, a jednocześnie wciąż niedostatecznie zbadanych grup zanieczyszczeń środowiska wodnego. Powszechna dostępność, wzrastająca konsumpcja i niewystarczające mechanizmy oczyszczania ścieków są główną przyczyną występowania substancji czynnych leków jak i ich pochodnych w wodach powierzchniowych na całym świecie (Daughton i Ruhoy, 2009; Pereira i in., 2020). Mimo rosnącej świadomości tego problemu, nadal brakuje szczegółowych regulacji prawnych dotyczących obecności substancji leczniczych w środowisku. Problem ten ma szczególne znaczenie ekologiczne, ponieważ nawet związki zaprojektowane jako bezpieczne i skuteczne w terapii człowieka mogą wywoływać nieprzewidywalne skutki u organizmów niedocelowych, w tym u producentów pierwotnych (Świacka i in., 2021; Hejna i in., 2022).

Zielenice planktonowe, jako autotrofy, stanowią podstawę wodnych sieci troficznych, uczestniczą w obiegu materii, a jednocześnie należą do organizmów wrażliwych na obecne w środowisku zanieczyszczenia (Samson i Popovic, 1988). Odgrywają także istotną rolę w procesach samooczyszczania zbiorników wodnych. W badaniach ekotoksykologicznych uznawane są za modelowe organizmy do analizy wpływu zanieczyszczeń na procesy fotosyntetyczne (Ball, 2005; Majewska i in., 2018, 2021; Hejna i in., 2022). Poznanie odpowiedzi mikroglonów na zanieczyszczenia farmaceutyczne ma znaczenie nie tylko poznawcze, ale także aplikacyjne, ponieważ organizmy te są rozważane jako potencjalne narzędzie fitoremediacji i oceny ryzyka ekotoksykologicznego (Doran, 2009; Dhir, 2013). Mimo rosnącej liczby doniesień dotyczących obecności NLPZ w środowisku, wiedza o ich wpływie na mikroglony pozostaje nadal fragmentaryczna, a dostępne dane koncentrują się częściej na organizmach zwierzęcych oraz kilku najczęściej badanych NLPZ, takich jak diklofenak (DCF) czy ibuprofen (IBU) (Caban i in., 2016; Świacka i in., 2021). Istotnym ograniczeniem jest także fakt, że toksyczność NLPZ bywa określana głównie na podstawie zahamowania wzrostu populacji i wyznaczenia wartości EC (ang. *effective concentration*), czyli stężenia powodującego zahamowanie wzrostu na określonym poziomie. Takie podejście nie pozwala jednak pokazać pełnego profilu zmian wywołanych przez te związki w komórkach mikroglonów, ponieważ hamowanie wzrostu populacji może wynikać z odmiennych zaburzeń fizjologicznych i biochemicznych.

Przyczyną podjęcia niniejszego tematu w dysertacji doktorskiej była więc potrzeba lepszego zrozumienia, w jaki sposób różne NLPZ wpływają na zielenice planktonowe oraz w jakim stopniu charakter tego wpływu zależy od właściwości chemicznych związku. Istotnym punktem wyjścia były także wyniki wcześniejszych badań dotyczących działania diklofenaku na *Chlamydomonas reinhardtii*, które wskazywały, że substancje należące do NLPZ wyraźnie zaburzają funkcjonowanie komórek (Majewska i in., 2018, 2021). Równie ważne było także porównanie odpowiedzi dwóch gatunków zielenic różniących się cechami morfologicznymi, a także charakteryzujących się odmienną wrażliwością na różne rodzaje zanieczyszczeń.

6. Cele pracy

Celem rozprawy doktorskiej było określenie fizjologiczno-biochemicznych podstaw odpowiedzi wybranych zielenic planktonowych na wybrane niesteroidowe leki przeciwzapalne, powszechnie obecne w środowisku wodnym. Cel ten realizowano poprzez zbadanie odpowiedzi *Chlamydomonas reinhardtii* oraz *Desmodesmus armatus* na wybrane NLPZ, ze szczególnym uwzględnieniem zmian dotyczących funkcjonowania aparatu fotosyntetycznego, zaburzeń równowagi redoks i odpowiedzi antyoksydacyjnej.

7. Omówienie publikacji wchodzących w skład rozprawy

Publikacja 1

W pierwszym artykule **“Nabumetone and flufenamic acid pose a serious risk to aquatic plants: A study with *Chlamydomonas reinhardtii* as a model organism”** przeanalizowano toksyczność dwóch NLPZ, nabumetonu (NBT) i kwasu flufenamowego (FFA) wobec modelowej zielenicy planktonowej *C. reinhardtii*. W pracy przyjęto założenie, że różnice w budowie chemicznej badanych związków mogą przekładać się na różnice zarówno w sile ich toksycznego działania jak i w charakterze indukowanych zaburzeń fizjologicznych. Analizowano wpływ tych substancji leczniczych na wzrost populacji, zawartość barwników fotosyntetycznych oraz parametry fluorescencji chlorofilu *a in vivo*, a także na przebieg fotosyntezy i oddychania. Uzupełnieniem były ocena ultrastruktury komórek oraz zastosowanie wskaźnika zintegrowanej odpowiedzi biomarkera (IBR, ang. *Integrated Biomarker Response*), pozwalającego na całościową ocenę toksycznego oddziaływania badanych substancji.

Wykazano, że oba związki hamowały wzrost *C. reinhardtii*, przy czym NBT charakteryzował się ponad sześciokrotnie wyższą toksycznością niż FFA. NLPZ powodowały także wzrost zawartości chlorofilu i karotenoidów w komórkach glonów, jednak ich wpływ na przebieg fotosyntezy był odmienny. FFA silnie ograniczał intensywność fotosyntezy i oddychania komórkowego, a analiza parametrów OJIP wskazała, że jego działanie mogło wiązać się z przekształcaniem części centrów reakcji PSII w tzw. „ciche centra” (ang. *“silent centers”*), których zadaniem jest rozpraszanie energii w postaci ciepła. Świadczy to więc o tym, że energia, która początkowo została zaabsorbowana, nie mogła być efektywnie wykorzystana w procesach fotochemicznych. W przypadku NBT stwierdzono natomiast zaburzenia transportu elektronów poza PSII, przy jednoczesnym utrzymaniu wysokiej aktywności części parametrów związanych z pierwotnymi reakcjami fotochemicznymi. Analiza ultrastruktury komórek nie wykazała drastycznej degradacji chloroplastów ani mitochondriów. W komórkach traktowanych FFA obserwowano autofagiczne wakuole i mniejszą niż w kontroli ilość ziaren skrobi, natomiast po ekspozycji na NBT ziarna skrobi były liczniejsze, występowały także organelle komórkowe o kwasowym charakterze, bogate w wapń (ang. *acidocalcisomes*). Wartości IBR potwierdziły toksyczność obu związków, przy czym była ona wyższa dla FFA, co wskazuje, że zasadniczym celem działania tego związku był aparat fotosyntetyczny. Uzyskane wyniki dowodzą, że oba badane NLPZ stanowią realne zagrożenie dla *C. reinhardtii*, jednak ich działanie ma odmienny charakter. Choć NBT wykazał wyraźnie wyższą toksyczność ocenianą na podstawie EC50, to FFA silniej zaburzał przebieg fotosyntezy i oddychania komórkowego, co wskazuje, że ocena działania substancji leczniczych nie może opierać się wyłącznie na analizie hamowania wzrostu. Różne typy odpowiedzi fizjologicznej uruchamiane przez związki należące do tej samej grupy terapeutycznej mają istotne znaczenie dla interpretacji ryzyka środowiskowego związanego z obecnością tych substancji.

Publikacja 2

Wnioski uzyskane w pierwszej publikacji pozwoliły na sformułowanie kolejnego pytania badawczego: czy obserwowane różnice mają związek z nasileniem stresu oksydacyjnego i akumulacją reaktywnych form tlenu? W związku z tym, że nadtlenek wodoru (H_2O_2) stanowi jeden z podstawowych wskaźników zaburzeń równowagi redoks, dalsze badania wymagały zastosowania odpowiednio czułego i wiarygodnego podejścia analitycznego, co stało się przedmiotem drugiej publikacji. Dostępne metody oznaczania stężenia H_2O_2 , choć używane w badaniach prowadzonych na komórkach zwierzęcych, roślinnych i bakteryjnych, nie zawsze mogą być bezpośrednio wykorzystywane w badaniach mikroglonów, ze względu na ich specyficzne właściwości biologiczne oraz cechy optyczne komórek, silnie wpływające na odczyt sygnału fluorescencji. Praca „**A sensitive and reliable method for the quantitative determination of hydrogen peroxide produced by microalgae cells**” jest ważną podstawą metodologiczną dla dalszej części cyklu badań.

Jej celem było opracowanie i optymalizacja czułej oraz powtarzalnej metody ilościowego oznaczania H_2O_2 produkowanego przez komórki *C. reinhardtii* z wykorzystaniem testu fluorometrycznego opartego na Amplex UltraRed. Zakres prac obejmował dobór odpowiedniego buforu reakcyjnego, optymalizację długości fali wzbudzenia i emisji, ocenę wpływu gęstości biomasy oraz stosunku ilości mieszaniny reakcyjnej do próbki biologicznej na wiarygodność wyników. Szczególną uwagę poświęcono również sprawdzeniu, czy fortyfikacja próbek, polegająca na dodaniu określonej ilości H_2O_2 , oraz usunięcie biomasy z analizowanej zawiesiny przed pomiarem mogą poprawiać dokładność i powtarzalność oznaczeń.

Wykazano, że najlepsze warunki pomiaru zapewniały bufor fosforanowo-potasowy oraz odczyty wykonywane przy fali wzbudzenia 560 nm i fali emisji 585 nm, co pozwalało ograniczyć problemy z autofluorescencją badanych komórek. Jednocześnie stwierdzono, że pomiar zależy od gęstości zawiesiny komórek i jej proporcji względem mieszaniny reakcyjnej, a także od obecności samej biomasy, która może modyfikować parametry odczytu nawet w próbkach zawierających martwe komórki. Istotnym wynikiem było wykazanie, że usunięcie komórek przed inkubacją z mieszaniną reakcyjną oraz zastosowanie fortyfikacji próbki znaną ilością H_2O_2 pozwalają ograniczyć efekt matrycy (ang. *matrix effect*) i poprawić wiarygodność obliczeń.

Aplikacyjny charakter pracy potwierdzono w doświadczeniu z NBT, wykazując wzrost ilości H_2O_2 produkowanego przez komórki *C. reinhardtii* wraz ze wzrostem stężenia badanego NLPZ. Metoda charakteryzowała się wysoką czułością, powtarzalnością i niskim błędem pomiarowym, a zastosowanie egzogennej peroksydazy chrzanowej znacząco poprawiło jakość sygnału w porównaniu z układem pozbawionym tego komponentu. Opracowany protokół umożliwiał wiarygodne, ilościowe oznaczanie H_2O_2 uwalnianego przez komórki mikroglonów, dzięki czemu stanowił zaplecze metodologiczne dla kolejnych prac, w których ocena stresu oksydacyjnego stała się jednym z głównych elementów analizy wpływu NLPZ na komórki zielenic.

Publikacja 3

Trzecia publikacja „**Differential antioxidant pathways displayed by *Chlamydomonas reinhardtii* exposed to selected NSAID**” stanowiła rozwinięcie wcześniejszych badań, wskazujących, że działanie NLPZ wobec *C. reinhardtii* nie ogranicza się do prostego efektu toksycznego, lecz obejmuje bardziej złożoną odpowiedź komórek mikroglonów, zależną od właściwości danej substancji leczniczej. Zasadne stało się więc rozszerzenie analiz o kolejne NLPZ będące częstymi zanieczyszczeniami akwenów, ibuprofen (IBU) i naproksen (NPX). Celem badań było określenie, które elementy odpowiedzi komórkowej mają ogólny charakter, a które są związkowo-specyficzne w odpowiedzi antyoksydacyjnej.

W pracy analizowano ilość H_2O_2 produkowanego przez komórki, aktywność głównych enzymów antyoksydacyjnych, dysmutazy ponadtlenkowej (SOD) i jej izoform, peroksydazy askorbinianowej (APX) oraz katalazy (CAT). Dodatkowo oceniono poziom ekspresji genów kodujących badane enzymy. Uzupełnienie pracy stanowiły analiza korelacji oraz określenie wskaźnika IBR.

Uzyskane wyniki wykazały, że wszystkie badane NLPZ stymulowały produkcję H_2O_2 i jednocześnie ograniczały wzrost populacji mikroglonów, potwierdzając zaburzenie homeostazy komórek. Strategie ograniczania skutków stresu oksydacyjnego uruchamiane przez komórki *C. reinhardtii* różniły się w zależności od stosowanych substancji. W przypadku mniej toksycznych związków, IBU i NPX, obserwowano bardziej skoordynowaną aktywację SOD i CAT, natomiast bardziej toksyczne NLPZ, FFA i NBT silniej angażowały APX, zlokalizowaną m. in. w chloroplastach, co sugerowało większe znaczenie mechanizmów detoksykacyjnych tych organelli w ograniczaniu skutków stresu oksydacyjnego. Na poziomie molekularnym wykazano wzrost ekspresji *APX1* we wszystkich wariantach badawczych. Mimo wzrostu aktywności katalazy zaobserwowano obniżenie poziomu transkryptu *CAT1*, co sugeruje występowanie potranskrypcyjnych lub potranslacyjnych modyfikacji tego białka. Stwierdzono również, że ekspresja genu *MSD3*, kodującego jedną z izoform SOD, wzrastała we wszystkich wariantach doświadczalnych, co pozwoliło wskazać ten gen jako potencjalny molekularny marker stresu indukowanego NLPZ. Na podstawie analizy IBR wykazano, że wraz ze wzrostem toksyczności badanego związku malała efektywność odpowiedzi adaptacyjnej komórek. Analiza korelacji wykazała ścisłe zależności między produkcją H_2O_2 a aktywnością SOD i CAT, co potwierdziło funkcjonalne powiązanie podstawowych elementów systemu antyoksydacyjnego.

Praca pozwoliła więc wykazać, że ogólny charakter odpowiedzi *C. reinhardtii* na badane NLPZ obejmuje indukcję stresu oksydacyjnego oraz wzrost ekspresji *MSD3*, natomiast o związkowo-specyficznym charakterze decyduje zróżnicowany udział enzymów antyoksydacyjnych w tej odpowiedzi.

Manuskrypt 4

Uzyskane w Pracy 1 i 3 wyniki skłaniały do poszerzenia badań o kolejny gatunek mikroglonów, aby ocenić, czy obserwowane wcześniej wzorce odpowiedzi na NLPZ są charakterystyczne wyłącznie dla *C. reinhardtii*, czy występują również u innych zielenic. Do dalszych analiz wybrano *Desmodesmus armatus*, stanowiący wartościowy model porównawczy, charakteryzujący się wysoką odpornością na różne zanieczyszczenia środowiskowe, zdolnością do tworzenia cenobiów i dużą plastycznością fizjologiczną i morfologiczną.

W manuskrypcie „**Distinct biochemical effects of naproxen and nabumetone drive their differential toxicity in *Desmodesmus armatus***” skupiono się na porównaniu działania dwóch NLPZ, NBT i NPX, a więc substancji leczniczych o zbliżonej budowie chemicznej. Celem badań było określenie, czy podobieństwo strukturalne tych NLPZ powoduje jednakowe działanie w komórkach badanej zielenicy, czy też prowadzi do odmiennych efektów biochemicznych.

W badaniach określono stopień zahamowania wzrostu, wyznaczono efektywne stężenia (EC) analizowanych substancji, a następnie oceniono ich wpływ na funkcjonowanie aparatu fotosyntetycznego, aktywność metaboliczną, poziom stresu oksydacyjnego i aktywność enzymów antyoksydacyjnych.

Podobnie jak u *C. reinhardtii* (Prace 1 i 3), NBT okazał się bardziej toksyczny niż NPX, o czym świadczyły wartości EC₅₀ wynoszące odpowiednio 6,57 mg/L i 41,2 mg/L. Toksyczność obu związków nie wynikała jednak z zaburzania tych samych procesów. NPX wpływał głównie na funkcjonowanie aparatu fotosyntetycznego, prowadząc do obniżenia maksymalnej wydajności kwantowej PSII (F_v/F_m), spadku ogólnego wskaźnika wydajności fotosyntetycznej (PI_{abs}) oraz zmniejszenia puli aktywnych centrów reakcji (RCM). Ponadto zmniejszał wydajność transportu elektronów między PSII a PSI (ϕE_0) oraz obniżał redukcję końcowych akceptorów elektronów po stronie PSI (ϕR_0). Zmianom tym towarzyszyły spadek zawartości barwników fotosyntetycznych w komórkach oraz spadek intensywności fotosyntezy, co wskazywało na wystąpienie zjawiska fotoinhibicji. Drugi z badanych związków, NBT nie powodował silnych zmian w parametrach związanych z fotosyntezą, prowadził natomiast do odpowiedzi związanej z reorganizacją procesów metabolicznych i aktywacją systemu antyoksydacyjnego. W komórkach traktowanych tym związkiem zaobserwowano zwiększoną zawartość karotenoidów oraz nasilenie oddychania ciemniowego. Jednocześnie stwierdzono podwyższoną produkcję nadtlenu wodoru oraz wzrost aktywności CAT i APX, a także akumulację proliny.

Analizy korelacji i dyskryminacji pozwalają stwierdzić, że zbliżona struktura badanych NLPZ nie determinuje podobieństwa w mechanizmie ich działania. Co więcej, uzyskane wyniki umożliwiły osiągnięcie założonego celu i pokazały, że odpowiedź zielenic na NLPZ ma gatunkowo swoisty i złożony charakter. Zatem ocena oddziaływania NLPZ obecnych w środowisku wodnym wymaga uwzględnienia nie tylko parametrów wzrostu, ale również zintegrowania wskaźników fotosyntetycznych, metabolicznych i antyoksydacyjnych.

Manuskrypt 5

Dotychczasowe wyniki coraz wyraźniej wskazywały, że działanie NLPZ nie ma uniwersalnego charakteru, a o kierunku odpowiedzi komórkowej decydują nie tylko właściwości chemiczne samego związku, lecz także cechy biologiczne badanego organizmu. Kontynuacją badań specyficzności gatunkowej w odpowiedzi mikroglonów na NLPZ jest manuskrypt **NSAID-induced stress triggers physiological and morphological plasticity in *Desmodesmus armatus*: a multi-biomarker approach.**

W pracy oceniono wpływ FFA, DCF i IBU na odpowiedź fizjologiczną i morfologiczną komórek. Punktem wyjścia dla tej pracy była hipoteza, że *D. armatus*, jako gatunek o dużej plastyczności fizjologicznej, będzie reagował na NLPZ poprzez odmienną, zależną od badanego związku regulację fotosyntezy i równowagi redoks. Założono, że odpowiedź ta obejmie zmiany w efektywności fotochemicznej, aktywności enzymów antyoksydacyjnych oraz akumulacji metabolitów ochronnych.

Działanie FFA wiązało się z odpowiedzią wskazującą na kosztowną energetycznie aklimatyzację komórek do warunków stresu, obejmującą zwiększenie aktywności APX, akumulację proliny, nasilenie oddychania ciemniowego oraz przekształcenie struktury populacji w kierunku form 4-komórkowych. Jednoczesne zachowanie względnie stabilnej intensywności fotosyntezy pomimo zmian w parametrach OJIP sugerowało skuteczne działanie mechanizmów kompensacyjnych. Komórki *D. armatus* znacznie łagodniej reagowały na IBU, co przejawiało się umiarkowanymi zmianami w funkcjonowaniu PSII, wzrostem rozpraszania energii oraz akumulacją proliny przy relatywnie niewielkim wpływie na pozostałe analizowane parametry. Z kolei najsilniejsze zaburzenia w funkcjonowaniu komórek stwierdzono po ekspozycji *D. armatus* na DCF, który wyraźnie zahamował fotosyntezę, prowadził do degradacji barwników fotosyntetycznych, wzrostu produkcji H_2O_2 oraz spadku aktywności CAT. Jednoczesne zaburzenia oddychania ciemniowego wskazywały na wielopoziomowe zaburzenia równowagi komórkowej.

Analizy korelacji i dyskryminacji potwierdziły, że każdy z badanych NLPZ wywoływał odrębny profil odpowiedzi fizjologicznej, a za różnicowanie wariantów odpowiadały odmienne zestawy parametrów związanych z fotosyntezą, metabolizmem, stresem oksydacyjnym i organizacją morfologiczną populacji. Uzyskane wyniki potwierdziły tym samym, że odpowiedź *D. armatus* na NLPZ ma charakter wielowymiarowy, zależny od właściwości danego związku, a wysoka zdolność adaptacyjna tego gatunku nie oznacza jednakowej odporności na wszystkie badane substancje lecznicze, lecz wyznacza związkowo specyficzny przebieg odpowiedzi komórkowej. Praca ta istotnie rozwinęła wcześniejsze obserwacje, potwierdzając, że plastyczność fizjologiczna i morfologiczna tej zielenicy jest jednym z elementów adaptacji do stresu indukowanego NLPZ.

8. Podsumowanie

Przedstawiony cykl publikacji dostarcza nowych danych na temat wrażliwości wybranych gatunków zielenic planktonowych na działanie NLPZ oraz stanowi istotny wkład w wyjaśnienie uruchamianych przez nie mechanizmów odpowiedzi komórkowej. Przeprowadzone badania wykazały, że:

1. **Poszczególne NLPZ nie wywołują u mikroglonów jednakowej odpowiedzi, lecz indukują odmienne, związkowo specyficzne wzorce zmian fizjologicznych i biochemicznych.**
2. **Charakter odpowiedzi, a tym samym wrażliwość zielenic na NLPZ, zależy zarówno od właściwości chemicznych substancji leczniczej, jak i cech biologicznych badanego organizmu.**
3. **Ocena toksyczności NLPZ wobec mikroglonów wymaga wielopoziomowej analizy i nie może opierać się wyłącznie na określeniu wartości EC na podstawie zahamowania wzrostu populacji.**

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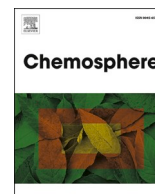
10. Praca nr 1.

Nabumetone and flufenamic acid pose a serious risk to aquatic plants: A study with *Chlamydomonas reinhardtii* as a model organism.

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Nabumetone and flufenamic acid pose a serious risk to aquatic plants: A study with *Chlamydomonas reinhardtii* as a model organism

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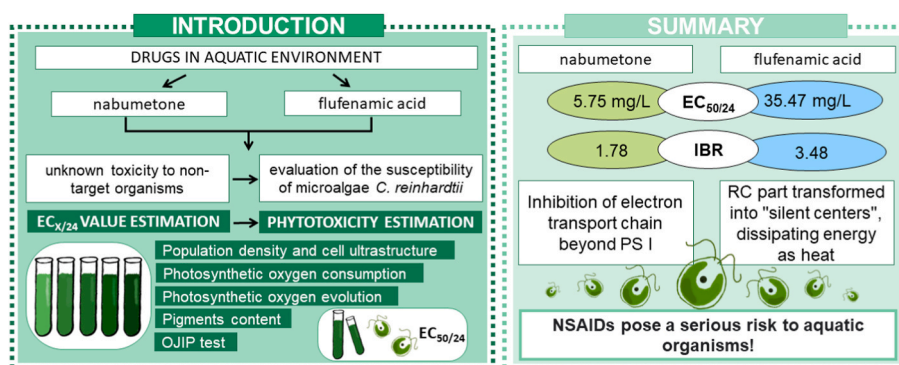
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HIGHLIGHTS

- Nabumetone and flufenamic acid effects on *Chlamydomonas reinhardtii* were evaluated.
- Nabumetone was over six times more toxic than flufenamic acid.
- Nabumetone inhibits electron transport beyond the PSII.
- Flufenamic acid probably transformed some PSII reaction centers into "silent centers".
- This is the first report of nabumetone and flufenamic acid toxicity to green algae.

GRAPHICAL ABSTRACT



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ABSTRACT

The aquatic environment is constantly under threat due to the release of numerous pollutants. Among them, pharmaceuticals constitute a huge and diverse group. Non-steroidal anti-inflammatory drugs (NSAIDs) are increasingly found in water bodies, but knowledge about their potential toxicity is still low. In particular, there is a lack of information about their influences on aquatic plants and algae. We estimated the susceptibility of the

Abbreviations: ABS/RC, the specific energy fluxes per reaction center (RC); DCF, diclofenac; DI₀/RC, dissipation; DIF, diflunisal; DMSO, dimethyl sulfoxide; EC, effective concentration; ET, etodolac; ET₀/RC, electron transport; FFA, flufenamic acid; F_v/F₀, the activity of the water-splitting complex; HSM, high salt medium; IB, ibuprofen; IBR, integrated biological response index; KET, ketoprofen; MEF, mefenamic acid; NBT, nabumetone; NPX, naproxen; NSAID, non-steroidal anti-inflammatory drugs; OJIP test, the fluorescence of chlorophyll *a* *in vivo*; PS I, photosystem I; PS II, photosystem II; RC₀, amount of active PS II reaction centers in the sample when the basic fluorescence was measured; RC_M, amount of active PS II reaction centers in the sample when the maximum fluorescence was reached; PI_{abs}, an expression indicating algal 'photosynthetic vitality'; RE₀/RC, reduction of end acceptors at the PS I electron acceptor side per RC; TR₀/RC, trapping; δR₀, efficiency with which an electron can move from the reduced intersystem electron acceptors to the PS I end electron acceptors; φE₀, the maximum yield of the electron transport; φP₀, the maximum yield of primary photochemistry; φR₀, quantum yield of electron transport from Q_A to the PS I end electron acceptors; ψ₀, probability with which a PS II-trapped electron is transferred from Q_A to Q_B.

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Microalgae
Nonsteroidal anti-inflammatory drugs

microalgae *Chlamydomonas reinhardtii* to nabumetone (NBT) and flufenamic acid (FFA), focusing on photosynthesis. Due to the differences in the structures of these compounds, it was assumed that these drugs would have different toxicities to the tested green algae. The hypothesis was confirmed by determining the effective concentration values, the intensity of photosynthesis, the intensity of dark respiration, the contents of photosynthetic pigments, the fluorescence of chlorophyll *a* *in vivo* (OJIP test), and cell ultrastructure analysis. Assessment of the toxicity of the NSAIDs was extended by the calculation of an integrated biomarker response index (IBR), which is a valuable tool in ecotoxicological studies. The obtained results indicate an over six times higher toxicity of NBT compared to FFA. After analysis of the chlorophyll *a* fluorescence *in vivo*, it was found that NBT inhibited electron transport beyond the PS II. FFA, unlike NBT, lowered the intensity of photosynthesis, probably transforming some reaction centers into “silent centers”, which dissipate energy as heat. The IBR estimated based on photosynthetic parameters suggests that the toxic effect of FFA results mainly from photosynthesis disruption, whereas NBT significantly affects other cellular processes. No significant alteration in the ultrastructure of treated cells could be seen, except for changes in starch grain number and autophagic vacuoles that appeared in FFA-treated cells. To the best of our knowledge, this is the first work reporting the toxic effects of NBT and FFA on unicellular green algae.

1. Introduction

In recent years, questions have been raised about the possible negative effects of the high and continuous use of pharmaceuticals on natural ecosystems. Drugs and their metabolites are released into the environment mainly from wastewater treatment plants, hospital and veterinary waste, wastewaters from drug production, agriculture, and aquaculture (Daughton and Ruhoy, 2009; Hamscher et al., 2003; Larsson et al., 2007; Papageorgiou et al., 2016; Pereira et al., 2020). Worldwide, different groups of drugs are continually detected in the aquatic environment, such as non-steroidal anti-inflammatory drugs (NSAIDs), which are known for their antipyretic, analgesic, and anti-inflammatory action (Li et al., 2019; Pereira et al., 2020). Despite the determined activity and efficacy of NSAIDs in humans and animals, still not enough is known about their toxicity for aquatic organisms. Recently, several studies have confirmed that these compounds can be accumulated in the aquatic environment (Caban et al., 2016; Corcoran et al., 2010; Izadi et al., 2020). As a result of the exposure of non-target aquatic organisms to pharmaceuticals, both acute and chronic toxicity have been observed. In many invertebrates, such as rotifers, crustaceans, chironomids, bivalves, and gastropods, disorders of growth and respiration processes as well as oxidative stress symptoms have been reported (Boisseaux et al., 2017; Du et al., 2016; Gagné et al., 2006; Sarma et al., 2014). Further, although many physiological and morphological changes have been described for NSAID-exposed fish (Li et al., 2016; Ogueji et al., 2018; Sehonova et al., 2017), relatively little is known about the effects of those drugs on higher plants and algae. Moreover, the published reports are usually focused on the most popular drugs such as diclofenac, paracetamol, or ibuprofen (Badar et al., 2022; Siemieniuk et al., 2021; Vannini et al., 2018), whereas far too little attention has been paid to less common NSAIDs such as nabumetone (NBT) and flufenamic acid (FFA).

NBT belongs to the 2,6-disubstituted aryl alkanones, and its activity is based on the metabolite 6-methoxy-2-naphthyl acetic acid. Structurally, it is similar to another non-steroidal anti-inflammatory drug, naproxen (Carryl and Spangler, 1995; Hedner et al., 2004). NBT is considered to be a less toxic pharmaceutical agent and to have fewer side effects compared to other NSAIDs (Confino-Cohen and Goldberg, 2003), it is mainly used to treat osteoarthritis or rheumatoid arthritis, which affects more than 350 million people (Global RA Network, 2021; Hedner et al., 2004). The safety of its use in humans and animals has been investigated (see e.g., Bannwarth, 2008; Khan and McLean, 2012), but information about NBT effects on non-target water organisms is completely lacking. This knowledge gap should be urgently filled because in the last few years, NBT has been detected in the environment, e.g., in seawater close to the coastline of China where its concentration was 1.94 ng/L (Peng et al., 2019). Further, according to studies by Tu et al. (2023) nabumetone and its major metabolite, 6-methoxy-2-naphthylacetic acid, are frequently detected in surface waters in China, with

concentrations ranging from 17.3 to 290.1 ng/L and from 14.2 to 355.6 ng/L, respectively.

FFA derived from a fenamic acid, belongs to the subgroup fenamates, which also includes other clinically prescribed NSAIDs such as mefenamic acid, tolafenamic acid, and meclofenamic acid (Hill and Zawia, 2021). Fenamates, originally used as painkillers, have also been studied and implemented for other clinical applications, including cancer, stroke, Alzheimer's disease, Huntington's disease, and epilepsy (Hill and Zawia, 2021), which has increased their use and environmental deposition. Fenamates excreted by humans and animals are only partially removed in wastewater treatment plants, making effluents a real source of these drugs in surface waters (Davis et al., 2017). FFA has been found in wastewater influent and effluent in Spain, with a higher concentration of the drug in the effluent as compared to the inflow to the treatment plant (Carmona et al., 2014; Chavez-Dozal et al., 2014). On the island of Gran Canaria, monitoring of reclaimed water used for irrigation showed that it is an important source of emerging pollutants, including 42 pharmaceuticals, one of which was FFA (Estévez et al., 2012). This substance has also been found in lakes of Switzerland (Chiaia-Hernandez et al., 2014) and in raw and treated landfill leachates in Greece (Nika et al., 2020). In addition, in the official database supervised by the German Environment Agency (Umwelt Bundesamt, 2022), the presence of FFA was confirmed, among others, in wastewater and surface waters in Spain, Germany, Hungary, Romania, and Serbia (Umwelt Bundesamt, 2022), in concentrations ranged from 0.2 ng/L to 1334.0 ng/L.

Flufenamic acid detection in the water environment has raised questions about its possible effects on non-target organisms. Toxicity studies on *Danio rerio* (zebrafish) embryos have shown that FFA affects many phenotypic endpoints associated with hepatic and gastrointestinal toxicity that would be masked if death had occurred at low drug concentrations (Nadanaciva et al., 2013). The above could be partially caused by respiration disorder since FFA can cause mitochondrial uncoupling by a protonophoric mechanism in rat liver mitochondria (Jordani et al., 2000; Nadanaciva et al., 2013). Since respiratory processes on the molecular level are similar in both animal and plant cells, it is likely that FFA will cause adverse effects also in plants and green algae. However, such investigations are not reported in the literature.

For many years, green algae have been used in research on the phytotoxicity of environmental pollutants (Samson and Popovic, 1988). They are also a promising tool for the detoxification and removal of contaminants, including pharmaceutical substances and their derivatives, from the environment (Hejna et al., 2022). However, to successfully use this tool, it is necessary to accurately determine the ability of different algal strains to tolerate, detoxify, or accumulate toxic substances and to understand the physiological and biochemical processes that underlie these phenomena (Ding et al., 2017; Guo et al., 2016). *Chlamydomonas reinhardtii*, selected for analysis in this study, is a model organism for physiological, molecular, and toxicological studies (Ball, 2005; Grossman et al., 2010; Harshkova et al., 2021; Majewska et al.,

2018). This study estimated the sensitivity of *C. reinhardtii* to FFA and NBT, with emphasis on the influence of these substances on the photosynthesis process. The research hypothesis is that pharmaceuticals differing in their structure exhibit different toxicities to *Chlamydomonas reinhardtii*. To confirm this assumption, the effective concentration values were determined, and the efficiency of photosynthesis and dark respiration, the contents of photosynthetic pigments, chlorophyll *a* fluorescence *in vivo*, and cell ultrastructure were analyzed.

2. Materials and methods

2.1. Chemicals

The studied nonsteroidal anti-inflammatory drugs were nabumetone (NBT, 4-(6-Methoxy-2-naphthyl)-2-butanone; 97% purity, CAS number 42924-53-8; Alfa Aesar, USA) and flufenamic acid (FFA, *N*-(3-[Tri-fluoromethyl]phenyl) anthranilic acid, 97% purity, CAS number 530-78-9; Alfa Aesar, USA). Both substances were dissolved in DMSO (dimethyl sulfoxide, analytical grade, Acros Organics, USA) and supplied to high salt medium (HSM) to obtain the desired concentrations. The concentration of solvent in all experimental variants did not exceed 0.1% (v/v); such a DMSO concentration has previously been shown to have no impact on the physiological parameters of the algae (Aksmann and Tukaj, 2004, 2008).

2.2. Algae cultures

Chlamydomonas reinhardtii cc-1690 (wild type) used in this study originated from the Chlamydomonas Resource Center (USA, <http://www.chlamy.org/>). The algae kept on the slants were transferred to culture vessels with a liquid HSM (Harris, 2009) and grown under white fluorescent light of $125 \pm 5 \mu\text{mol photons/m}^2 \times \text{s}$, measured inside the culture vessels (Quantitherm QRT1 light meter, Hansatech Ltd., UK) at a temperature of 30 °C. Cultures were bubbled with sterilized air (bacteriological filter 0.2 mm PTFE; Sartorius, Germany) and enriched with 2.5% CO₂ (v/v) (Aksmann and Tukaj, 2008). The population in the mid-logarithmic growth phase was used as inoculum for the toxicity testing. The initial cell density in each experiment was 0.5×10^6 cells/mL (Aksmann et al., 2014; Aksmann and Tukaj, 2008), and the experiments lasted 24 h.

2.3. Phytotoxicity estimation and actual exposure concentrations

In the first phase of experiments the toxicity of the substance was tested, and EC concentrations were calculated based on growth inhibition curves presented in the supplementary section, Fig. S1. In the main part of the experiments, algae cultures (see 2.2) were exposed to toxicants at nominal concentrations corresponding to the EC₅₀₋₂₄ values, i.e., 5.75 mg/L for NBT and 35.47 mg/L for FFA. The actual concentrations of the tested pharmaceuticals in the growth media determined using HPLC (for method description see supplementary section Text S2, Table S3) were 2.97 mg/L for NBT and 31.8 mg/L for FFA (Table S2). The cultures without tested chemicals served as controls. After 24 h (t₂₄), cultures were sampled for physiological parameter analyses, as described below.

2.4. Analyzed parameters

2.4.1. Population density and cell ultrastructure

An electronic particle counter Z2 (Beckman Coulter, USA), run by a dedicated Z2 AccuComp software, was used to count cells in each algae population.

To analyze the cell ultrastructure, algae suspension was centrifuged (1.000 g, 10 min) and pellets were fixed overnight with 2.5% glutaraldehyde (Polysciences, USA) in 0.1 M sodium cacodylate buffer pH 7.2 (Sigma-Aldrich, USA), and post-fixed with 2% osmium tetroxide

(Polysciences, USA), in 0.1 M sodium cacodylate buffer. Further, the cells were dehydrated with increasing concentrations of ethyl alcohol (99.8%, POCH, Poland) and after that infiltrated and embedded in Epon 812 resin (Sigma-Aldrich, USA). Ultrasections (approximately 65 nm) were cut on ultramicrotome Leica UC7 (Leica, Germany). Sections were stained with 3% uranyl acetate (BDH Chemicals, UK) and 3% lead citrate (Delta Microscopies, France). All reagents used for this analysis were of EM grade, high purity. They were examined using a Tecnai Spirit BioTWIN transmission electron microscope (FEI, USA).

2.4.2. Photosynthetic oxygen evolution and consumption

Photosynthetic oxygen consumption and evolution were determined using a Clark-type oxygen electrode run with a dedicated software (Oxyview, Hansatech Ltd., UK) as described in Harshkova et al. (2021). In brief, 1-mL culture samples were placed in the measurement chamber equipped with a Clark-type oxygen electrode unit. The cell suspension was stirred continuously, and oxygen evolution/consumption was measured at 30 °C, using the same light conditions as in the cultures or under total darkness. Photosynthesis efficiency and respiration intensity were calculated and expressed as nmol O₂/mL cells $\times$ min and nmol O₂/pg (chlorophyll *a*) $\times$ min.

2.4.3. OJIP test

Chlorophyll *a* fluorescence *in vivo* (OJIP test) was measured using a Handy Pea fluorometer (Hansatech Ltd., UK) run by the Handy Pea software, according to the method described in Pokora et al. (2017). In brief, before each fluorescence measurement, the cells suspension was diluted to OD₆₈₀ = 0.2 ± 0.01 and dark-adapted for 15 min. Next, aliquots with a volume of 2 mL were placed in the measuring chamber using a glass vial. All measurements were carried out at room temperature under saturated light pulse ($3000 \mu\text{mol photons/m}^2 \times \text{s}$). The analyzed parameters are described in supplementary section (Table S4).

2.4.4. Photosynthetic pigment content in the cells

The photosynthetic pigments content in the cells were determined using a spectrophotometric method after extraction with 80% acetone, as described in Majewska et al. (2021). A detailed description of the method is provided in the supplementary section (Text S3).

2.5. Statistical analysis

Statistical analysis was performed using MS Excel 2010 (Microsoft) and Statistica 9.0 (StatSoft). All data were expressed as the means of at least three independent experiments, with at least four replications for each experimental variant $\pm$ standard error (SE). The accuracy of the measurement was determined by observing the principle of using such units so that the difference between the highest and the lowest measurement value was within the range of 30–300 units (Łomnicki, 2003). The Grubbs test was performed to remove outliers, and non-linear curve fitting was performed using the Origin Pro 7.5 program (OriginLab Corp., USA). Statistica 9.0 (StatSoft Europe, Germany) was used to calculate a correlation matrix to measure the strength of associations that differed between the variables. The same software was applied to compute basic statistics. Data were analyzed by one-way ANOVA or Kruskal-Wallis test, followed by the Tukey or Dunn's post-tests. Statistical analyses were performed at a significance level of 0.05, where the differences are marked using letters. The same software was used for canonical discriminant analysis, in which population density, photosynthetic pigment content, photosynthesis, and dark respiration intensity were included. Additionally, for the control group and the algae treated with pharmaceuticals, the integrated biological response index (IBR) was calculated as described elsewhere (Beliaeff and Burgeot, 2002). Biomarkers related to photosynthesis were selected for analysis, i.e., the contents of chlorophylls *a* and *b*, carotenoids, the intensity of photosynthesis, and the performance index (PI). The IBR index allows for a comprehensive assessment of the impact of pollutants on living

organisms. The higher the IBR value, the greater the effect of the contaminant (Caliani et al., 2021).

3. Results

3.1. Toxic effects of NBT and FFA on population growth

After 24 h, the control cultures of *C. reinhardtii* had reached a density up to 12.5×10^6 cells/mL (data not shown). Both NBT and FFA caused a concentration-dependent inhibition of population growth (Fig. S1, Table S1). Based on the algal growth inhibition, the effective concentrations (ECs) values were calculated (Table S2). The nominal EC₅₀₋₂₄ was 5.75 mg/L for NBT and 35.47 mg/L for FFA. The actual concentrations of FFA in the growth media, determined by HPLC analysis, were close to nominal and amounted to 31.8 mg/L. The determined actual concentration of NBT was 2.97 mg/L and represented 51.7% of the nominal concentration (Table S2).

3.2. Photosynthetic pigments and photosynthetic oxygen evolution and consumption

The cellular contents of chlorophyll *a* and *b* increased significantly in *C. reinhardtii* after the application of both substances compared to the control (Fig. 1A). These effects were more pronounced in NBT-treated cells, where the chlorophyll *a* content increased by an average of 76% compared to the control and by 23% compared to the FFA treatment. In the case of chlorophyll *b*, these values were higher by 92% and 21%, respectively. Both pharmaceuticals used had a similar effect on the carotenoid content in the cells. The amount of these pigments was almost 50% higher in the treated cells as compared to the control (Fig. 1A).

Measurement of the oxygen evolution/consumption revealed that the intensity of photosynthesis and dark respiration, expressed as nmol O₂ evolved/consumed by mln of cells for min, was significantly higher in NBT-treated populations as compared to either the control or FFA-treated cultures (Fig. 1B). However, photosynthesis intensity recalculated per pg of chlorophyll *a* did not exhibit significant differences between the control and NBT-treated cultures (Fig. 1C).

The opposite trend was observed after applying FFA to the *C. reinhardtii* culture (Fig. 1B). The amount of O₂ evolved/consumed by FFA-treated algal cells was extremely low as compared with the control, independent of whether the oxygen concentration was recalculated per mln of cells or per pg of chlorophyll *a* (Fig. 1C). The photosynthesis and respiration processes were inhibited after FFA treatment.

3.3. Chlorophyll *a* fluorescence in vivo (OJIP test)

The comprehensive determination of the changes occurring in light-dependent photosynthetic processes in toxicant-treated cells was possible because of the analysis of chlorophyll *a* fluorescence *in vivo* (Fig. 2, Table S4 and Fig. S2). The original OJIP transients of the control, NBT-, and FFA-treated cells of *C. reinhardtii*, plotted on a logarithmic time scale, are shown in Fig. 2. The OJIP curves for the control cultures show the typical shape of chlorophyll *a* fluorescence rises kinetics. The curves obtained for toxicant-treated cultures had a shape similar to those of the control, but the values of particular marks of the fluorescence rise (J, I, P) reached different levels (Fig. 2A). Fluorescence was significantly higher in steps J, I, and P for NBT as opposed to FFA, where this level was decreased in these steps (Fig. S2). However, when the curves were plotted as the relative variable fluorescence at any time, the differences were more apparent in the section between steps J and I (Fig. 2B), which reflects the accumulation of Q_A- (around 2 ms) and an accumulation of Q_A- and Q_B- (around 30 ms).

The general parameter of photosynthetic “vitality” (PI_{abs}) did not differ in the experimental variants. However, the maximum yield of primary photochemistry (φP₀) decreased in FFA-treated cells (97% of

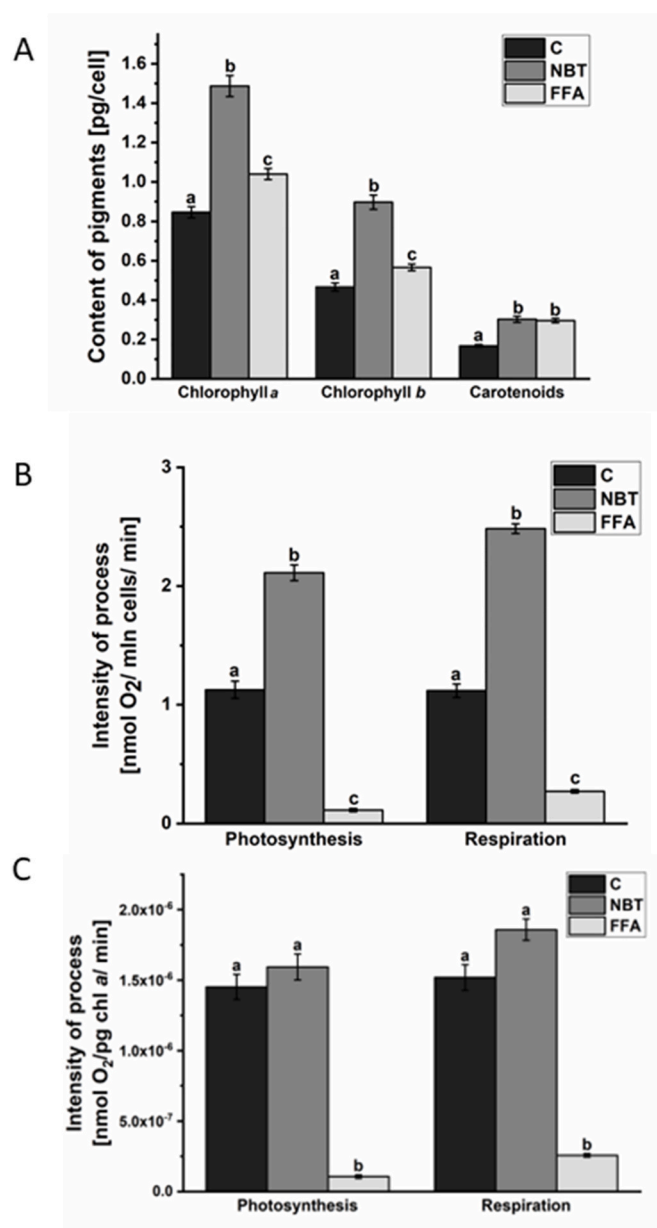


Fig. 1. Effects of nabumetone and flufenamic acid, applied to *C. reinhardtii* cultures at concentrations corresponding to EC₅₀₋₂₄, on photosynthetic pigment contents in the cells (A), intensity of photosynthesis, and intensity of dark respiration (B, C). Intensities of photosynthesis and dark respiration were expressed as nmol O₂ evolved/consumed by mln of cells within min (B) or as nmol O₂ evolved/consumed per pg chlorophyll *a* within min (C). Data are presented as means of at least three biological replicates ± SE. Different letters indicate significant differences ($p \leq 0.05$) between groups (ANOVA, Tukey's HSD test).

the control). The opposite effect was observed for NBT-treated cells, where this parameter reached 107% of the control. The quantum efficiency of electron transport (ϕE_0), the efficiency with which a trapped electron is transferred from Q_A to Q_B (ψ_0), the probability with which an electron from Q_B is transferred to PSI acceptors (δR_0), and the quantum yield of electron transport from Q_A to the PS I end electron acceptors (ϕR_0) decreased by 4%–30% in NBT-treated cells compared to the control. The opposite result was obtained for the FFA-treated cells, where the levels of these parameters were higher (ϕE_0 , ψE_0 , δR_0 , ϕR_0) than those in the control. A significant reduction in the fraction of active reaction centers PSII (RC_M) and the activity of the water-splitting

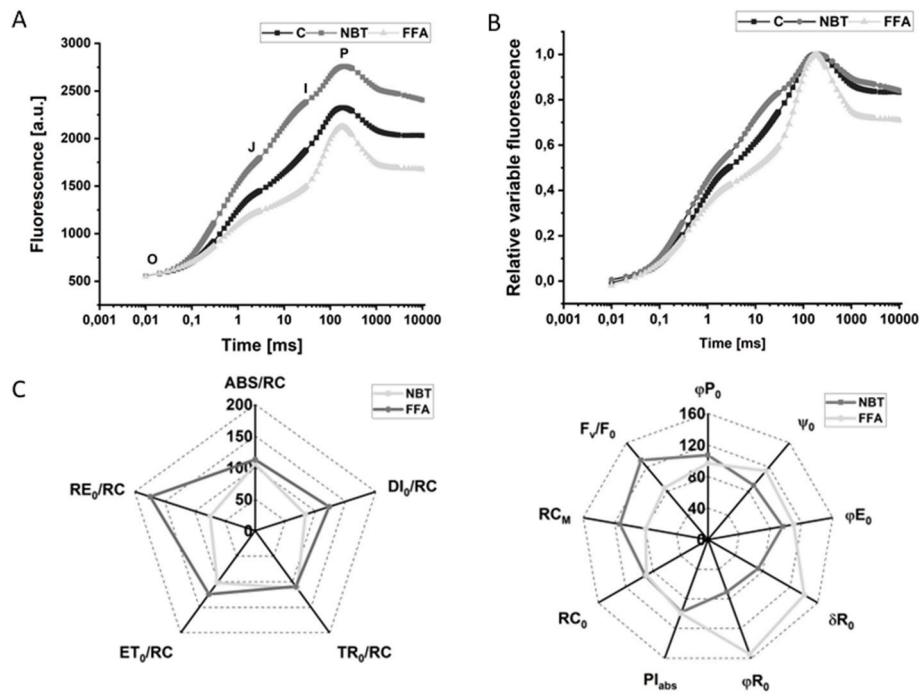


Fig. 2. Influences of nabumetone (NBT) and flufenamic acid (FFA), applied at EC₅₀₋₂₄ concentrations, on chlorophyll *a* fluorescence transient (A), relative variable fluorescence (B), and the selected parameters of chlorophyll *a* fluorescence *in vivo* (C) in *C. reinhardtii* cells after 24-h treatment. Data are presented as averages of at least three biological replicates or expressed as a percentage of the control. For exact parameter values and statistics, see supplementary data Fig. S2 and Tab. S3.

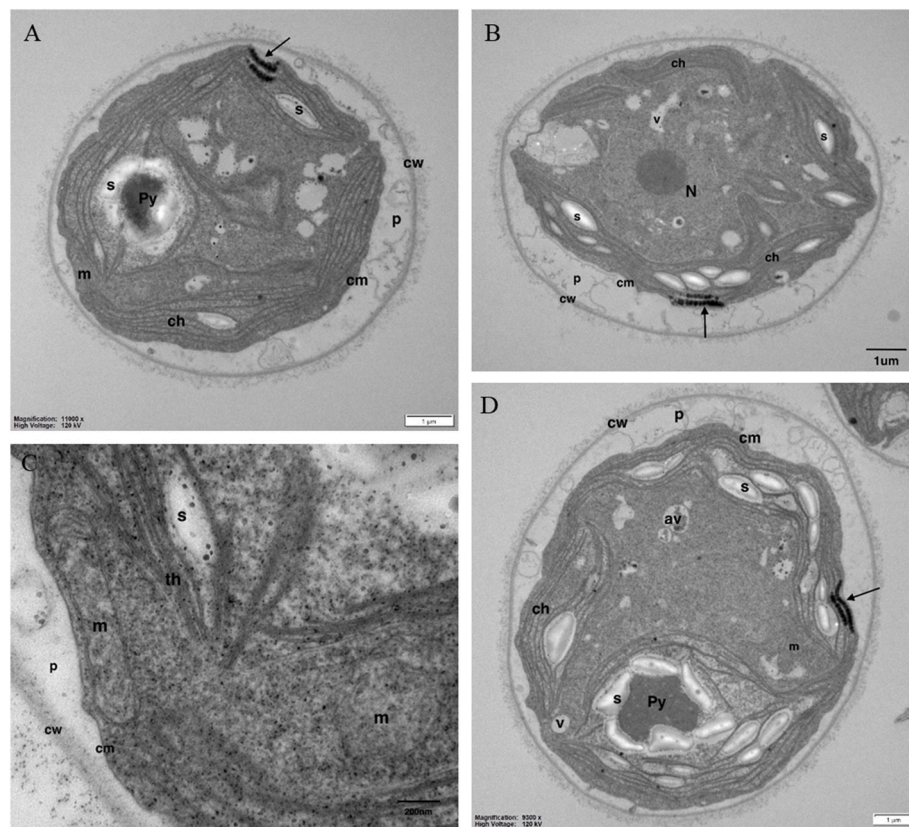


Fig. 3. Transmission electron micrographs of *C. reinhardtii* control samples. (A) Pyrenoid, eye spot (black arrow), and spherically arranged chloroplasts visible in section. (B) Centrally arranged cell nucleus and starch granules are located at the chloroplasts. (C) Mitochondria with the internal structure of mitochondrial cristae and thylakoids. (D) Structure of the autophagic vacuole and starch granules located next to the chloroplast and pyrenoid. Abbreviations: cw – cell wall, cm – cell membrane, p – periplast, N – nucleus, Ch – chloroplast, s – starch grain, th – thylakoid, Py – pyrenoid, m – mitochondria, v – vacuole, av – autophagic vacuole.

complex (F_V/F_0) in FFA-treated cells was observed. A different effect was provoked by NBT since a higher fraction of active reaction centers and the higher efficiency of water-splitting complexes were observed in the cells treated with this drug (Fig. 2C, Table S4).

Regarding the specific energy fluxes per one active reaction center, for NBT- and FFA-treated cells, an increase (106% and 113% of the control, respectively) in energy absorption per one active reaction center (ABS/RC) was recorded. Also, energy trapping by one active reaction center (TR_0/RC) increased to 113% and 109% of the control for NBT and FFA, respectively. In the case of FFA-treated cells, a significantly higher (by 24%) level of the efficiency of electron transport by one active reaction center (ET_0/RC) was observed. Compared to non-treated algae cells, FFA caused an increase (by 23%) in nonphotochemical energy dissipation, as recalculated per one active reaction center (DI_0/RC), and

in the reduction of final electron acceptors in PSI (RE_0/RC was higher by 74%). In contrast, in NBT-treated cells, the nonphotochemical energy dissipation (DI_0/RC) and the reduction of electron acceptors (RE_0/RC) were lower by 17% and 25%, respectively, than in the control (Fig. 2C, Table S4).

3.4. Ultrastructure

The ultrastructure analysis showed that the cells in all experimental variants had properly organized chloroplast with regular thylakoids and a pyrenoid (Figs. 3–5). However, in FFA-treated cells, a lower amount of starch grains was observed (Fig. 4) compared to the control. In opposite, in NBT-treated cells, the number of starch grains was the highest among the experimental variants and they were relatively large (Fig. 5). No

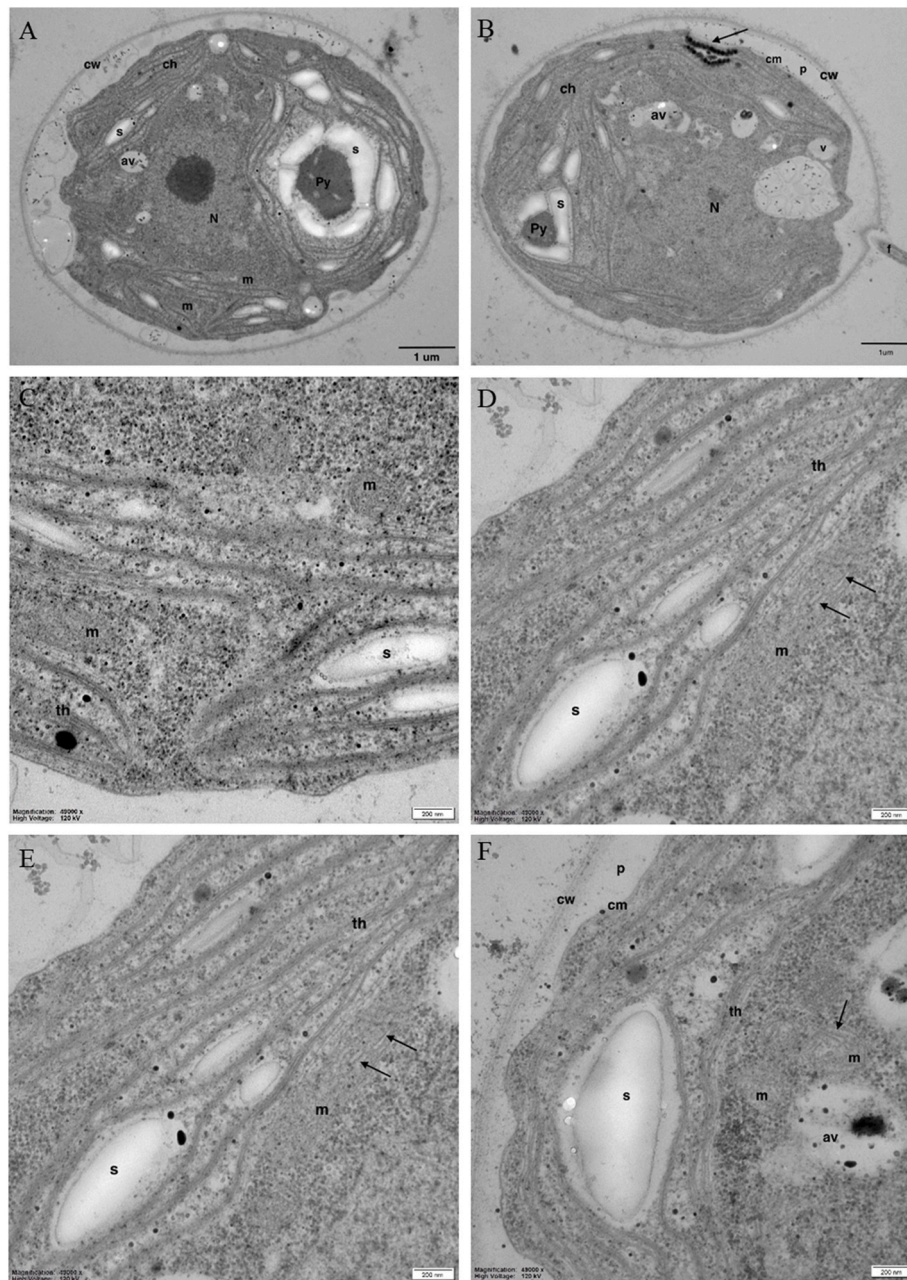


Fig. 4. Transmission electron micrographs of *C. reinhardtii* treated with FFA. (A, B) The cross-section shows the cell nucleus, pyrenoid surrounded by starch granules, mitochondria, chloroplasts, eye spot (black arrow), flagellum, and autophagic vacuoles. (C, D, E) Mitochondria with the internal structure of mitochondrial cristae (black arrows), thylakoids, and starch granules. (F) Structure of the autophagic vacuole and periplast. Abbreviations: cw – cell wall, cm – cell membrane, p – periplast, N- nucleus, Ch-chloroplast, s – starch grain, th – thylakoid, Py – pyrenoid, m – mitochondria, v – vacuole, av – autophagic vacuole, f – flagella.

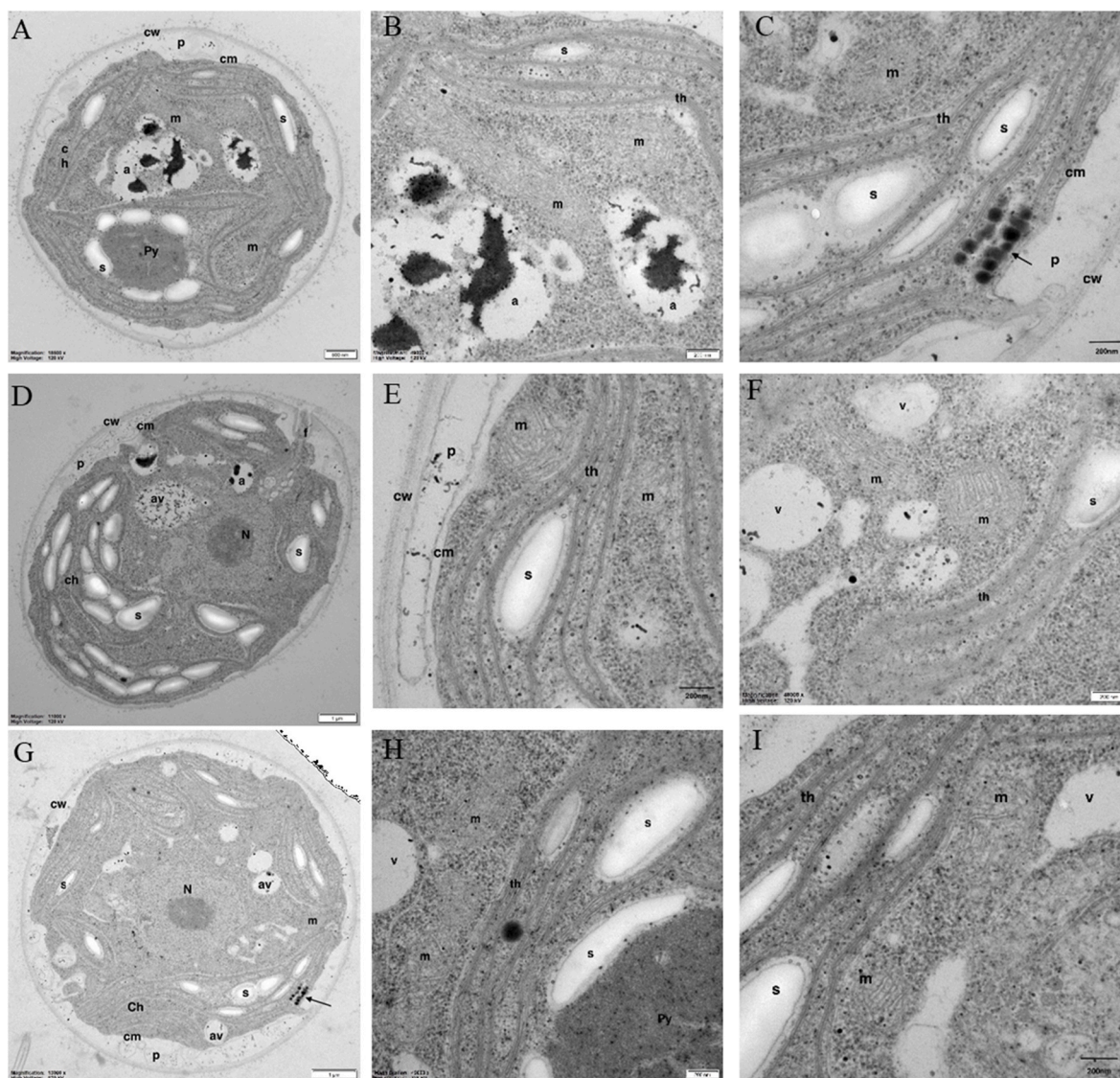


Fig. 5. Transmission electron micrographs of *C. reinhardtii* treated with NBT. (A, D, G) In cross-section visible cell nucleus, pyrenoid, mitochondria, chloroplasts, eye spot (black arrow), flagella, and autophagic vacuoles, starch grains are very numerous and larger than in other samples. (B) High electron density acidocalcisomes. (C, E) Structure of mitochondria and thylakoids, eye spot (black arrow), starch granules, and periplasm. (F, H, I) Mitochondria with well-defined mitochondrial cristae, thylakoids, and vacuoles. Abbreviations: a – acidocalcisome, f – flagella, cw – cell wall, cm – cell membrane, p – periplast, N- nucleus, Ch-chloroplast, s – starch grain, th – thylakoid, Py – pyrenoid, m – mitochondria, v – vacuole, av – autophagic vacuole, f – flagella.

major changes in the structure of mitochondria were observed, however, NBT-treated cells seemed to have better developed mitochondrial cristae. Autophagic vacuoles were present in algal cells treated with FFA (Fig. 4). In NBT-exposed cells acidocalcisomes were present, which are similar to autophagic vacuoles, but their content is more electron-dense (Fig. 5).

4. Discussion

Recently, pharmaceuticals have become one of the most common pollutants in the environment, triggering a growing interest among scientists. Despite numerous studies focusing on the identification of these compounds in the environment, their transformations, the assessment of their impact on non-target organisms, and possible ways of their removal (Almeida et al., 2017; Baker and Kasprzyk-Hordern, 2013; DeLorenzo and Fleming, 2008; Gracia-Lor et al., 2011; Hejna et al., 2022; López-Serna et al., 2013), this complex subject leaves many uncertainties.

The results obtained in the present study indicate a huge risk posed

to aquatic organisms by popular NSAIDs, namely NBT and FFA. Most of the studies considering NSAIDs toxicity apply to animals (Brozinski et al., 2013; Gonzalez-Rey and Bebianno, 2014; Sehonova et al., 2017), although plants constitute one of the most important elements of the food chain, and the potential accumulation of NSAIDs in their organisms poses a threat to the higher trophic levels (Hejna et al., 2022).

Our results indicate that both tested drugs exhibit toxicity towards *C. reinhardtii*, with a stronger effect of NBT with nominal $EC_{50-24} = 5.75$ mg/L, than FFA with nominal $EC_{50-24} = 35.47$ mg/L (Fig. S1; Tab. S2). Considering that the bioavailable NBT concentration in the culture media was almost two times lower than the nominal concentration (Table S2), the toxicity of this drug seems to be of special concern. It should be considered that the chemical structure of nabumetone makes it relatively susceptible to both biotic (metabolic) and abiotic transformation (Tu et al., 2023). *In vivo*, prodrug NBT is metabolized to active 6-methoxy-2-naphthylacetic acid, while in aqueous solution mainly abiotic photolysis occurs (Tu et al., 2023). The above processes could be responsible for the differences between the nominal and actual NBT concentrations but at this stage of our study it is not possible to clearly

determine which process we are dealing with. Whatever the case, it seems reasonable to assume that similar processes occur in the environment and that NBT toxicity may be a sum of toxic effects of parent compound and its degradation or transformation products. Because there is no literature data about FFA and NBT phytotoxicity, we compared our results with ECs reported for other green algae species and other NSAIDs (Table S6). Bearing in mind that the EC₅₀ value strongly depends not only on the toxicant type and the organism tested but also on the experimental conditions, we first confront the ECs estimated for FFA and NBT with our previous work (Majewska et al., 2018), in which the toxicity of diclofenac (DCF) towards *C. reinhardtii* was estimated under the same conditions as applied here. Interestingly, DCF (EC₅₀₋₂₄ = 134.5 mg/L) appeared to be 4 to 22 times less toxic compared to NBT and FFA, respectively (Table S6). In another study (see Table S6), the EC₅₀ was estimated within 24 h (EC₅₀₋₂₄), and naproxen exhibited similar toxicity towards *Cymbella* sp. (EC₅₀₋₂₄ = 29.20 mg/L) as FFA towards *C. reinhardtii*, whereas it seemed to be extremely toxic towards *Scenedesmus quadricauda* (EC₅₀₋₂₄ = 0.7 mg/L) (Table S6). Such a high susceptibility to NSAIDs has also been noticed for *Haematococcus pluvialis* exposed to mefenamic acid, DCF, and diflunisal (Table S6). The possible reasons for such differences in organisms' susceptibility to drugs can be related to the specificity of the cell wall architecture or the antioxidant enzyme activity of particular strains (Moreno-Sánchez et al., 1999). Also, the toxicity of particular pharmaceuticals was connected to the exposure time. Considering the study by Ding et al. (2017), it can be assumed that chronic toxicity is much lower than the acute one, which can be explained by the inducibility of defense mechanisms in algal cells. On the other hand, ibuprofen toxicity towards *Chlorella vulgaris* seemed to be time-independent (Table S6). It should be taken into account that nominal EC values for pharmaceuticals may differ from the bioavailable concentrations (actual ones), due to low solubility, interaction with biomass, possible photodegradation, and the fact that some of the compounds are in the form of prodrugs. Analysis of the literature data leads, therefore, to the conclusion that NSAIDs pose a real and hard-to-estimate threat to aquatic plants and that one of their possible targets on the cellular level is photosynthesis (see Table S6, Grzesiuk et al., 2016; Harshkova et al., 2021; Majewska et al., 2018). In this study, no pronounced effect of NBT or FFA on chloroplast ultrastructure was observed (Figs. 3–5). Moreover, both NBT and FFA caused the increase of chlorophyll content in *C. reinhardtii* cells (Fig. 1A). Numerous studies reported in literature demonstrate that low-level stress provoke increase in chlorophyll content in plant cells (the so-called chlorophyll hormesis), while high-level stress cause a decrease in its content (for review see Agathokleous et al., 2020). In the present study, NBT and FFA concentrations were relatively high, thus high level of chlorophyll in the algal cells was an unexpected result. On the other hand, stimulatory or inhibitory effect of a stressor is known to be dependent also on exposure time (Agathokleous et al., 2020). Thus, it is possible that in the case of our experiment toxicant dose/exposure time combination triggers chlorophyll accumulation, similarly as it was demonstrated for algae *Cymbella* sp. and *S. quadricauda* treated with naproxen (Ding et al., 2017). Maintaining of high chlorophyll level in the pharmaceuticals-treated cells was supported by elevated content of carotenoids (Fig. 1A), which are the first line of defense against oxidative stress in chloroplasts (Ramel et al., 2012). The inability to scavenge ROS would damage photosynthetic pigments (Hindarti and Larasati, 2019) and the increase in cellular carotenoid content in drug-treated cultures is a common marker of cells' response to photosynthetic disturbances (Bar et al., 1995; Rise et al., 1994).

Investigating the above problem, we found significant differences between the effects of NBT and FFA on photosynthetic processes. At first glance, it seemed that NBT caused a strong stimulation of photosynthesis in a single cell, whereas FFA acted as an inhibitor (Fig. 1B), which was supported also by higher starch content in the NBT-treated cells and the lower starch content in FFA-exposed cells, as compared to control (Figs. 3–5). However, the expression of oxygen evolution per unit of

chlorophyll content in the cell showed no significant differences between control and NBT-treated cells (Fig. 1C), most likely because in a single cell, the chlorophyll content was higher than in control cells (Fig. 1A). The same trend was seen in the case of oxygen consumption connected with dark respiration (Fig. 1B and C). Moreover, in NBT-treated cells mitochondria seemed to have highly developed cristae, suggesting intensive mitochondrial metabolism. Opposite effects were observed in FFA-treated cultures, in which a dramatic inhibition of both oxygen evolution and consumption was noticed (Fig. 1B). Since the chlorophyll level in FFA-treated cells was only about 20% higher than that in the control, the inhibitory effect expressed per one cell was similar to the results expressed per chlorophyll unit. The observed respiration disorder in FFA-treated cells is in line with research based on animals, where the uncoupling of the electron transport in rat liver mitochondria by FFA has been reported (Jordani et al., 2000; Nadanaciva et al., 2013). Since electron transport and H⁺ gradient generation are similar in both respiration and photosynthesis, FFA will probably cause the disruption of both processes.

To develop a deeper understanding of the abovementioned differences between NBT and FFA action, we examined the chlorophyll *a* fluorescence *in vivo* parameters (OJIP test). Analysis of raw OJIP curves revealed differences between NBT- and FFA treatments, similar to the O₂ evolution results (Fig. 2). The NBT increased steps J, I, and P of the fluorescence curve but did not influence step O. Since the O–J phase reflects mainly Q_A → Q_A⁻ reduction, J–I represents the Q_A⁻ → Q_B electron transfer (first phase of PQ pool reduction with the maximal rate of PQ pool re-oxidation by Cyt *b/f*), and the I–P phase represents reduction of the PQ pool (due to the decrease in its re-oxidation, as the electron carriers beyond Cyt *b/f* become reduced) (Antal and Rubin, 2008; Schansker et al., 2006; Tsimilli-Michael and Strasser, 2008), it can be assumed that NBT inhibited the electron transport chain beyond PS II. The above was also confirmed when the data were expressed as relative variable fluorescence – the differences were most pronounced between steps J and I of the control and the NBT curves (Fig. 2B), suggesting inhibition of PQ pool reoxidation by Cyt *b6/f* (Antal and Rubin, 2008). On the other hand, step O was not shifted in NBT-treated cells as compared with the control, which indicates that neither energy quenching within antenna complexes nor energy transfer between antenna complexes and PS II reaction centers was influenced by the drug (Krause and Weis, 1984; Tsimilli-Michael and Strasser, 2008).

The lack of a shift in step O was a common feature for both NBT- and FFA-treated cells, whilst step P was lowered in the FFA curve as compared to both the control and the NBT curve (Fig. 2A). In contrast to the NBT curves, neither in the raw FFA curves nor in the relative-variable-fluorescence FFA curves, an increase in steps J–I step was detected, suggesting that FFA does not influence electron transport between PQ pool and PS I, as NBT did. Further, the relatively low P step (F_M value) indicates that some of the RCs become inactive, i.e., non-Q_A-accumulating centers ("silent centers") (Tsimilli-Michael and Strasser, 2008). Thus, to further evaluate the events reflected by the drugs' influence on photosynthetic processes, chlorophyll *a* fluorescence parameters were calculated based on OJIP curves (Fig. 2C, Table S4).

Surprisingly, neither of the two drugs tested seemed to have had a significant adverse effect on the "photosynthetic vitality" of the cells, since the value of the performance index (PI) was at the control level in both NBT- and FFA-treated cultures (Fig. 2C, Table S4). However, a detailed analysis of the OJIP parameters revealed effects caused by the tested drugs as well as differences in their action. First, one should consider that PI consists of three parameters: the density of the active reaction centers (RC), the quantum yield of primary photochemistry (φP₀), and the efficiency of moving electrons beyond quinone Q_A (Ψ₀) (functioning of electron chain between PS II and PS I) (Strasser et al., 2000). Our results indicate that in NBT-treated cells, both RC_M and φP₀ increase, whereas Ψ₀ decreases (Fig. 2C, Table S4). This means that although the electron transport beyond Q_A was disrupted, the cells were able to maintain their "photosynthetic vitality", increasing the fraction

of reaction centers that are active under saturating light (RC_M), along with a high performance of the water splitting complex (F_v/F_0) and, thus, increasing the quantum yield of primary photochemistry (ϕP_0). The above assumptions are also supported by the observation that energy absorption and trapping (ABS/RC , TR_0/RC) per one active RC in NBT-cells were higher than in the control cells (Fig. 2C, Table S4). The increased fraction of active RCs of PS II that become closed under saturated light can explain the high fluorescence signal at step P of the OJIP curve and is in a line with the high chlorophyll content in the cells (Figs. 1A and 2, Tab. S4). Further, the high performance of the water splitting complexes connected with these RCs can explain the high level of O_2 evolution in the samples (Fig. 1B). On the other hand, the decreased efficiency of electron transport beyond Q_A (ϕE_0 , Ψ_0) could be the reason for the low efficiency of electron transport to end acceptors of PS I (ϕR_0 , δR_0), assuming that PS I was not effectively fed with electrons from PS II. It cannot be also excluded that NBT influenced electron transport beyond both PS II and PS I in a similar way. In contrast to the above, the relatively high PI value in FFA-treated cells resulted mainly from the effective functioning of the electron chain between PS II and PS I (Ψ_0), since both the density of active reaction centers and the quantum yield of primary photochemistry were diminished in FFA-treated cells (Fig. 2C, Table S4), which was in a line with the low P step (F_M value) seen on the raw FFA curve of fluorescence (Fig. 2A). Interestingly, the energy fluxes per one active reaction center (ABS/RC , TR_0/RC , ET_0/RC) were increased in FFA-treated cells as compared with the control, which leads us to infer that some RCs are transformed into “silent centers”, dissipating energy as heat, while the other ones retain a high activity (Strasser et al., 2004). The efficient electron transport between active PS II and PS I is in a line with the high values of the parameters of PS I functioning (RE_0/RC , δR_0 , ϕR_0). On the other hand, silencing a part of PS II RCs can explain the low efficiency of water splitting (F_v/F_0) as well as the inhibition of oxygen evolution in FFA-treated cells (Fig. 1B and C).

Searching for possible chloroplast damages that could be seen in the ultrastructure of NBT- or FFA-treated algae, we have found in the cytoplasm stress-related vacuoles (Figs. 4 and 5). In the cells treated with FFA they were most probably autophagic (Fig. 4), while in the cells treated with NBT they were electron-dense, which suggested acidocalcisomes (Fig. 5) (Aksoy et al., 2014; Goodenough et al., 2019). The presence of autophagic vacuoles in FFA-treated cells, along with relatively low starch content, is in a line with the reports that autophagy is inevitable for the regulation of C/N metabolism in *C. reinhardtii* (Kajikawa and Fukuzawa, 2020) and that its autophagy-deficient mutant develops lipid droplets rather than starch granules under stress conditions (Kajikawa et al., 2019). Further, autophagy seems to be an important factor for maintaining cell viability under stress conditions (Kajikawa and Fukuzawa, 2020) which can partially explain the lower toxicity of autophagosome-inducing FFA as compared to NBT. On the other hand, in NBT-treated cells, the other stress-related organelles, namely acidocalcisomes were observed (Fig. 5). Acidocalcisomes have numerous functions, e.g. the storage of phosphorus and many cations, calcium signaling, osmoregulation, or cells response to stress. It seems that in stressed *C. reinhardtii* cells several acidocalcisome classes with diverse functions can exist (Goodenough et al., 2019) thus their exact role in drug-exposed cells needs to be investigated. To further confirm our observation that NBT and FFA differed in their phytotoxic effects, canonical discriminant analysis was performed (Fig. S3; Tab. S5). Analysis based on selected physiological and biochemical parameters revealed that the differentiation of the pharmaceutical type was significant and that all parameters were characterized by high discriminant power (low Wilks' lambda value). The greatest contribution to the discrimination between NBT and FFA was found for the intensity of photosynthesis, the dark respiration, and the population density (the lowest partial Wilks' lambda values) (Table S5).

Because our results strongly indicate that both NBT and FFA influence photosynthesis, the IBR was estimated based on the values of photosynthetic parameters (Fig. S3). This analysis confirmed that both

drugs are toxic to *C. reinhardtii* cells, but a higher IBR index was obtained for FFA (IBR = 3.48) than for NBT (IBR = 1.71), although FFA had a higher EC_{50-24} value than NBT. This suggests that the toxic effect of FFA is mainly a result of photosynthesis disruption, whereas the NBT affects other cellular processes, e.g. cells proliferation, as it was demonstrated for NBT-treated animal cells (Grande et al., 2021) or for diclofenac-treated *C. reinhardtii* cells (Harshkova et al., 2021). Considering the similar structure of nabumetone and naproxen, it cannot be also excluded that both pharmaceuticals act similarly, influencing processes related to the antioxidant activity of the cell, including the peroxidation of lipids present in cell membranes (Ding et al., 2017) or causing genotoxic effects, as it is reported for animal cells (see Hejna et al., 2022 and reference herein); however, in the case of higher plants and microalgae this question is still open.

Regarding particular markers used for analysis in our work, the highest values were recorded for the contents of chlorophyll *a* and *b* and carotenoids. These biomarkers are therefore most susceptible to the presence of pharmaceuticals in the environment (Fig. S3).

It is worth noting that the effects of FFA on photosynthesis are similar to those observed in diclofenac (DCF)-treated *Chlamydomonas* cells (Majewska et al., 2018). Similarities between both chemicals can be considered not only in photosynthesis-related aspects; one study showed that DCF blocks the NMDA receptor enabling the Ca^{2+} transport to cells (Gan, 2010). Alternatives of these channels, i.e., glutamate receptor-like channels (GLRs), have been determined in plants and algae (Connorton et al., 2011). Similarly, it has been confirmed that FFA influences calcium and sodium uptake in lymphoid cells (Famaey and Whitehouse, 1976). Currently, FFA is used to study many types of channels such as chloride, sodium, potassium, and non-selective cation channels (Guinamard et al., 2013). The multiplicity of effects of FFA on the membrane channels may be in part an explanation for the complex inhibitory effects on photosynthetic and respiratory processes; however, this requires further investigations.

5. Conclusions

The obtained results indicate a serious ecotoxicological risk resulting from the presence of NBT and FFA in the environment. To the best of our knowledge, this is the first report about the toxic effects of these drugs on green algae. The toxicity of NBT and FFA is confirmed by the calculated index of the integrated biomarker response (IBR), which is a valuable tool for assessing the susceptibility of living cells to environmental pollutants. Although NBT was found to have higher toxicity, estimated as the EC_{50} value, it was the influence of FFA on photosynthetic parameters that was stronger, according to the IBR. The effect of this drug thus seems to be mainly connected to the inhibition of photosynthesis. In addition, FFA lowers the number of active reaction centers and increases energy dissipation. Thus, the mechanism of action is similar to that demonstrated for diclofenac (Majewska et al., 2018). It is worth noting that the chemical structures of these compounds are similar; both of them have two aromatic rings separated by an NH-bond/skeleton. Moreover, both of them are halogenated since diclofenac contains two chlorines and FFA has three fluorides. The negative effect of NBT on photosynthesis was related to the disruption of the electron transport chain beyond PS II; however, photosynthesis inhibition does not seem to be the direct cause of the high toxicity of NBT toward *C. reinhardtii* cells. Discriminatory analysis can further confirm different mechanisms of action of the investigated drugs, making these pharmaceuticals an interesting object for further investigations. Understanding the mechanism of their toxicity will be an important contribution to the development of ecotoxicology, as it will enable prediction of the phytotoxicity of drugs with a chemical structure similar to nabumetone and flufenamic acid.

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CRediT authorship contribution statement

Dominika Kapuścińska: Investigation, (Main Investigator), Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Magdalena Narajczyk:** Investigation, Visualization. **Ivan Liakh:** Investigation, Formal analysis. **Bartosz Wielgomas:** Conceptualization, Supervision, Data curation. **Anna Aksmann:** Conceptualization, Validation, Writing - original draft, Writing - review & editing, Supervision, Data curation, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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Supplementary Material for:

Nabumetone and flufenamic acid pose a serious risk to aquatic plants: a study with *Chlamydomonas reinhardtii* as a model organism

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The supporting data contains:

- **Text S1.** Toxicological effective concentration parameter estimation.
- **Figure S1.** Density of *C. reinhardtii* populations exposed to nabumetone (NBT) or flufenamic acid (FFA) at various concentrations for 24 h.
- **Table S1.** Density of *C. reinhardtii* populations exposed to nabumetone (NBT) or flufenamic acid (FFA) at various concentrations for 24 h.
- **Table S2.** Nominal and measured values of toxicological EC parameters for *C. reinhardtii* exposed to nabumetone or flufenamic acid for 24 h.

- 24 • **Text S2.** Chromatography.
- 25 • **Table S3.** Gradient programm for chromatographic analysis.
- 26 • **Table S4.** Photosynthetic parameters estimated for *C. reinhardtii* treated for 24 h with
27 nabumetone (NBT) or flufenamic acid (FFA) at concentrations corresponding to EC₅₀₋₂₄.
- 28 • **Text S3.** Photosynthetic pigments content in the cells.
- 29 • **Figure S2.** Effects of nabumetone (NBT) and flufenamic acid (FFA), applied at EC₅₀₋₂₄
30 concentrations, on steps O, J, I, and P (A–D) of the fluorescence curve.
- 31 • **Figure S3.** Discriminant analysis (A), biomarker plots, and IBR values (B) in *C.*
32 *reinhardtii* control cells and cells exposed for 24 h to flufenamic acid (FFA) or nabumetone
33 (NBT), applied at EC₅₀₋₂₄ concentrations.
- 34 • **Table S5.** Discriminant analysis of physiological parameters in *Chlamydomonas*
35 *reinhardtii* control cells and cells exposed for 24 h to flufenamic acid (FFA) or nabumetone
36 (NBT) at EC₅₀₋₂₄ concentrations.
- 37 • **Figure S4.** Structures of selected non-steroidal anti-inflammatory drugs (based on
38 <https://pubchem.ncbi.nlm.nih.gov/>).
- 39 • **Table S6.** The toxicological EC/IC values for selected NSAIDs estimated for different
40 algae species.
- 41 • **Figure S5.** A visualization of nabumetone and flufenamic acid effects on *C. reinhardtii*.

Text S1. Toxicological effective concentration parameters estimation.

Chlamydomonas reinhardtii cc-1690 (wild type; Chlamydomonas Resource Center USA, <http://www.chlamy.org/>) cultures were grown in liquid HSM under white fluorescent light of $125 \pm 5 \mu\text{mol photons/m}^2 \times \text{s}$, measured inside the culture vessels (Quantitherm QRT1 light meter, Hansatech Ltd.) at a temperature of 30°C. Cultures were bubbled with sterilized air (bacteriological filter 0.2 mm PTFE; Sartorius) enriched with 2.5% CO₂ (v/v). The population in the mid-logarithmic growth phase was used as inoculum for the toxicity testing. The initial cell density in each experiment was 0.5×10^6 cells/mL, and the experiments lasted 24 h.

Algae were exposed to nominal nabumetone (NBT) concentrations of 8.8, 17.5, 35, 70.1, 140.2, and 280.3 μM (4, 8, 16, 32, 64 $\text{mg} \cdot \text{dm}^{-3}$) and flufenamic acid (FFA) concentrations of 7.1, 14.2, 28.4, 56.9, and 113.8 μM (4, 8, 16, 32, 64 $\text{mg} \cdot \text{dm}^{-3}$), applied at the beginning of the experiment (t_0). Cultures without the tested chemicals served as controls. After 24 h (t_{24}), cultures were sampled for population density measurements (see chapter 2.4 in the main text). The effective concentrations were calculated by regression analysis based on exponential models of the dependency between the relative population density (cells number) versus the concentrations of the pharmaceutical, using the following equation:

$$Y = A1 * e^{(x/t1)} + y0,$$

where $A1 = 97.44 \pm 2.19$ is the amplitude, $t1 = -7.17 \pm 0.39$ is the time constant, $y0 = 6.30 \pm 1.21$ is the lower asymptote for NBT, and $A1 = -1.74$, $t1 = -1.33$, $y0 = -1.74$ for FFA.

The calculated EC₅₀₋₂₄ concentrations were subsequently used for phytotoxicity estimation along with actual exposure concentrations verification.

Figure S1. Density of *C. reinhardtii* populations exposed to nabumetone (NBT) or flufenamic acid (FFA) at various concentrations for 24 h. Data are expressed as percentages of the control. Independent experiments are indicated by individual symbols. The exponential curves were fitted to the data (for curve equation, see Text S1). The graph also shows the 95% confidence intervals for the function value.

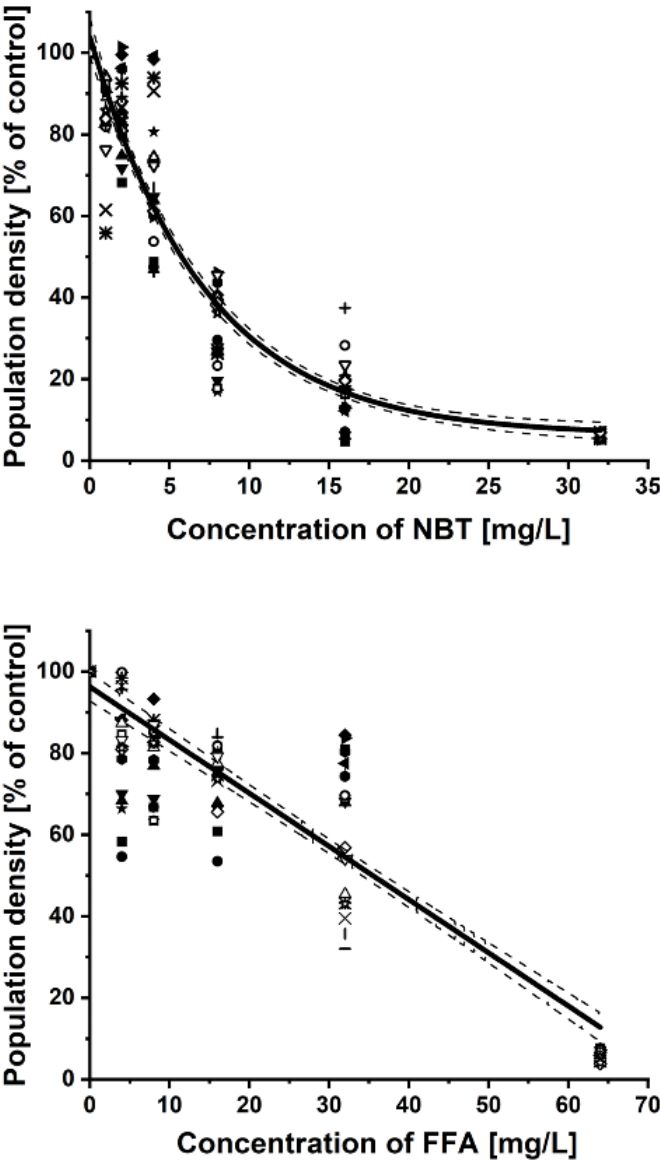


Table S1. Density of *C. reinhardtii* populations exposed to nabumetone (NBT) or flufenamic acid (FFA) at various concentrations for 24 h. Data are presented as the means of at least three

biological replicates $\pm$ SE. The initial population density in each experiment was 0.5×10^6 cells/mL.

Variant	Concentration [mg/L]	Population density $\pm$ SE [cell/mL]
Control	0	11 646 000 $\pm$ 112 000
Nabumetone	1	9 589 000 $\pm$ 311 000
	2	10 010 000 $\pm$ 193 000
	4	8 104 000 $\pm$ 417 000
	8	3 588 000 $\pm$ 251 000
	16	1 858 000 $\pm$ 203 000
	32	742 000 $\pm$ 58 000
Flufenamic acid	4	9 456 000 $\pm$ 376 000
	8	9 052 000 $\pm$ 239 000
	16	8 468 000 $\pm$ 214 000
	32	7 085 000 $\pm$ 413000
	64	917 000 $\pm$ 212 000

Table S2. Nominal and measured values of toxicological EC parameters for *C. reinhardtii* exposed to nabumetone or flufenamic acid for 24 h. The nominal concentration calculated on the basis of inhibition of population growth (cell number). A 95% confidence interval (C.I.) is given for each nominal value.

EC values	Nabumetone				Flufenamic acid			
	Nominal concentration [mg/L]	C. I. (95%)	Nominal concentration [μM]	C. I. (95%)	Nominal concentration [mg/L]	C. I. (95%)	Nominal concentration [μM]	C. I. (95%)
EC ₉₀	23.46	21.14-28.54	102.8	92.61-125.02	--	--	--	--
EC ₇₅	11.84	11.21-12.49	51.9	49.11-54.72	54.64	52.66-56.89	194.3	187.25-202.29
EC ₅₀	5.75	5.45-6.09	25.2	23.85-26.66	35.47	34.15-36.84	126.1	121.42-130.98
EC ₂₅	2.51	2.27-2.72	11.0	9.96-11.93	16.30	14.48-17.94	58.0	51.48-63.78
EC ₁₀	1.09	0.94-1.35	4.8	4.12-5.89	4.80	2.37-7.05	17.1	8.43-25.06
	Measured concentration [mg/L]	SD	SE	Percentage of the nominal concentration [%]	Measured concentration [mg/L]	SD	SE	Percentage of the nominal concentration [%]
EC ₅₀	2.97	0.28	0.11	51.7	31.8	1.80	0.73	89.7

84 **Text S2. Chromatography**

85 **Materials and Reagents**

86 NSAIDs standards: flufenamic acid and nabumetone were from ThermoFisher (Kandel) (GmbH,
87 Germany). Methanol, acetonitrile, and water (HPLC grade) were purchased from Sigma-Aldrich
88 Chemie (GmbH, Germany). Analytical-grade water was prepared using Hydrolab (Wislina,
89 Poland) Milli-Q water purification system. Algae cultures were prepared as described in section
90 2.3 Toxicological effective concentration parameter estimation.

91 **Apparatus**

92 The HPLC system consisted of a DIONEX P580 pump coupled to a DIONEX ASI-100
93 autosampler, DIONEX TCC-100 thermostatted column compartment, and DIONEX UltiMate
94 3000 photodiode array detector. The wavelengths used for the detection were 200, 225, and 275
95 nm. Data processing was carried out with Chromeleon 6.8 software (Dionex).

96 **Chromatographic conditions**

97 The chromatographic separation was performed using an ACE Excel 3 C18-AR analytical column
98 (75 x 4.6mm) from Advanced Chromatography Technologies (Aberdeen, Scotland) at 40 °C. The
99 mobile phase was composed of water (A) and acetonitrile (B) acidified with 0.1% (v/v) formic
100 acid with gradient elution at a flow rate of 1 mL/min. (Suppl. Table S2)

101 **Preparation of standard solutions and real samples**

102 Stock solutions of NSAIDs (1 mg/mL) were prepared in methanol and stored at -20 °C in a freezer.
103 Standard solutions were prepared by diluting stock solution. Using this stock solution, serial
104 dilutions were made to get 8 different concentrations (0.5, 1, 2.5, 5, 10, 50, 100, and 200 mg/L) to
105 construct a calibration curve. Samples of HSM with algae were centrifuged at 3000 rpm, and the
106 supernatants were filtered using a 0.22 µm syringe microfilter and were further analyzed. Samples

107 of HSM without algae and methanol after tube washing were prepared in the same manner
108 excluding the centrifugation step.

109

110 **Table S3.** Gradient programm for chromatographic analysis.

	Time (min.)	Flow (mL/min.)	%B
1	0	1	30
2	1	1	30
3	20	1	55
4	21	1	90
5	24	1	90
6	25	1	30
7	30	1	30

111

Table S4. Photosynthetic parameters estimated for *C. reinhardtii* treated for 24 h with nabumetone (NBT) or flufenamic acid (FFA) at concentrations corresponding to EC₅₀₋₂₄. Data are presented as the means of at least three biological replicates $\pm$ SE. Different letters indicate significant differences between groups ($p \leq 0.05$). Data were analyzed by one-way ANOVA or Kruskal-Wallis test (indicated as *), followed by the Tukey or Dunn's tests.

Variant	Control			Nabumetone			Flufenamic acid			Means of parameters
Parameters	ME	SE	Label	ME	SE	Label	ME	SE	Label	
the specific energy fluxes per one active reaction center:										
ABS/RC	1.91	0.01	a	2.02	0.02	b	2.15	0.01	c	energy absorption
DI ₀ /RC*	0.480	0.012	a	0.400	0.007	b	0.589	0.011	c	energy dissipation
TR ₀ /RC*	1.43	0.01	a	1.62	0.01	b	1.56	0.01	b	energy trapping (Q _A reduction)
ET ₀ /RC	0.751	0.043	a	0.765	0.005	a	0.933	0.006	b	efficiency of electron transport
RE ₀ /RC	0.363	0.003	a	0.271	0.006	b	0.632	0.004	c	reduction of final electron acceptors in PSI
the quantum yields:										
φP ₀	0.752	0.005	a	0.8042	0.002	b	0.7268	0.004	c	maximum yield of primary photochemistry
ψ ₀	0.525	0.003	a	0.472	0.005	b	0.597	0.003	c	efficiency/probability with which electron trapped in PSII is transferred from Q _A to Q _B
φE ₀	0.395	0.004	a	0.379	0.004	b	0.434	0.004	c	the maximum yield of the electron transport, quantum yield of the electron transport flux from Q _A to Q _B
ΔR ₀ *	0.483	0.002	a	0.353	0.006	b	0.678	0.001	c	efficiency/ probability with which an electron from Q _B is transferred until PSI acceptors
φR ₀	0.190	0.002	a	0.134	0.003	b	0.294	0.003	c	yield of electron transport from Q _A to the PS I end electron acceptors
PI abs	1.877	0.068		1.835	0.047		1.867	0.058		performance index
RC ₀	263	2	a	240	3	b	237	2	b	fraction (density) of active reaction centers referring to time when minimal fluorescence is reached
RC _M	1090	30	a	1230	20	b	870	20	c	fraction (density) of active reaction centers referring to time when maximal fluorescence is reached
Fv/F ₀	3.14	0.07	a	4.12	0.04	b	2.68	0.05	c	the activity of the water-splitting complex on the donor side of PSII

Text S3. The contents of chlorophyll *a*, chlorophyll *b*, and carotenoids in the cells were determined using a spectrophotometric method, after extraction with 80% acetone. For this, cell suspension samples were centrifuged (2,000 *g*, 10 min, RT), the supernatant was discarded, and the cell pellets were disintegrated with glass beads using a homogenizer (FastPrep-24 instrument, MP Biomedicals, 5 m/s, 1 min). The extracts were clarified by centrifugation (2,000 *g*, 10 min) and applied to a 96-well plate. Absorbance was measured at 447, 647, 664, and 740 nm using the spectrofluorometer Varioskan Flash Multimode Microplate Reader (Thermo Fisher Scientific). The concentrations of photosynthetic pigments were calculated according to Jeffrey & Humphrey (1975) and Lichtenthaler & Wellburn (1983). Correction on the path length was applied according to Warren (2008).

Figure S2. Effects of nabumetone (NBT) and flufenamic acid (FFA), applied at EC_{50-24} concentrations, on steps O, J, I, and P (A–D) of the fluorescence curve. O – minimal fluorescence when all photosystem II reaction centers are open; J – fluorescence intensity measured after 2 ms, I – fluorescence intensity measured after 30 ms; P – maximal fluorescence when all photosystem II reaction centers are closed. Data are presented as means of at least three biological replicates $\pm$ SE. Different letters indicate significant differences between groups ($p \leq 0.05$). (ANOVA, Tukey's HSD test).

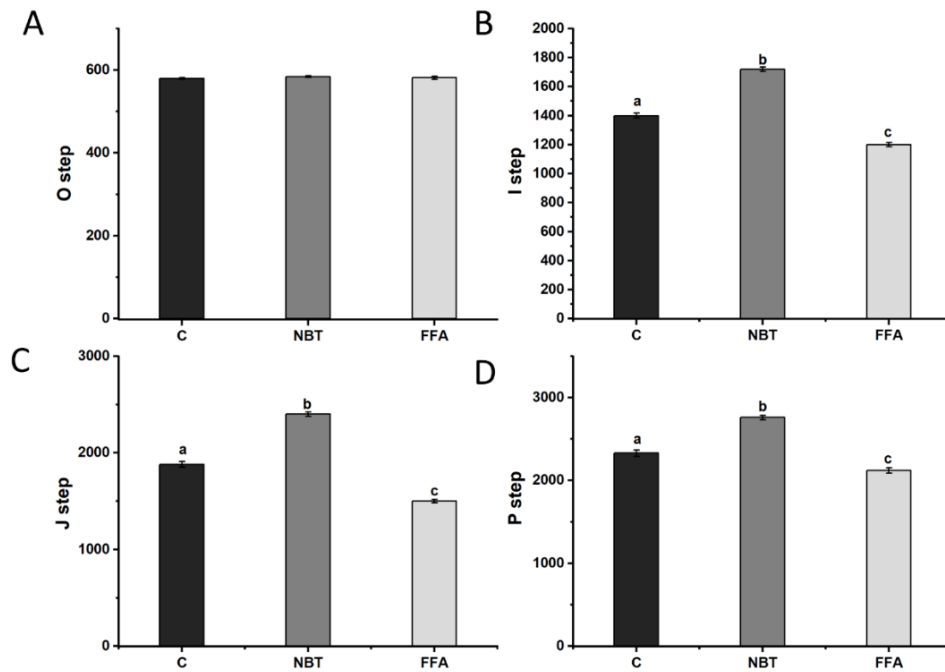


Figure S3. Discriminant analysis (A), biomarker plots, and IBR values (B) in *C. reinhardtii* control cells and cells exposed for 24 h to flufenamic acid (FFA) or nabumetone (NBT), applied at EC₅₀₋₂₄ concentrations. In discriminant analysis population density, photosynthetic pigment contents, photosynthesis intensity, and dark respiration intensity were included. For IBR estimation, chlorophyll *a* and *b*, carotenoids, PI_{abs}, and the intensity of photosynthesis were selected.

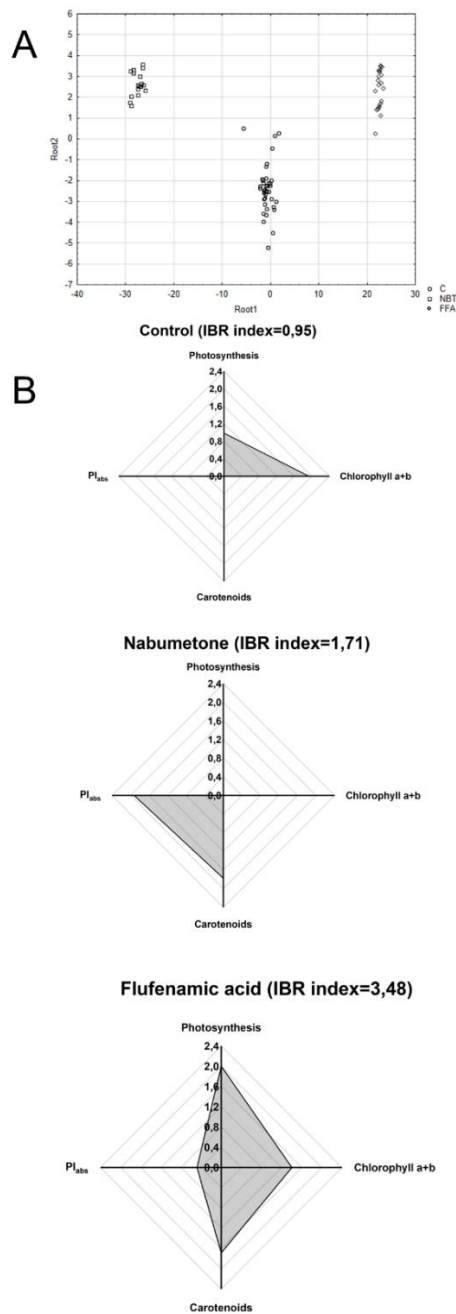
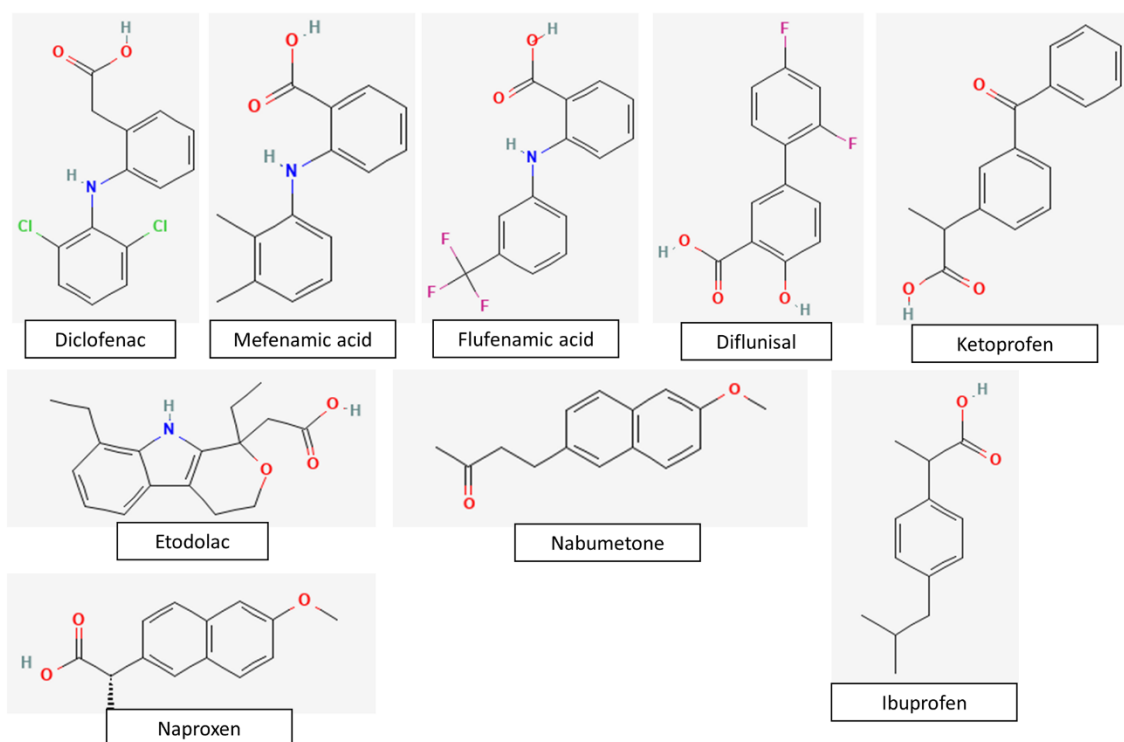


Table S5. Discriminant analysis of physiological parameters in *Chlamydomonas reinhardtii* control cells and cells exposed for 24 h to flufenamic acid (FFA) or nabumetone (NBT) at EC₅₀₋₂₄ concentrations.

Parameters	Wilks' - Lambda	Partial Lambda	F- remove	P- level
Cell density	0.001355	0.323142	74.359	p<0.0001
Chlorophyll <i>a</i>	0.000470	0.931121	2.626	0.079381
Chlorophyll <i>b</i>	0.000445	0.985111	0.537	0.587117
Carotenoids	0.000480	0.911873	3.431	0.037816
Photosynthesis	0.006364	0.068810	480.416	p<0.0001
Dark respiration	0.019811	0.022103	1570.611	p<0.0001

151 **Figure S4.** Structures of selected non-steroidal anti-inflammatory drugs (based on
152 <https://pubchem.ncbi.nlm.nih.gov/>).



156 **Table S6.** The toxicological EC/IC values for selected NSAIDs estimated for different algae
157 species.

NSAID	Species	EC/IC	Ref.
NBT	<i>Chlamydomonas reinhardtii</i>	EC ₅₀₋₂₄ =5.75 mg/L	this study
FFA	<i>Chlamydomonas reinhardtii</i>	EC ₅₀₋₂₄ =35.47 mg/L	this study
MEF	<i>Haematococcus pluvialis</i>	EC ₅₀₋₉₆ =0.106 mg/mL	Bácsi et al. (2018)
DCF	<i>Chlamydomonas reinhardtii</i>	EC ₅₀₋₂₄ =134.5 mg/L	Majewska et al. (2018)
	<i>Desmodesmus subspicatus</i>	EC ₅₀₋₇₂ =71.9 mg/L	Cleuvers (2004)
	<i>Pseudokirchneriella subcapitata</i>	EC ₅₀₋₉₆ =6 mg/L	Zind et al. (2021)
	<i>Haematococcus pluvialis</i>	EC ₅₀₋₉₆ =0.103 mg/mL	Bácsi et al. (2018)
DIF	<i>Haematococcus pluvialis</i>	EC ₅₀₋₉₆ =0.12 mg/mL	Bácsi et al. (2018)
NPX	<i>Desmodesmus subspicatus</i>	EC ₅₀₋₇₂ =625.5 mg/L	Cleuvers (2004)
	<i>Raphidocelis subcapitata</i>	EC ₅₀₋₇₂ = 27.1 mg/L	Grabarczyk et al. (2020)
	<i>Cymbella</i> sp.	EC ₅₀₋₂₄ = 29.20 mg/L EC ₅₀₋₄₈ = 37.93 mg/L EC ₅₀₋₇₂ = 102.76 mg/L	Ding et al. (2017)
	<i>Scenedesmus quadricauda</i>	EC ₅₀₋₂₄ = 0.7 mg/L EC ₅₀₋₄₈ = 69.86 mg/L EC ₅₀₋₇₂ = 101.45 mg/L	Ding et al. (2017)
IB	<i>Chlorella vulgaris</i>	IC ₅₀₋₄₈ =82.25 mg/L IC ₂₀₋₄₈ =48.20 mg/L IC ₅₀₋₇₂ =86.43 mg/L IC ₂₀₋₇₂ =51.05 mg/L IC ₅₀₋₉₆ =89.65 mg/L IC ₂₀₋₉₈ =54.84 mg/L	Geiger, Hornek-Gausterer, and Saçan (2016)

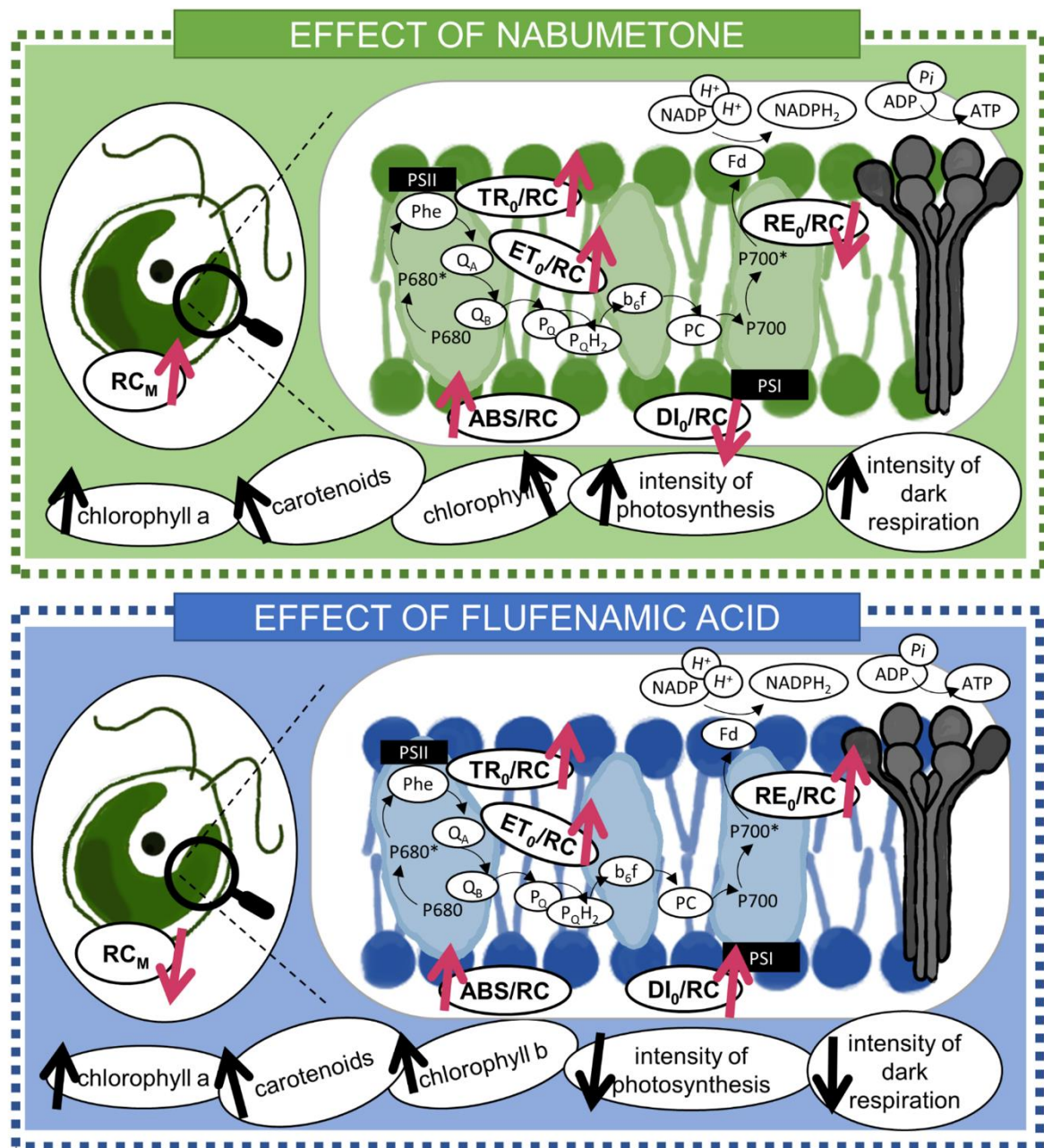
	<i>Pseudokirchneriella subcapitata</i>	EC ₅₀₋₇₂₋₉₆ =232.64 mg/L	González-Naranjo and Boltes (2014)
		EC ₅₀₋₇₂ =93.26 mg/L	Grabarczyk et al. (2020)
	<i>Desmodesmus subspicatus</i>	EC ₅₀₋₇₂ =342.2 mg/L	Cleuvers (2004)
	<i>Acutodesmus obliquus</i>	EC ₅₀₋₅ =174/288 ¹ mg/L	Grzesiuk et al. (2016)
	<i>Chlamydomonas reinhardtii</i>	EC ₅₀₋₅ =403-622 ¹ mg/L	Grzesiuk et al. (2016)
	<i>Nannochloropsis limnetica</i>	EC ₅₀₋₅ =215-965 ¹ mg/L	Grzesiuk et al. (2016)
KET	<i>Chlorella</i>	IC ₅₀₋₇₂ =85.20 mg/L	Gomaa et al. (2021)
	<i>Desmodesmus spinosus</i>	IC ₅₀₋₇₂ =71.91 mg/L	Gomaa et al. (2021)
	<i>Pseudokirchneriella subcapitata</i>	IC ₅₀₋₇₂ =24.6 mg/L	Watanabe et al. (2016)
ET	<i>Pseudokirchneriella subcapitata</i>	IC ₅₀₋₇₂ =102 mg/L	Watanabe et al. (2016)

158 DCF – diclofenac; NPX – naproxen; IB- ibuprofen; KET-ketoprofen; MEF- mefenamic acid; DIF-

159 diflunisal, ET- etodolac

160 ¹-depending on the availability of phosphorus, based on maximum quantum yield

161



163

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polegał na:

(i) przeprowadzeniu eksperymentów obejmujących hodowlę glonów, wyznaczanie wartości EC oraz oznaczanie liczebności komórek; (ii) wykonaniu analiz fotosyntetycznych, w tym intensywności fotosyntezy, analizy fluorescencji chlorofilu *a in vivo*, oznaczeniu zawartości barwników, oznaczeniu intensywności oddychania ciemniowego oraz przeprowadzeniu analiz statystycznych (iii) udziale w opracowaniu i interpretacji wyników; (iv) przygotowaniu grafik i rycin przedstawiających wyniki badań; (v) przygotowaniu pierwotnej wersji manuskryptu; (vi) udziale w redagowaniu manuskryptu; oraz (vii) udziale w przygotowaniu odpowiedzi na uwagi recenzentów.

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<https://doi.org/10.1016/J.CHEMOSPHERE.2023.140853>*

polegał na:

(i) wykonaniu analiz ultrastrukturalnych z zastosowaniem mikroskopii elektronowej oraz przygotowaniu dokumentacji mikroskopowej; oraz (ii) analizie i interpretacji uzyskanych obrazów.

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<https://doi.org/10.1016/J.CHEMOSPHERE.2023.140853>

polegał na:

(i) oznaczeniu rzeczywistych stężeń badanych związków metodą HPLC; (ii) przeprowadzeniu formalnej analizy uzyskanych danych; oraz (iii) udziale w redagowaniu manuskryptu.

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Chlamydomonas reinhardtii as a model organism. Chemosphere 2024;349:140853.
<https://doi.org/10.1016/J.CHEMOSPHERE.2023.140853>

polegał na:

(i) udziale w opracowaniu koncepcji części analitycznej badań; (ii) sprawowaniu nadzoru nad poprawnością analiz wykonanych za pomocą HPLC; (iii) udziale w opracowaniu i walidacji wyników HPLC; (iv) udziale w redagowaniu manuskryptu; oraz (v) przygotowaniu odpowiedzi na uwagi recenzentów.



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polegał na:

(i) opracowaniu koncepcji badań; (ii) walidacji wyników; (iii) nadzorze nad realizacją badań; (iv) udziale w opracowaniu i interpretacji wyników; (v) administracji projektem; (vi) pozyskaniu finansowania; (vii) udziale w przygotowaniu pierwotnej wersji manuskryptu; (viii) udziale w redagowaniu manuskryptu; oraz (ix) udziale w przygotowaniu odpowiedzi na uwagi recenzentów.



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PODPIS

11. Praca nr 2.

A sensitive and reliable method for the quantitative determination of hydrogen peroxide produced by microalgae cells.

Hejna M[#], **Kapuścińska D[#]**, Aksmann A.

Journal of Phycology,

2024, 60(6), 1356–1370. <https://doi.org/10.1111/JPY.13524>

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A sensitive and reliable method for the quantitative determination of hydrogen peroxide produced by microalgae cells

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Short title: Analysis of H₂O₂ produced by microalgae

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Abstract

One of the reactive forms of oxygen is hydrogen peroxide (H₂O₂) which has been investigated as a key component of growth processes and stress response. Different methods for the determination of H₂O₂ production by animal and bacterial cells exist; however, its detection in

algal cell cultures is more complicated, due to the presence of photosynthetic pigments in the cells and the complex structure of cell walls. Considering these issues, a reliable, quick, and simple method for H₂O₂ detection is needed in phycological research. The aim of this methodological study was to optimize an Amplex UltraRed method for the fluorometric detection of H₂O₂ produced by microalgae cells, using a wild type strain of *Chlamydomonas reinhardtii* as a model. The results showed that (i) potassium phosphate is the most suitable reaction buffer for this method, (ii) a 560 nm wavelength variant is the most appropriate as the excitation wavelength for fluorescence spectra measurement, (iii) a 50:50 ratio for the reaction mixture to sample was the most suitable, (iv) the fluorescence signal was significantly influenced by density of the microalgae biomass, (v) sample fortification with H₂O₂ allowed for an increase of the method's reliability and repeatability. The proposed protocol of the Amplex UltraRed method for the fluorometric detection of H₂O₂ produced by microalgae cells can yield a sensitive and accurate determination of the content of the test compound, minimizing measurement errors, eliminating chlorophyll autofluorescence problem, and compensating for the matrix effect. This method can be applied to the study of other microalgae species.

Highlights

The H₂O₂ determination protocol with Amplex Ultra Red in microalgae was optimized
Biomass removal and sample fortification with H₂O₂ allow minimizing the matrix effect
Samples fortification increases the repeatability of the method
The usefulness of the method in ecotoxicological tests was confirmed

Keywords: hydrogen peroxide, reactive oxygen species, Amplex UltraRed, horseradish peroxidase, fortification, *Chlamydomonas reinhardtii*, microalgae.

1. Introduction

Reactive oxygen species (ROS) have a signaling function in controlling plant growth and development processes such as cells proliferation, pollen tube formation, root hair growth, establishing the primary and secondary cell wall structure, plant-pathogen interactions, survival under stress conditions etc. (Nafees et al. 2019; Peshev et al., 2013; Tripathy & Oelmüller, 2012). The production of ROS at low concentration is therefore important for proper cell functioning (Nafees et al. 2019; Schmitt et al., 2014), whereas, if they are formed in excess, ROS are responsible for the oxidation of biomolecules, including lipids, proteins, and nucleic acids (Halliwell, 1991; Halliwell & Gutteridge, 2015). ROS accumulation increases through biotic and abiotic stress-induced factors (Chokshi et al., 2017; Zhao et al., 2019). Abiotic stress induces cell apoptosis by manipulating cellular synthesis and the detoxification of ROS, while biotic stressors increase intracellular ROS by activating membrane-associated oxidases to synthesize extracellular superoxide (Apel & Hirt, 2004; Suzuki et al., 2014). The reactive forms of ROS include species such as: superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($\bullet OH$), alkoxy radical ($RO\bullet$), peroxy radicals ($RO_2\bullet$), singlet oxygen (1O_2), organic hydroperoxides ($ROOH$), and hydrogen peroxide (H_2O_2) (Chakraborty et al., 2016). Cellular ROS concentrations range from pico- to micromoles (Anjum et al., 2020).

Among different ROS species, H_2O_2 is the most often analyzed molecule, due to its vital role in signal transduction of living cells, function as the biomarker of oxidative stress, relative stability, and inter-compartmental mobility compared to other ROS (Xu et al. 2013; Wang et al. 2020). Hydrogen peroxide can be synthesized by plant and algal cells via both non-enzymatic and enzymatic reactions in which superoxide dismutases, peroxidases, oxidases, and flavin-containing enzymes can be involved (Chang & Tang, 2014; Cona et al., 2006; Francoz et al., 2015). Hydrogen peroxide plays a significant role in redox reactions and is needed to build the resistance of microalgae to oxidative stress caused by e.g., heavy metals (Wang et al.,

2014). On the other hand, H₂O₂ overproduction can lead to the production of other free radicals, which are harmful to organisms at high concentrations, causing oxidative damage to nucleic acids, amino acids, proteins, and membrane lipids; they can induce enzyme inhibition and the activation of programmed cell death (Chakraborty et al., 2016; Legrand et al., 2022; Urbina et al., 2019).

In ecotoxicological studies the assessment of ROS-related enzymes activity is commonly used as a marker of oxidative stress (Ding et al., 2017; Gomez-Olivan et al., 2014). Thus, activity of superoxide dismutase (SOD) is often regarded to be an equivalent of H₂O₂ production by the cells, while the real net production of this molecule depends also on its subsequent transformation by enzymes such as catalase, ascorbate peroxidase, and glutathione peroxidase (Rezayian et al., 2019). This approach of oxidative stress evaluation may lead to an overinterpreting of results and to drawing incorrect conclusions, since H₂O₂ serves as both a product and substrate in reactions catalyzed by the above-mentioned enzymes. Therefore, a more methodologically sound approach involves direct estimation of H₂O₂ production by the cells and its quantification provides a more precise insight into the potential occurrence of oxidative stress in investigated organisms. Different methods for the determination of extracellular and intracellular H₂O₂ have been developed for animal and bacterial cells, each with its advantages and drawbacks (Grisham, 2013; Ahmad et al., 2022). However, H₂O₂ detection in higher plant cells and algae cells populations is much more complicated due to (i) the presence of a cell wall containing numerous polysaccharides, proteoglycans, or aromatic polymers with various functional groups that can non-specifically interact with the dyes, (ii) the potential interference of enzymes like POX, leading to an underestimation or overestimation of the detected H₂O₂ (Domozych et al., 2012; B. Zhang et al., 2021), (iii) the possibility that some fluorochromes or other reaction components, which should enter the cell, can be blocked by the cell wall (Chen et al., 2009) , (iv) the necessity of selecting appropriate fluorochromes

in order to avoid the overlapping of the waves of fluorescence emission and the autofluorescence of plant cell components, e.g., chlorophylls (Ortega-Villasante et al., 2016). Therefore, protocols optimized for plant and algal cells are lacking in the literature, and commercial kits are not available to directly estimate the H₂O₂ levels produced by algae cells (Noctor et al., 2016; Queval et al., 2008), so a quick and simple method is still needed in research that focuses on plant and algal cell development and the stress response.

Methods used to detect hydrogen peroxide are basically divided into fluorometric and colorimetric approaches (Anjum et al., 2020; Rezende et al., 2018). One common colorimetric H₂O₂-indicator is 3,3'-diaminobenzidine (DAB), which causes the formation of a brown precipitate in a reaction catalyzed by peroxidase in plants (Thordal-Christensen et al., 1997). However, DAB is unsuitable for vital staining, and the method itself is time-consuming, multi-step and exhibits low specificity and relatively low sensitivity (Förster et al., 2005; Smirnoff and Arnaud, 2019). Thus, when rapid measurements of oxidative stress in ecotoxicological studies are needed, and differences between the tested samples are minimal, DAB does not provide a reliable result. In this context, the use of fluorescent probes for fluorometry, fluorescence microscopy, and cytometry seems to be a better approach (Halliwell and Gutteridge, 2015). A frequently used fluorochrome in ecotoxicological studies is dihydramine 123 (DHR), which is oxidized by H₂O₂, ONOO[−] and other NO₂ radicals to the fluorescent rhodamine 123 (Crow, 1997; Jamers and De Coen, 2010). Although recommended for oxidative stress assessment, DHR is susceptible to non-specific oxidation, and its lipophilicity may lead to membrane binding (Anjum et al., 2020). Therefore, substances that may interfere with DHR should be carefully monitored (Ortega-Villasante et. al, 2016).

Another fluorochrome dedicated to H₂O₂ detection is Amplex Red (AR) or its improved form, Amplex UltraRed (AUR). The method is based on the oxidation of 10-acetyl-3,7-dihydroxyphenoxazine, the derivative of which is AUR, by H₂O₂. The reaction is catalyzed by

peroxidases (POXs) and results in the formation of fluorescent resorufin in a stoichiometric ratio of AUR: resorufin = 1:1 (Debski et al., 2016; Mohanty et al., 1997). AUR provides a low background signal, while resorufin shows high fluorescence, resulting in a beneficially low signal-to-noise ratio (Kalyanaraman et al., 2012, Kolbert et al., 2012). AUR can be also oxidized by other single-electron transfer oxidants, however the electron transfer catalyzed by POXs is highly efficient, ensuring AUR high H₂O₂-specificity. Since this specificity, as well as method sensitivity, is mainly based on the activity of POXs, there is a risk that the results will be falsified by different levels of activity of these enzymes in various types of biological material (Smirnoff and Arnaud, 2019). Furthermore, POXs activity changes under the influence of various factors, as it was shown for *Chlamydomonas reinhardtii* cells exposed to oxidative stress caused by the herbicide paraquat (Chankova et al. (2014). Thus, the use of exogenous peroxidase, such as horseradish peroxidase (HRP) is highly recommended to increase fluorescence signal stability and repeatability of results (Towne et al., 2004). Studies using neutrophils have confirmed, that this approach enables researchers to detect H₂O₂ at concentrations as low as 5 pmol · L⁻¹ (Zhou et al., 1997). However, variations in protocols found in the literature (Veljovic-Jovanovic et al., 2002; Mattila et al., 2015) indicate the necessity for the method optimization to specific types of biological material. The problem can be especially seen in the case of ecotoxicological studies on unicellular green algae, where the specific and sensitive method for determining the amount of H₂O₂ produced in response to toxicants is indispensable.

In the light of the above, the main aim of this study was to modify and to optimize an Amplex UltraRed method for its application in ecotoxicological studies, especially for the fluorometric detection of H₂O₂ produced by microalgae cells. The wild-type strain of *C. reinhardtii*, the unicellular plant model possessing the features of an indicator organism, has been chosen for investigations. This organism is a widely used biomarker for the evaluation of

ecological risk assessments and a convenient tool for toxicological studies on chemical pollutants in an aquatic environment (Aksmann et al., 2014; Çelekli et al., 2016; Majewska et al., 2018). To optimize the method for assessment of H₂O₂ produced by *C. reinhardtii* cells, we have (i) selected the appropriate wavelengths of signal excitation and detection, considering the presence of natural pigments in analyzed material, (ii) selected of proper reaction buffers, to ensure a sufficiently high level of fluorescence signal, (iii), evaluated the possibility of signal scattering due to presence of biological matrix, and (iiii) analyzed the significance of samples fortification for reliability of the obtained results. To confirm the usefulness of the method for phytotoxicity testing, we used nabumetone, a non-steroidal anti-inflammatory drug that is found as a contaminant in water reservoirs (Peng et al., 2019).

2. Materials and methods

2.1. Algae cultures

C. reinhardtii, wild type strain (CC-1690), was purchased from the Chlamydomonas Resource Center at The University of Minnesota (USA). The algae were transferred from slants into a liquid high-salt medium (HSM; pH 6.9 ± 0.1) (Sueoka, 1960) and incubated for a few days in pre-culture to adapt to the conditions of the batch culture. Further, cultures with an initial cell density of $0.5 \times 10^6 \text{ cells} \cdot \text{mL}^{-1}$ were grown for 24 h in 200 mL glass test tubes in a water bath at 30 °C ± 1 °C under constant fluorescent light ($130 \pm 5 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) measured inside the test tube (Quantitherm QRT1 light meter, Hansatech Ltd, UK). Cultures were continuously bubbled with sterilized air (PTFE filter, Sartorius 2000) containing 2.5% (v/v) CO₂ (Aksmann and Tukaj, 2008). The population in the mid-logarithmic growth phase after 24 h was used to perform all tests. The cell number was determined using an electronic particle counter (Beckman Coulter Z2, USA). Observations of cells were performed under a light

microscope (JENAVAL, Carl Zeiss, Germany) at a magnification of 400× to minimize the risk that the results obtained from the Z2 counter were significantly misestimated.

2.2. Buffers and chemicals

2.2.1. Buffers

Three different reaction buffers (pH 7.0) were used for reaction mixture preparation: (A) 100 mM potassium phosphate buffer consisting of K_2HPO_4 and KH_2PO_4 , pH 7.0; (B) 100 mM HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) buffer consisting of 10 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.6 mM sorbitol, pH 7.0; (C) 100 mM Tris-HCl, pH 7.0. Further, an HRP-buffer, namely, 100 mM potassium phosphate buffer consisting of K_2HPO_4 and KH_2PO_4 , pH 6.0, was prepared for a stock solution of horseradish peroxidase. All of the chemicals for buffer preparation were of analytical grade (purity of above 95%).

2.2.2. Chemicals

Amplex UltraRed reagent and horseradish peroxidase (HRP) were purchased from Invitrogen (MA, USA) and Thermo Scientific (MA, USA), respectively, and 50% stabilized H_2O_2 solution was purchased from Sigma-Aldrich (MA, USA). Nabumetone (4-(6-methoxynaphthalen-2-yl)butan-2-one) was purchased from Thermo Scientific (MA, USA). All of the other chemicals used were of analytical grade (purity above 95%).

Amplex UltraRed was prepared according to the manufacturer's instruction. Briefly, to prepare a 10 mM stock solution of Amplex UltraRed reagent, 340 μ L of high-purity DMSO was added into one vial (1 mg) of Amplex UltraRed reagent. An HRP stock solution was prepared by dissolving 1 mg of salt-free, lyophilized HRP (activity = 307 U · mg⁻¹) powder in an HRP-buffer to a final activity of 10 U · mL⁻¹. These chemicals were stored in aliquots in the dark at -80 °C for further use.

Directly before each experiment, a 1 M stock solution of H₂O₂ was prepared using a 50% stabilized solution of H₂O₂ and distilled water. The reaction mixture (RMix) was also freshly prepared by mixing 0.5 µL of 10 mM Amplex UltraRed reagent stock solution with 1 µL of 10 U · mL⁻¹ HRP and 48.5 µL of 0.1 M buffer depending on the variant tested for one sample.

2.3. Experimental variants

2.3.1. Excitation/emission fluorescence spectra measurements in different buffers

To choose the best experimental conditions, an aliquot (50 µL) of reaction buffer, A, B, or C (see Subchapter 2.2.1.) was placed in the well of a black 96-well microplate and mixed with RMix (50 µL), and the fluorescence spectra were measured at excitation wavelengths between 550 and 580 nm, and an emission wavelength of 581 nm (at 25 °C), using a spectrofluorometer (Varioskan Flash-Spectral Scanning Multimode Microplate Reader, Thermo Fisher Scientific, Waltham, MA USA) that was operated using Varioskan Flash SkanIt Software. In the next step, the measurements were repeated as described above, with one modification, namely, before the measurement, the RMix was supplemented with 15 µmol · mL⁻¹ H₂O₂. For the next experiment, two buffers, namely A and C, were chosen, and the fluorescence spectra were measured at excitation wavelengths of 500 nm, 530 nm, and 560 nm, and emission wavelengths of 580-650 nm, as described above. The experiment was repeated with 15 µmol · mL⁻¹ H₂O₂ being added to the RMix. After the initial selection of the phosphate buffer and wavelengths, excitation wavelengths of 500-570 nm at emission wavelength of 585 nm, and emission wavelengths of 580-650 nm at an excitation wavelength of 560 nm were scanned again using the algal suspension, the supernatant after centrifugation of the algal biomass and the buffer.

2.3.2. Influence of algal suspension density on the fluorescence signal

The determination of the effect of the population density on the intensity of the fluorescence signal was performed by diluting the algae suspension with the medium to densities of 10.0, 5.0, 2.5, 1.0, and 0.5×10^6 cells $\cdot$ mL⁻¹. The reaction was then performed by adding 50 μ L of the RMix prepared on buffer C to 50 μ L of the cell suspension, and incubating for 25 min in the darkness at 25 °C. The fluorescence was measured at $\lambda_{\text{ex}} = 560$ nm and $\lambda_{\text{em}} = 585$ nm. The H₂O₂ concentration was determined using a standard curve (Ia) (see Appendix 1 and Fig. S1).

2.3.3. Influence of the ratio of an algal cell suspension to the reaction mixture on the fluorescence signal

Experimental variants with various ratios of algae cell suspension to the RMix were prepared to measure the possibility of reaction saturation. The starting density of the cell suspension (CS) in the growing media was 10 million cells $\cdot$ mL⁻¹. The following variants were used: (i) 10 μ L CS: 90 μ L of the RMix, (ii) 20 μ L CS: 80 μ L RMix, (iii) 30 μ L CS: 70 μ L RMix, (iv) 40 μ L CS: 60 μ L RMix, and (v) 50 μ L CS: 50 μ L RMix. The fluorescence signal and the H₂O₂ concentration were determined as described in Subchapter 2.3.2.

2.3.4. Quenching of the fluorescence signal by the dead algae biomass

To investigate the possibility of whether the biomass can significantly influence the fluorescence signal, the microalgae cultures of the density, approximately 11×10^6 cells $\cdot$ mL⁻¹, were incubated at 100 °C for 3 minutes. The suspension of the dead cells was diluted to a different density: 10.0, 5.0, 2.5, 1.0, and 0.5×10^6 cells $\cdot$ mL⁻¹. The aliquots of the samples (50 μ L) were placed in a 96-well black microplate, and 50 μ L of the RMix was added to the prepared samples. The fluorescence signal and the H₂O₂ concentration were determined as described in Subchapter 2.3.2. Then, the measurements were repeated with one modification, namely, 5 nmol $\cdot$ mL⁻¹ H₂O₂ was added to each sample. The H₂O₂ concentration was determined as described in Subchapter 2.3.2.

2.3.5. *Influence of biomass centrifugation on the fluorescence signal*

The influence of biomass centrifugation on the fluorescence signal was assessed by preparing two experimental variants. In the first one, 150 μL of the live algae suspension of a density of $10 \times 10^6 \text{ cells} \cdot \text{mL}^{-1}$ was mixed with 50 μL of the RMix, incubated for 25 min, and centrifuged (5 min., $1500 \times g$, RT). Then, 100 μL of the sample was transferred to a 96-well plate for fluorescence measurements. In the second variant, the live algae suspension of a density of $10 \times 10^6 \text{ cells} \cdot \text{mL}^{-1}$ was centrifuged (5 min., $1500 \times g$, RT); 150 μL of supernatant was mixed with 150 μL of the RMix and incubated for 25 min. Then, 100 μL of the sample was transferred to a 96-well plate for fluorescence measurements. The control sample was non-centrifuged algae, prepared as follows: 150 μL of the live algae suspension of a density of $10 \times 10^6 \text{ cells} \cdot \text{mL}^{-1}$ was mixed with 150 μL of the RMix and incubated for 25 min. Then, 100 μL of the sample was transferred to a 96-well plate for fluorescence measurements.

2.3.6. *Standard curves and calculations*

The standard curves (I). A 50% stabilized solution of H_2O_2 in phosphate buffer was used to prepare the first standard curve (Ia, Fig. S1) by mixing 50 μL of the H_2O_2 solution of the desired concentration and 50 μL of the RMix. The H_2O_2 concentration range was from 0 to 5 $\text{nmol} \cdot \text{mL}^{-1}$. The fluorescence signal was determined as described in Subchapter 2.3.2. A linear curve ($y = a + b \times x$) was fitted using Origin software, where $a = 4.695 \pm 0.899$ and $b = 118.277 \pm 0.309$ (OriginLab Corporation, USA). The standard curves (standard curves Ib – If) for calculation of H_2O_2 concentrations in the experiments with various ratios of algae cell suspension to the RMix (see subchapter 2.3.3) were prepared as described above, using proper ratios of H_2O_2 solution to RMix. Equations for standard curves used to calculate H_2O_2 concentration for different ratios of reaction mixture to algae suspension are provided in the supplemental material (Appendix 1; Tab. S1). Moreover, the limit of detection (LOD) and limit

of quantification (LOQ) were determined using the following formulas: $LOD = 3.3(SD/S)$ and $LOQ = 10(SD/S)$.

The matrix-matched standard curve (II) and equation for the H_2O_2 concentration calculation. To prepare the matrix-matched standard curve, the supernatant obtained from algae cultures with initial densities of 0.5, 2.5, 5.0, and $10.0 \times 10^6 \text{ cells} \cdot \text{mL}^{-1}$, centrifuged at $1500 \times g$, 5 min, was used. A solution of H_2O_2 (1 μL) was added to these supernatants (49 μL) to final concentrations of 0, 0.5, 1, 5.0, and $10.0 \text{ nmol} \cdot \text{mL}^{-1}$, and the RMix (50 μL). The fluorescence signal was determined as described in Subchapter 2.3.2. and the standard curves were fitted using Origin software (see Appendix 1, Fig. S2, and Tab. S2). The H_2O_2 concentration was also calculated using the equation adapted from the manual for colorimetric/fluorometric assays for ATP determination (Sigma Aldrich, n.d.) as follows (see also Appendix 2):

$$C \text{ } H_2O_2 = [ST \times F_{\text{scorr}}] / [(F_{\text{sscorr}} - F_{\text{scorr}}) \times Sv] [\text{nmol} \cdot \mu\text{L}^{-1}],$$

where:

ST – the amount of H_2O_2 spiked in the well (nmoles); F_{scorr} – the corrected sample fluorescence reading (unspiked well); F_{sscorr} – the corrected spiked sample fluorescence reading (spiked well); Sv – the sample volume (μL) added into the well.

2.3.7. *The usefulness of the method for phytotoxicity testing*

The suitability of the method for ecotoxicological analysis on algae was investigated by treating *C. reinhardtii* cells with nabumetone at EC values (EC means the effective concentration that reduces population growth by a specific percentage). Algae were cultured as described in Subchapter 2.1. The population with an initial cellular density of $0.5 \times 10^6 \text{ cells} \cdot \text{mL}^{-1}$ was exposed to nabumetone at concentrations corresponding to $EC_{10/24}$, $EC_{25/24}$, and $EC_{50/24}$ (manuscript in preparation). After 24 h, the cell suspensions were sampled, and the amount of H_2O_2 produced by the cells was determined according to the established protocol (see Appendix 2). The control sample consisted of algae without the addition of nabumetone.

To compare the sensitivity and effectiveness of the optimized method, the results of fluorescence measurements for microalgae suspensions with and without horseradish peroxidase in the reaction mixture were presented (see Appendix 1).

2.4. Statistical analysis

Statistical analysis was performed using MS Excel 2010 (Microsoft, Redmond, WA, USA) and Statistica 9.0 (StatSoft, Hamburg, Germany). All data were expressed as the mean of at least three independent experiments, with at least three replications for each experimental variant $\pm$ standard error (SE). In order to remove any outliers, the Grubbs test was performed. The graphs were prepared using the Origin Pro 7.5 program (Northampton, MA, USA).

3. Results and discussion

This methodological study describes the modification and optimization of the Amplex UltraRed-HRP (AUR-HRP) method for H_2O_2 detection in an algal cell suspension. This method, based on a fluorescence signal generated during fluorochrome interaction with a detected molecule, has been successfully used for animal-derived samples, while its use for plant samples is often problematic due to microalgae cells being rich in pigments that give strong fluorescence signals, called autofluorescence. Moreover, they are surrounded by a cell wall, which is a complex structure that can scatter the light signal, affecting the accuracy of measurements. Nevertheless, the AUR-HRP method can provide a high degree of sensitivity for H_2O_2 detection in complex biological matrices (Šnyrychová et al., 2009; Ye et al., 2011). Thus, finding the optimal conditions for microalgae samples analysis can bring undoubted benefits for phycologists, plant physiologists, and ecotoxicologists. Therefore, in the present work, the optimal conditions for applicable H_2O_2 determination in algae samples were selected and tested.

3.1. Excitation/emission fluorescence spectra measurements in different buffers

For the AUR-HRP method, different reaction buffers have been proposed in the literature (Prieto et al., 2017; Zhu et al., 2010). Due to the different compositions of the buffers and the potential reactivities of their components, their use can cause the falsification of the obtained fluorescence values, and an overestimation of changes in fluorescence signals and variability, and thus, this can lead to a misinterpretation of the obtained results (Tomasek et al., 2018). Moreover, since resorufin formation from Amplex Red is a pH-dependent reaction (Chakraborty et al., 2016), the maintenance of an appropriate pH in the reaction mixture is necessary in order to obtain reliable results. Therefore, three various reaction buffers of pH 7.0 (TRIS buffer, phosphate buffer, and HEPES buffer) within the reaction mixture were tested (Fig. 1 and Fig. 2).

We detected that the fluorescence signal obtained for each buffer used for the reaction mixture preparation was unequal, with the lowest values being for HEPES and the highest one for TRIS (Fig. 1 a). The experiment was repeated using $15 \mu\text{mol} \cdot \text{mL}^{-1}$ H_2O_2 to spike (to fortify) the samples (Fig. 1 b). In this case, the lowest fluorescence intensity was also recorded for HEPES, while the TRIS and phosphate buffers demonstrated similar results, with slightly higher values for phosphate buffer; thus, HEPES was excluded from further analyses. The excitation and emission waves recommended by the manufacturer for Amplex Red and Amplex UltraRed are 571/585 and 568/581 nm, respectively. In studies using mouse mitochondria, excitation wavelengths of 544 nm and emission wavelengths of 590 nm were used for Amplex Red (Miwa et al., 2016). For different studies using *C. reinhardtii*, the excitation and emission wavelengths used for AUR were 544/585 nm, 518/583nm, 490/585 nm, respectively (Çakmak et al., 2015, Roach et al., 2015, Aksmann et al., 2016). Peroxidase activity was also tested using Amplex UltraRed in *C. reinhardtii*, in this case the following wavelengths were used for

347 resorufin, excitation: 535 to 555 nm and emission: 580 to 620 nm (Lau et al., 2023). Thus, the
348 scanning of the fluorescence signal was used to optimize the excitation and emission
349 wavelengths for the experimental method (Fig. 2). Three different excitation wavelengths (500
350 nm, 530 nm, and 560 nm) were scanned. The results showed that the highest fluorescence
351 intensity was observed at 560 nm, with or without H₂O₂ spike to the samples (Fig. 2); therefore,
352 this wavelength was selected as an excitation wavelength for further analyses. Considering that
353 some buffers and commonly used extraction additives (activated charcoal (AC), saturated
354 ammonium sulfate, 1 M perchloric acid, polyvinylpolypyrrolidone (PVPP), and trichloroacetic
355 acid (TCA), except for phosphate buffer, interfered either directly with H₂O₂ or indirectly with
356 the Amplex Red (Chakraborty et al., 2016), a pure phosphate buffer was selected as an
357 appropriate reaction buffer for further H₂O₂ detection. To the best of our knowledge, the
358 excitation and emission wavelengths for Amplex UltraRed have not been optimized using
359 samples from the tested organism. Following the wavelength selection, the spectra of the RMix
360 fluorescence of the excitation and emission wavelength were scanned at 560 nm and 585 nm,
361 respectively, for the algae suspension, the supernatant after their centrifugation and the selected
362 buffer (Fig. 3). The results obtained validate appropriateness of these chosen wavelengths.
363 Fluorescence values are slightly lower for the supernatant than for the microalgae suspension,
364 which may suggest that the components of their cell walls may influence the measurement
365 results.

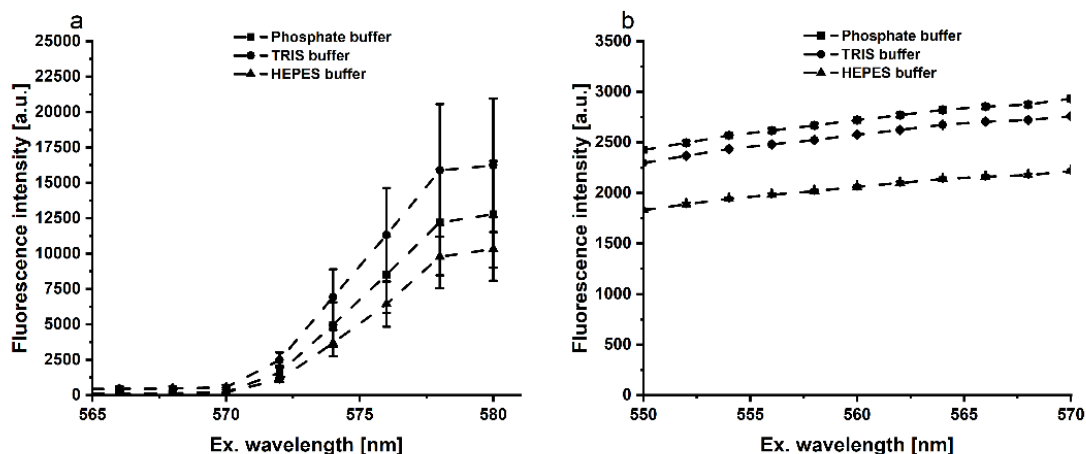


Figure 1. Spectra of the excitation wavelength scan between 550 and 580 nm of the reaction mixture (RMix) fluorescence, depending on the buffer used in the reaction mixture (a) and depending on the buffer used in the reaction mixture, with the addition of $15 \mu\text{mol} \cdot \text{mL}^{-1} \text{H}_2\text{O}_2$ (b) at an emission wavelength of 581 nm. RMix consisted of 10 mM Amplex UltraRed, 10 U $\cdot \text{mL}^{-1}$ HRP, and 0.1 M buffer, i.e. potassium phosphate buffer, HEPES or Tris-HCl with pH 7.0. Before measurement, the samples were incubated for 25 min in the dark at 25 °C.

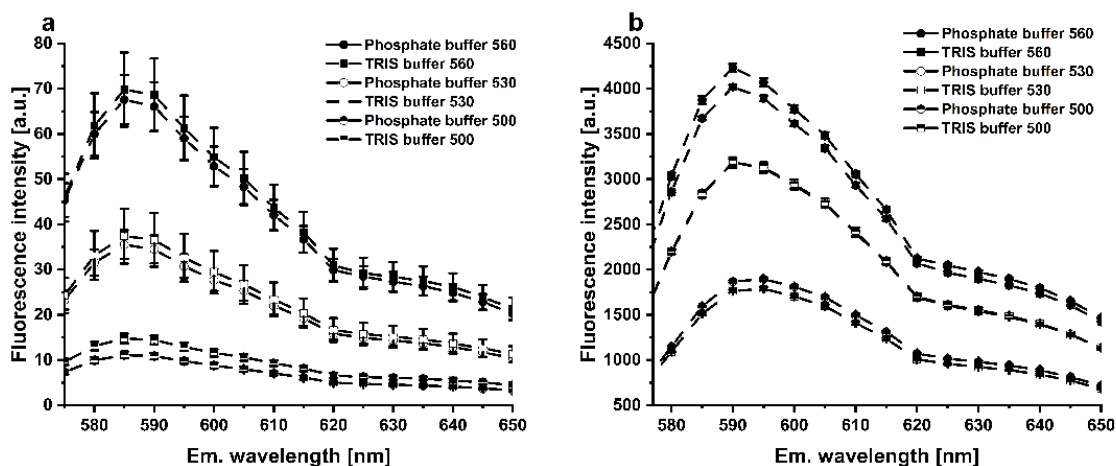


Figure 2. Spectra of the emission wavelength scan between 570 and 650 nm of the reaction mixture (RMix) fluorescence, depending on the buffer used in the reaction mixture (a) and depending on the buffer used in the reaction mixture with the addition of $15 \mu\text{mol} \cdot \text{mL}^{-1} \text{H}_2\text{O}_2$ (b) at excitation wavelengths of 500 nm, 530 nm, and 560 nm. Results are presented as an average of at least 3 repetitions $\pm$ SE. RMix consisted of 10 mM Amplex UltraRed, 10 U $\cdot \text{mL}^{-1}$

¹ HRP, and 0.1 M buffer, i.e. potassium phosphate buffer, or Tris-HCl with pH 7.0. Before measurement, the samples were incubated for 25 min in the dark at 25 °C.

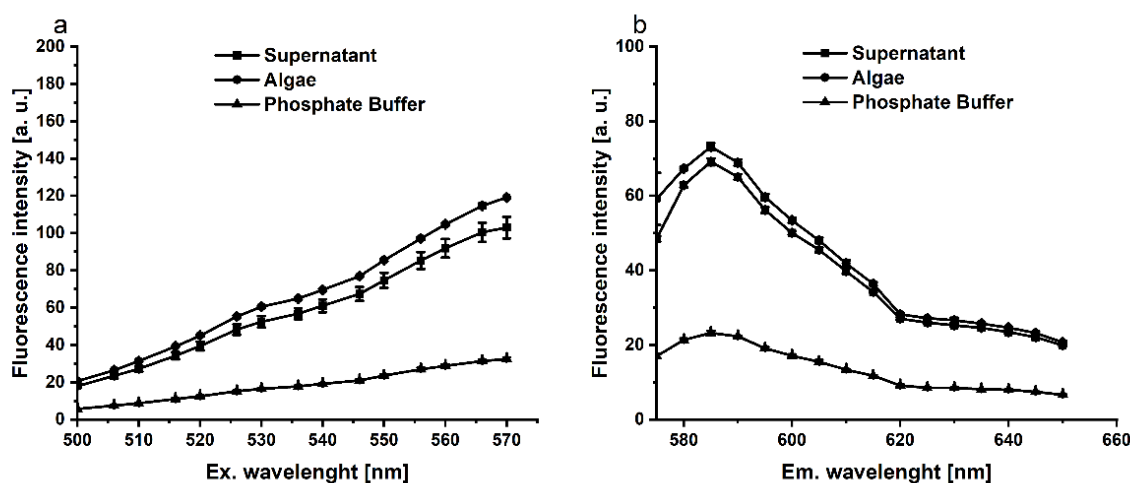


Figure 3. Spectra of the reaction mixture (RMix) fluorescence of (a) the excitation wavelength scan between 500-570 nm at emission wavelength of 585 nm, and (b) the emission wavelengths of 580-650 nm at an excitation wavelength of 560 nm for the algal suspension, the supernatant after centrifugation of the algal biomass and the buffer. RMix consisted of 10 mM Amplex UltraRed, 10 U · mL⁻¹ HRP, and 0.1 M potassium phosphate buffer with pH 7.0. Before measurement, the samples were incubated for 25 min in the dark at 25 °C.

3.2. Influence of algal suspension density and reaction mixture to sample ratio on the fluorescence signal

The next step in the optimization of the method was to measure the possible influence of population density (number of cells in the sample used for analysis) on the fluorescence signal, and, thus, on the reliability of H₂O₂ detection. We found that the fluorescence signal increased along with the increase in the cells number in the sample (Fig. 4), but an increase in the fluorescence signal was not directly proportional to the changes in biomass density. Different trends were seen when the hydrogen peroxide concentration per 1 million cells was expressed. The highest amount of H₂O₂ seemed to be produced by a single cell when the population density

in the sample was 0.5 million cells · mL⁻¹ (Fig. 4), while no significant differences were observed among the three highest algal population densities.

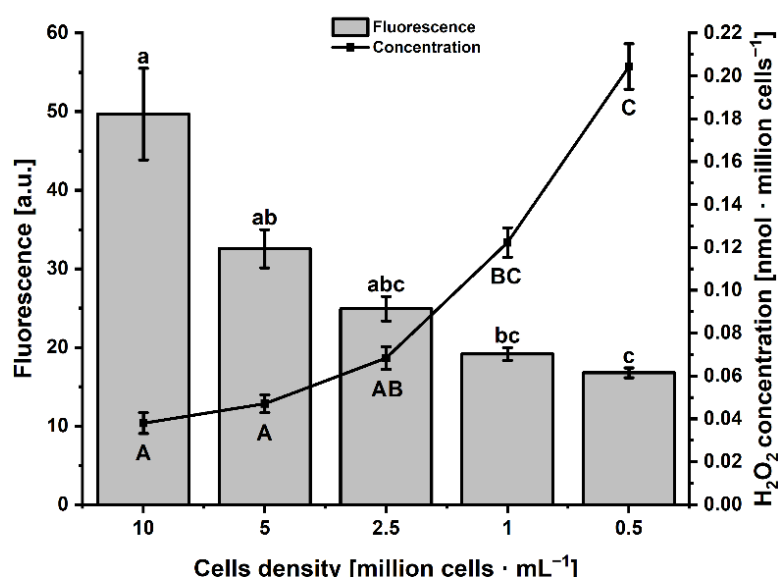


Figure 4. Amplex UltraRed fluorescence intensity for the different population densities of *C. reinhardtii* at $\lambda_{\text{ex}} = 560$ nm and $\lambda_{\text{em}} = 585$ nm, and the amount of H₂O₂ produced by the cells, expressed in nmoles per 1 million cells. H₂O₂ concentration was calculated on the basis of a standard curve (Ia). Results are presented as the average of at least 3 replicates $\pm$ SE. Different letters indicate significant differences ($p \leq 0.05$) between groups (Kruskal Wallis, Dunn's test).

The results thus suggest that in the presence of a high amount of biomass, the fluorescence signal is misestimated, and the detectability of the H₂O₂ that is released into the culture medium is significantly influenced when the number of cells in the sample exceeds approximately 2.5 million cells · mL⁻¹, since in the same experimental conditions, more cells should generate more H₂O₂. Also, diluting the algae before measurement may cause stress and the production of hydrogen peroxide. Moreover, the amount of H₂O₂ produced by a single cell should be equal for all density variants, which was not observed in our work (Fig. 4). This result may be explained by non-specific interactions between the biological matrix, particularly by the cell walls of the microalgae and the reaction mixture, as well as by possible light scattering by cells.

416 We observed that in very dense samples, the amount of the H_2O_2 and interfering substances
417 produced by cells seems to be high enough to saturate the reaction. This is important because
418 Amplex UltraRed, in the presence of HRP, reacts with H_2O_2 at a stoichiometric ratio of 1:1
419 (Quinlan et al., 2013); therefore, an appropriate amount of reagents in relation to H_2O_2 in the
420 sample mixture and any interfering substances must be estimated. To verify the above points,
421 in the next step of the experiments, the fluorescence signal and the H_2O_2 concentration were
422 compared at various ratios of the reaction mixture to the algae samples, where different volumes
423 of the cell suspension at a density of $10 \text{ million cells} \cdot \text{mL}^{-1}$ were mixed with the appropriate
424 volume of the reaction mixture (Fig. 5). We then discovered that the fluorescence intensity in
425 the tested samples significantly decreased with an increase in the ratio of the reaction mixture
426 (RMix) to the cell suspension (CS) volume (Fig. 5). The reverse trend was observed for the
427 H_2O_2 concentration expressed in $\text{nmol} \cdot \text{million cells}^{-1}$; this value increased as the volume of
428 the reaction mixture increased. The differences were not statistically significant among
429 experimental variants with CS:RMix ratios of 50:50, 40:60, 30:70, and 20:80. This suggests
430 that a ratio of 50:50 is reliable since the reaction is not saturated, even for relatively dense cell
431 suspensions, and that a further increase in the RMix volume is economically unprofitable.
432 Furthermore, too high an RMix-to-CS ratio might cause an overestimation of the H_2O_2 content,
433 and this has been noticed at a ratio of CS:RMix = 10:90 (Fig. 5).

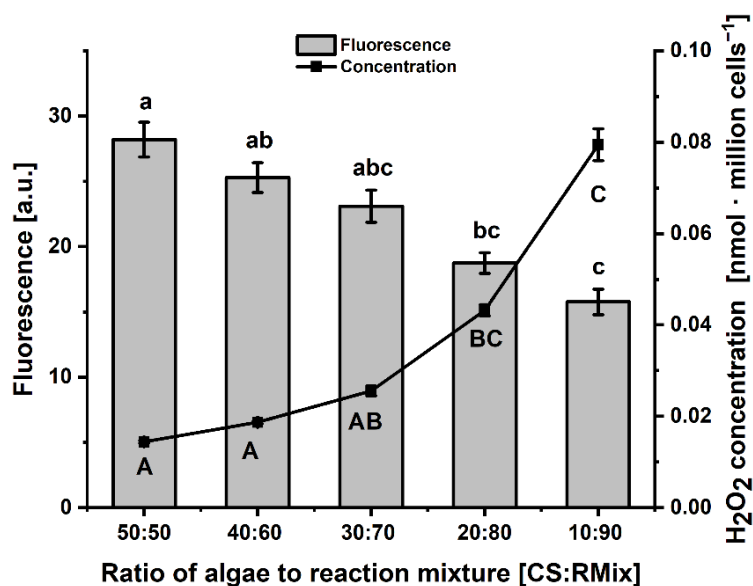


Figure 5. Fluorescence intensity in the sample, and the amount of H₂O₂ produced by the million of cells, detected in *C. reinhardtii* cell suspension, depending on the ratio of the cell suspension (CS) to the reaction mixture (RMix). H₂O₂ concentration was calculated based on standard curves Ib – If (Tab. S1). Results are presented as the average of at least 3 replicates $\pm$ SE. RMix consisted of 10 mM Amplex UltraRed, 10 U $\cdot$ mL⁻¹ HRP, and 0.1 M potassium phosphate buffer with pH 7.0. Before measurement, the samples were incubated for 25 min in the dark at 25 °C. Different letters indicate significant differences ($p \leq 0.05$) between groups (Kruskal Wallis, Dunn's test).

3.3. Quenching of the fluorescence signal by the dead algae biomass

The experiments described above indicated that biological matrix in this case, algae cells, can significantly influence the fluorescence signal. To eliminate the possibility that the observed effect was caused by molecules derived from living algae that fluoresced or were able to react with the fluorochrome, the impact of the biological matrix on the fluorescence signal was measured using dead algal biomass. Similarly, as for the live algae suspension (Fig. 4), the highest fluorescence signal was observed for a cell suspension density of 10 million cells $\cdot$ mL⁻¹

451 ¹ (Fig. 6 a), and the fluorescence signal decreased as the density of the microalgal cell
452 suspension declined. In an opposite manner, the H₂O₂ concentration recalculated per 1 million
453 cells tended to increase along with a decrease in cell numbers, and the highest value was noticed
454 for samples containing 0.5 million cells · mL⁻¹ (approximately 0.5 nmol H₂O₂ · million cells⁻¹
455 ¹), while the lowest concentration of approximately 0.15 nmol H₂O₂ · million cells⁻¹ was
456 calculated for samples of a density of 10 million cells · mL⁻¹ (Fig. 6 a). Since many substances
457 in the dead biomass such as proteins, dyes, and sugars lose their properties and cannot influence
458 the fluorescence signal, the results support our previous observation that the biomass per se
459 affects the measurement results. To further assess this problem, the samples were spiked with
460 H₂O₂ (5 nmol · mL⁻¹), and no significant differences between the fluorescence signal values in
461 samples with different densities of dead microalgae spiked with an exogenous H₂O₂ solution
462 were observed (Fig. 6 a, b). Significant differences were observed in the calculated H₂O₂
463 concentration per 1 million cells between samples with different biomass densities. The
464 concentration of H₂O₂ in samples with 0.5×10^6 cells · mL⁻¹ was about 13 nmol · million cells⁻¹
465 ¹, while in the samples with a density of 10×10^6 cells · mL⁻¹, it was less than 1 nmol · million
466 cells⁻¹ (Fig. 6 b).

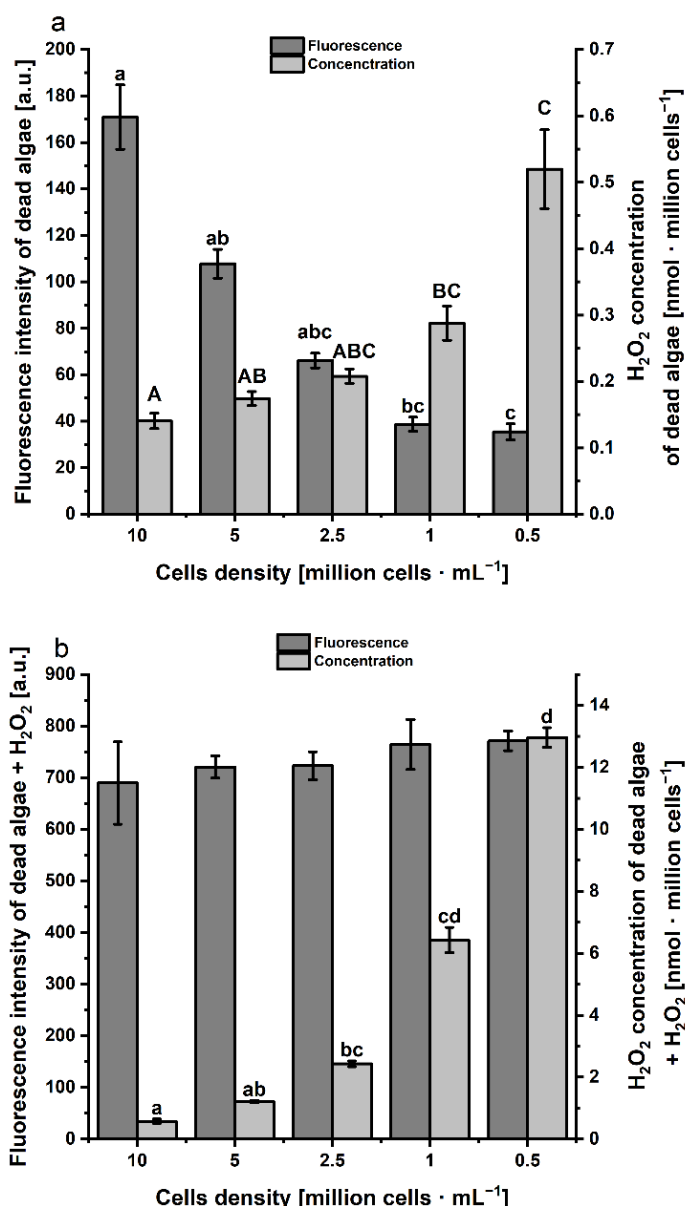


Figure 6. The effect of different densities of dead biomass (a) and biomass spiked with 5 nmol · mL⁻¹ H₂O₂ (b) on the fluorescence signal of Amplex UltraRed with RMix, and on the calculated concentration of H₂O₂, expressed as nmoles per 1 million cells. H₂O₂ concentration was calculated based on a standard curve (1a). The results are presented as the average of at least 3 replicates ± SE. RMix consisted of 10 mM Amplex UltraRed, 10 U · mL⁻¹ HRP, and 0.1 M potassium phosphate buffer with pH 7.0. Before measurement, the samples were incubated

for 25 min in the dark at 25 °C. Different letters indicate significant differences ($p \leq 0.05$) between groups (Kruskal Wallis, Dunn's test).

The results indicated that in the samples with a high H_2O_2 concentration (Fig. 6 b), the biomass density had no effect on the fluorescence signal, while in the samples with low H_2O_2 concentrations, as is usually observed for biological samples, this effect was significant (Fig. 6 a). The interference of the biological matrix is one of the major problems for the determination of endogenous substances produced in many types of cells, and it is particularly inconvenient in plant cell research due to the heterogeneous structure of the cell wall with the presence of different types of extracellular matrix (Domozych et al., 2012). To eliminate the matrix effect, we propose that the microalgae biomass could be removed via centrifugation, and/or the fortification of the tested samples with the established amount of H_2O_2 (see above) can be used to increase the stability and repeatability of the results.

3.4. Influence of cell removal from the samples on the measurements results

To establish the best method of cell removal from the tested sample, two variants were assessed. In the first one, the cell suspension was incubated with the RMix, the cells were removed by centrifugation (centrifugation with the RMix), and the fluorescence signal was immediately determined as described above. In the second one, cells were removed from the samples via centrifugation, the obtained supernatant was mixed with RMix (centrifugation without the RMix), and the fluorescence signal was measured after required incubation time. As a control, uncentrifuged samples were used. A decrease in the fluorescence signal in the second variant compared to the control and the first variant was observed (Fig. 7). This may indicate that part of the used fluorochrome non-specifically reacts with cell-derived substances, causing an increase in the fluorescence signal. Therefore, the higher density of algae could

result in greater deviations in fluorescence readings. On the other hand, diluting the algae to the desired densities before the measurement is not recommended because it might generate additional stress in the cells and cause the production of H₂O₂. Moreover, Amplex UltraRed reacts only with the H₂O₂ molecules released into the medium (X. Zhang and Soldati, 2013); thus, diluting the algae cell suspension before the measurement would cause the dilution of the released H₂O₂ and an underestimation of the measurements. Thus, separating the incubation medium from the cellular components before adding the reaction mix would increase the accuracy of the assay and reduce interactions between the reagents used and the interfering substances (Grisham, 2013). A similar approach, based on the separation of *C. reinhardtii* cells from the culture medium, is used for the determination of extracellular carbonic anhydrase (Coleman et al., 1984).

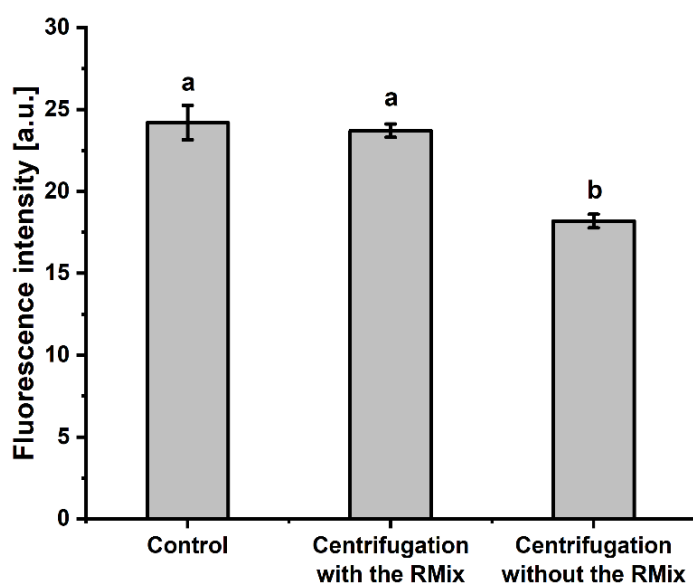


Figure 7. Fluorescence intensity measured in the uncentrifuged control sample, in the sample centrifuged after incubation with RMix, and in the sample centrifuged before incubation with RMix. Results are presented as the average of at least 3 replicates $\pm$ SE. RMix consisted of 10 mM Amplex UltraRed, 10 U $\cdot$ mL⁻¹ HRP, and 0.1 M potassium phosphate buffer with pH 7.0. Before measurement, the samples were incubated for 25 min in the dark at 25 °C.

516

517 Despite the cell separation from the culture medium significantly improving the accuracy
518 of the H₂O₂ concentration measurements, it can be insufficient due to the fact that it is
519 impossible to predict the specific properties of a sample, such as pH, ionic strength,
520 temperature, viscosity, or the presence of impurities or interfering substances (Bader, 1980).
521 Bacterial and eukaryotic cells grown in liquid media release various molecules outside the cells
522 (Liu et al., 2016; Maffei et al., 2017). Regarding microalgae cells, these are mainly various
523 types of sugars (Babich et al., 2022). Because of that, the tested samples in particular, those
524 derived from the cultures of living cells, significantly differ from the standards based on which
525 a simple calibration curve is usually created to calculate the content of H₂O₂, as has been
526 assessed in the current work (Fig. 4, 5, and 6). The use of the established concentration of
527 analyte to spike the samples (sample fortification) helps to reduce the differences between the
528 tested sample and the standards, and, thus, to reduce the measurement errors (Mirmont et al.,
529 2022; van de Merbel, 2008). Matrix-matched standard curves are commonly used in the
530 detection of molecules via chromatographic methods to compensate for matrix effects (Yang et
531 al., 2011). The single-point standard addition method has been applied in the quantitative
532 analysis of pesticide residues in food samples via gas chromatography and tandem mass
533 spectrometry (GC-MS/MS) (Garrido Frenich et al., 2009). Samples fortification, commonly
534 used in chromatography and mass spectrometry, has also been used in colorimetric and
535 fluorometric measurements for some time. In the studies of Bertozzini et al. (2011) the
536 combination of the standard addition method and Nile Red fluorochrome staining may be a
537 good approach for the quantification of neutral lipids in diatoms and dinoflagellates. This
538 analysis method has had reliable results for the determination of the content of neutral lipids at
539 very low concentrations. Therefore, we decided to prepare a matrix-matched standard curve
540 (see Appendix S2, Fig. 2, Tab. S2). However, the matrix-matched standard curves, prepared on

the basis of the supernatant from the centrifuged culture of algae with different densities, indicated that the measurements depend on the initial density of the algae (Appendix 1, Fig. S2). It means that for a proper H_2O_2 calculation in the analyzed sample, a matrix-matched standard curve should be prepared separately for each experimental variant, making the analysis complicated, time-consuming, and laborious. Thus, we decided to adapt for our purposes the calculation method used in the manuals for the determination of ATP concentrations in biological samples (Sigma Aldrich, n.d). These calculations considered the concentration of hydrogen peroxide used for spiked samples as well as the fluorescence of spiked and non-spiked samples (for details of calculations see Appendix 2).

3.5. The usefulness of the method for phytotoxicity testing

To confirm the accuracy and usefulness of the optimized method for the determination of H_2O_2 , microalgae cells were exposed to nabumetone. This compound belongs to a group of non-steroidal anti-inflammatory drugs (NSAIDs), which have been a serious threat to living organisms in the aquatic environment. NSAIDs, including nabumetone, are known to induce ROS production in cells of microalgae and higher plants (Pawłowska et al., 2021, Harshkova et al., 2021, Kapuścińska et al., 2024). The toxicity of NSAIDs to microalgae is related to growth inhibition, photosynthesis, respiration disorders, and the disturbance of the redox balance (Hejna et al., 2022). The use of different concentrations of nabumetone, corresponding to the $\text{EC}_{10/24}$, $\text{EC}_{25/24}$, and $\text{EC}_{50/24}$ values, which were determined based on population growth inhibition (Kapuścińska et al., 2024), allowed us to confirm the high precision and repeatability of the method. The amount of H_2O_2 produced by one cell was significantly increased after the treatment of microalgae with increasing nabumetone concentrations. In the cultures treated with nabumetone in the concentrations corresponding to $\text{EC}_{25/24}$ and $\text{EC}_{50/24}$, 0.06 and 0.1 nmol H_2O_2 per 1 million cells, were measured, respectively (Fig. 8), and the standard error of the

measurements did not exceed 5%. To compare the effectiveness of the proposed method, the results obtained for *C. reinhardtii* cell suspensions using HRP and without its addition are presented (Tab. S3, Fig. S3). The method based on fluorescence measurement without HRP addition exhibits a high background signal, surpassing the fluorescence values observed for the tested samples. The exogenous addition of peroxidase enhances the fluorescence signal in the tested samples by approximately 3-4 times compared to the blank. However, after converting the fluorescence value into the number of microalgae cells, the measurement sensitivity is low, and no discernible differences are observed between the control and algae treated with nabumetone at a concentration corresponding to EC_{10/24}.

To compare our results to those of other research teams, we analyzed data available in the literature. Unfortunately, such a comparison turned out to be impossible. First, the H₂O₂ concentration in literature is recalculated rather per chlorophyll concentration, fresh weight, protein concentration, or optical density, than per the number of cells. Secondly, in many publications the H₂O₂ concentration is not given as absolute concentrations but as relative units, e.g., arbitrary fluorescence units. This approach can be misleading and unprecise because it fails to account for variability in cell numbers. Moreover, chlorophyll content or OD may change in a non-linear way depending on the stressor used. Presenting data on a per-cell basis is, in our opinion, more precise and allows for direct comparison of results across different studies and experimental conditions. This allows for a more accurate understanding of the biological significance of the observed changes in H₂O₂ concentration.

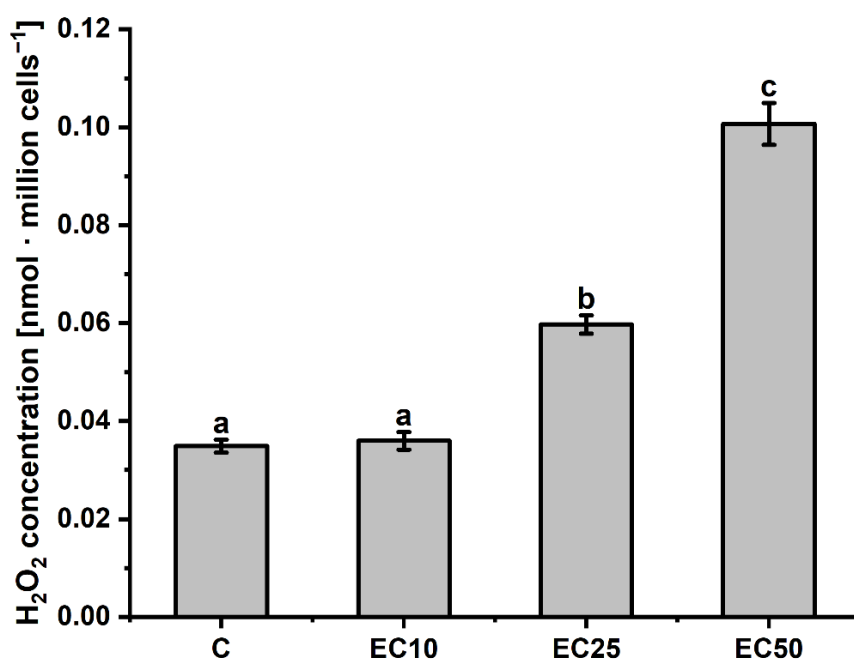


Figure 8. Effect of nabumetone used in the concentrations corresponding to EC_{10/24}, EC_{25/24}, and EC_{50/24}, on the production of H₂O₂ by *C. reinhardtii* cells. Measurements and calculations were performed according to the protocol described in Appendix 2, in short, RMix was added to the centrifuged supernatant and spiked samples were prepared. Then, the samples were incubated for 25 min in the dark at 25 °C. The results are presented as the average of at least 3 replicates $\pm$ SE. Different letters indicate significant differences ($p \leq 0.05$) between groups (ANOVA, Tukey's HSD test). RMix consisted of 10 mM Amplex UltraRed, 10 U · mL⁻¹ HRP, and 0.1 M potassium phosphate buffer with pH 7.0.

Since current trends in plant stress biology and toxicology demand for the sensitive and selective detection of H₂O₂ *in vivo*, this modified and optimized AUR-HRP method for the fluorometric detection of H₂O₂ released by stressed microalgae cells can be successfully applied in phycological and ecotoxicological research. The proposed protocol reduces the variability caused by culture conditions, minimizes measurement errors, eliminates the problem of chlorophyll autofluorescence, compensates for the matrix effect, and using AUR-HRP

detection system significantly increases the fluorescence signal as compared to system based on AUR only, suggesting that much lower H₂O₂ concentrations could be detected using AUR-HRP detection system (Tab. S2). In addition, this method is efficient and can be successfully applied to the study of different species of microalgae, as well as to the cells of other organisms grown in liquid cultures. Moreover, statistical analyses have also confirmed the reproducibility, sensitivity, and applicability of this method for ecotoxicological studies.

Conclusion

In the present work, we proposed a reliable and sensitive method of detection of H₂O₂ produced by microalgal cell suspension. We have proven that this method is appropriate for measuring H₂O₂ concentration in the samples with a significant matrix effect, because it helps to minimize the variability caused by culture conditions, to eliminate the problem of chlorophyll autofluorescence, and to minimize measurement errors. Moreover, statistical analyses have confirmed the reproducibility, sensitivity, and applicability of this method for ecotoxicological studies, compared to methods where cells were also used for measurement, the matrix of which could influence the obtained results. Based on the results, we developed a complete protocol for the detection of H₂O₂ produced by microalgal cells, which can be applied to the study of many species of microalgae, as well as to the cells of other organisms grown in liquid cultures.

Abbreviations

ROS: Reactive oxygen species, H₂O₂: hydrogen peroxide, HRP: Horseradish peroxidase, RMix: reaction mix, AUR: Amplex UltraRed, CS: cell suspension, NSAIDs: non-steroidal anti-inflammatory drugs.

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions:

Conceptualization: MH., AA.; data curation: DK., MH.; formal analysis: MH., DK.; funding acquisition: AA.; investigation: MH., DK.; methodology: MH., DK.; project administration: AA.; resources: MH., DK.; software: DK.; supervision: AA.; validation: MH., DK.; visualization: DK.; roles/writing - original draft: MH., DK.; writing - review&editing: MH., DK., AA. All authors read and approved the final manuscript.

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918

Appendix S1 for:

A sensitive and reliable method for the quantitative determination of hydrogen peroxide produced by microalgae cells

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Standard curve calculations and microalgae analysis.

The supporting data contains:

- **Figure S1.** Standard calibration curve for quantitation of hydrogen peroxide.
- **Table S1.** Linear curves ($y = a + b \times x$) fit with Origin software used to calculate hydrogen peroxide concentration in an experiment with different ratios of cell suspension (CS) to reaction mixture (RMix) along with limit of detection (LOD) and limit of quantification (LOQ).
- **Figure S2.** The standard curve on *C. reinhardtii* cell matrix.
- **Table S2.** Linear curves on *C. reinhardtii* cells matrix ($y = a + b \times x$) fit with Origin software for each algae density along with limit of detection (LOD) and limit of quantification (LOQ).
- **Text S1.** Sensitivity of the non-optimized hydrogen peroxide determination method using Amplex UltraRed in ecotoxicological studies.
- **Table S3.** Fluorescence intensity of Amplex UltraRed and H₂O₂ concentration produced by *C. reinhardtii* cells treated with nabumetone used in the concentrations corresponding to EC_{10/24} (NBT10), EC_{25/24} (NBT25), and EC_{50/24} (NBT50); C – control.
- **Text S2.** Analysis of microalgae using a flow cytometer.
- **Figure S2.** Cytometric analysis of microalgae cells incubated with RMix containing HRP or without HRP.

1 Supplementary Figures and Tables

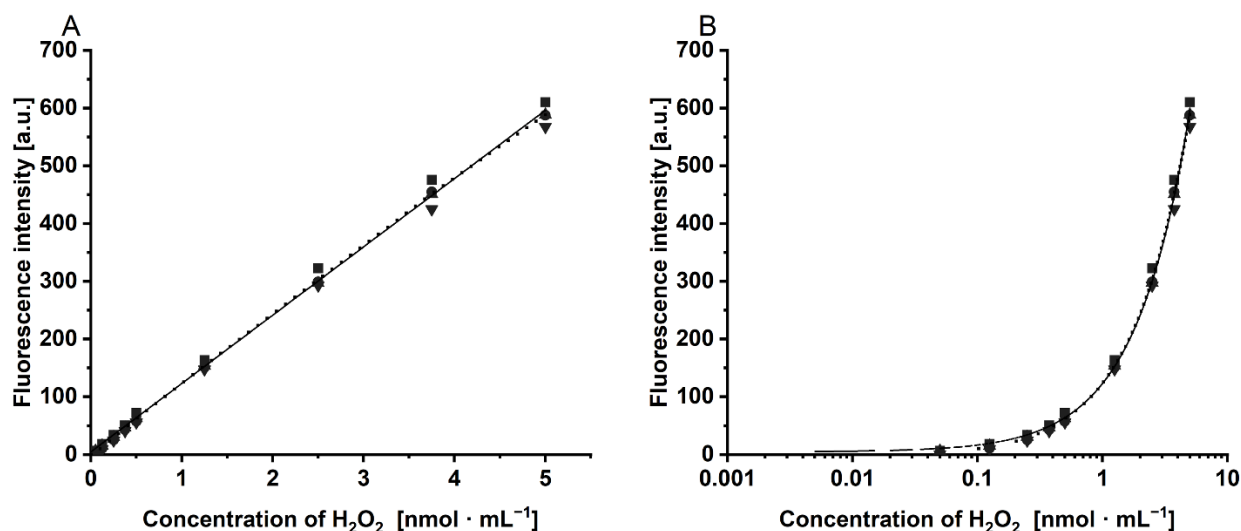


Figure S1. Standard calibration curve for quantitation of hydrogen peroxide (I, 1a) on a linear (A) and logarithmic scale (B). The limit of detection (LOD) was 0.025 nmol · mL⁻¹, and the limit of quantification (LOQ) was 0.076 nmol · mL⁻¹. RMix consisted of 10 mM Amplex UltraRed, 10 U · mL⁻¹ HRP, and 0.1 M potassium phosphate buffer with pH 7.0. Before measurement, the samples were incubated for 25 min in the dark at 25°C.

The volume ratio of H ₂ O ₂ to reaction mixture [H ₂ O ₂ :RMix]	a	SE	b	SE	LOD	LOQ
50:50 (1b)	-0.17108	4.78988	171.3154	4.70713	0.092266	0.279594
40:60 (1c)	-3.86162	0.96129	113.5779	0.78409	0.02793	0.084637
30:70 (1d)	-2.45545	1.53862	72.95061	1.08111	0.069601	0.210913
20:80 (1e)	-6.22842	2.06637	42.29037	1.18738	0.161243	0.488615
10:90 (1f)	-3.72457	1.20596	17.89386	0.61535	0.222404	0.673952

Table S1. Linear curves ($y = a + b \times x$) fit with Origin software used to calculate hydrogen peroxide concentration in an experiment with different ratios of cell suspension (CS) to reaction mixture (RMix) along with limit of detection (LOD) and limit of quantification (LOQ).

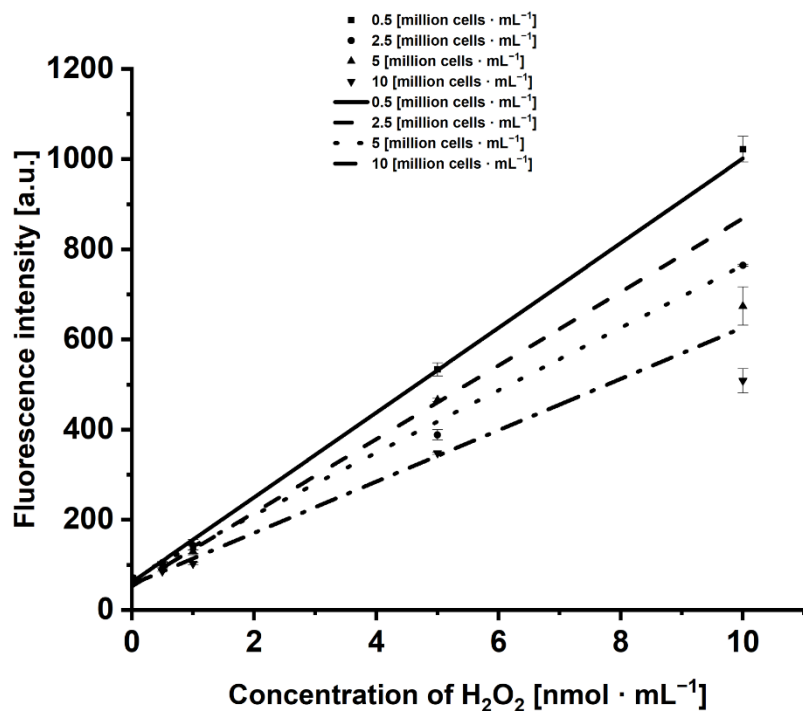


Figure S2. The standard curve on *C. reinhardtii* cell matrix with starting densities of 0.5, 2.5, 5 and 10 million cells · mL⁻¹ (II). Results presented as the average ± SD.

Cell density [million cells · mL ⁻¹]	a	SE	b	SE	LOD	LOQ
0.5	61.8314	1.02049	93.97548	2.51681	0.035835	0.108591
2.5	72.00213	2.35771	69.22259	0.66744	0.112397	0.340598
5	52.66436	1.72056	81.55934	2.50069	0.069616	0.210958
10	57.06397	1.94932	56.92713	2.91407	0.113	0.342424

Table S2. Linear curves on *C. reinhardtii* cells matrix ($y = a + b \times x$) fitted with Origin software for each algae density along with limit of detection (LOD) and limit of quantification (LOQ).

Text S1. Sensitivity of the non-optimized hydrogen peroxide determination method using Amplex UltraRed in ecotoxicological studies.

C. reinhardtii cells were treated with nabumetone with EC values (EC means the effective concentration that reduces population growth by a specific percentage). Algae were cultured as described in Subchapter 2.1. The population with an initial cellular density of 0.5×10^6 cells $\cdot$ mL⁻¹ was exposed to nabumetone at concentrations corresponding to EC_{10/24}, EC_{25/24}, and EC_{50/24} (Kapuścińska et al., 2024). The control sample consisted of algae without the addition of nabumetone. After 24 hours, 50 μ L of cell suspensions from each variant were taken for measurement fluorescence intensity of Amplex UltraRed (AUR) using two types of reaction mixture. The first one consisted of 0.5 μ L of 10 mM Amplex UltraRed, while the second one additionally contained 1 μ L of 10 U $\cdot$ mL⁻¹ horseradish peroxidase (HRP). Both mixtures were made up to 50 μ L with 0.1M phosphate buffer. The results were expressed in arbitrary units (a. u.) in the tested variants and recalculated per one million of cells. A linear curve for AUR ($y = a + b \times x$) was fitted using Origin software, where $a = 0.5072$ and $b = 16.299$ (OriginLab Corporation, USA). A linear curve for AUR+HRP shown in Table S1 (50:50).

AUR					
Unit	Buffer	C	NBT10	NBT25	NBT50
a.u.	8.60	3.99	4.69	5.10	5.33
nmol $\cdot$ million cells ⁻¹	ME	0.026a	0.033a	0.045a	0.079b
	SD	0.007	0.011	0.009	0.024
	SE	0.003	0.005	0.004	0.011
AUR + HRP					
Unit	Buffer	C	NBT10	NBT25	NBT50
a.u.	11.13	39.70	35.85	41.12	39.67
nmol $\cdot$ million cells ⁻¹	ME	0.028a	0.027a	0.038b	0.062c
	SD	0.003	0.003	0.005	0.004
	SE	0.001	0.001	0.002	0.002

Table S3. Fluorescence intensity of AmplexUltra Red and H₂O₂ concentration produced by *C. reinhardtii* cells treated with nabumetone used in the concentrations corresponding to EC_{10/24} (NBT10), EC_{25/24} (NBT25), and EC_{50/24} (NBT50); C – control. Two types of reaction mixtures were used to carry out the reactions, as described in Text S1: AUR – reaction mixture containing Amplex UltraRed; AUR+HRP - reaction mixture containing Amplex UltraRed and horseradish peroxidase. Different letters indicate significant differences ($p \leq 0.05$) between groups (ANOVA, Tukey's HSD test).

Text S2. Analysis of microalgae using a flow cytometer.

Algae and two types of reaction mixtures were prepared, as described in Text S1. A total of 1000 events of each sample, triplicate measurements, were acquired in a Amnis® FlowSight® flow cytometer, after

daily quality control procedures using The Amnis® FlowSight® Calibration beads. The H₂O₂ was detected by measuring the fluorescence on channel 03 (Em = 560-595 nm) and was used also channel 09 (Brightfield) for general cell's observation, Ex = 488 nm (intensity = 60 mW). All data analyses were performed using the IDEAS® Image Analysis software.

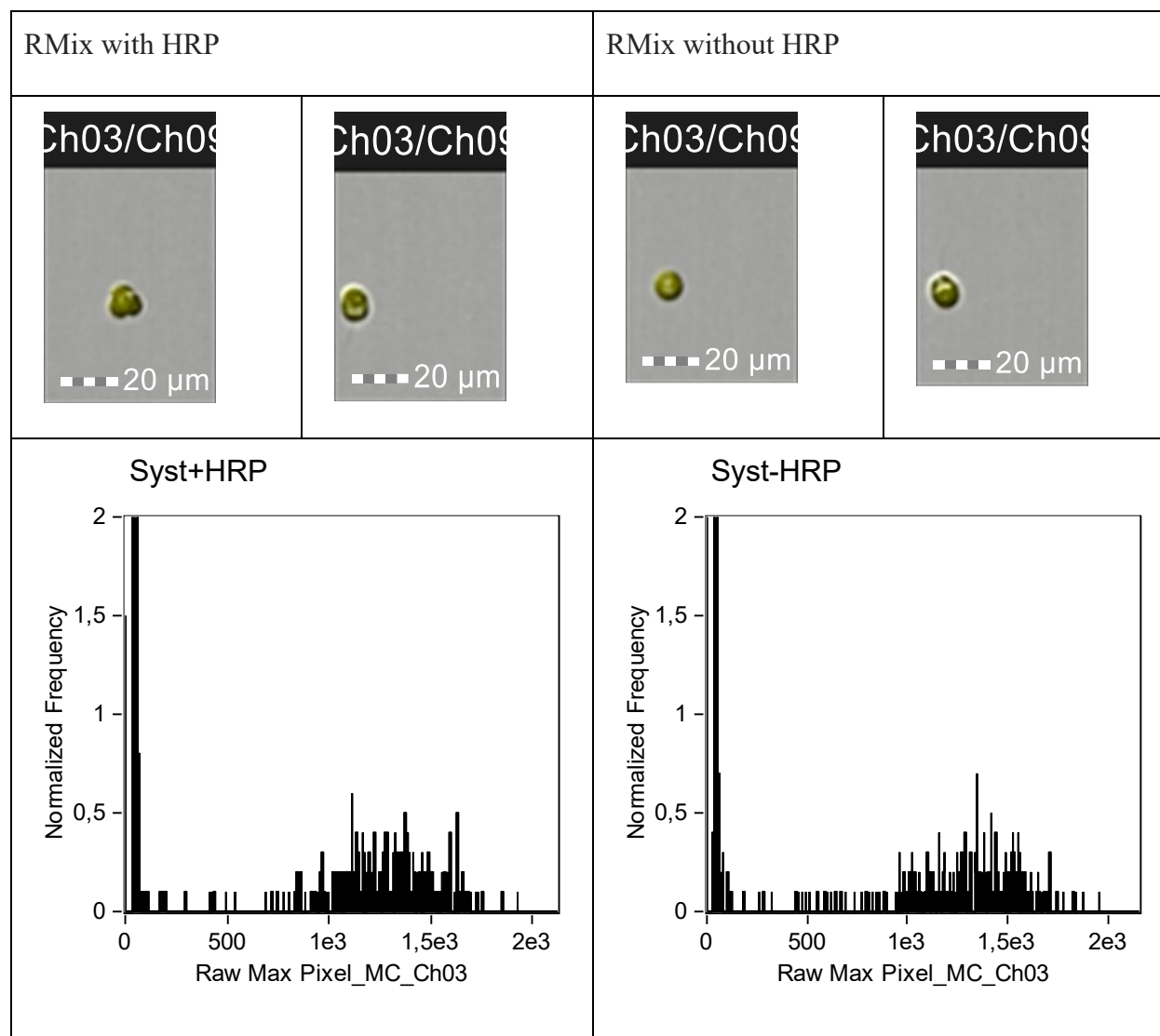


Figure S3. Cytometric analysis of microalgae cells incubated with RMix containing HRP or without HRP.

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Kapuścińska, D., Narajczyk, M., Liakh, I., Wielgomas, B. & Aksmann, A. (2024). Nabumetone and flufenamic acid pose a serious risk to aquatic plants: A study with *Chlamydomonas reinhardtii* as a model organism. *Chemosphere*, 349, 140853.

*Appendix S2 for:***A sensitive and reliable method for the quantitative determination of hydrogen peroxide produced by microalgae cells****Monika Hejna^{1,†,*}, Dominika Kapuścińska^{2,†}, Anna Aksmann^{2,*}**¹ Department of Biotechnology and Nutrigenomics, Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences, Jastrzębiec, Postępu 36A, 05-552 Magdalenka, Poland² Department of Plant Physiology and Biotechnology, Faculty of Biology, University of Gdańsk, Wita Stwosza 59, 80-308 Gdańsk, Poland

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Protocol for determining the concentration of hydrogen peroxide released by microalgae.**1. Supplies and reagents**

- Buffer – Potassium phosphate buffer stock, 0.1 M, pH 7.0;
- Amplex UltraRed Reagent (AUR):
 - Pink coloring in Amplex UltraRed Reagent is an early indicator of compromised material (Molecular Probes, Inc., 2009a),
 - Amplex UltraRed Reagent exposure to strong light should be avoided during all steps; the preparation and handling of AUR stock solutions is recommended under minimum ambient light/darkroom conditions within 625–740 nm of the visible spectrum;
- Horseradish peroxide (HRP), (307 U · mL⁻¹);
- Fresh, high-quality dimethyl sulfoxide (DMSO);
- Stabilized 50% H₂O₂;
- Double-distilled water (ddH₂O);
- 96-well microplates (black for fluorescence measurements);
- Microplate reader.

2. Preparation of reagents***2.1. Buffer solutions:*****0.1 M potassium phosphate buffer stock, pH 7.0 (1 L):**

- Dissolve 4.672 g of K_2HPO_4 in 800 mL of ddH_2O ,
- Add 3.154 g of KH_2PO_4 to the solution,
- Add ddH_2O until the volume is 1 L.

2.2. Amplex UltraRed reagent

Prepare a 10 mM stock solution of Amplex UltraRed Reagent by adding 340 μ L of fresh, high-quality DMSO to one vial (1 mg) of Amplex UltraRed Reagent. Vortex well to dissolve. Protect this solution from light, air, and moisture. Store the remaining solution in the dark with a desiccant at -80°C for future use. When stored properly, this solution should be stable for at least 6 months (Zhu et al., 2010).

2.3. Horseradish peroxidase (HRP) stock solution

Dissolve 1 mg of pure, salt-free, lyophilized horseradish peroxidase (EC 1.11.1.7) powder (307 $\text{U} \cdot \text{mL}^{-1}$) in 1.0 mL of fresh 0.1 M potassium phosphate buffer, pH 7.0. Store at -15°C and use within 6 months from the date of storage. Prepare a **10 $\text{U} \cdot \text{mL}^{-1}$** (1 mg) stock solution of HRP in **potassium phosphate buffer**.

2.4. Hydrogen peroxide (H_2O_2) stock solution

This solution is needed to fortify the samples to determine the amount of hydrogen peroxide released into the culture medium. Each sample is fortified with 1 μ L of the prepared solution. The final concentration of hydrogen peroxide in the well should be within the range of the expected result. In the case of *Chlamydomonas reinhardtii* cultures, it is $3 \text{ nmol} \cdot \text{mL}^{-1}$.

Solutions should be prepared freshly before the measurement!

3. Fluorescence measurement parameters*

- Shaking: Duration: 5 s, Speed: 600 rpm;
- Incubation: time: 25 min, Temperature: 25°C ;
- Fluorometric Scanning: Optics: Top (for black 96-well microplates), $\lambda_{\text{em}} = 585 \text{ nm}$; $\lambda_{\text{ex}} = 560 \text{ nm}$, step size: 2 nm, excitation bandwidth: 5, measurement time: 1000 ms.

* Parameters were used to prepare a program for measurements in a Varioskan™ LUX Multimode Microplate Reader (ThermoFisher Scientific). For other multiplate readers, the parameters should be controlled and optimized if needed.

4. Assay protocol

4.1. Prepare a reaction mixture (RMix) working solution of Amplex UltraRed/HRP. To prepare enough RMix to perform 1 assay for the detection of H_2O_2 , mix 0.5 μL of 10 mM Amplex UltraRed reagent stock solution with 1 μL of $10 \text{ U} \cdot \text{mL}^{-1}$ HRP, and 48.5 μL of 0.1 M potassium phosphate buffer. It is recommended to prepare more of the reaction mixture in a black Eppendorf/Falcon tube, which will then be added to the test samples.

4.2. Prepare a suspension of the analyzed algae. Centrifuge 1 mL of algal cell suspension at 291 RCF for 5–10 min. Endogenous compounds in the sample may interfere with the reaction; thus, use samples spiked with an established amount of H_2O_2 to ensure an accurate determination of H_2O_2 in the test samples (see Point 4).

4.3. For the fluorescence measurements, pipette 50 μL of the supernatant obtained in Point 2 (the analyzed samples) into the well of a black 96-well plate and add 50 μL of reaction mixture (prepare each sample in triplicate).

4.4. For the spiked samples, pipette 50 μL of supernatant obtained in Point 4.2 into the well of a black 96-well plate and add 1 μL hydrogen peroxide solution prepared according to Point 2.4. Add 50 μL of reaction mix into a black 96-well plate. Each sample is prepared in duplicate. Remember to apply the reaction mix as a last step after all the samples have been pipetted.

4.5. Prepare a sample background control if the sample contains substances that can give significant autofluorescence signals (50 μL supernatant + 50 μL reaction buffer) and a treatment background control, if the algae will be treated with any substance that can possibly interfere with the reaction mixture (50 μL reaction buffer containing the tested substance + 50 μL reaction mixture). The fluorescence values obtained for these samples should be further subtracted from the analyzed samples signal (corrected samples).

4.6. Measure the fluorescence after incubation at $\lambda_{\text{ex}} = 560 \text{ nm}$ and $\lambda_{\text{em}} = 585 \text{ nm}$ using a microplate reader.

4.7. Calculate the H_2O_2 concentration in the samples using the following equation:

$$C_{\text{H}_2\text{O}_2} = [\text{ST} \times F_{\text{Scorr}}] / [(F_{\text{SScorr}} - F_{\text{Scorr}}) \times S_v] [\text{nmol} \cdot \mu\text{L}^{-1}],$$

where:

ST – amount of H₂O₂ spiked in well (nmol); F_{S_{corr}} – corrected sample fluorescence reading (unspiked well); F_{SS_{corr}} – corrected spiked sample fluorescence reading (spiked well); S_v – sample volume (uL) added into the well.

4.8. In order to unify the result, the amount of hydrogen peroxide produced can be converted to the density of cells that were subjected to centrifugation or another appropriate conversion factor.

References:

Molecular Probes, Inc., 2009a, Amplex® UltraRed Reagent Retrieved October, 23, 2024, from <https://assets.fishersci.com/TFS-Assets/LSG/manuals/mp36006.pdf>

Zhu, A., Romero, R. & Petty, H. R. (2010). Amplex UltraRed enhances the sensitivity of fluorimetric pyruvate detection. *Analytical Biochemistry*, 403(1–2), 123–125. <https://doi.org/10.1016/J.AB.2010.04.008>

Gdańsk, 12.06.2026

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OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

Hejna M., Kapuścińska D., Aksmann A.

A sensitive and reliable method for the quantitative determination of hydrogen peroxide produced by microalgae cells. Journal of Phycology, 2024, 60(6), 1356–1370.

<https://doi.org/10.1111/JPY.13524>

polegał na:

(i) udziale w przeprowadzeniu eksperymentów związanych z opracowaniem i optymalizacją metody ilościowego oznaczania nadtlenu wodoru produkowanego przez komórki zielenic jednokomórkowych; (ii) udziale w przygotowaniu metodologii badań, ocenie warunków pomiarowych oraz gromadzeniu i opracowaniu danych badawczych; (iii) udziale w walidacji i interpretacji wyników; (iv) statystycznym i graficznym opracowaniu wyników; (v) udziale w przygotowaniu pierwotnej wersji manuskryptu; (vi) udziale w redagowaniu manuskryptu; oraz (vii) udziale w przygotowaniu odpowiedzi na uwagi recenzentów.

okapuścińska Dominika
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PODPIS

Warszawa, 14.06.2026

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Raport z weryfikacji podpisu

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SZCZEGÓŁY WERYFIKACJI:

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Signed by /
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Monika Hejna
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Uwagi	Weryfikowany podpis został uznany za kwalifikowany. Zgodnie z Art. 25. Punkt 2 rozporządzenia Parlamentu Europejskiego i Rady (UE) nr 910/2014 z 23 lipca 2014 r. kwalifikowany podpis elektroniczny ma skutek prawny równoważny podpisowi własnoręcznemu.

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OŚWIADCZENIE

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polegał na:

(i) udziale w przygotowaniu koncepcji badań; (ii) koordynowaniu realizacji badań; (iii) nadzorze nad realizacją badań; (iv) pozyskaniu finansowania; (v) udziale w redagowaniu manuskryptu; oraz (vi) udziale w przygotowaniu odpowiedzi na uwagi recenzentów.



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PODPIS

12. Praca nr 3.

Differential antioxidant pathways displayed by *Chlamydomonas reinhardtii* exposed to selected NSAIDs

Kapuścińska D., Hejna M., Pokora W., Aksmann A.

Scientific Reports,

2026, **16**, 27289

<https://doi.org/10.1038/s41598-026-57688-8>



OPEN

Differential antioxidant pathways displayed by *Chlamydomonas reinhardtii* exposed to selected NSAIDs

Dominika Kapuścińska¹, Monika Hejna^{2✉}, Wojciech Pokora¹ & Anna Aksmann^{1✉}

The chemically diverse group of nonsteroidal anti-inflammatory drugs (NSAIDs) is a significant source of aquatic pollution, inducing oxidative stress in algae. The aim of this study was to evaluate the antioxidant response of *Chlamydomonas reinhardtii* exposed to four NSAIDs differing in toxicity and structure: flufenamic acid (FFA), nabumetone (NBT), ibuprofen (IBU), and naproxen (NPX). This study demonstrated that all pharmaceuticals significantly increased H₂O₂ production, confirming redox imbalance in cells. The antioxidant defense also showed compartment- and compound-specific signatures depending on the NSAID toxicity level. Less toxic IBU and NPX induced coordinated SOD isoforms and catalase activation, while more toxic FFA and NBT triggered chloroplast-targeted H₂O₂ scavenging via APX pathways. Notably, *MSD3* transcript levels increased in all treatments, indicating its potential as an NSAID stress biomarker. IBR analysis demonstrated that antioxidant efficiency decreased with increasing NSAID toxicity. These findings demonstrate that NSAID toxicity shapes compartment- and isoform-specific antioxidant strategies in *C. reinhardtii*. We believe that future studies with a broader range of NSAIDs would enable us to investigate subcellular redox dynamics using compartment-specific ROS reporters and to identify NSAID-sensitive biomarkers for aquatic ecotoxicology monitoring.

Keywords Microalgae, Oxidative stress, Nonsteroidal anti-inflammatory drugs, *Chlamydomonas reinhardtii*, Gene expression, Antioxidative enzymes

Pharmaceuticals are one of the most important components of modern healthcare. The effectiveness of their active compounds in the treatment of various diseases and their widespread availability contribute to their increasing consumption. However, the uncontrolled release of pharmaceutical residues and their metabolic derivatives into the environment through sewage, improper disposal, and the absence of appropriate treatment methods, as well as the insufficient legal regulations governing their remediation, poses a major challenge for worldwide ecosystems¹.

Nonsteroidal anti-inflammatory drugs (NSAIDs), and their metabolites have been detected in the aquatic environment for many years. The range of their environmental concentrations varies depending on country-specific consumption patterns and ranges from ng/L to even several dozen of µg/L in analyzed samples^{2,3}. Because NSAIDs occur in aquatic environments as diverse mixtures of parent compounds and their derivatives, their toxic effects on non-target organisms are often difficult to determine⁴. Most studies focus on animal organisms, and knowledge about the effects of NSAIDs on plant organisms, such as green microalgae, is still insufficient^{3,5}.

The need for comprehensive studies on microalgae is related to their important role in aquatic ecosystems and their ability to remove many pharmaceuticals from polluted environment⁶. Therefore, to assess the potential impact of NSAIDs on microalgae communities in water bodies and to select the suitable organisms for phycoremediation, it is necessary to determine the sensitivity and resistance of green microalgae, such as *Chlamydomonas reinhardtii*, to NSAIDs with diverse chemical structures and properties, such as ibuprofen (IBU), naproxen (NPX), nabumetone (NBT), or flufenamic acid (FFA)².

One of the most extensively studied eukaryotic microalgal platforms for phycoremediation is the unicellular green alga *C. reinhardtii*, and it is also recognized as a valuable biotechnological platform with applications

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in biofuel production, and the synthesis of high-value bioproducts due to its high proliferation rate, ease of cultivation, broad environmental adaptability, and ease of genetic and physiological manipulation^{7,8}. Furthermore, its simple cellular structure is an attractive cell factory for bioenergy production. A recent study demonstrated that bicarbonate enhancement can improve bioenergy generation from *C. reinhardtii*, and acetate assimilation was identified as a promising strategy to support sustainable biofuel production. Therefore, *C. reinhardtii* has established itself as a model organism for biofuel research, including bioethanol, biodiesel, and photobiological hydrogen^{8,9}. *C. reinhardtii* has also emerged as a platform for high-value bioproduct synthesis, such as the production of therapeutic antibodies, vaccines, antimicrobial peptides, and industrial enzymes¹⁰.

In addition to its well-established biotechnological applications, *C. reinhardtii* also serves as a valuable model organism for ecotoxicological studies, particularly in assessing the impact of emerging contaminants. The toxicity of chemicals, including NSAIDs, is typically determined based on their effective concentrations, most commonly EC50 values, which refer to the inhibition of population growth^{11,12}. In our previous study on *C. reinhardtii*, NBT exhibited more than sixfold higher toxicity than FFA, with EC50 values of 5.75 mg/L and 35.47 mg/L, respectively¹³. On the other hand, ibuprofen (IBU) demonstrated relatively low toxicity to *C. reinhardtii*, with an EC50/24 of 250 mg/L¹⁴. Another NSAID, naproxen (NPX), exhibited a clear toxic effect on green algae, with a EC50/24 value of 29.2 mg/L for *Scenedesmus quadricauda*, and 0.7 mg/L for *Cymbella* sp.¹⁵. At the cellular level, these compounds induce a range of physiological disruptions that additionally explain their inhibitory effects on algal growth. Further, many cellular-level disorders were detected in microalgae cells treated with NSAIDs. For instance, NPX caused changes in photosynthetic pigments content in the algal cells¹⁵, IBU depolarized mitochondrial and cytoplasmic membranes¹⁴, NBT and FFA disrupted photosynthesis¹³. However, the toxicity of NSAIDs depends on multiple factors, including the structure of the compound, exposure time, as well as the activity of oxidative enzymes, and the cell wall architecture, which varies among the microalgae species^{13,16}.

Moreover, numerous studies have reported that pharmaceuticals induce oxidative stress in microalgal cells by enhancing ROS production^{12,14,17,18}. ROS are standard byproducts of aerobic metabolism which are formed in almost all cellular compartments, with chloroplasts, mitochondria, and peroxisomes as primary sources. At low concentrations, ROS act as signaling molecules, however when overproduced due to biotic and abiotic stressors, they cause redox imbalance in cells^{19,20}. One of the most stable forms among ROS is hydrogen peroxide, which is highly mobile, and can be synthesized in multiple cellular compartments²¹ leading to disturbances in metabolic pathways²². The majority of H₂O₂ in algal cells is produced by superoxide dismutase (SOD) while decomposing superoxide radical. In *C. reinhardtii*, the iron isoform (Fe-SOD) resides in the chloroplast, whereas the manganese isoform (Mn-SOD) is distributed in the chloroplast and mitochondria²³. Removal of excess hydrogen peroxide generated by SOD is carried out by antioxidant enzymes such as catalase (CAT) and ascorbate peroxidase (APX)²⁴. The antioxidant system not only protects cells against stress, but is also recognized as a key element responsible for cellular adaptation and increased resistance of plant organisms to environmental pollutants. Investigating the antioxidant response enables a comparison of microalgal tolerance to the tested pharmaceuticals, which may have potential applications in the biodegradation of these compounds. Moreover, considering the activity of individual enzymes and their cellular location, it is possible to indicate cellular compartments that are particularly sensitive to the applied stress factor.

In light of this, despite the growing awareness of the environmental risks associated with NSAIDs, significant gaps in current knowledge still persist. In many cases, findings obtained for individual compounds are generalized to the entire class of NSAIDs, despite the substantial structural and physicochemical diversity within this group. Therefore, comparative analyses of selected NSAIDs differing in chemical structure are essential for a more comprehensive understanding of their ecotoxicological effects. Moreover, although numerous studies report the induction of oxidative stress by NSAIDs, relatively few have addressed the intracellular sources of reactive oxygen species (ROS) or their detoxification at the molecular level. Importantly, comparative studies at this level, particularly those examining NSAIDs with distinct chemical properties, remain scarce.

Therefore, the present study aimed to compare the antioxidant response of *C. reinhardtii* to selected NSAIDs, such as flufenamic acid (FFA), nabumetone (NBT), ibuprofen (IBU) and naproxen (NPX), differing in their structure and properties. The research hypothesis assumed that each of the selected NSAIDs would cause oxidative stress in *C. reinhardtii* cells, but the mechanisms involved in minimizing its negative effects would differ. The transcriptional responses were studied in genes related to antioxidant defense, enzymatic biomarkers such as SOD, CAT, and APX were analyzed, and hydrogen peroxide produced by microalgae was determined.

Results

Population density and hydrogen peroxide production by the cells

The pharmaceuticals used in the study inhibited the growth of *C. reinhardtii* population, compared to the control, where the cell number was about 12.4×10^6 cells/mL. Population density for FFA-, NBT-, IBU-, and NPX-treatment reached 44, 49, 57, and 58% of the control, respectively, and was statistically significantly lower than the control group, with no differences observed among the treated cultures (Fig. 1a). Consistently, the growth rate was significantly reduced in all treated groups from 3.13 ± 0.12 day⁻¹ in the control to 2.38–2.66 day⁻¹ following exposure to the tested NSAID (Tab. S2). In the control cultures, the concentration of H₂O₂ released by the cells was 0.034 nmol/million cells (Figs. 1 and 5). All NSAIDs caused a significant increase in H₂O₂ production by microalgae, except for FFA, where this increase did not reach statistical significance compared to the control (Figs. 1b and 5). The amount of H₂O₂ released by *C. reinhardtii* treated with NPX, IBU, and NBT reached 180, 252, and 290% of the control, respectively (Fig. 5).

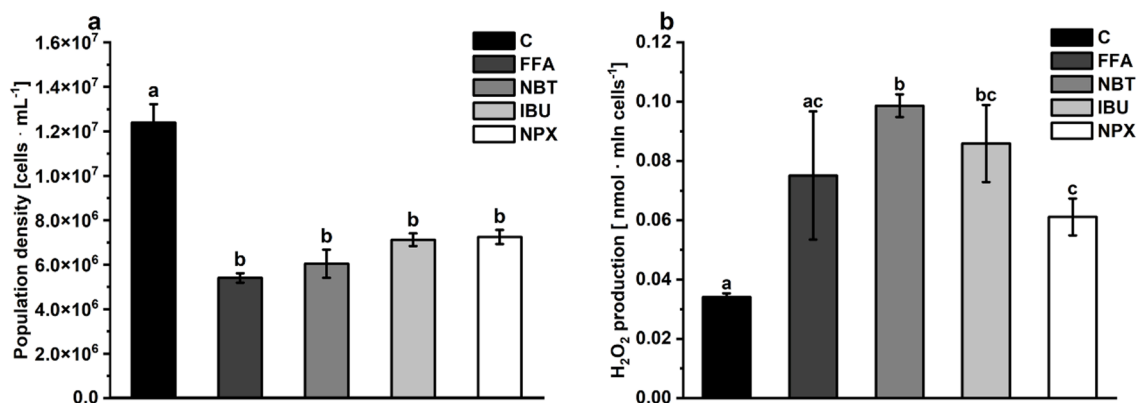


Fig. 1. Effect of flufenamic acid (FFA), nabumetone (NBT), ibuprofen (IBU), and naproxen (NPX), applied at EC50 concentrations on population density (a), and hydrogen peroxide production by *C. reinhardtii* cells (b). Data are presented as averages ± SE. Different letters indicate significant differences ($p \leq 0.05$) between groups (a- ANOVA followed by the Tukey's HSD test, b- Kruskal-Wallis followed by the Dunn's test).

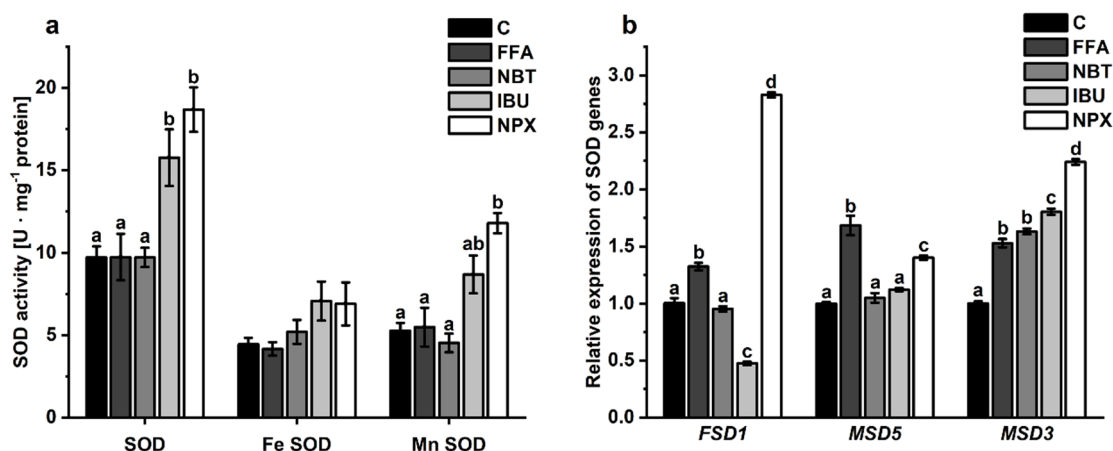


Fig. 2. Effect of flufenamic acid (FFA), nabumetone (NBT), ibuprofen (IBU), and naproxen (NPX), applied at EC50 concentrations on activity of superoxide dismutase (SOD, a), and relative level of SOD genes transcription in *C. reinhardtii* cells (b). Data are presented as averages ± SE. Different letters indicate significant differences ($p \leq 0.05$) between groups (ANOVA followed by the Tukey's HSD test). *FSD1*: gene encoding for Fe-SOD, *MSD5*, *MSD3*: genes encoding for Mn-SODs.

SOD activity and gene expression

Considering the high stimulation of hydrogen peroxide production by *C. reinhardtii* treated with NSAIDs, our focus was on determining the activity of superoxide dismutase, which is one of the main sources of H₂O₂. During PAGE analysis, two isoforms of superoxide dismutase were detected, such as Fe-SOD, known to be located in chloroplasts, and Mn-SOD, which is present in the chloroplast and mitochondria (Fig. S3)^{23,25}. The activity of both SOD isoforms, and total SOD activity did not differ from the control in cells treated with FFA and NBT (Figs. 2a and 5). A significant increase in total SOD activity was observed upon exposure to IBU and NPX, reaching 15.8 U/mg and 18.7 U/mg, respectively, while the untreated cells exhibited an activity of 9.7 U/mg (Figs. 2a and 5). In cells treated with NBT, IBU, and NPX, Fe-SOD activity was enhanced, achieving 117%, 159%, and 155% of the control, respectively (Figs. 2a and 5). However, no statistically significant differences were observed. Exposure to NPX resulted in a pronounced 223% increase in Mn-SOD activity, compared to the control group. The activity of these isoforms for other NSAIDs was not statistically significant and reached 104% for FFA, 86% for NBT, and 165% for IBU (Figs. 2a and 5). To identify the transcriptional regulation of enzyme expression, we performed a more detailed analysis of the abundance of transcripts encoding superoxide dismutase. The presence of *MSD4* was not detected in any of the tested variants (data not shown). *FSD* transcript abundance was significantly increased only in FFA- and NPX-treated microalgae (132%, and 282% of control, respectively). These values for NBT and IBU were 95% and 47% of the level determined in the control (Fig. 2b). Similarly, FFA and NPX showed a similar trend for *MSD5* transcript, where the relative gene expression was 168% and 140%, respectively, compared to the control (Figs. 2b and 5). The increase observed for cells treated with NBT and IBU was not statistically significant. In contrast to other genes, the relative expression of

the *MSD3* gene significantly increased in response to all tested NSAIDs (Figs. 2b and 5). Additionally, Pearson correlation analyses showed a strong positive and significant correlation between total SOD activity, and H_2O_2 production in all tested NSAIDs (Fig. 6a-d). Mn-SOD activity demonstrated positive and significant correlations with H_2O_2 and total SOD activity for FFA and IBU, with Fe-SOD for NBT, IBU, and NPX, and also with *FSD1* for NPX (Fig. 6a-d). A similar relationship was found between *MSD5* and *FSD1* transcripts in the case of FFA, NBT, and NPX (Fig. 6a, b and d). Negative correlations were observed only for IBU between *MSD3* and H_2O_2 , Fe-SOD, Mn-SOD, and total SOD activity, although only the correlation with Fe-SOD activity reached statistical significance.

APX activity and gene expression

We also examined ascorbate peroxidase activity, a key enzyme involved in the decomposition of hydrogen peroxide produced in chloroplasts²⁶. APX activity in untreated *C. reinhardtii* cells was about 0.13 U/ 100 million cells (Fig. 3a). The activity of APX was the lowest in IBU-treated cells, where it accounted for 81% of the control and was not statistically different (Figs. 3a and 5). All other pharmaceuticals tested increased APX activity in *C. reinhardtii* cells. The increase in APX activity was the least pronounced for NPX, reaching 140% of the control cells (Figs. 3a and 5). Meanwhile, the highest significant activity levels were recorded for FFA and NBT, achieving 212%, and 195% of the activity observed in untreated cells (Figs. 3a and 5). The expression of the gene encoding ascorbate peroxidase was also analyzed. All NSAIDs used in the study increased the relative transcript abundance of the *APX1* gene (Fig. 3b). The obtained values were 144% (NBT), 169% (FFA), 231% (NPX), and 351% (IBU) of the control (Figs. 3b and 5). Significant positive correlations were observed only for *APX1* with *MSD5* for IBU and with *MSD3* for NBT (Fig. 6b, c). Compared with *APX1*, *APX2* and *APX4* showed more variable expression profiles and did not allow clear interpretation of treatment-related changes. Therefore, further interpretation focused on *APX1*.

CAT activity and gene expression

The activity of catalase, which is involved in the decomposition of H_2O_2 into O_2 and H_2O outside chloroplasts, was increased in *C. reinhardtii* cells treated with each of the tested NSAIDs. In FFA- and NBT-treated cells, CAT activity reached 191%, and 259% of the control, respectively (Figs. 4a and 5). Treatment with IBU, and NPX resulted in a significant increase in the activity of this enzyme, reaching 432%, and 311% of the level observed in untreated cells, respectively (Figs. 4a and 5). Analysis of *CAT1* transcript abundance encoding catalase demonstrated the opposite effect to the activity of CAT observed in *C. reinhardtii* cells. All tested NSAIDs caused a significant decrease in the relative transcript levels of *CAT1* (Figs. 4b and 5). This value ranged from 9% to 46% of the level obtained in the control group (Figs. 4b and 5). Interestingly, all tested substances exhibited a significant and very strong positive correlation between CAT activity and Mn-SOD activity. Moderate, strong, and very strong correlations were also observed between CAT activity and H_2O_2 production, total SOD, and Fe-SOD activity in the cells exposed to the tested NSAIDs, although not all were statistically significant (Fig. 6a-d). We also detected a very strong positive correlation between *CAT1* and *FSD1* and *MSD3* levels in cells treated with FFA. A strong correlation between *CAT1* and *MSD3* transcript was also observed after NBT treatment (Fig. 6a, b).

Integrated Biomarkers Response (IBR)

The IBR analysis allowed for a comprehensive evaluation of the antioxidant system activity of *C. reinhardtii* cells treated with the tested NSAIDs (Fig. 7). We observed that with increasing NSAID toxicity (EC₅₀), the IBR index value decreased (Fig. 7a). Among the tested biomarkers, APX and CAT showed the highest biological effects in cells treated with FFA (Fig. 7b). In the case of NBT, the production of H_2O_2 and APX activity played the most

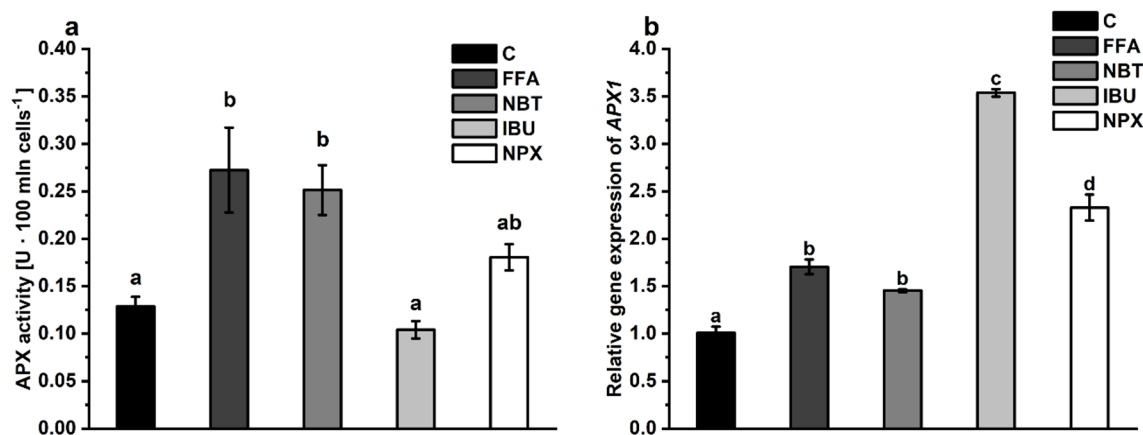


Fig. 3. Effect of flufenamic acid (FFA), nabumetone (NBT), ibuprofen (IBU), and naproxen (NPX), applied at EC₅₀ concentrations on the activity of ascorbate peroxidase (APX, **a**), and relative gene transcription level of *APX1* (**b**) in *C. reinhardtii* cells. Data are presented as averages $\pm$ SE. Different letters indicate significant differences ($p \leq 0.05$) between groups (ANOVA followed by the Tukey's HSD test).

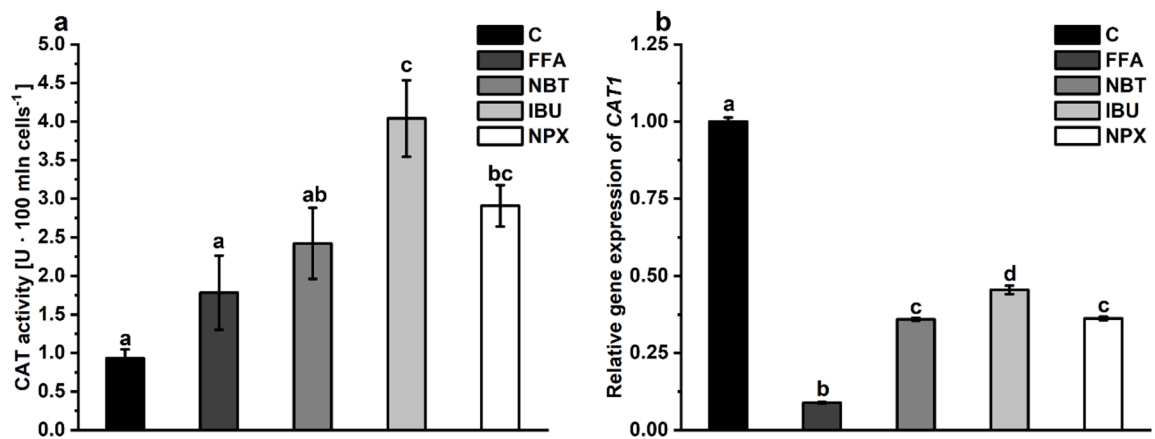


Fig. 4. Effect of flufenamic acid (FFA), nabumetone (NBT), ibuprofen (IBU), and naproxen (NPX), applied at EC50 concentrations on activity of catalase (CAT, **a**), and relative gene transcription level of *CAT1* (**b**) in *C. reinhardtii* cells. Data are presented as averages $\pm$ SE. Different letters indicate significant differences ($p \leq 0.05$) between groups (ANOVA followed by the Tukey's HSD test).

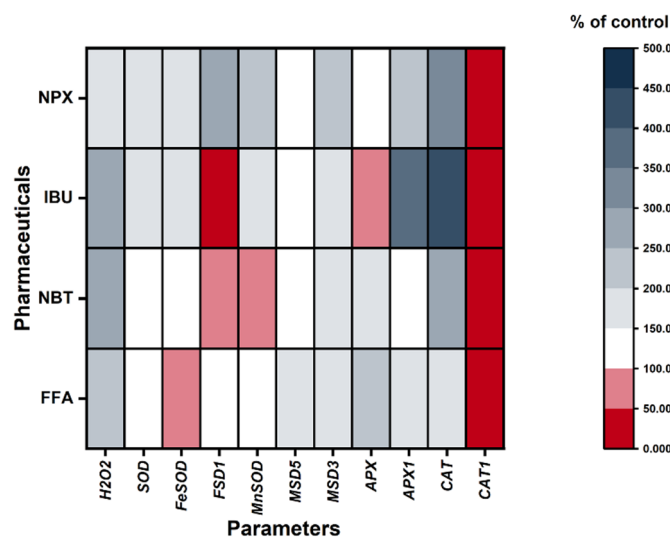


Fig. 5. Overview of NSAIDs effect on analyzed parameters, relative to control values.

important role in IBR (Fig. 7c). The most sensitive biomarkers in cells treated with IBU were CAT and Fe-SOD (Fig. 7d). Interestingly, the highest S-values observed for the activity of SOD and its isoforms indicate that these biomarkers were most involved in the response of *C. reinhardtii* cells to NPX (Fig. 7e).

Discussion

Abiotic stress caused by the exposure of living organisms to toxic substances is a major factor triggering the release of reactive oxygen species (ROS), potentially leading to oxidative stress when scavenging mechanisms are insufficient²⁷. This research compared the antioxidant response of *C. reinhardtii* to selected NSAIDs, such as FFA, NBT, IBU, and NPX, and proved that each of the selected NSAIDs caused oxidative stress in *C. reinhardtii* cells, but the mechanisms involved in minimizing their negative effects differ.

Firstly, all tested NSAIDs increased H₂O₂ production in *C. reinhardtii* cells, confirming the induction of redox imbalance, growth inhibition, and reduced photosynthesis efficiency. This result is in line with our previous work focusing on the toxicity of diclofenac (DCF) to *C. reinhardtii*, where the amount of H₂O₂ produced by these microalgae was 200% of the control¹². In this work, we observed that FFA, NBT, NPX, and IBU caused a similar or even greater overproduction of H₂O₂. This underscores the potential for these common NSAIDs to induce significant oxidative stress in aquatic photoautotrophs. Moreover, our observations align with the results of other research teams focusing on the effects of pharmaceuticals on ROS levels in algal cells, including increased superoxide anion production in *C. reinhardtii* cells treated with ibuprofen and the antibiotic oxytetracycline¹⁴. *Chlorella* sp. and *Desmodesmus spinosus* are other freshwater microalgae in which increased levels of ROS (hydrogen peroxide) have been observed after treating cells with amoxicillin, ciprofloxacin,

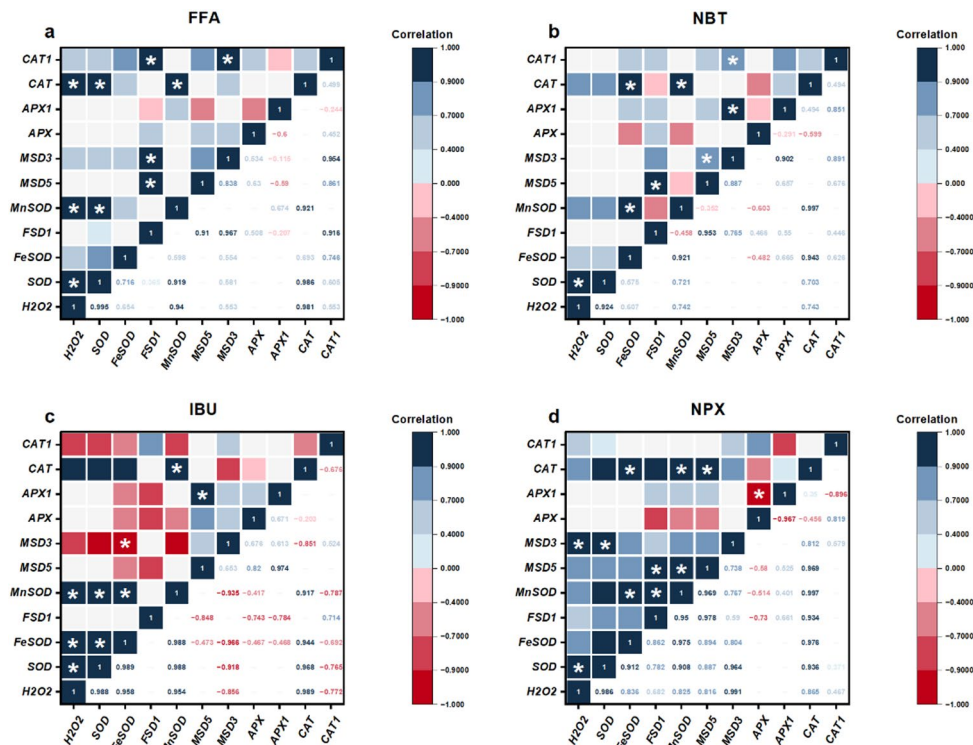


Fig. 6. Pearson correlation matrices between H_2O_2 production, antioxidant enzyme activity, and gene expression in *C. reinhardtii* cells treated with FFA (a), NBT (b), IBU (c), and NPX (d). Positive correlations are shown in blue and negative correlations in red; color intensity is proportional to the correlation coefficient; white stars indicate statistically significant correlations ($p \leq 0.05$). Grey squares indicate no linear relationship among the analyzed parameters.

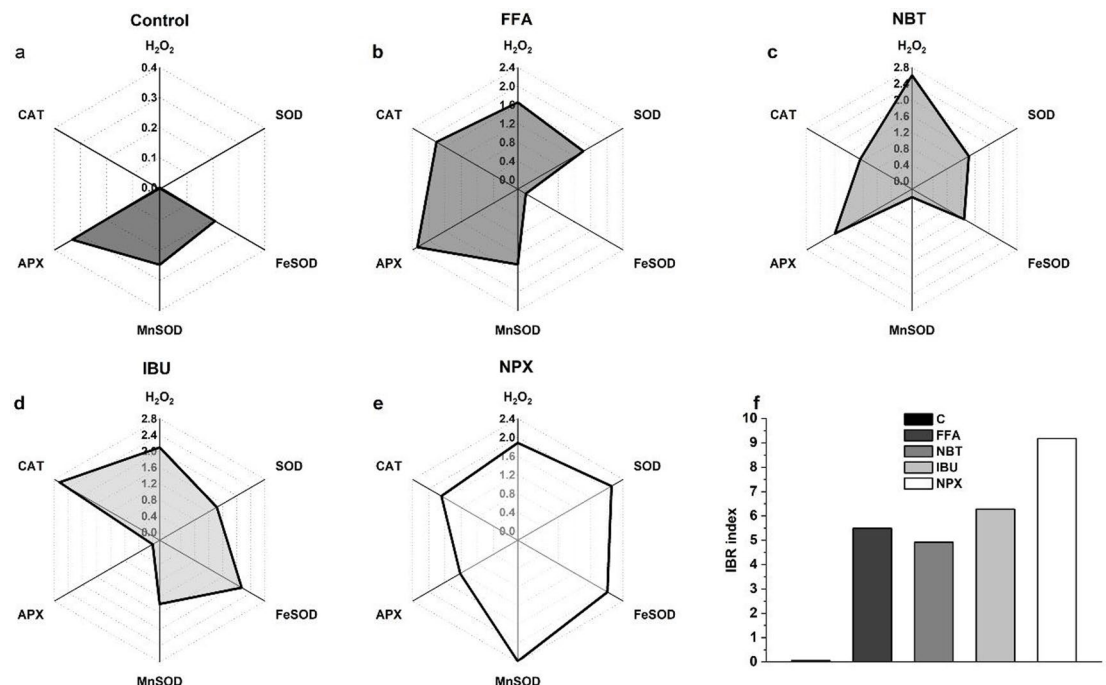


Fig. 7. Biomarker star plots (a-e) and integrated biomarker response (IBR) index (f) of *C. reinhardtii* cells exposed to FFA, NBT, IBU, and NPX, applied at EC₅₀ concentrations. H₂O₂- hydrogen peroxide production, SOD- total SOD activity, Fe-SOD- iron superoxide dismutase activity, Mn-SOD- manganese superoxide dismutase activity, APX- ascorbate peroxidase activity, CAT- catalase activity.

tetracycline, paracetamol, ketoprofen, and diclofenac²⁸. This strongly suggests that ROS overproduction is a common response of green algal cells to pharmaceutical-induced stress. Therefore, investigating cells' capacity to scavenge ROS seemed to be a sensible choice for the research. The chemical properties of many NSAIDs, particularly their ability to perturb mitochondrial oxidative phosphorylation or interfere with photosynthetic electron flow^{6,17,29}, suggest that multiple compartments (chloroplasts, mitochondria, peroxisomes) can serve as ROS sources during NSAIDs exposure. This compartment-specific ROS generation indicates that *C. reinhardtii* must activate a coordinated antioxidant response spanning several organelles to maintain redox homeostasis.

Secondly, the observed increases in H₂O₂ were accompanied by strong activation of the superoxide dismutases (SODs), which are often described as the first-line of defense in stressed cells. Total SOD activity increased significantly in IBU- and NPX-treated cells (Fig. 2a) and was strongly positively correlated with H₂O₂ production under all treatments. This confirms that SOD activity is tightly coupled to ROS accumulation; it was also shown using Pearson correlation matrices (Fig. 6), and is in line with the literature^{30,31}.

The part of our study examining the expression of SOD-encoding genes, and the activity of SOD isoforms was particularly noteworthy, as studies analyzing SOD in NSAID-treated algae cells are scarce, and usually report only a total SOD activity. Such limited SOD-level analyses were reported, for example, in *Desmodesmus obliquus* exposed to ibuprofen, aspirin, and ketoprofen, where oxidative stress markers were measured, but isoenzyme-specific SOD responses were not assessed⁶. Similarly, studies on NSAIDs toxicity to *Chlorella* and *Desmodesmus* species have focused mainly on cellular stress biomarkers and total antioxidant enzyme activities, without resolving individual SOD isoforms²⁸.

In this research, we found that the SOD response was highly isoform-specific at the isoenzyme level. Mn-SOD activity increased markedly after NPX exposure (~ 223% of control), indicating a strong involvement of mitochondria, and possibly peroxisomes in ROS detoxification. In contrast, Fe-SOD, localized mainly in chloroplasts, showed moderate, non-significant increases in NBT-, IBU-, and NPX-treated cells (Fig. 2a; Tab. S4; Tab. S5). These findings highlight that NSAIDs induce compartment-dependent ROS production/detoxification, with NPX in particular stimulating mitochondrial/peroxisomal antioxidant defenses. This organelle-specific ROS formation agrees with observations that several NSAIDs disrupt photosynthetic processes and stimulate ROS production, as shown for DCF in *C. reinhardtii* and ibuprofen/ketoprofen in *Scenedesmus obliquus*^{6,17}.

At the transcriptional level, the expression of *FSD1*, encoding the chloroplast-located iron superoxide dismutase (chl-Fe-SOD)³², increased significantly after FFA and NPX treatment. A comparable effect on *FSD1* expression was observed previously in *C. reinhardtii* treated with DCF, which enhanced expression of photosynthesis-, and redox-related genes, including *FSD1*, in response to oxidative stress¹⁷. In the present study, in NPX-treated cells, we found a positive correlation between *FSD1* expression and chl-Fe-SOD activity (Figs. 2b and 6d). Moreover, chl-Fe-SOD activity was very strongly positively correlated with the activity of Mn-SOD (predicted to localize to the secretory pathway; sp-Mn-SOD)³², and strongly but not statistically significantly correlated with expression of its genes (*MSD3*, *MSD5*, *FSD1*) (Fig. 6a-d). Importantly, *MSD3* expression increased significantly in all NSAID-treated cultures, making it a promising molecular biomarker of NSAID-induced oxidative stress in *C. reinhardtii*. Similar NSAID-induced oxidative stress signaling has been documented for DCF in algae and other aquatic organisms, where oxidative stress responses and DNA damage are consistently reported under chronic NSAID exposure^{33–35}. Moreover, correlations between *FSD1* expression and Mn-SOD activity, as well as between *MSD5* expression and Mn-SOD activity, indicate coordinated regulation of chloroplast and extrachloroplast SOD isoenzymes under NPX stress. Overall, these results demonstrate that *C. reinhardtii* activates a compartmentalized, isoform-specific SOD response, essential for early adaptation to NSAID-induced oxidative stress.

Thirdly, although SOD is considered the first line of defense because it converts superoxide into hydrogen peroxide, the subsequent hydrogen peroxide detoxification in *C. reinhardtii* primarily depends on two enzymes, namely chloroplast-localized ascorbate peroxidase (APX), and catalase (CAT), which function in peroxisomes, the endoplasmic reticulum, and the cytoplasm^{36,37}. Our results indicated that APX activity increased in all treatments except IBU, with the strongest induction observed for FFA and NBT (Fig. 3a). NPX induced a moderate increase (to ~ 140% of control), whereas in IBU-treated cells, APX activity was comparable to the control. Despite these differences, *APX1* transcript levels increased significantly under all treatments, especially in IBU and NPX-treated cells. This highlights that APX is consistently upregulated at the transcriptional level, suggesting that chloroplasts are an early and sensitive target of NSAID-induced redox perturbation. This interpretation is further supported by the chlorophyll *a* fluorescence data and pigment analyses, which revealed clear, compound-specific alterations in PSII performance, electron transport, and content of chlorophylls and carotenoids (Tab. S6)¹³. Taken together, these changes may reflect a partial acclimatory adjustment of photosynthetic apparatus, potentially contributing to stress mitigation. On the other hand, discrepancies between transcript level and enzyme activity indicate post-transcriptional regulation of APX activity. Such transcript-activity mismatches are well documented for APX. For instance, Lopez et al.,³⁸ reported that salt stress in *Raphanus sativus* caused an increase in APX activity without a proportional rise in APX mRNA levels. Various reasons for such discrepancies have been suggested in the literature. First, post-translational modifications (PTMs) regulate APX activity *in vivo*^{39,40}. In higher plants and some green algae, cysteine S-nitrosylation enhances, whereas tyrosine nitration diminishes APX activity. These changes occur independently of transcript levels and integrate redox signals from ROS, RNS, and thiol-disulfide interchange^{39,40}. An additional recognized control mechanism involves isoform diversity and processing, including alternative splicing of genes⁴⁰. Furthermore, APX activity depends on cofactors/substrates (such as ascorbate availability) and on compartment-specific oxidative environment⁴¹. Given the highest H₂O₂ levels in NSAID-exposed cells, the self-inactivation of APX through its product-catalyzed reaction provides a mechanistic explanation for the observed divergence⁴². The above-mentioned reports may explain the observed discrepancy between APX transcript levels and enzyme activity in NSAIDs-treated algal cells, especially pronounced in the case of IBU treatment (Fig. 3a-b).

While considering the role of APX in the cell, it should be noted that beyond ROS detoxification, this enzyme plays several important functions. It is distributed across various cellular compartments, such as chloroplasts, cytosol, mitochondria, peroxisomes, and even the apoplast in plant and algal cells^{40,43,44}, and participates in redox signaling that coordinates stress responses and cell development. The second important enzymatic component of stress signaling and mitigation machinery is catalase. In contrast to APX, CAT activity increased in all NSAID-treated cells, with the highest values observed for IBU and NPX treatments. Surprisingly, *CAT1* transcript levels decreased in all treatments (to 9–46% of control). This striking divergence between transcriptional suppression and enzymatic activity remains difficult to interpret due to the limited availability of literature data, and thus further study is needed. Some reports have suggested that a decrease in the expression of certain nucleus-encoded genes, including CAT, may be caused by a retrograde signal originating from the chloroplast⁴⁵. While the enzymatic activity of CAT could be regulated post-transcriptionally through enzyme stabilization, activation, or post-translational modifications^{46–48}. In our opinion, strong, positive and significant correlations between Mn-SOD and CAT activity in all NSAIDs treatment (Fig. 6a–b) indicate a functional coupling between mitochondrial/peroxisomal superoxide detoxification (Mn-SOD) and downstream H₂O₂ removal (CAT). This supports a model in which NSAIDs enhance mitochondrial/peroxisomal ROS production, triggering compensatory activation of CAT despite transcriptional downregulation.

Analysis of our results directs our attention to structurally similar drugs, namely NPX and NBT. Although NBT and NPX share similar chemical structures (methoxynaphthalene derivatives)⁴⁹, their biological effects differed considerably. Despite both substances causing H₂O₂ overproduction (Fig. 1b), the antioxidative system response was different. NPX strongly activated both Fe-SOD and Mn-SOD (gene expression and activity), and greatly increased CAT activity (Figs. 2a–b and 4a), indicating a multi-compartment antioxidant response. NBT, despite generating high H₂O₂ levels, did not significantly increase SOD activity and instead triggered a stronger APX response (Figs. 1a, 2a and 3a), highlighting a chloroplast-centered reaction. Data reporting direct comparisons of the phytotoxic effects of NBT and NPX are scarce, but those that are available indicate higher toxicity of NPX than NBT⁵⁰. In our opinion, this observation may be explained by the higher reactivity of NPX and its greater photosensitizing potential due to the electron-donating effect of the additional methyl group present in NPX. ROS are expected to be formed at different steps during the photodegradation pathways of both drugs, which give rise to their effects on biomolecules, such as lipid peroxidation⁵¹.

Given the complex and not yet fully understood of NSAIDs' effects, we decided to analyze IBR, which provides a comprehensive evaluation of the integrated antioxidant system activity. The IBR analysis (Fig. 7) revealed that the IBR index decreased with increasing NSAID toxicity (EC50), indicating that highly toxic compounds reduce the overall efficiency of antioxidant adaptation in *C. reinhardtii*. Particularly, for FFA, APX, and CAT exhibited the highest contributions to the stress response, whereas for NBT, H₂O₂ production and APX activity had the largest biological effects. For IBU, CAT, and Fe-SOD were most responsive, and for NPX, SOD isoenzymes had the strongest influence (highest S-values). Thus, these results suggest that the most successful adaptive strategy, characteristic of less toxic compounds such as IBU and NPX, involves simultaneous activation of SOD isoenzymes and strong CAT activity, supporting efficient removal of both superoxide and hydrogen peroxide. Treatments with more toxic compounds (NBT, FFA) were mainly characterized by APX activation, which indicates a strong shift to chloroplast detoxification pathways, probably reflecting stronger chloroplast-targeted stress. To our knowledge, no published studies have reported NSAID-specific antioxidant-response patterns that could explain the differential activation of SOD/CAT versus APX pathways observed here. Thus, our findings provide a novel and unique insight into how chemically diverse NSAIDs shape compartment-specific redox strategies.

Conclusions and perspectives

In conclusion, this study offers a comprehensive analysis of the antioxidant response in *C. reinhardtii* cells exposed to selected NSAIDs, which form a significant group of aquatic contaminants. Our findings also highlight the need for monitoring NSAID-induced oxidative stress in microalgae and their potential as indicators of pharmaceutical pollution. It is also proved that each of the selected NSAIDs caused oxidative stress in *C. reinhardtii* cells, but the mechanisms involved in minimizing their negative effects differ. Therefore, the key outcomes include: (i) identification of *MSD3* transcript level as a universal molecular biomarker of NSAID stress; (ii) evidence for compartment-specific adaptation, with APX serving as the primary chloroplast-localized H₂O₂ scavenger, particularly under FFA and NBT exposure, and with CAT playing a dominant role in extra chloroplast compartments, especially under IBU and NPX exposure; and (iii) recognition that NSAIDs induce compound-specific antioxidant signatures, reflecting their chemical properties. We also recommend that future studies should investigate subcellular redox dynamics using compartment-specific ROS reporters. Proteomic analysis is also recommended to reveal post-translational modifications of APX, CAT, and SOD proteins. Additionally, examining a wider range of pharmaceuticals and environmental mixtures to map chemotype-specific adaptation strategies would be highly valuable.

Materials and methods

Tested compounds

In this study four substances belonging to the group of non-steroidal anti-inflammatory drugs (NSAIDs) were examined, namely ibuprofen (IBU, 4-Isobutyl- α -methylphenylacetic acid, 99% purity, CAS number 15687-27-1), flufenamic acid (FFA, N-(3-[Trifluoromethyl]phenyl) anthranilic acid, 97% purity, CAS number 530-78-9), nabumetone (NBT, 4-(6-Methoxy-2-naphthyl)-2-butanone; 97% purity, CAS number 42924-53-8), and naproxen (NPX, (S)-6-Methoxy- α -methyl-2-naphthaleneacetic acid sodium salt, 98% purity, CAS number: 26159-32-2). All substances were purchased from Alfa Aesar (Ward Hill, Massachusetts, USA). Ibuprofen and

naproxen were dissolved in ddH₂O to obtain the stock solutions of 100 mg/mL. For preparing stock solutions of FFA (70 mg/mL) and NBT (100 mg/mL), DMSO (dimethyl sulfoxide, analytical grade, BioShop, Canada) was used as the carrier solvent. In the experiments, where FFA and NBT were used, the final DMSO concentration did not exceed 0.1% (v/v). This DMSO concentration has been verified in our laboratory, and previous studies have shown that DMSO at this level does not affect the vital parameters of the green alga *C. reinhardtii*^{12,52–54}. NSAID solutions were added to the HSM medium to obtain the desired concentrations (see 5.2).

Algae cultures and treatment

C. reinhardtii (CC-1690, WT) was purchased from the Chlamydomonas Resource Center (University of Minnesota, Minnesota, USA). The algae were transferred to a liquid HSM medium from slants used for long-term storage⁵⁵ and pre-cultured for several days at 30 ± 1 °C, with continuous bubbling with sterilized air (bacteriological filter 0.2 mm PTFE; Sartorius, Germany), enriched with 2.5% CO₂ (v/v), and at white fluorescent light (125 ± 5 μmol photons/m² × s)⁵³. The pre-cultures which were at the logarithmic growth phase were used to establish experimental sets for acute toxicity testing. The appropriate volume of cell suspension taken from pre-culture was mixed with HSM to achieve an initial algal population density of 0.5 × 10⁶ cells/mL, and divided into several culture vessels (200 mL volume each)^{25,53}. The cultures were treated for 24 h with FFA, NBT, IBU, and NPX at 35.47, 5.75, 87.04, or 137.44 mg/L, respectively. The concentrations used represent nominal EC50 values that were determined from population growth inhibition curves, for FFA and NBT¹³, for IBU and NPX the equations are provided in the Supplement (see Text S1, Fig. S1, and Tab. S1). Cultures without the tested chemicals were used as controls. At the beginning of the experiment (0 h) and after 24 h of cultivation the algae cells were collected for analysis, as described below.

To unify the results to comparable units, the population density was determined in each experimental variant. The number of cells was counted using a particle counter Z2 (Beckman Coulter, USA), run by dedicated software. Additional measurements of chlorophyll *a* fluorescence *in vivo* and photosynthetic pigments content were analyzed, as described in the supplementary materials (Text S2).

Hydrogen peroxide production by cells

The amount of hydrogen peroxide produced by microalgae cells was determined using the Amplex UltraRed[®] fluorochrome (Molecular Probes, USA), according to the established protocol⁵⁶. Briefly, the algae cells suspension was centrifuged at 291 RCF for 5 min., at room temperature, the cells' pellet was discarded, and supernatant was used for H₂O₂ estimation. 50 μL of the supernatant was placed in a 96-well black plate, and mixed with a 50 μL reaction mixture comprising 10 mM Amplex UltraRed, and 10 U/mL horseradish peroxidase in 0.1 M potassium phosphate buffer (pH 7.0). For the preparation of fortified samples, a freshly prepared H₂O₂ solution (stabilized, analytical grade, Sigma, USA) of a concentration of 3 nmol/mL was used. Fortified samples were prepared by introducing hydrogen peroxide of known concentration into the supernatant obtained as described above, followed by the addition of the reaction mixture. The samples were incubated in the darkness for 25 min. at 25 °C, the fluorescence was measured at λ_{ex} = 560 nm and λ_{em} = 585 nm using a Varioskan Flash-Spectral Scanning Multimode Microplate Reader (ThermoFisher Scientific, USA). The concentration of H₂O₂ was expressed as the amount of compound produced by millions of cells and calculated using equation:

$$\text{CH}_2\text{O}_2 = [\text{ST} \times \text{Fscorr}] / [(\text{Fsscorr} - \text{Fscorr}) \times \text{Sv}] \text{ [nmol/}\mu\text{L]},$$

where:

ST – the amount of H₂O₂ spiked in the well (nmol); Fscorr – corrected sample fluorescence reading (unspiked well); Fsscorr – corrected spiked sample fluorescence reading (spiked well); Sv – sample volume (μL) added into the well.

The activity of antioxidative enzymes

All chemicals used for the enzymes' activity analyses were of analytical grade purity (Sigma-Aldrich, Missouri, USA). To analyze the activity of antioxidative enzymes, 100 mL of algae suspension was collected by centrifugation (5 min., 3 000 × g), and the sedimented material was re-suspended in 1 mL of appropriate extraction buffer. For superoxide dismutase (SOD) and catalase (CAT), 0.05 M potassium phosphate buffer (pH 7.0) was used, while for ascorbic peroxidase (APX), 0.05 M phosphate buffer (pH 7.6), containing 0.86 mM AsA (ascorbic acid), 2.4 mM EDTA (ethylenediaminetetraacetic acid), 20% sorbitol and 2% PVP (polyvinylpyrrolidone) was used. Briefly, the pellet was homogenized with glass beads (30 s, 4.5 RPS) in 1 mL of the appropriate buffer using FastPrep-24 (MP Biomedicals, USA), after which the samples were centrifuged (20 min., 20,000 × g, at 4 °C). The obtained supernatants were used to determine protein concentration and enzyme activities.

Protein concentration was examined according to the method of Bradford⁵⁷. Briefly, 300 μL of Bradford reagent was added to 10 μL of sample or bovine albumin standard and incubated for 10 min. in room temperature. Measurements were made at a wavelength of 595 nm using a Varioskan Flash-Spectral Scanning Multimode Microplate Reader (ThermoFisher Scientific, USA). The protein concentration in the analyzed supernatant samples was calculated using the equation derived from the standard curve. The results were used for enzyme activity calculations.

The method involving the reduction of nitroblue tetrazolium (NBT) was used to determine the activity of SOD isoforms^{58,59}. Briefly, non-denaturing polyacrylamide gel electrophoresis (PAGE) was performed, and the resulting gels were immersed in a 2.45 mM NBT solution for 20 min., followed by immersion in a solution containing 28 mM TEMED and 2.8 × 10^{−6} M riboflavin in 36 mM potassium phosphate buffer at pH 7.8 for 15 min. The gels were exposed to illumination until a uniform blue color was obtained, with areas containing SOD remaining colorless. SOD isoforms were resolved on the gels⁶⁰. The activity of individual SOD isoform

bands was determined by comparing the relative band intensity in mm^2 to that of the Cu/Zn-SOD reference pattern with a defined activity of 4.4 U/mg protein (Sigma-Aldrich, Missouri, USA). Densitometric analysis was performed using ImageLab 6.0 (Bio-Rad Laboratories, Inc., California, USA).

APX activity was determined according to the method of Nakano and Asada⁶¹, as described in Aksmann et al.,⁵⁴ with a slight modification. A reaction mixture containing 50 mM phosphate buffer (pH 7.0), 18 mM pyrogallol, and 0.1 mM H_2O_2 was prepared. To 50 μL of enzyme extract, 150 μL of the reaction mixture was added, and the decrease in absorbance at 430 nm corresponding to pyrogallol oxidation was monitored for 1 min at 25 °C (by Hitachi U-2900 spectrophotometer, Japan). Enzyme activity was quantified using the molar extinction coefficient for pyrogallol ($\epsilon = 2.47 \text{ mM}^{-1} \text{ cm}^{-1}$), and the results were expressed as U per 100 million cells.

CAT activity was determined spectrophotometrically (Hitachi U-2900, Japan), as described in Aksmann et al.,⁵⁴. Briefly, after adding the reaction mixture (50 mM sodium phosphate buffer pH = 7.0, 10 mM H_2O_2) to the enzyme extract, the decrease in absorbance ($\lambda = 240 \text{ nm}$) was monitored⁶². Results were expressed as U per 100 million cells.

RNA extraction and RT-qPCR

Transcript levels of selected genes related to oxidative stress in NSAIDs-treated cells were determined, as described by Aksmann et al., and Pokora et al.,^{54,63,64}. Total RNA was isolated using the Total RNA Mini Kit (A&A Biotechnology, Poland) according to the manufacturer's instructions, after centrifugation of algal cells (5 min, 3000 $\times$ g). Reverse transcription (RT) was carried out using an iScript™ cDNA Synthesis Kit 170–8891 (Bio-Rad Laboratories, Inc.) from 200 ng of total RNA with oligohexamer (dT) primers. Real-time PCR was performed using DNA-binding dye SYBR Green I (iTaQ™ Universal SYBR™ Green Supermix, Bio-Rad Laboratories, Inc.), following the manufacturer's protocol. Gene-specific primer pairs of *FSD1* (iron superoxide dismutase Fe-SOD), *MSD3*, *MSD4*, *MSD5* (isoforms of manganese superoxide dismutase Mn-SOD), *CAT1* (catalase, CAT), and *APX1*, *APX2*, *APX4* (isoforms of ascorbate peroxidase, APX) are listed in Table S3. The 18 S transcript was used to standardize the results as an internal quantifying control. The levels of relative gene expression were calculated using Pfaffl method⁶⁵. All PCR reactions and data analysis were performed on the CFX96 Touch™ Real-Time PCR Detection System using CFX Maestro™ Software (Bio-Rad Laboratories, Inc., California, USA).

In silico prediction of enzymes' subcellular localization

Computational predictions of the subcellular localization of *CAT1*, *APX1*, *APX2*, *APX4*, *FSD1*, *MSD3*, *MSD4*, and *MSD5* gene products were performed using web-based interfaces provided by each prediction program. The sequences of the nucleotide, or protein from algae, were retrieved in the FASTA format from the NCBI (<https://www.ncbi.nlm.nih.gov/>). Subcellular localizations were predicted using online tools such as UniPort⁶⁶, deeploc2.1⁶⁷, and PredAlgo⁶⁸.

Statistical analysis

The presented data were prepared in at least three independent experiments, each including a minimum of three biological replicates per treatment and two technical replicates. Microsoft Excel (Microsoft, 2023, USA), Origin Pro 9.6 (OriginLab Corp., USA), and Statistica 9.0 (StatSoft Europe, Germany) were used for experimental data processing, statistical analysis and graphics preparation. The data were expressed as the means $\pm$ SE. Data were analyzed by one-way ANOVA or Kruskal-Wallis test, followed by the Tukey or Dunn's post-tests. Statistical analyses were performed at a probability value of $p < 0.05$, where the differences are marked using letters. The same software was used to calculate the Pearson correlation matrix to determine the relationship between H_2O_2 production, the activity of antioxidant enzymes, and the expression of the analyzed genes. The integrated biological response index (IBR) was also calculated for the control group and algae treated with NSAIDs, following the method⁶⁹. The H_2O_2 production, and activity of SODs isoforms, CAT, and APX were selected as the biomarkers to analyze the antioxidant response of *C. reinhardtii* cells.

Data availability

All data generated or analysed during this study are included in this published article and its Supplementary Information files. Raw datasets are available from the corresponding author upon reasonable request.

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Author contributions

Conceptualization: AA.; data curation: DK, WP, AA.; formal analysis: DK, AA.; funding acquisition: AA.; investigation: DK, MH, WP.; methodology: DK, MH, WP, AA.; project administration: AA.; resources: DK, MH.; software: DK.; supervision: AA.; validation: DK, AA.; visualization: DK.; roles/writing - original draft: DK, AA.; writing - review&editing: DK, MH, WP, AA.

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Declarations

Competing interests

The authors declare no competing interests.

Consent for publication

This study does not contain any individual personal data including any individual's name, images, or videos.

Additional information

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Supplementary Material for:

Differential antioxidant pathways displayed by *Chlamydomonas reinhardtii* exposed to selected NSAIDs

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The supporting data contains:

Text S1. Determination of effective concentrations for ibuprofen and naproxen.

Algae were exposed to nominal ibuprofen (IBU) concentrations of 17.5, 30.6, 53.6, 93.8, 164.1 mg * dm⁻³ and naproxen (NPX) concentrations of 30, 52.5, 91.9, 160.8, 281.4 mg * dm⁻³ applied at the beginning of the experiment (t₀). Cultures without the tested chemicals served as controls. After 24 h (t₂₄) cultures were sampled for population density measurements. The effective concentrations for IBU and NPX were calculated by regression analysis based on linear models of the dependency between the relative population density (cells number) versus the pharmaceutical concentrations using the following equation: $y = a + b \cdot x$, where a is intercept and b is slope. The values of these variables are presented in the table S1.

Table S1. Variables in the equations for determining EC values for IBU and NBT.

Variant	a	b
IBU	89.23517 ± 1.24703	-0.45079 ± 0.01305
NPX	79.42202 ± 2.35328	-0.21407 ± 0.01436

Figure S1. Concentration-dependent growth inhibition curves of *C. reinhardtii* exposed to ibuprofen and naproxen for 24 h. Data are presented as averages (± SE).

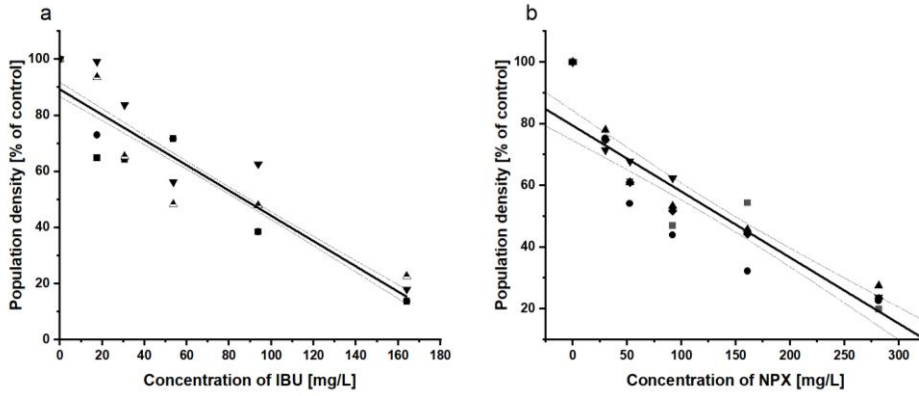


Table S2. Specific growth rate of *C. reinhardtii* after 24 h exposure to NSAIDs at EC50 concentration. The growth rate was calculated using the formula $K = [\ln(N_1) - \ln(N_0)]/t$, where N_1 is the number of cells after 1 day and N_0 is the initial number of cells. Data are presented as averages $\pm$ SE. Different letters indicate significant differences at $p \leq 0.05$ (ANOVA followed by Tukey's HSD test).

	C	FFA	NBT	IBU	NPX
Growth rate [day⁻¹]	$3.13 \pm 0.12a$	$2.38 \pm 0.04b$	$2.45 \pm 0.11b$	$2.65 \pm 0.04b$	$2.66 \pm 0.04b$

Table S3. Sequences of the primers for *Chlamydomonas reinhardtii* genes (according to <https://www.ncbi.nlm.nih.gov/genbank/>) analyzed in the control. and NSAIDs treated cultures.

Gene	Protein encoded	Primer sequence from 5' end to 3' end
<i>MSD3</i>	Isoform of manganese superoxide dismutase (Mn-SOD)	ACCAGACCCACCCAGGAG : CAGCCCCAACCAGGATAAC
<i>MSD5</i>	Isoform of manganese superoxide dismutase (ι Mn-SOD)	CACAGCCAACCAGGACAACC : CGCGGACCAGTTTATCAG
<i>MSD4</i>	Isoform of manganese superoxide dismutase (Mn-SOD)	CCATGAGTGGGTTGTCCTG : GACACTGGAACCACCAGATG
<i>FSD1</i>	Chloroplastic isoform of iron superoxide dismutase (Fe-SOD)	CCGTGGTGGAGGGCAAGACCCCAT : TCGCTGGCGCCGACATGCTTGTGA
<i>APX1</i>	Isoform of ascorbate peroxidase (APX)	TCAAGGAGATCAAGGCCAAG : GCCGCTCAGTCCAGAGTAAC
<i>APX2</i>	Isoform of ascorbate peroxidase (APX)	GTTCGCACCGTAGTCACAAA : GTGACAGGTCCCCTAGCTTG
<i>APX4</i>	Ascorbate peroxidase (APX 4)	AGGTGGCTGGGTTGATAACG : CAAAATCGGAGCACCTTGCC
<i>CAT1</i>	Mono-functional catalase (CAT 1)	CACGAGGGCTTCATGAACTT : CTTGGCGATCACCATCTTCT
<i>18S</i> (reference)	18S ribosomal RNA (18S)	GTAGTCATATGCTTGTCTC : GGCTGCTGGCACCAGACTTGC

Table S4. *In silico* analysis of protein localization in *Chlamydomonas reinhardtii* with PredAlgo. Darker blue indicates the predicted location. Lighter blue indicates the location where $p > 0.5$.

Gene	Signals	Membrane types	Cytoplasm	Nucleus	Extracellular	Cell membrane	Mitochondrion	Plastid	Endoplasmic reticulum	Lysosome/Vacuole	Golgi apparatus	Peroxisome
<i>APX1</i>	Chloroplast transit peptide	Soluble	0.1082	0.0864	0.0362	0.0274	0.1983	0.9247	0.0143	0.0217	0.0173	0.0017
<i>CAT1</i>	Peroxisomal targeting signal	Soluble	0.7118	0.2398	0.3313	0.4196	0.2591	0.0356	0.1272	0.1710	0.1446	0.6791
<i>FSD1</i>	Chloroplast transit peptide	Peripheral Soluble	0.0974	0.0715	0.0331	0.0368	0.1634	0.8936	0.0073	0.0240	0.0312	0.0039
<i>MSD4</i>	Signal peptide	Soluble	0.1259	0.0680	0.7527	0.1548	0.0318	0.1666	0.3267	0.3807	0.2426	0.0058
<i>MSD3</i>	Signal peptide	Soluble	0.1586	0.0322	0.8173	0.1480	0.0450	0.1069	0.2724	0.6811	0.3861	0.0020
<i>MSD5</i>	Peroxisomal targeting signal	Soluble	0.3775	0.0325	0.6425	0.1470	0.5835	0.4677	0.1942	0.5154	0.1729	0.5692

Table S5. The table summarizes the subcellular localization predictions of *Chlamydomonas reinhardtii* enzymes generated using various bioinformatics tools, including DeepLoc2.1 UniProt and PredAlgo.

Gene	deeploc2.1	UniProt	PredAlgo
<i>APX1</i>	Plastid	-	Chloroplast
<i>APX2</i>	Plastid	-	-
<i>APX4</i>	Plastid	-	-
<i>CAT1</i>	Cytoplasm/peroxisome	Peroxisome	Cytoplasm/peroxisome
<i>FSD1</i>	Plastid	Chloroplast nucleoid	Chloroplast
<i>MSD4</i>	Extracellular	-	Secretory pathway
<i>MSD3</i>	Extracellular. Lysosome/Vacuole	Cytoplasm	Secretory pathway
<i>MSD5</i>	Extracellular/ Mitochondrion/ Peroxisome	-	-

Figure S2 . Effects of flufenamic acid (FFA), nabumetone (NBT), ibuprofen (IBU), and naproxen (NPX), applied at EC50 concentrations on protein content in *C. reinhardtii* cells. Data are presented as averages $\pm$ SE and expressed as a percentage of the control.

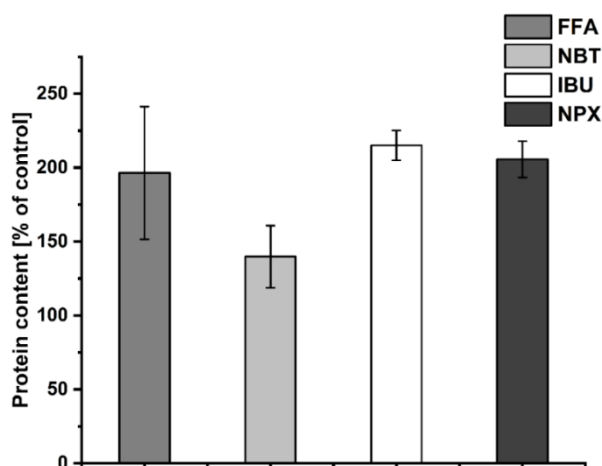
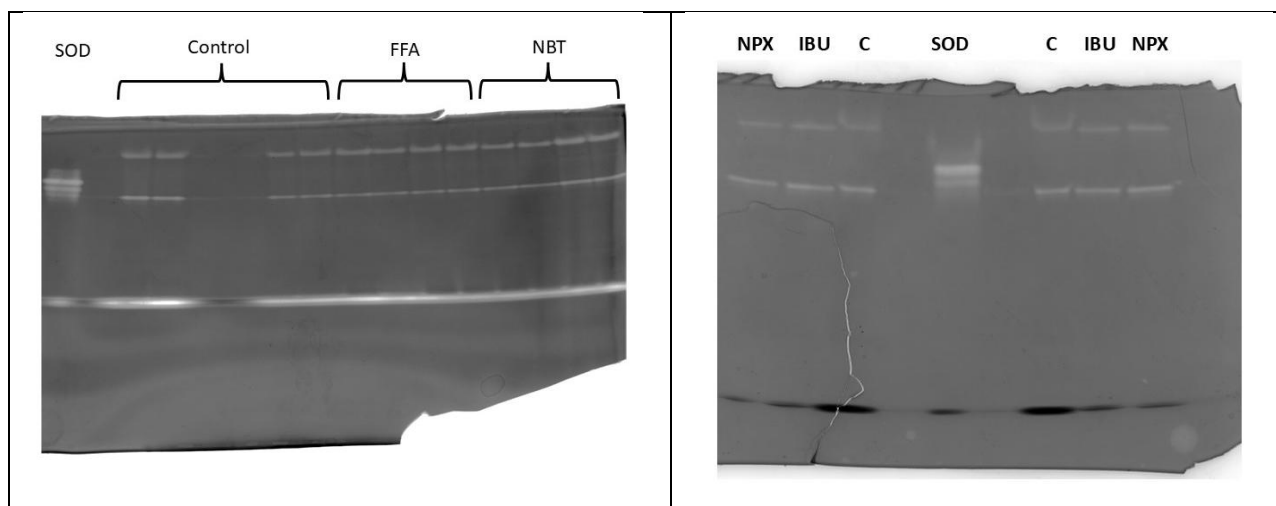


Figure S3. SOD isoenzymes PAGE analysis of *C. reinhardtii* cells treated for 24 h with (A) flufenamic acid (FFA), nabumetone (NBT), and (B) ibuprofen (IBU), naproxen (NPX). SOD- Cu/Zn-SOD reference pattern of defined activity 4.4 U·mg⁻¹ protein (Sigma-Aldrich).



Text S2. Chlorophyll *a* fluorescence *in vivo* and photosynthetic pigments content.

Chlorophyll *a* fluorescence *in vivo* was measured using Handy Pea fluorometer with dedicated software PEA+ (Hansatech Ltd., UK), following Pokora et al. (2017). Before measurement, cell suspensions were adjusted to OD₆₈₀ = 0.2 $\pm$ 0.01, dark-adapted for 15 min, and measured at room temperature under a saturated light pulse (3000 μ mol photons m⁻² s⁻¹). Photosynthetic pigments were determined spectrophotometrically after three extractions with 80% acetone (Majewska et al., 2021). Briefly, cell suspensions were centrifuged (2000 x g, 10 min, room temperature) and the pellets were disrupted with

glass beads using a FastPrep-24 homogenizer (MP Biomedicals, USA). After clarification by centrifugation, the extracts were collected and analyzed in a 96-well plate at 447, 647, 664, and 740 nm using a Varioskan Flash Multimode Microplate Reader (Fisher Scientific, USA). Pigment concentrations were calculated according to Jeffrey & Humphrey (1975) and Lichtenthaler & Wellburn (1983), with path length correction according to Warren (2008).

Table S6. Chlorophyll *a* fluorescence parameters and photosynthetic pigments content in control, IBU- and NPX-treated cells of *C. reinhardtii* after 24 h exposure. Data are presented as average $\pm$ SE. Different letters indicate significant differences among groups ($p \leq 0.05$; ANOVA or Kruskal-Wallis test followed by Tukey's HSD or Dunn's test).

Parameters	C	IBU	NPX	Parameter description
ABS/RC	$1.94 \pm 0.04a$	$4.38 \pm 0.29b$	$2.99 \pm 0.12c$	energy absorption per active reaction center
DI₀/RC	$0.38 \pm 0.02a$	$2.12 \pm 0.26b$	$1.24 \pm 0.10c$	energy dissipation per active reaction center
TR₀/RC	$1.55 \pm 0.01a$	$2.26 \pm 0.10b$	$1.73 \pm 0.02c$	energy trapping (Q _A reduction) per active reaction center
ET₀/RC	$0.84 \pm 0.00a$	$0.86 \pm 0.03a$	$0.83 \pm 0.01a$	efficiency of electron transport per active reaction center
ϕP_0	$0.81 \pm 0.01a$	$0.53 \pm 0.03b$	$0.61 \pm 0.02b$	maximum quantum yield of primary photochemistry
ψE_0	$0.54 \pm 0.01a$	$0.38 \pm 0.01b$	$0.48 \pm 0.01c$	probability that a trapped exciton transfers an electron from Q _A to Q _B
ϕE_0	$0.44 \pm 0.01a$	$0.20 \pm 0.01b$	$0.30 \pm 0.01b$	the maximum quantum yield of electron transport further than Q _A ,
Chlorophyll <i>a</i> [pg/cell]	$0.59 \pm 0.02a$	$0.83 \pm 0.05b$	$0.77 \pm 0.05b$	chlorophyll <i>a</i> content
Chlorophyll <i>b</i> [pg/cell]	$0.39 \pm 0.01a$	$0.56 \pm 0.03b$	$0.48 \pm 0.03b$	chlorophyll <i>b</i> content
Carotenoids [pg/cell]	$0.159 \pm$ $0.003a$	$0.282 \pm$ $0.012b$	$0.253 \pm$ $0.015b$	carotenoids content

Related data for FFA and NBT are available in our previous publication (Kapuścińska et. al., 2024).

Gdańsk, 12.06.2026

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OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

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polegał na:

(i) przeprowadzeniu eksperymentów obejmujących hodowle mikroglonów, oznaczenia liczebności komórek, aktywności enzymów, oraz zawartości nadtlenu wodoru i przeprowadzeniu analiz *in silico* dotyczących lokalizacji subkomórkowej badanych enzymów; (ii) udziale w przygotowaniu metodologii badań; (iii) udziale w gromadzeniu i opracowaniu danych badawczych; (iv) walidacji i interpretacji wyników; (v) przeprowadzeniu analiz statystycznych; (vi) przygotowaniu grafik i rycin przedstawiających wyniki badań; (vii) przygotowaniu pierwotnej wersji manuskryptu; (viii) udziale w redagowaniu manuskryptu; oraz (ix) udziale w przygotowaniu odpowiedzi na uwagi recenzentów.

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polegał na:

(i) udziale w przeprowadzeniu eksperymentów obejmujących hodowlę glonów oraz oznaczenia liczebności komórek, aktywności enzymów oraz zawartości nadtlenku wodoru; (ii) udziale w przygotowaniu metodologii badań; (iii) udziale w gromadzeniu i opracowaniu danych badawczych; (iv) udziale w redagowaniu manuskryptu; oraz (v) udziale w przygotowaniu odpowiedzi na uwagi recenzentów.

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selected NSAIDs. Scientific Reports, 2026, <https://doi.org/10.1038/s41598-026-57688-8>

polegał na:

(i) udziale w przeprowadzeniu eksperymentów obejmujących hodowlę glonów oraz oznaczenia liczebności komórek, aktywności enzymów oraz zawartości nadtlenku wodoru; (ii) udziale w przygotowaniu metodologii badań; (iii) udziale w gromadzeniu i opracowaniu danych badawczych; (iv) udziale w redagowaniu manuskryptu; oraz (v) udziale w przygotowaniu odpowiedzi na uwagi recenzentów.



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OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w publikację:

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Differential antioxidant pathways displayed by Chlamydomonas reinhardtii exposed to selected NSAIDs. Scientific Reports, 2026, <https://doi.org/10.1038/s41598-026-57688-8>

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(i) udziale w przeprowadzeniu eksperymentów obejmujących oznaczenie ekspresji genów; (ii) udziale w przygotowaniu metodologii badań; (iii) udziale w gromadzeniu i opracowaniu danych badawczych; oraz (iv) udziale w redagowaniu manuskryptu.



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polegał na:

(i) przygotowaniu koncepcji badań; (ii) udziale w przygotowaniu metodologii badań; (iii) udziale w walidacji i interpretacji wyników; (iv) nadzorze nad realizacją badań; (v) pozyskaniu finansowania; (vi) udziale w przygotowaniu pierwotnej wersji manuskryptu; (vii) udziale w redagowaniu manuskryptu; oraz (viii) udziale w przygotowaniu odpowiedzi na uwagi recenzentów.



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PODPIS

13. Praca nr 4.

Distinct biochemical effects of naproxen and nabumetone drive their differential toxicity in *Desmodemus armatus*

Kapuścińska D., Harshkova D., Shams M., Liakh I., Wielgomas B., Aksmann A.
(2026)

1 Distinct biochemical effects of naproxen and nabumetone drive their differential toxicity in
2 *Desmodesmus armatus*

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16

17 Abstract
18

19 Nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently detected in aquatic
20 environments and are considered an important group of emerging contaminants. However, their
21 mechanisms of toxicity toward primary producers remain insufficiently understood. In this
22 study, the comparative toxicity and physiological responses of the freshwater microalga
23 *Desmodesmus armatus* exposed to two commonly used NSAIDs, nabumetone (NBT) and
24 naproxen (NPX), were investigated. To clarify their potential modes of action, growth inhibition
25 tests were combined with chlorophyll fluorescence measurements, pigment analysis, oxygen
26 evolution and consumption, antioxidant enzyme activity, and multivariate statistical analysis.

27 The results showed a clear difference in toxicity between the tested compounds. NBT exhibited
28 approximately six-fold higher toxicity ($EC_{50} = 6.57$ mg/L) than NPX ($EC_{50} = 41.2$ mg/L).

Despite this higher toxicity, NBT exposure did not significantly affect photosystem II photochemistry or major chlorophyll *a* fluorescence parameters. Instead, cells exposed to NBT showed increased carotenoid content and significantly elevated dark respiration rates, suggesting activation of protective metabolic and antioxidant responses. In contrast, NPX exposure resulted in a dose-dependent deterioration of the photosynthetic apparatus, reflected by decreases in maximum quantum yield (F_v/F_m), performance index (PI_{abs}), the proportion of active reaction centers, and pigment content, indicating photoinhibition and impaired electron transport.

Chemical stability analysis showed that NBT rapidly dissipated in the exposure medium (>96% reduction within 24h), whereas NPX remained comparatively stable. Multivariate analysis identified maximum quantum yield, hydrogen peroxide, and carotenoid content as the most sensitive variables distinguishing treatment conditions. These findings indicate that structurally related NSAIDs may affect microalgae through distinct physiological pathways and suggest that compound stability and transformation processes should be considered when evaluating the environmental risks of pharmaceutical contaminants in aquatic ecosystems.

Keywords

Microalgae, oxidative stress, ecotoxicity, nonsteroidal anti-inflammatory drugs, photosystem II inhibition

Highlights:

Nabumetone (NBT) is approximately six times more potent than Naproxen (NPX) in inhibiting *Desmodesmus armatus* growth

Naproxen (NPX) demonstrates high photochemical stability in the medium, whereas Nabumetone (NBT) shows a significant environmental concentration decrease

Rapid NBT dissipation (>97% in 24 h) suggests transformation-driven toxicity.

Chemical stability and transformation dynamics critically influence observed NSAID toxicity in microalgae.

Integrated biomarkers revealed distinct toxicity pathways of two NSAIDs.

NPX induced photoinhibition by impairing PSII efficiency and electron transport.

NBT triggered metabolic stress responses without direct PSII inhibition.

1. Introduction

Unlike conventional environmental pollutants, such as heavy metals or pesticides, pharmaceuticals, substances originally designed to modulate biological processes, constitute a specific category of contamination (Gupta et al., 2024). Their molecular structure is optimized to interact with strictly defined biochemical pathways, ensuring efficacy, stability, and bioavailability in clinical and veterinary applications (H. Wang et al., 2021). However, these same properties make them particularly problematic from an ecotoxicological perspective. Once released into the environment, pharmaceuticals may undergo incomplete degradation and transformation, forming multi-component, difficult to define mixtures of parent substances and their derivatives, leading to their accumulation and long-term circulation within aquatic food webs (Estrada-Almeida et al., 2024; Wilkinson et al., 2022). Their interactions with non-target organisms can trigger unexpected physiological and molecular responses, often at concentrations far below those used therapeutically (Estrada-Almeida et al., 2024; Hejna et al., 2022; Parikh et al., 2025).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most frequently detected pharmaceuticals in the aquatic environment, with concentrations ranging from ng/L to µg/L in surface waters globally (Caban et al., 2016; Hejna et al., 2022). Many studies have been devoted to the effects of NSAIDs on non-target organisms, such as microalgae, showing that these substances pose a threat to the aquatic environment (Ortúzar et al., 2022). There are reports on the disruption of numerous metabolic pathways in microalgae cells treated with NSAIDs, but it is such a heterogeneous group of substances that the described toxic effects also vary significantly depending on the specific drug. Since microalgae form the foundation of aquatic food webs, such unpredictable toxic effects on these organisms may affect higher trophic levels, changing nutrient cycling, energy flow, and ecosystem functioning (S et al., 2024).

Despite the growing number of studies on the environmental occurrence and toxicity of NSAIDs, the mechanisms responsible for their varying effect on microalgae remain insufficiently understood (Hejna et al., 2022; Świacka et al., 2021). Differences in structure, physicochemical properties, and transformation pathways may highly influence their environmental persistence and biological activity (S et al., 2024). While ecotoxicological research on NSAIDs provides important reference data, it often does not fully account for how chemical transformation mechanisms modulate toxicity.

Within the NSAIDs group, naproxen (NPX) and nabumetone (NBT) are structurally related drugs with different transformation and activation pathways. NPX undergoes photochemical

modification caused by ultraviolet radiation, which leads to the formation of various hydroxylated, carboxylated, and dimeric photoproducts (Cazzaniga et al., 2020; Kawabata et al., 2019). Seven major photoproducts have been identified, including compounds that may exhibit potentially higher persistence and toxicity than the original compound (Cazzaniga et al., 2020). The formation of persistent and potentially more toxic photoproducts may interfere with essential components of the microalgal photosynthetic apparatus, consequently impairing overall photosynthetic performance. In contrast, NBT is a pharmacologically inactive prodrug that requires enzymatic transformation to 6-methoxy-2-naphthylacetic acid (6-MNA) to exert biological effects. The bioactivation process of NBT involves an initial Baeyer-Villiger oxidation catalyzed by non-cytochrome P450 enzymes, mainly FMO5, followed by further modification by CYP enzymes (Matsumoto et al., 2020, 2021; Varfaj et al., 2014). Its neutral and lipophilic character facilitates uptake and accumulation in algal cells. These delayed mechanisms of toxicity may result in persistent sublethal effects, including growth inhibition and oxidative stress. The contrasting transformation and action mechanisms of NPX and NBT suggest that these pharmaceuticals may differently influence microalgal physiology, photosynthetic performance, and oxidative balance.

Despite the growing number of studies on the toxicity of NSAIDs, the mechanisms responsible for their varying effect on microalgae remain insufficiently understood (Hejna et al., 2022; Świacka et al., 2021). Therefore, the present study aimed to assess and compare the short-term (24h) toxicity of naproxen and nabumetone to the green algae *Desmodesmus armatus*. The experiments were designed to answer the question of whether NBT and NPX, which are structurally related, show similar toxicity towards green algae *D. armatus*, or, on the contrary, whether the effect on algal cells is significantly different. The experiments, for the first time, included the determination of ecotoxicological EC10 and EC50 values for both NSAIDs and the evaluation of their effects on photosynthetic efficiency, oxidative stress indicators, and antioxidant enzyme activities. Understanding the mechanisms that underlie the algae's response to tested substances will significantly expand knowledge about the threat posed by NSAIDs in the aquatic environment and will support evidence-based management of pharmaceutical pollution in aquatic ecosystems.

2. Materials and methods

2.1 Algae cultures

The green microalga *Desmodesmus armatus* (strain 276-4d, SAG, Germany) was transferred from agar slants to HSM liquid medium (Harris, 2009). Experimental cultures were maintained

in glass vessels aerated with filtered air (0.2 μm PTFE membrane, Sartorius), enriched with 2.5% CO_2 , under constant fluorescent light ($125 \pm 5 \mu\text{mol photons/m}^2 \times \text{s}$), and at 30 °C. Cultures in the mid-logarithmic phase, with an initial density of 0.5×10^6 cells/mL, were used for toxicity testing (Aksmann et al., 2014; Aksmann & Tukaj, 2008; Kapuścińska et al., 2024).

2.2 Examined NSAIDs

Nabumetone (NBT, 4-(6-Methoxy-2-naphthyl)-2-butanone; 97% purity, CAS number 42924-53-8, Alfa Aesar, USA) was dissolved in dimethylsulfoxide (DMSO, BioShop Canada Inc., Canada) to a concentration of 100 mg/mL. Naproxen (NPX, (S)-6-Methoxy- α -methyl-2-naphthaleneacetic acid sodium salt, 98% purity, CAS number: 26159-32-2, Alfa Aesar, USA) was dissolved in water to a concentration of 100 mg/L. At the beginning of experiments, the stock solutions of NBT or NPX were added to the culture media to obtain the required concentration range (see 2.3). During the experiments, DMSO concentration in the treated cultures was limited to 0.1 % (v/v) to ensure no measurable solvent impact on algal physiology (Aksmann and Tukaj, 2004).

2.3 ECs estimation

To estimate toxicological EC (effective concentration) parameters, 24-hour growth inhibition experiments were conducted using exponentially growing algal cells exposed to NBT at nominal concentrations of 4, 6, 8, 10, 12 mg/L or NPX at nominal concentrations of 10, 20, 40, 80, 160 mg/L. Population density was measured (see 2.4.1), and the obtained data were fitted to a five-parameter logistic model to determine EC10 and EC50 values using Origin2024 (OriginLab) (see Tab. S1). In the next step, algal cultures were treated for 24 hours with previously established EC10 and EC50 concentrations, NBT: 4.38 and 6.57 mg/L, NPX: 11.03 and 41.2 mg/L. In the 24th hour of the experiment, samples were taken for analysis of both physiological and oxidative stress-related parameters. Control (non-treated) cultures were set up and proceeded along with the treated cultures. To check the real concentrations of the NSAIDs in the culture medium and their stability during the experiment, high-performance liquid chromatography (HPLC) analysis was performed. The analytical procedure and results are given in the Supplementary material, Text S1, Tab. S3.

2.4 Physiological responses

2.4.1 Population density

Population density was assessed by direct microscopic cell counting using a Bürker chamber under a light microscope (magnification 200x; Leica, Germany). To perform this analysis, 10

156 μL of each *D. armatus* culture was placed into a hemocytometer. Cells were counted in multiple
157 fields, and the final cell number was averaged and expressed as cells per mL of culture
158 (Andersen, 2005).

159 2.4.2 Photosynthetic performance

160 Fast chlorophyll fluorescence induction kinetics were applied to evaluate the photochemical
161 efficiency of PSII in *D. armatus* cells (Kapuścińska et al., 2024; Majewska et al., 2021).
162 Samples of cell suspension (2 mL) were collected directly from the culture vessels, diluted to
163 $\text{OD}_{680} = 0.2 \pm 0.01$, dark-adapted for 15 min, and placed in a measuring chamber of Handy
164 PEA+ fluorometer (Hansatech Ltd., UK). The fluorescence signal was measured under a high
165 intensity red light pulse ($3000 \mu\text{mol photons} \times \text{m}^2 \times \text{s}^{-1}$). The resulting OJIP curves were
166 analyzed to extract JIP-test parameters, including F_v/F_m (the maximum quantum yield of
167 photosystem II), PI_{abs} (the performance index), F_v/F_0 (the activity of the water-splitting complex
168 on the donor side of PSII), RCM (fraction (density) of active reaction centers referring to time
169 when maximal fluorescence is reached), ϕP_0 (maximum yield of primary photochemistry), ψ_0
170 (efficiency/probability with which electron trapped in PSII is transferred from Q_A to Q_B), ϕE_0
171 (the maximum yield of the electron transport, quantum yield of the electron transport flux from
172 Q_A to Q_B), ΔR_0 (efficiency/ probability that an electron from the intersystem electron carriers
173 is transferred to reduce final electron acceptors at the PSI acceptor side), ϕR_0 (quantum yield
174 for the reduction of end electron acceptors at the PSI acceptor side), ABS/RC (energy
175 absorption per active PSII reaction center), DI_0/RC (energy dissipation per active PSII reaction
176 center), TR_0/RC (energy trapping per active PSII reaction center), ET_0/RC (efficiency of
177 electron transport per active PSII reaction center), RE_0/RC (reduction of final electron acceptors
178 in PSI calculated per active PSII reaction center).

179 2.4.3 Clark electrode measurements of oxygen

180 Oxygen evolution and consumption were measured using a Clark-type electrode (Oxygraph+
181 system, Hansatech Ltd., UK) equipped with a thermostated chamber and magnetic stirrer. One
182 milliliter of algal cell suspension was taken directly from the culture, placed in the measuring
183 chamber, and oxygen evolution was recorded under light and temperature conditions matching
184 those of the culture. Oxygen consumption was measured in darkness. Oxygen
185 evolution/consumption rates were normalized to cell density (Kapuścińska et al., 2024).

186 2.4.4 Photosynthetic pigments content

187 Photosynthetic pigments were isolated from algal culture as described in Kapuścińska et al.,
188 (2024). Briefly, the cells were collected by centrifugation ($4000 \times g$, 10 min, RT), followed by

homogenization with glass beads (5 m/s, 1 min, FastPrep-24, MP Biomedicals, USA), and extraction from the pellet using 80% acetone. Absorbance readings at 447, 647, 664, and 740 nm were collected using a Varioskan Flash Microplate Reader (Thermo Fisher Scientific, USA). The contents of chlorophyll *a*, chlorophyll *b*, and carotenoids were calculated using Jeffrey & Humphrey, (1975) and Lichtenthaler & Wellburn, (1983) formulas, applying the path length correction described by Warren, (2008).

2.4.5 Mitochondrial Membrane Potential (MMP)

Mitochondrial membrane potential (MMP) was assessed *in vivo* using the fluorescent dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide, Sigma-Aldrich, Germany), according to Harshkova et al., (2019). Briefly, cells were collected by centrifugation ($460 \times g$, 5 min), and pellets were resuspended in HSM medium to a final density of 1 mln cells mL⁻¹. Following 20 min incubation with JC1 in black 96-well plates, fluorescence was measured using a Varioskan Flash Microplate Reader (Thermo Fisher Scientific, USA) at 488 nm excitation and 538/596 nm emission (monomers/oligomers). MMP was expressed as the red/green fluorescence ratio (a.u).

2.5 Oxidative stress and antioxidant defense

2.5.1 Hydrogen peroxide

Hydrogen peroxide production by algae cells was assessed using the AmplexUltraRed reagent (Molecular Probes, USA) as described by Hejna et al., (2024). The reaction mixture contained 10 mM fluorescent dye, 10 U/mL horseradish peroxidase, and phosphate buffer (0.1 M, pH 7.0). After centrifugation of algae cells ($291 \times g$, 5 min), an equal volume of the obtained supernatant was added to the reaction mixture. To validate the assay and correct the results for matrix effects, H₂O₂ was added to some samples (spiked samples) to a final concentration of 3 nmol/mL. Fluorescence was measured after 25-minute incubation of samples at 25 °C, at λ_{ex} =560 nm and λ_{em} =585 nm using a Varioskan Flash Microplate Reader (Thermo Fisher Scientific, USA). The final concentration of H₂O₂ produced by the cells was expressed per million cells.

2.5.2 Proline level in the cells

Proline level in the cells was quantified using the acid ninhydrin colorimetric method adapted from Bates et al., (1973), with modifications by Carillo et al., (2011). Algal cells were collected by centrifugation ($4000 \times g$, 10 min), disrupted by homogenization (5 m/s, 1 min, FastPrep-24, MP Biomedicals, USA) with glass beads in 70% ethanol, then incubated at 70°C for 20 minutes. Following centrifugation at $12000 \times g$ for 5 min, 150 μ L of the supernatant was mixed with 300

μL of freshly prepared ninhydrin reagent. The samples were incubated at 95°C for 20 minutes, and absorbance was measured at 520 nm using a Varioskan Flash Microplate Reader (Thermo Fisher Scientific, USA). Proline content in the cells was calculated using a standard curve with known concentrations of L-proline.

2.5.3 Protein extraction

To isolate protein for enzyme activity assays, algal cells were pelleted by centrifugation (3000 × g, 5 min) and resuspended in dedicated extraction buffers: a phosphate buffer (pH 7.0) for CAT, and a phosphate buffer (pH 7.6) containing ascorbic acid, EDTA, PVP, and sorbitol for APX (Aksmann et al., 2014). The cell suspension was disrupted mechanically using glass beads in a FastPrep-24 (5 m/s, 1 min, MP Biomedicals, USA). The homogenates were centrifuged (20000 × g, 20 min, 4 °C), and the supernatants were collected for further enzymatic assays.

2.5.4 Catalase activity

CAT activity was determined spectrophotometrically (Hitachi U-2900, Japan). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.0) and 10 mM H₂O₂. To monitor the consumption of H₂O₂, 500 μL of supernatant was added to 2500 μL of reaction mixture in a quartz cuvette. The decrease in absorbance at 240 nm ($\epsilon = 43.6 \text{ M}^{-1} \text{ cm}^{-1}$) was recorded (Aebi, 1984). Enzyme activity was standardized based on cell number.

2.5.5 Ascorbate peroxidase activity

APX activity was assayed according to Nakano & Asada, (1981) with slight modifications. The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 18 mM pyrogallol, and 0.1 mM H₂O₂. The reaction was initiated by adding 50 μL supernatant to 150 μL of the reaction mixture. The increase in absorbance at 430 nm was monitored for 5 minutes at 25°C using a spectrofluorometer Varioskan Flash-Spectral Scanning Multimode Microplate Reader (Thermo Fisher Scientific, USA). Enzyme activity was calculated using the molar extinction coefficient of pyrogallol ($\epsilon = 2.47 \text{ mM}^{-1} \text{ cm}^{-1}$). Final activity values were normalized to million algal cells.

2.6 Statistical analysis

The obtained data were statistically analyzed using Statistica 13.1 (StatSoft Europe, Germany) and Origin Pro 10.2 (OriginLab Corp., USA). Results were shown as mean values ± standard error (SE); all experiments were replicated at least three times. Outliers were excluded based on interquartile range analysis. Data were tested for normal distribution (Shapiro-Wilk test) and homogeneity of variance (Levene's test). Since the data did not meet the assumptions for parametric tests, the Kruskal-Wallis with post-hoc Dunn's test was applied. To further deepen

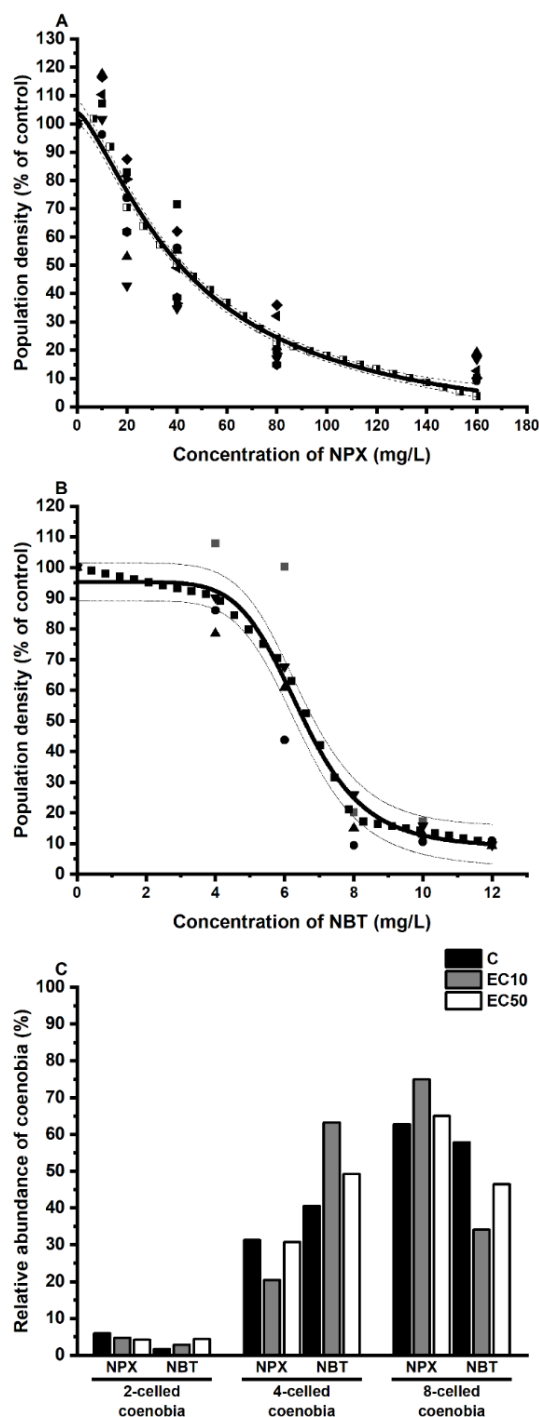
the analysis and strengthen the inferences regarding differences in the mechanism of action of individual substances, we used advanced statistical methods, such as canonical discriminant analysis and correlation matrices.

3. Results

3.1. Phytotoxicity

Both naproxen (NPX) and nabumetone (NBT) induced concentration-dependent growth inhibition of *Desmodemus armatus* 276-4d population after 24 h exposure, although their toxic potency differed (Fig. 1A, B). The concentration-response curves for NBT showed a steep response ($p = 6.96$), indicating threshold-like toxicity, whereas NPX exhibited a more gradual response ($p = 1.41$), consistent with progressive toxicity (Tab. S1). NBT exhibited higher toxicity, with EC50 of 6.57 mg/L (referred to further as EC50_{NBT}), compared to 41.2 mg/L for NPX (referred to further as EC50_{NPX}). EC10 values were 4.38 mg/L for NBT (referred to further as EC10_{NBT}) and 11.03 mg/L for NPX (referred to further as EC10_{NPX}).

Differences between NPX and NBT effects were also visible in population structure (Fig. 1C; Tab. S2). In the control group, the most numerous fractions were eight-celled coenobia, followed by four-celled ones. The fraction of 2-celled coenobia was the smallest. Exposure to EC10_{NPX} increased the disproportion between four- and eight-celled coenobia in favor of eight-celled, while the EC50_{NPX}-treated population remained a control-like structure. In contrast, EC10_{NBT} concentration caused a statistically significant increase in the four-celled coenobia fraction, while the relative abundance of eight-celled coenobia was reduced relative to the control. This reduction in eight-celled forms persisted at both NBT concentrations (Fig. 1C, Tab. S2).



275

276 Fig.1 Growth inhibition and population structure of *Desmodesmus armatus* 276-4d treated with
 277 naproxen (NPX) and nabumetone (NBT); (A, B) concentration-response curves used to
 278 determine EC10 and EC50 values after 24 h exposure to NPX and NBT, respectively; (C)
 279 Distribution of 2-, 4- and 8-celled coenobia in microalgae exposed to EC10 and EC50
 280 concentrations of the NSAIDs. Data are presented as means $\pm$ SE of at least three biological
 281 replicates. Asterisks indicate significant differences compared to the control ($p \leq 0.05$; Kruskal-
 282 Wallis test followed by Dunn's post hoc test).

3.2 Photosynthesis and dark respiration

Exposure to NPX and NBT differently affected the photosynthetic activity of the cells (Fig. 2). In NPX-treated cells, the rate of photosynthetic oxygen evolution was significantly reduced at EC50 concentration (0.740 ± 0.055 nm/mln cell $\times$ min), compared to control (1.507 ± 0.170 nm/mln cell $\times$ min), whereas EC10 concentration showed no significant impact on this parameter (Fig. 2A). These observations were convergent with a significant reduction in chlorophyll *a* (0.572 ± 0.035 pg/mln cell), chlorophyll *b* (0.166 ± 0.018 pg/mln cell), and carotenoid (0.238 ± 0.014 pg/mln cell) content at EC50_{NPX}, while the pigments level remained unchanged at EC10_{NPX}, compared to control (0.848 ± 0.062 ; 0.343 ± 0.016 ; 0.242 ± 0.025 pg/mln cell, for chlorophyll *a*, chlorophyll *b*, and carotenoids, respectively)(Fig. 2B).

Dark respiration intensity did not change under NPX-treatment, suggesting proper mitochondrial functioning at both NPX concentrations, which was in line with unchanged mitochondrial membrane potential (MMP) at both EC10_{NPX} and EC50_{NPX} (Fig. 2C-D).

In NBT-exposed cells, photosynthetic rate was maintained in both EC10- and EC50-treatments (Fig. 2A). Chlorophyll *a* and *b* content remained stable, whereas carotenoid levels increased significantly at EC50_{NBT} (Fig. 2B). Dark respiration was significantly stimulated at EC50_{NBT} (2.066 ± 0.230 nm/mln cell $\times$ min) relative to untreated cells (1.195 ± 0.079 nm/mln cell $\times$ min in control), while MMP showed no changes, indicating preserved mitochondrial function (Fig. 2C-D).

These results indicate that NPX primarily inhibited photosynthetic activity and reduced pigment content at higher concentrations, whereas NBT triggered compensatory increases in carotenoids and elevated dark respiration without strongly impairing photosynthetic rates.

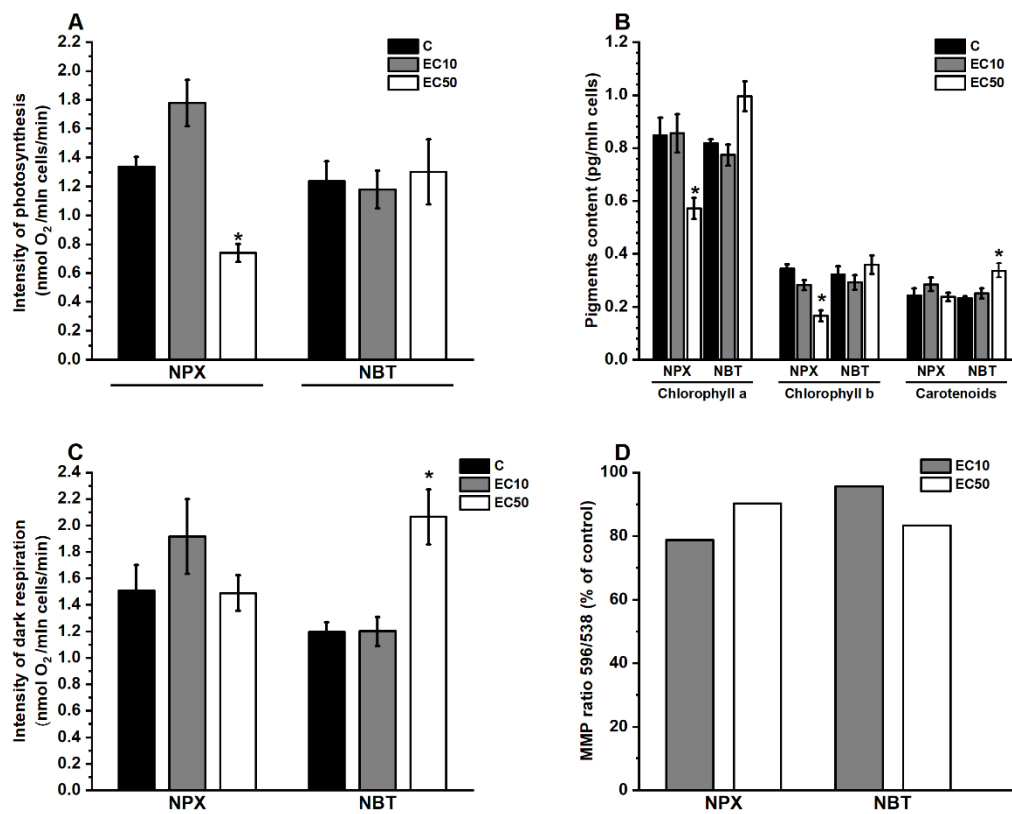


Fig. 2. Cellular responses of *Desmodemus armatus* 276-4d after 24 h exposure to naproxen (NPX) and nabumetone (NBT) at EC10 and EC50 concentrations; (A) photosynthetic performance, (B) photosynthetic pigment content, (C) dark respiration, and (D) mitochondrial membrane potential. Data are presented as means $\pm$ SE of at least three biological replicates. Asterisks indicate significant differences compared to the control ($p \leq 0.05$; Kruskal-Wallis test followed by Dunn's post hoc test).

To further investigate the effects of NPX and NBT on photosynthetic performance, we analyzed chlorophyll *a* fluorescence parameters, which allowed assessment of PSII efficiency, energy fluxes, and photochemical performance under different exposure concentrations (Fig. 3, Tab. S4). NPX caused a concentration-dependent impairment of PSII primary photochemistry. At EC50_{NPX}, the maximum quantum yield of PSII photochemistry (F_V/F_M) decreased to 93% of the control value. This response was accompanied by significant alterations in other parameters, including a reduction in the performance index (PI_{abs} : 47% of the control), a decrease in F_V/F_0 (70% of the control), and an increase in F_0/F_M (133% of the control) (Fig. 3B, Tab. S4). Moreover, a reduced fraction of active PSII reaction centers (RCM, 63% of the control) was noticed. At EC10 concentration, NPX caused more subtle, selective effects, mainly reflected by decreases in F_V/F_0 (87% of the control), PI_{abs} (71% of the control), and RCM (83% of the control) (Fig. 3A, Tab. S4). In the case of NBT, both concentrations had no

significant effect on PSII efficiency. At the EC50 concentration, NBT caused slight, non-significant reductions in F_V/F_0 , RCM, and PI_{abs} (87% of the control) (see Fig. 3 A-B, Tab. S4).

Analysis of parameters of specific energy flux per one active reaction center revealed distinct responses to NPX and NBT (Fig. 3C-D; Tab. S4). Exposure to NPX led to non-significant increases in energy absorption (ABS/RC) and dissipation (DI_0/RC), with only minor changes in energy trapping (TR_0/RC), which were 109%, 124%, and 106% of control at EC10_{NPX} (Fig. 3C; Tab. S4). At EC50_{NPX}, ABS/RC and DI_0/RC increased further to 121% and 162% of the control, respectively, with the increase in DI_0/RC being statistically significant (Fig. 3D; Tab. S4). These results indicate a progressive redistribution of excitation energy toward dissipation under higher NPX exposure. NBT effects on specific energy fluxes were generally minor (Fig. 3C- D, Tab. S4). At EC10_{NBT}, only ABS/RC decreased significantly to 95% of the control, whereas DI_0/RC , TR_0/RC , and ET_0/RC remained essentially unchanged. At EC50_{NBT}, only modest increases in energy dissipation were observed (114% of control, non-significant) (Fig. 3D, Tab. S4).

Subsequently, the effect of NPX and NBT on downstream electron transport and PSI efficiency was assessed. Even at the lower concentration, NPX decreased ψE_0 (92% of the control), ϕE_0 (90% of the control), and ϕR_0 (90% of the control), indicating a decline in the probability and efficiency of electron transport between PS II and PS I and limited reduction of PSI end acceptors (Fig. 3E, Tab. S4). At EC50_{NPX}, the effect was markedly stronger, RE_0/RC decreased to 92%, ψE_0 to 89%, ϕE_0 to 82%, ΔR_0 to 92%, and ϕR_0 to 77% of control, although these changes were not statistically significant (Fig. 3F, Tab. S4). In contrast to NPX, NBT caused only minor deviations in these parameters. At EC10_{NBT}, most of these parameters remained close to the control level (RE_0/RC 102%, ψE_0 101%), while ϕE_0 , ΔR_0 , and ϕR_0 significantly increased to 104, 104, and 107% of the control, suggesting a compensatory response or maintained PSI acceptor function (Fig. 3E, Tab. S4). At EC50_{NBT}, all parameters remained close to the control values, with no significant changes, confirming minimal impact on PSI and electron transport relative to NPX (Fig. 3F, Tab. S4).

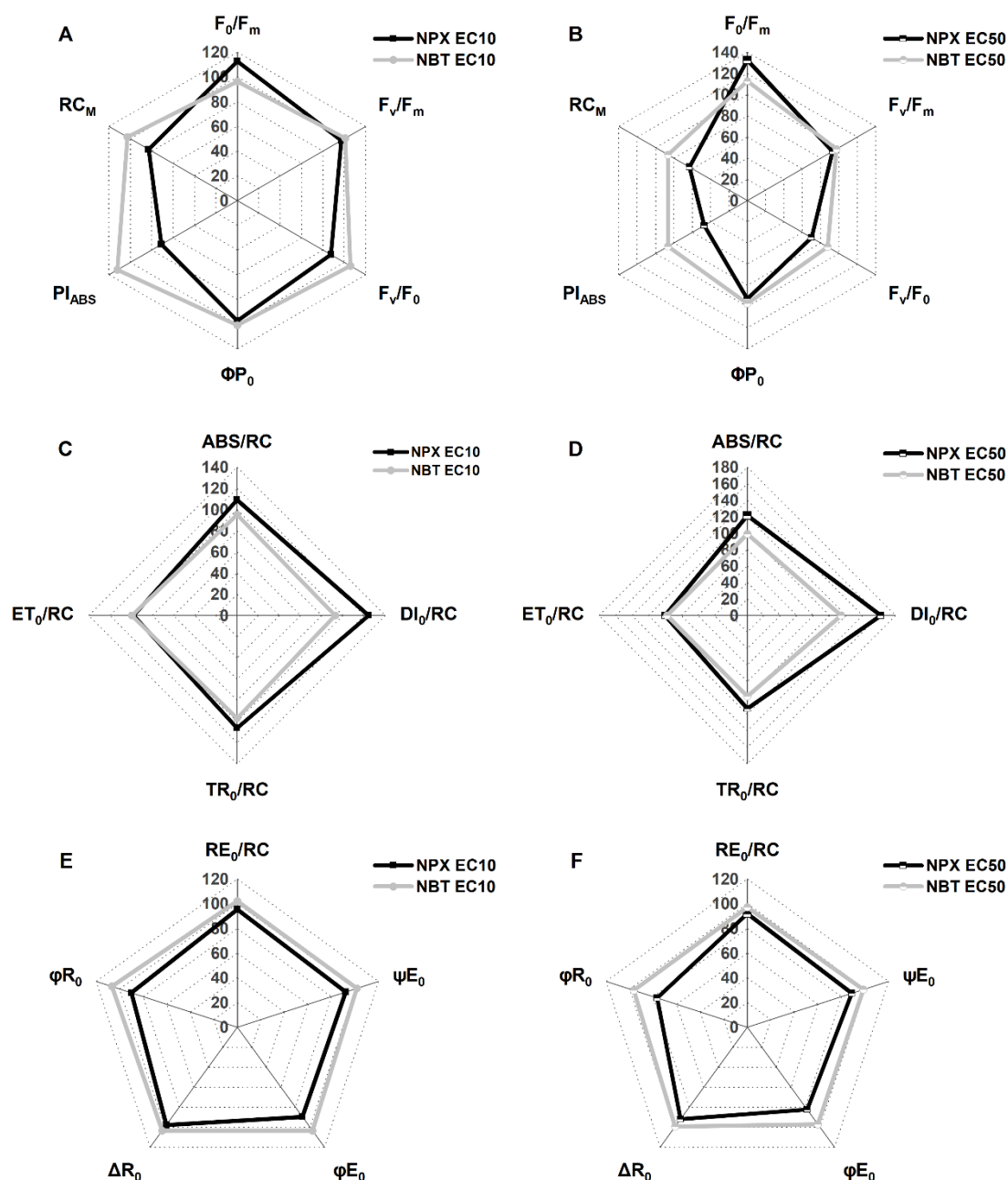


Fig. 3. Radar plots of OJIP-derived photosynthetic parameters obtained for *Desmodemus armatus* 276-4d after NPX and NBT treatment at EC10 and EC50 concentrations; (A-B) PSII photochemistry; (C-D) Specific energy fluxes per reaction center; (E-F) Electron transport efficiencies. Data are presented as means of at least three biological replicates, expressed as % of control. Dark line - NPX; grey line - NBT. For detailed values with statistical analysis, see Supplementary Table S4.

3.2. Oxidative stress and antioxidant defense

Since OJIP analysis revealed dysfunction of the photosynthetic apparatus, the next step was to assess selected stress biomarkers to link photosynthetic disruption with stress response. It was found that exposure to NPX induced a moderate increase in H₂O₂ production at EC₁₀, whereas EC₅₀ triggered pronounced oxidative stress, as significantly evidenced by a rise in H₂O₂ levels to 0.137 ± 0.009 nmol/mln cell, compared to 0.067 ± 0.007 nmol/mln cell in the control cultures (Fig. 4A). In NBT-treated microalgae, H₂O₂ production followed a similar concentration-dependent trend, reaching statistical significance only at EC₅₀ (Fig. 4A).

Analysis of proline content in the cells has shown that the amount of this amino acid in algae treated with EC₅₀_{NPX} was higher than in the control group (Fig. 4B), but this difference was not statistically significant. Proline accumulation was more pronounced in NBT-treated cells, increasing to 1.02 ± 0.094 μ mol/mln cell at EC₁₀_{NBT} and 1.37 ± 0.131 μ mol/mln cell at EC₅₀_{NBT}, with only the EC₅₀ values differing significantly from the control group (0.929 ± 0.094 μ mol/mln cell; Fig. 4B).

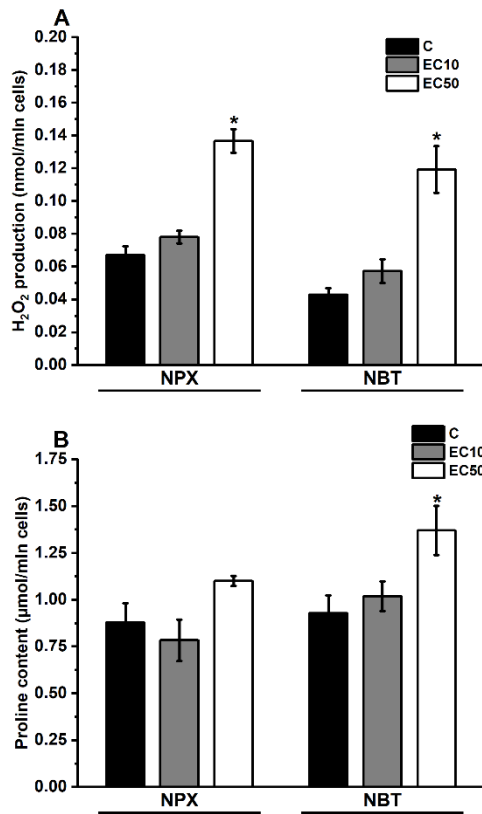


Fig.4. Oxidative stress responses of *Desmodesmus armatus* 276-4d after 24 h exposure to NPX and NBT; (A) Hydrogen peroxide levels; (B) Proline accumulation. Data are presented as means

± SE of at least three biological replicates. Asterisks indicate significant differences compared to the control ($p \leq 0.05$; Kruskal-Wallis test followed by Dunn's post hoc test).

To assess the enzymatic antioxidant response associated with the observed H_2O_2 production, the activities of catalase (CAT) and ascorbate peroxidase (APX) were subsequently analyzed. In NPX-treated cells, APX activity increased from 0.008 U/min $\times$ mln cells in the control group to 0.011 ± 0.002 U/min $\times$ mln cells at $EC_{10_{NPX}}$, and 0.02 ± 0.006 U/min $\times$ mln cells at $EC_{50_{NPX}}$ (Fig. 5A). In contrast, CAT activity decreased from 0.240 U/min $\times$ mln cells in the control to 0.226 U/min $\times$ mln cells at $EC_{10_{NPX}}$ and 0.148 U/min $\times$ mln cells at $EC_{50_{NPX}}$ (Fig. 5B). Under NBT treatment, both APX and CAT activities increased (Fig. 5). APX activity, that in the control amounted to 0.009 U/min $\times$ mln cell, increased in $EC_{10_{NBT}}$ to 0.012 U/min $\times$ mln cells and 0.017 U/min $\times$ mln cells at $EC_{50_{NBT}}$ (Fig. 5A). CAT activity was significantly elevated at $EC_{50_{NBT}}$, amounted to 0.468 U/min $\times$ mln cells, while its activity of 0.284 U/min $\times$ mln cells was observed in the control group. At $EC_{10_{NBT}}$, only a slight increase in CAT activity was noted (0.332 U/min $\times$ mln cells; Fig. 5A).

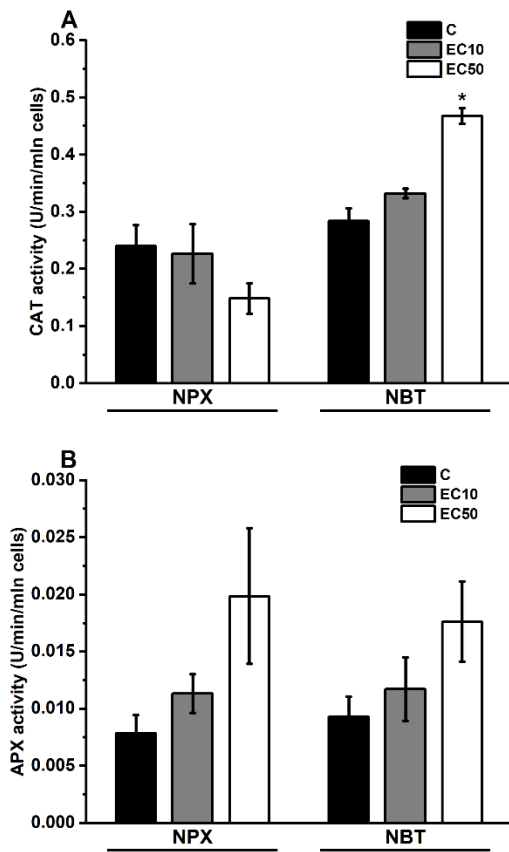


Fig. 5. Antioxidant enzymes activity in *Desmodesmus armatus* 276-4d exposed to NPX and NBT; (A) Catalase activity; (B) Ascorbate peroxidase activity. Data are presented as means

± SE of at least three biological replicates. Asterisks indicate significant differences compared to the control ($p \leq 0.05$; Kruskal-Wallis test followed by Dunn's post hoc test).

3.3. Canonical discriminant and correlation analysis

Following the assessment of photosynthetic performance, oxidative stress, and antioxidant parameters, multivariate analyses were conducted. Canonical discriminant analysis and Spearman correlation were applied to integrate the measured parameters and identify the main factors driving the cellular response to NPX and NBT.

In the NPX-exposed groups, Spearman's test correlation analysis revealed a consistent pattern of statistically significant relationships between oxidative stress markers, photosynthetic performance indices, and photosynthetic energy flux parameters (Fig. 6, Tab. S7). Chlorophyll *a* and *b* contents showed strong negative correlations with key indicators of PSII photochemical efficiency, including F_v/F_m , F_v/F_0 , ϕP_0 , while being positively correlated with parameters associated with energy dissipation, such as DI_0/RC and ABS/RC .

Chlorophyll *a* fluorescence performance indices (PI_{abs}) were positively correlated with maximum quantum efficiency parameters (F_v/F_m , F_v/F_0), whereas negative correlations were observed with DI_0/RC and with electron transport-related variables (TR_0/RC), indicating coordinated changes between photochemical performance and non-photochemical energy dissipation.

Dark respiration showed positive correlations with performance indices (PI_{abs}), while it showed negative correlations with DI_0/RC . Pigment concentrations, particularly chlorophyll *a* and carotenoids, exhibited strong negative correlations with photosynthetic and electron transport parameters (ψE_0 , ΔR_0).

Parameters characterizing population structure, including higher proportions of larger coenobia (4- and 8-celled forms) were strongly associated with reduced dark respiration and electron transport parameter efficiency.

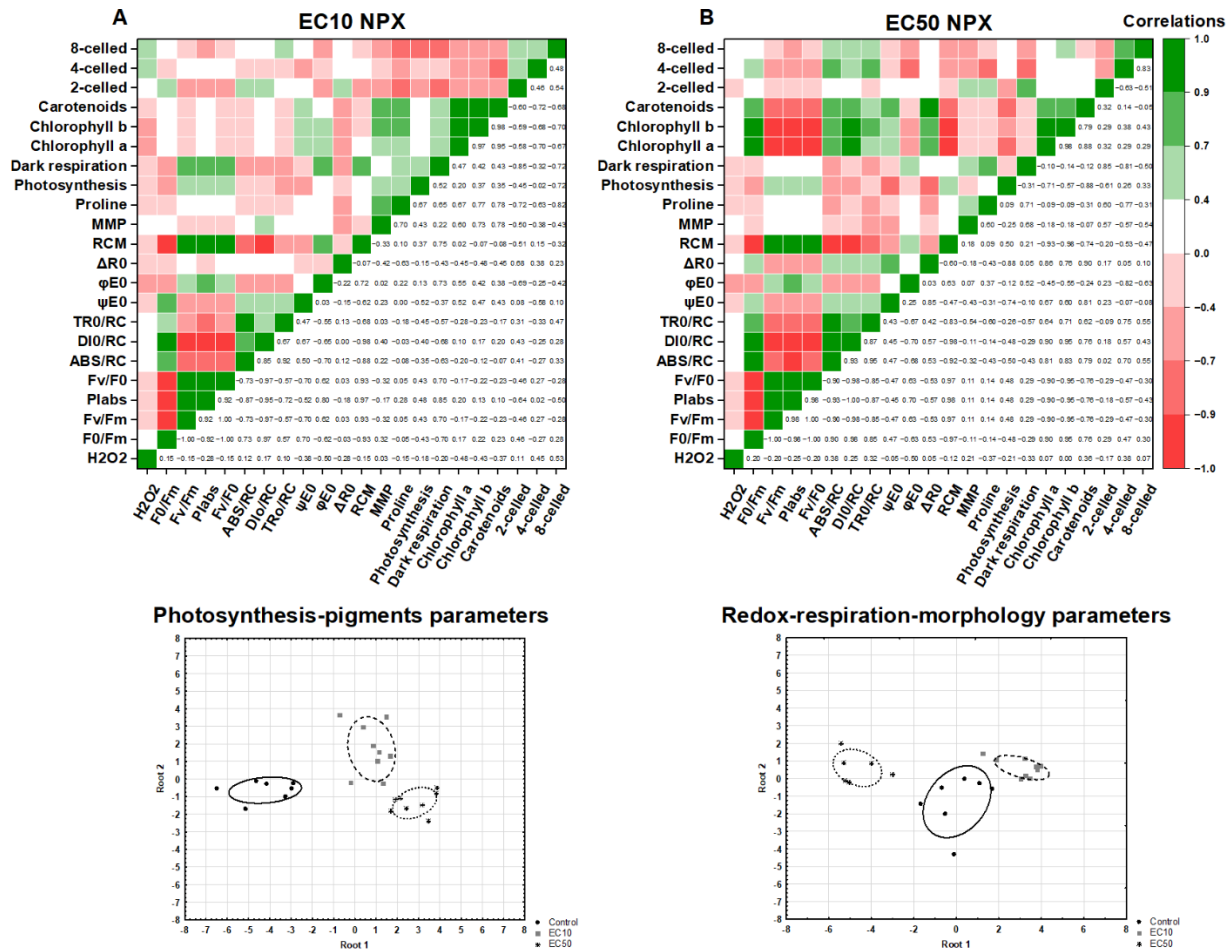


Fig. 6. Multivariate analyses summarizing relationships among the measured parameters under NPX exposure of *Desmodemus armatus* 276-4d. Spearman correlation matrices are shown for the EC10_{NPX} and EC50_{NPX} groups (upper panels); colors indicate correlation coefficient (green = positive, red = negative). Lower panels show canonical discriminant score plots for EC10 and EC50 groups based on photosynthesis/pigment parameters (left; F_v/F_m , Pl_{abs} , photosynthesis intensity, chlorophyll *a*, and chlorophyll *b*) and redox-respiration-population structure parameters (right; H_2O_2 , proline, dark respiration, carotenoids, and proportions of 2-, 4-, and 8-celled coenobia). The ellipses in the figure show the data distribution with a range of coefficients of 0.95.

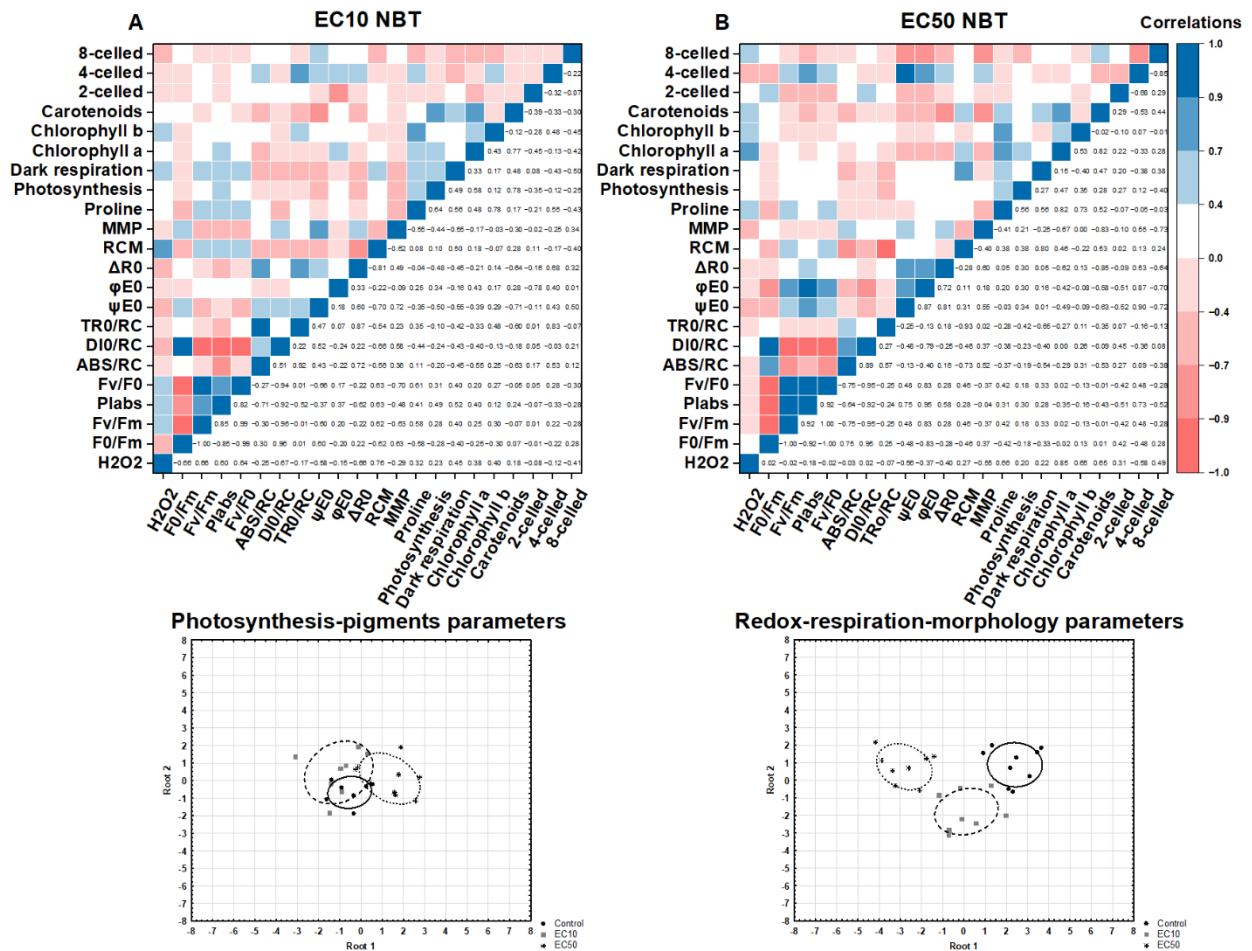


Fig. 7. Multivariate analyses summarizing relationships among the measured parameters under NBT exposure of *Desmodium armatum* 276-4d. Spearman correlation matrices are shown for EC10_{NBT} and EC50_{NBT} groups (upper panels); colors indicate the correlation coefficient (blue = positive, red = negative). Lower panels show canonical discriminant score plots for Control, EC10, and EC50 groups based on photosynthesis-pigment parameters (left; F_v/F_m , PI_{abs} , photosynthesis intensity, chlorophyll *a*, and chlorophyll *b*) and redox-respiration-population structure parameters (right; H_2O_2 , proline, dark respiration, carotenoids, and proportions of 2-, 4-, and 8-celled coenobia). The ellipses in the figure show the data distribution with a range of coefficients of 0.95.

Discriminant analysis was created for a clear separation between the control, EC10, and EC50 groups based on photosynthetic efficiency and respiration parameters (Fig. 6, Tab. S6). The first canonical root was predominantly defined by a very strong positive contribution of F_v/F_m (56.23), indicating its primary role in group discrimination along this axis. Additional contributions to the first function were provided by chlorophyll *a* content (4.116), whereas chlorophyll *b* and PI_{abs} contributed negatively (-14.58 and -4.798, respectively). Contributions of chlorophyll *a* and *b* and PI_{abs} to the second axis were comparatively minor, while F_v/F_m played a limited role in the discrimination along with the second function (5.403). In the respiration intensity context, the first canonical root was primarily defined by strong negative

contributions of H₂O₂ concentration (-26.61) and proline content (-15.43), together with a strong positive contribution of carotenoid content (19.44). The second canonical function was mainly associated with H₂O₂ concentration (13.80) and carotenoid content (5.956), both contributing positively, while the remaining variables exhibited very low discriminative power along this axis.

Overall, the discriminant analysis emphasizes oxidative stress intensity (H₂O₂), stress-related metabolite accumulation (proline), antioxidant pigment content (carotenoids), and maximum quantum yield in photosynthetic chain as dominants in discriminating among the control, EC10, and EC50 groups under NBT exposure.

In the NBT-treated samples, correlation analysis (Spearman's test) demonstrated a highly coherent structure of statistically significant associations among photosynthetic efficiency parameters, oxidative stress markers, and metabolic activity (Fig. 7, Tab. S7). H₂O₂ concentration was negatively correlated with chlorophyll *a* content, indicating an inverse relationship between oxidative stress intensity and photosynthetic efficiency. Strong positive correlations were observed among primary fluorescence parameters (F_v/F_m , F_v/F_0 , ϕP_0) with performance indice (PI_{abs}). These parameters were simultaneously negatively correlated with F_0/F_m and DI_0/RC , reflecting coordinated shifts between excitation energy utilization and dissipation.

Mitochondrial membrane potential (MMP) exhibited significant negative correlations with carotenoid content. Proline content was positively correlated with chlorophyll *a* and *b* and H₂O₂ concentrations, indicating that the main source of free radicals is chloroplasts.

Dark respiration correlated positively with a fraction of active photosynthetic reaction centers (RCM), while decreases in total cell number and higher proportions of larger coenobia (4-cells) were associated with increased photosynthetic indices (PI_{abs}) and electron transport parameters (ψE_0 , ϕE_0 , and ϕR_0).

Like NPX, for a clear separation between the control, EC10, and EC50 groups under NBT treatment, discriminant analysis was created based on photosynthetic efficiency and respiration parameters (Fig. 6, Tab. S6). The first canonical root was primarily defined by strong contributions of F_v/F_m and PI_{abs} , chlorophyll *a* and chlorophyll *b* content, as well as photosynthetic rate. Among these variables, F_v/F_m showed the highest absolute standardized coefficient (-43.88), indicating its dominant role in group discrimination along the first axis. Chlorophyll *a* content (7.393) also contributed strongly and positively to this function. The second canonical function was mainly associated with F_v/F_m (-96.95) and PI_{abs} (4.116), with F_v/F_m again exhibiting a very high absolute coefficient, followed by a positive contribution of

PI_{abs} and chlorophyll *a* content (4.611). According to respiration parameters, the first canonical root was primarily defined by strong negative contributions of carotenoid content (-12.21) and H₂O₂ concentration (-7.578), followed by dark respiration rate (-1.893). The second canonical function was mainly associated with proline accumulation (2.454), which showed a strong positive contribution, and to a lesser extent with H₂O₂ content (-11.04) and dark respiration (0.811).

Overall, the discriminant analysis indicates that the dominant role in discriminating among the control, EC10, and EC50 groups under NBT exposure belongs to maximum quantum yield, H₂O₂ concentration, and carotenoid content.

4. Discussion

Pharmaceuticals are designed to interact with specific molecular targets in living cells, which explains why, even at relatively low environmental concentrations, they may elicit unexpected responses in non-target species such as microalgae (Wojcieszynska and Guzik, 2020). In the present study, to determine whether such effects also occur in *Desmodesmus armatus* 276-4d, we assessed the short-term impact of two chemically similar NSAIDs: naproxen and nabumetone. Although both compounds inhibited population growth over a period of 24 hours, their physiological and biochemical effects differed substantially. The growth inhibition assays revealed that the toxicity of NBT (EC₅₀ = 6.57 mg/L) was about six times higher compared to NPX (EC₅₀ = 41.2 mg/L) (Tab. S3). NPX, in addition to having a higher EC₅₀ value, also had a less steep concentration-response curve ($p=1.41$) compared to NBT, for which the curve was steeper ($p=6.96$) (Fig. 1A-B, Tab. S1). This indicates a more progressive development of toxicity with increasing concentration of NPX, whereas for NBT, a threshold effect was observed. These results were linked to compound-specific changes in photosynthetic inhibition, induction of antioxidant defense mechanisms, and cell stress symptoms. Such varying responses suggest differences in metabolic activation or environmental transformation pathways of these structurally related NSAIDs, providing experimental support for structure-dependent toxicity among pharmaceutical contaminants. Taking the above into account, we decided to check the stability of both substances in the medium during the experiments. Analytical verification by HPLC showed a significant decrease in NBT concentrations in the culture medium, so that after 24 h it was only 4-5% of the initial value (Tab. S3). This result is consistent with the study showing that NBT can be photodegraded under both UV and visible light (Valero and Costa, 2003; Musa and Eriksson, 2008), forming 6-methoxy-2-naphthalene aldehyde and 4-(6-methoxy-2-naphthyl)-3-buten-2-one as the major

products, most likely *via* a NBT radical cation formation, with singlet oxygen generated during photolysis. However, NBT concentration decrease in the culture medium is probably a combination of abiotic degradation, uptake, transformation, and biological degradation.

Nabumetone, as a relatively hydrophobic substance, is expected to be taken up by plants and microalgae primarily *via* passive diffusion across membranes, a process largely controlled by compound lipophilicity and environmental conditions (Carvalho et al., 2014). Although direct data on nabumetone uptake are scarce, studies on NSAIDs indicate that both higher plants and microalgae are capable of accumulating and transforming these compounds (Mulkiewicz et al., 2021; Hejna et al., 2022). In mammals, NBT is transformed to 6-methoxy-2-naphthylacetic acid (6-MNA). This process occurs through oxidative bioactivation catalyzed by flavin-containing monooxygenase (FMO) and non-cytochrome P450 enzymes. Since in microalgae an analogous detoxification system is present, the possibility of NBT transformation by microalgal cells should be considered (Wu et al., 2025). Consequently, environmental assessments relying exclusively on the parent compound may underestimate the true exposure risk, since transformation products of nabumetone with retained or altered biological activity may persist beyond the disappearance of the parent molecule.

In contrast to NBT, the high stability of NPX in the culture medium (Tab. S3) suggests that observed biological effects reflect exposure to the original pharmaceutical rather than its metabolite. NPX caused a concentration-dependent disruption of photosynthesis performance (Fig. 3 Tab. S4). Although NPX toxicity is believed to be mainly connected to UV-mediated photodegradation (Gong et al., 2022; Li et al., 2024), our data show that NPX can reduce photosynthetic activity under constant visible light, indicating that its toxicity is not fully dependent on UV-driven photochemical reactions (Bracchitta et al., 2013). This is of particular relevance in the environment, as UV radiation poorly penetrates the water column of reservoirs where phytoplankton exists. In our study, changes in OJIP parameters showed that NPX treatment induced stress responses associated with the disruption of PSII activity despite UV not being present in the light source. Exposure of algae to EC10_{NPX} resulted in a slight decrease in photosynthetic vitality (PI_{abs}) and in the density of active PSII reaction centers (RCM) (Fig. 3A), and these nonsignificant changes suggest a slight disturbance of cell homeostasis. A modest increase in the photosynthesis intensity (Fig. 2A) may indicate an acclimatory response, e.g., energy redistribution and photosynthesis regulation. According to the literature, low concentrations of NSAIDs may trigger subtle physiological changes described as hormesis. Such changes are often manifested as modifications of cell growth and PSII fluorescence

parameters, rather than chlorophyll degradation (Wang et al., 2020). Our results support these findings, showing that low concentrations of NPX exposure affected PSII activity without pigment loss (Fig. 2B), while at the EC50 concentration NPX led to pronounced PSII dysfunction. A decrease in the photosynthetic vitality parameter (PI_{abs}), integrating information on density of PSII reaction centers, quantum yield of primary photochemistry, and the efficiency of electron transport beyond quinone Q_A , indicates the multifaceted influence of NPX on the photosynthetic apparatus. Indeed, a decrease in RCM with increased ABS/RC and DI_0/RC values suggests the partial deactivation of PSII reaction centers, consistent with the formation of “silent” centers, which increase non-photochemical dissipation of energy. Such effects have also been observed in our previous study on DCF and FFA in *C. reinhardtii*, suggesting constitutive responses to different NSAIDs (Majewska et al., 2018; Kapuścińska et al., 2024). Further, changes caused by NPX in primary photochemistry efficiency (ϕP_0) indicated that the electron transport restriction beyond Q_A (ψE_0 , ϕE_0) resulted from insufficient electron donation by PS II rather than from electron transport chain disruption. As a result, PSI terminal acceptors (ϕR_0) were affected. These photochemical disturbances resemble the processes occurring in plant cells exposed to light stress, where excitation pressure originating from limited electron transport decreased overall photochemical activity and promoted ROS accumulation within the chloroplast (Morgan-Kiss et al., 2006; L. Wang et al., 2023). This provides an explanation for the observed significant reductions in chlorophyll *a* and *b* levels (Fig. 2B) and a decrease in oxygen evolution at $EC50_{NPX}$. Although the increase in the chlorophyll *a/b* ratio indicates a proportionally stronger reduction in chlorophyll *b* level (Tab. S5), suggesting a remodeling of the antenna complex to avoid over-excitation under NPX conditions, carotenoids level were maintained (Maroneze et al., 2019). This indicates a selective impact on chlorophyll maintenance while the photoprotective role of carotenoids was preserved (Pérez-Gálvez et al., 2020; Shi et al., 2020).

The oxidative biomarker profile is consistent with the abovementioned observations. In NPX-treated cells, H_2O_2 production was moderately increased at EC10 and strongly increased at EC50, whereas proline accumulation was practically unchanged, reflecting insufficient osmotic and redox regulation. The specific differences in APX and CAT activity indicate different levels of ROS production in particular cellular organelles (L. Wang et al., 2023). Together, these findings point to a limited compensatory capacity, in which photosynthetic and enzymatic adjustments reduce, but do not prevent, oxidative pressure and loss of photosynthetic efficiency.

Integrated statistical analyses, performed as the next step of our investigations, further supported the interpretation presented above, showing that NPX induces a coordinated response centered on photosynthesis (Fig 6). Within the EC50_{NPX} group, Spearman correlations were consistent with PSII disruption, where a strong negative correlation between F_v/F_m and DI_0/RC was observed. Positive associations of F_v/F_m with RCM suggest that a smaller pool of active PSII reaction centers may contribute to the observed decrease in photochemical efficiency in *D. armatus* treated with NPX_{EC50}. Another important parameter influencing the response profile of NPX-treated cells was the content of chlorophyll *a* and *b*. Both pigments correlated inversely with the main parameters of PSII performance and positively with ABS/RC and DI_0/RC , which may reflect potential reorganization of the light-harvesting antenna rather than nonspecific chlorophyll degradation. Consistent with these patterns, canonical discriminant analysis clearly separated control, EC10, and EC50 groups of NPX. In the photosynthesis-pigment analysis, groups were separated by variation in PSII function and pigment content, implying NPX toxicity is primarily related to photosynthesis and pigment levels disruption. A more distinct separation of treatment groups was observed in the redox/respiration/morphology model of analysis, highlighting the significant role of oxidative stress in the NPX response in algae, mainly due to changes in H_2O_2 concentration and proline content.

In opposite to NPX, NBT did not significantly affect the intensity of photosynthesis or chlorophyll *a* and *b* concentrations in *D. armatus* cells, neither at EC10 nor EC50 concentration. This distinguished it from NPX and indicated that PSII activity was not the dominant target of NBT toxicity. However, at the EC50_{NBT}, a significant increase in carotenoid level and dark respiration intensity was observed, while mitochondrial membrane potential was not affected. To the best of our knowledge, data on the toxicity of NBT in microalgae are limited to our previous studies in *C. reinhardtii*, where enhanced dark respiration and developed cristae were observed (Kapuścińska et al., 2024). This could be a result of the activation of compensatory processes, in which the algae cell intensifies mitochondrial metabolic activity and carotenoid accumulation to maintain redox balance during stress. The OJIP analysis also supported the relatively slight effect of NBT on PSII activity, as F_v/F_m , PI_{abs} , and RCM values were maintained close to those of the untreated cells at both NBT concentrations, with minimal, mostly non-significant changes. Notably, at EC10_{NBT}, a decrease in ABS/RC to approximately 95% of the control value was observed, in line with a modest increase in ϕE_0 , ΔR_0 , and ϕR_0 . These differences probably indicate subtle adjustments in energy absorption and electron transport beyond Q_A , rather than photoinhibition, unlike the PSII dysfunction observed in NPX-

treated cells. Comparing the results with our previous study on *C. reinhardtii*, at $EC_{50_{NBT}}$ concentration, where a reduced efficiency of electron transport beyond Qa (ϕE_0) as well as low efficiency of electron transport to final acceptors of PSI (ΔR_0 , ϕR_0) were observed, it seems that *D. armatus* may reflect differences in physiological plasticity between species (Kapuścińska et al., 2024). Although photosynthetic efficiency remained stable, NBT at EC_{50} caused a significant increase in H_2O_2 production and accumulation of proline, which indicated oxidative and osmotic stress in algal cells. In contrast to NPX, the activity of APX and CAT was higher, which suggests more effective detoxification of H_2O_2 . Furthermore, it appears that changes in coenobia morphology (including an increase in the proportion of 4-celled forms and a decrease in 8-celled forms) may be related to the effect on cell division. This conclusion is supported by studies in animal cells, where NBT's effect on cell proliferation was observed (Grande et al., 2021). However, the potential effect of NBT on cell division remains unclear and requires further detailed analysis. In discriminant analyses, H_2O_2 , proline, and carotenoids had higher potential to differentiate the groups, which indicates that NBT exposure is associated with redox- and metabolic regulation rather than primary photochemical damage. Spearman correlation analysis confirmed that cellular response to NBT is related to oxidative stress, and H_2O_2 and proline showed associations with pigments.

In summary, NPX mainly affected the photosynthesis process, including PSII dysfunction, pigment loss, and oxidative imbalance at EC_{50} . By contrast, NBT showed higher toxicity, as indicated by lower EC_{10} and EC_{50} values, but its effect on primary photochemistry was minimal. The response of NBT-treated cells was characterized by increased carotenoid content, stimulated dark respiration, elevated oxidative and osmotic stress markers, and coordinated APX and CAT activity. These findings indicate that the toxicity of NSAIDs for primary producers like microalgae should be assessed using an integrated set of endpoints, including photosynthetic performance and redox-related biomarkers, rather than only growth inhibition. The difference between NBT and NPX highlights that structurally similar NSAIDs can cause toxicity via distinct physiological pathways, which has direct implications for environmental monitoring and mechanism-based ecological risk assessment.

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CRediT authorship contribution statement

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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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Supplementary material for:

Distinct biochemical effects of naproxen and nabumetone drive their differential toxicity in *Desmodesmus armatus*

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The supporting data contain:

Table S1. Parameters of concentration-response curve fitting for NPX and NBT.

Parameter	NPX	NBT
Model	Logistic	
Equation	$y = A2 + (A1-A2)/(1 + (x/x0)^p)$	
A1	103.9879 ± 2.25102	95.32069 ± 0.91131
A2	-10.55181 ± 4.78283	8.6017 ± 1.52427
x0	44.72341 ± 2.99837	6.48503 ± 0.07147
p	1.40926 ± 0.12148	6.96074 ± 0.48762
Reduced Chi-Sqr	6.44645	8.18647
R-Square (COD)	0.99372	0.99452
Adj. R-Square	0.99282	0.99389

Table S2. Distribution of coenobia number and relative abundance in NPX- and NBT-treated culture. Data are presented as means $\pm$ SE of at least three biological replicates.

NSAID	Concentration	Number of coenobia (per 10 μ L)			Relative abundance of coenobia (%)		
		2-celled coenobia	4-celled coenobia	8-celled coenobia	2-celled coenobia	4-celled coenobia	8-celled coenobia
NPX	C	4.2 $\pm$ 0.8	22.2 $\pm$ 4.8	44.5 $\pm$ 3.4	6	31.2	62.8
	EC10	2.6 $\pm$ 0.4	11.3 $\pm$ 1.6	41.4 $\pm$ 3.5	4.7	20.4	74.9
	EC50	2.9 $\pm$ 0.5	21.9 $\pm$ 3.0	46.4 $\pm$ 2.5	4.1	30.8	65.1
NBT	C	1.2 $\pm$ 0.5	28.5 $\pm$ 2.8	40.7 $\pm$ 1.9	1.7	40.5	57.8
	EC10	2.1 $\pm$ 1.0	49.1 $\pm$ 3.9*	26.5 $\pm$ 1.4	2.8	63.2	34.1
	EC50	2.8 $\pm$ 1.0	31.8 $\pm$ 4.3	30.1 $\pm$ 4.6	4.3	49.2	46.5

Text S1. High-performance liquid chromatography (HPLC) analysis was performed using a Dionex UltiMate 3000 HPLC system (Germering, Germany) equipped with UV detector set at 225 nm. Separation was achieved on an Agilent Zorbax Extend-C18 column (150 mm $\times$ 4.6 mm, 3.5 μ m; Agilent Technologies, Santa Clara, CA, USA) maintained at 40 $^{\circ}$ C. The mobile phase consisted of (A) water acidified with 0.1% (v/v) formic acid and (B) acetonitrile. A linear gradient elution program was applied as follows: 40% B held for 1 min, then increased linearly to 70% B from 1 to 17 min, maintained at 70% B from 17 to 18 min, returned to 40% B in 0.1 min (18–18.1 min), and finally equilibrated at 40% B for 3,9 min (total run time: 22 min). The flow rate was 1.0 mL/min, and the injection volume was 5 μ L. Data acquisition and processing were performed using Chromeleon software (version 7.2.10, Thermo Fisher Scientific). Analytical verification of actual concentrations by HPLC confirmed the high stability of NPX after 24h, while NBT concentrations decreased rapidly, with more than 97% loss observed by 24 h, consistent with its prodrug character

Table S3. Nominal and measured concentrations at EC10 and EC50 for naproxen (NPX) and nabumetone (NBT) in *D. armatus* culture. Data are presented as means $\pm$ SE of at least three biological replicates.

	Expected concentration (mg/L)	Real concentration at 0 h (mg/L)	Real concentration at 24h (mg/L)	Concentration remaining after 24 h (%)
NPX EC10	11.03	10.79 $\pm$ 0.18	8.87 $\pm$ 0.16	82.2
NPX EC50	41.2	36.97 $\pm$ 1.22	33.65 $\pm$ 0.70	91.0
NBT EC10	4.38	2.64 $\pm$ 0.10	0.10 $\pm$ 0.01	3.8
NBT EC50	6.57	3.59 $\pm$ 0.20	0.19 $\pm$ 0.04	5.3

Table S4. Summary of PSII photochemistry, energy fluxes, and electron transport parameters (OJIP test) in *Desmodesmus armatus* treated with NPX and NBT at EC10 and EC50 concentrations. Data are presented as means $\pm$ SE of at least three biological replicates. Bold values indicate significant differences compared to the control ($p \leq 0.05$; Kruskal-Wallis test followed by Dunn's post hoc test).

Parameter	Control	NPX EC10	NPX EC50	Control	NBT EC10	NBT EC50
Primary PSII photochemistry						
Fo/Fm	0.181 $\pm$ 0.01	0.203 $\pm$ 0.013	0.239$\pm$0.013	0.177 $\pm$ 0.005	0.17 $\pm$ 0.005	0.199 $\pm$ 0.007
Fv/Fm	0.819 $\pm$ 0.01	0.797 $\pm$ 0.013	0.761$\pm$0.013	0.824 $\pm$ 0.005	0.83 $\pm$ 0.005	0.801 $\pm$ 0.007
Fv/Fo	4.696 $\pm$ 0.299	4.108 $\pm$ 0.299	3.286$\pm$0.223	4.728 $\pm$ 0.154	4.996 $\pm$ 0.205	4.133 $\pm$ 0.171
$\Phi P0$	0.819 $\pm$ 0.01	0.797 $\pm$ 0.013	0.761$\pm$0.013	0.823 $\pm$ 0.005	0.83 $\pm$ 0.005	0.801 $\pm$ 0.007
PI abs	3.715 $\pm$ 0.251	2.636 $\pm$ 0.212	1.745$\pm$0.138	3.451 $\pm$ 0.174	3.872 $\pm$ 0.133	2.986 $\pm$ 0.19
RCM	1673 $\pm$ 94	1387 $\pm$ 109	1055$\pm$81	1669 $\pm$ 67	1714 $\pm$ 94	1444 $\pm$ 23
Energy fluxes						
ABS/RC	1.629 $\pm$ 0.02	1.777 $\pm$ 0.054	1.978 $\pm$ 0.053	1.641 $\pm$ 0.022	1.561$\pm$0.021	1.623 $\pm$ 0.016
Dl _o /RC	0.296 $\pm$ 0.019	0.367 $\pm$ 0.035	0.48$\pm$0.04	0.287 $\pm$ 0.01	0.266 $\pm$ 0.01	0.328 $\pm$ 0.015
TR _o /RC	1.326 $\pm$ 0.007	1.409 $\pm$ 0.02	1.498 $\pm$ 0.014	1.337 $\pm$ 0.02	1.295 $\pm$ 0.017	1.312 $\pm$ 0.006
ET _o /RC	0.765 $\pm$ 0.012	0.747 $\pm$ 0.018	0.764 $\pm$ 0.016	0.721 $\pm$ 0.01	0.716 $\pm$ 0.01	0.702 $\pm$ 0.009
Electron transport and PSI performance						
RE _o /RC	0.372 $\pm$ 0.008	0.354 $\pm$ 0.008	0.342 $\pm$ 0.01	0.344 $\pm$ 0.004	0.351 $\pm$ 0.006	0.334 $\pm$ 0.008
$\psi E0$	0.574 $\pm$ 0.007	0.53 $\pm$ 0.006	0.51 $\pm$ 0.007	0.544 $\pm$ 0.003	0.553 $\pm$ 0.003	0.537 $\pm$ 0.006
$\phi E0$	0.47 $\pm$ 0.007	0.422 $\pm$ 0.004	0.387$\pm$0.005	0.444 $\pm$ 0.002	0.459$\pm$0.003	0.43 $\pm$ 0.007
$\Delta R0$	0.486 $\pm$ 0.004	0.474 $\pm$ 0.006	0.446 $\pm$ 0.004	0.478 $\pm$ 0.002	0.495$\pm$0.002	0.475 $\pm$ 0.006
$\phi R0$	0.225 $\pm$ 0.003	0.203 $\pm$ 0.001	0.173$\pm$0.003	0.212 $\pm$ 0.002	0.226$\pm$0.002	0.205 $\pm$ 0.006

Tab. S5. Chlorophyll a/b ratio in microalgae exposed to NPX and NBT at EC10 and EC50 concentrations.

Parameter	Control	NPX EC10	NPX EC50	Control	NBT EC10	NBT EC50
Chl a/b ratio	2.47	3.03	3.44	2.54	2.65	2.77

Tab. S6. Canonical coefficients for discriminant analysis of physiological variables in *D. armatus* cells treated with NPX and NBT.

NBT					
Variable	Raw coefficients for canonical variables		Variable	Raw coefficients for canonical variables	
	Root 1	Root 2		Root 1	Root 2
F_v/F_m	-43.884	-96.948	H ₂ O ₂ (nmol/mln cells)	-7.578	-11.043
PI _{abs}	0.196	4.116	Proline (μmol/1 mln cells)	-0.613	2.454
Photosynthesis (nm/1 mln cell/min)	-0.891	-1.418	Dark respiration (nm/1 mln cells/min)	-1.893	0.811
chlorophyll <i>a</i> (pg/1 mln cells)	7.393	4.611	carotenoids (pg/1 mln cells)	-12.211	-6.917
chlorophyll <i>b</i> (pg/1 mln cells)	1.859	-1.970	2coenobia count	-0.111	-0.249
			4coenobia count	-0.068	-0.131
			8coenobia count	0.027	-0.027
Constant	29.666	63.637	Constant	9.431	3.170
Eigenvalue	1.299	0.237	Eigenvalue	5.237	1.696
Cumulative proportion	0.846	1.000	Cumulative proportion	0.755	1.000
NPX					
Variable	Raw coefficients for canonical variables		Variable	Raw coefficients for canonical variables	
	Root 1	Root 2		Root 1	Root 2
F_v/F_m	56.228	5.403	H ₂ O ₂ (nmol/mln cells)	-26.606	13.805
PI _{abs}	-4.798	-1.067	Proline (μmol/1 mln cells)	-15.426	-0.258
Photosynthesis (nm/1 mln cell/min)	0.602	3.347	Dark respiration (nm/1 mln cells/min)	1.236	-0.213
chlorophyll <i>a</i> (pg/1 mln cells)	4.115	0.827	carotenoids (pg/1 mln cells)	19.443	5.956
chlorophyll <i>b</i> (pg/1 mln cells)	-14.578	0.956	2coenobia count	-0.233	-0.360
Constant	-31.845	-6.711	4coenobia count	0.056	-0.068
Eigenvalue	9.203	2.092	8coenobia count	-0.121	0.052
Constant	-31.845	-6.711	Constant	13.740	-0.479
Eigenvalue	9.203	2.092	Eigenvalue	11.302	0.937
Cumulative proportion	0.815	1.000	Cumulative proportion	0.923	1.000

Table S7. Spearman correlation coefficients for measured parameters in *D. armatus* exposed to nabumetone and naproxen.

Control for NBT

Parameters	H ₂ O ₂	F ₀ /F _m	F _v /F _m	PI _{abs}	F _v /F ₀	ABS/RC	DI ₀ /RC	TR ₀ /RC	ψE ₀	φE ₀	ΔR ₀	RCM	MMP	Proline	Photosynthesis	Dark respiration	Chlorophyll _a	Chlorophyll _b	carotenoids	2-celled	4-celled	8-celled
H ₂ O ₂	1.00	-0.87	0.87	0.44	0.87	-0.13	-0.79	0.25	-0.24	0.32	0.25	0.65	-0.13	0.87	0.59	0.43	-0.21	0.48	0.13	0.00	-0.47	0.40
F ₀ /F _m	-0.87	1.00	-1.00	-0.69	-0.99	0.05	0.93	-0.13	0.21	-0.45	-0.28	-0.72	0.34	-0.73	-0.39	-0.50	0.00	-0.15	0.32	-0.14	0.70	-0.76
F _v /F _m	0.87	-1.00	1.00	0.69	0.99	-0.05	-0.93	0.13	-0.21	0.45	0.28	0.72	-0.34	0.73	0.39	0.50	0.00	0.15	-0.32	0.14	-0.70	0.76
PI _{abs}	0.44	-0.69	0.69	1.00	0.66	-0.65	-0.83	-0.44	-0.04	0.65	0.45	0.69	-0.44	0.38	0.50	0.25	-0.39	-0.33	-0.50	0.12	-0.82	0.95
F _v /F ₀	0.87	-0.99	0.99	0.66	1.00	-0.03	-0.91	0.14	-0.27	0.32	0.24	0.73	-0.36	0.69	0.36	0.50	0.00	0.15	-0.32	0.14	-0.70	0.76
ABS/RC	-0.13	0.05	-0.05	-0.65	-0.03	1.00	0.40	0.80	-0.30	-0.28	-0.62	-0.43	0.27	-0.04	-0.75	0.22	0.86	0.31	0.26	0.05	0.43	-0.56
DI ₀ /RC	-0.79	0.93	-0.93	-0.83	-0.91	0.40	1.00	0.21	0.12	-0.60	-0.44	-0.79	0.43	-0.70	-0.59	-0.50	0.39	0.15	0.30	-0.27	0.87	-0.91
TR ₀ /RC	0.25	-0.13	0.13	-0.44	0.14	0.80	0.21	1.00	0.06	-0.22	-0.64	-0.26	0.43	0.27	-0.38	-0.06	0.79	0.75	0.23	-0.37	0.40	-0.45
ψE ₀	-0.24	0.21	-0.21	-0.04	-0.27	-0.30	0.12	0.06	1.00	0.25	0.37	-0.19	0.52	-0.10	0.12	-0.55	0.14	0.26	-0.36	-0.54	0.05	-0.19
φE ₀	0.32	-0.45	0.45	0.65	0.32	-0.28	-0.60	-0.22	0.25	1.00	0.47	-0.03	0.08	0.67	0.48	0.48	0.14	-0.14	0.60	0.00	-0.37	-0.03
ΔR ₀	0.25	-0.28	0.28	0.45	0.24	-0.62	-0.44	-0.64	0.37	0.47	1.00	0.22	0.10	0.22	0.60	0.33	-0.14	-0.64	-0.36	0.50	-0.29	0.00
RCM	0.65	-0.72	0.72	0.69	0.73	-0.43	-0.79	-0.26	-0.19	-0.03	0.22	1.00	-0.48	0.56	0.61	0.25	-0.46	-0.08	-0.47	0.27	-0.70	0.88
MMP	-0.13	0.34	-0.34	-0.44	-0.36	0.27	0.43	0.43	0.52	0.08	0.10	-0.48	1.00	0.06	-0.07	-0.10	0.50	0.27	0.05	-0.24	0.82	-0.90
Proline	0.87	-0.73	0.73	0.38	0.69	-0.04	-0.70	0.27	-0.10	0.67	0.22	0.56	0.06	1.00	0.60	0.50	0.14	0.62	0.38	-0.05	-0.17	0.12
Photosynthesis	0.59	-0.39	0.39	0.50	0.36	-0.75	-0.59	-0.38	0.12	0.48	0.60	0.61	-0.07	0.60	1.00	0.10	-0.93	-0.02	0.00	0.14	-0.18	0.16
Dark respiration	0.43	-0.50	0.50	0.25	0.50	0.22	-0.50	-0.06	-0.55	0.48	0.33	0.25	-0.10	0.50	0.10	1.00	0.21	-0.22	0.32	0.41	-0.17	0.21
Chlorophyll _a	-0.21	0.00	0.00	-0.39	0.00	0.86	0.39	0.79	0.14	0.14	-0.14	-0.46	0.50	0.14	-0.93	0.21	1.00	0.57	-0.11	-0.63	0.39	-0.38
Chlorophyll _b	0.48	-0.15	0.15	-0.33	0.15	0.31	0.15	0.75	0.26	-0.14	-0.64	-0.08	0.27	0.62	-0.02	-0.22	0.57	1.00	-0.02	-0.56	0.38	-0.26
carotenoids	0.13	0.32	-0.32	-0.50	-0.32	0.26	0.30	0.23	-0.36	0.60	-0.36	-0.47	0.05	0.38	0.00	0.32	-0.11	-0.02	1.00	0.07	0.07	-0.38
2-celled	0.00	-0.14	0.14	0.12	0.14	0.05	-0.27	-0.37	-0.54	0.00	0.50	0.27	-0.24	-0.05	0.14	0.41	-0.63	-0.56	0.07	1.00	-0.22	0.03
4-celled	-0.47	0.70	-0.70	-0.82	-0.70	0.43	0.87	0.40	0.05	-0.37	-0.29	-0.70	0.82	-0.17	-0.18	-0.17	0.39	0.38	0.07	-0.22	1.00	-0.85
8-celled	0.40	-0.76	0.76	0.95	0.76	-0.56	-0.91	-0.45	-0.19	-0.03	0.00	0.88	-0.90	0.12	0.16	0.21	-0.38	-0.26	-0.38	0.03	-0.85	1.00

NBT at EC10

Parameters	H ₂ O ₂	F ₀ /F _m	F _v /F _m	PI _{abs}	F _v /F ₀	ABS/RC	DI ₀ /RC	TR ₀ /RC	ψE ₀	φE ₀	ΔR ₀	RCM	MMP	Proline	Photosynthesis	Dark respiration	Chlorophyll _a	Chlorophyll _b	carotenoids	2-celled	4-celled	8-celled
H ₂ O ₂	1.00	-0.66	0.66	0.60	0.64	-0.25	-0.67	-0.17	-0.58	-0.15	-0.66	0.76	-0.29	0.32	0.23	0.45	0.38	0.40	0.18	-0.08	-0.12	-0.41
F ₀ /F _m	-0.66	1.00	-1.00	-0.85	-0.99	0.30	0.96	0.01	0.60	-0.20	0.22	-0.62	0.63	-0.58	-0.28	-0.40	-0.25	-0.30	0.07	-0.01	-0.22	0.28
F _v /F _m	0.66	-1.00	1.00	0.85	0.99	-0.30	-0.96	-0.01	-0.60	0.20	-0.22	0.62	-0.63	0.58	0.28	0.40	0.25	0.30	-0.07	0.01	0.22	-0.28
PI _{abs}	0.60	-0.85	0.85	1.00	0.82	-0.71	-0.92	-0.52	-0.37	0.37	-0.62	0.63	-0.48	0.41	0.49	0.52	0.40	0.12	0.24	-0.07	-0.33	-0.28
F _v /F ₀	0.64	-0.99	0.99	0.82	1.00	-0.27	-0.94	0.01	-0.66	0.17	-0.22	0.63	-0.70	0.61	0.31	0.40	0.20	0.27	-0.05	0.05	0.28	-0.30
ABS/RC	-0.25	0.30	-0.30	-0.71	-0.27	1.00	0.51	0.92	0.43	-0.22	0.72	-0.56	0.36	0.11	-0.20	-0.45	-0.55	0.25	-0.63	0.17	0.53	0.12
DI ₀ /RC	-0.67	0.96	-0.96	-0.92	-0.94	0.51	1.00	0.22	0.52	-0.24	0.22	-0.66	0.58	-0.44	-0.24	-0.43	-0.40	-0.13	-0.18	0.05	-0.03	0.21
TR ₀ /RC	-0.17	0.01	-0.01	-0.52	0.01	0.92	0.22	1.00	0.47	0.07	0.87	-0.54	0.23	0.35	-0.10	-0.42	-0.33	0.48	-0.60	0.01	0.83	-0.07
ψE ₀	-0.58	0.60	-0.60	-0.37	-0.66	0.43	0.52	0.47	1.00	0.18	0.60	-0.70	0.72	-0.35	-0.50	-0.55	-0.39	0.29	-0.71	-0.11	0.43	0.50
φE ₀	-0.15	-0.20	0.20	0.37	0.17	-0.22	-0.24	0.07	0.18	1.00	0.33	-0.22	-0.09	0.25	0.34	-0.16	0.43	0.17	0.28	-0.78	0.40	0.01
ΔR ₀	-0.66	0.22	-0.22	-0.62	-0.22	0.72	0.22	0.87	0.60	0.33	1.00	-0.81	0.49	-0.04	-0.48	-0.45	-0.21	0.14	-0.64	-0.16	0.68	0.32
RCM	0.76	-0.62	0.62	0.63	0.63	-0.56	-0.66	-0.54	-0.70	-0.22	-0.81	1.00	-0.62	0.08	0.10	0.50	0.18	-0.07	0.28	0.11	-0.17	-0.40
MMP	-0.29	0.63	-0.63	-0.48	-0.70	0.36	0.58	0.23	0.72	-0.09	0.49	-0.62	1.00	-0.55	-0.44	-0.55	-0.17	-0.03	-0.30	-0.02	-0.25	0.34
Proline	0.32	-0.58	0.58	0.41	0.61	0.11	-0.44	0.35	-0.35	0.25	-0.04	0.08	-0.55	1.00	0.64	0.56	0.48	0.78	0.17	-0.21	0.55	-0.43
Photosynthesis	0.23	-0.28	0.28	0.49	0.31	-0.20	-0.24	-0.10	-0.50	0.34	-0.48	0.10	-0.44	0.64	1.00	0.49	0.58	0.12	0.78	-0.35	-0.12	-0.25
Dark respiration	0.45	-0.40	0.40	0.52	0.40	-0.45	-0.43	-0.42	-0.55	-0.16	-0.45	0.50	-0.55	0.56	0.49	1.00	0.33	0.17	0.48	0.08	-0.43	-0.50
Chlorophyll <i>a</i>	0.38	-0.25	0.25	0.40	0.20	-0.55	-0.40	-0.33	-0.39	0.43	-0.21	0.18	-0.17	0.48	0.58	0.33	1.00	0.43	0.77	-0.45	-0.13	-0.42
Chlorophyll <i>b</i>	0.40	-0.30	0.30	0.12	0.27	0.25	-0.13	0.48	0.29	0.17	0.14	-0.07	-0.03	0.78	0.12	0.17	0.43	1.00	-0.12	-0.28	0.48	-0.45
carotenoids	0.18	0.07	-0.07	0.24	-0.05	-0.63	-0.18	-0.60	-0.71	0.28	-0.64	0.28	-0.30	0.17	0.78	0.48	0.77	-0.12	1.00	-0.39	-0.33	-0.30
2-celled	-0.08	-0.01	0.01	-0.07	0.05	0.17	0.05	0.01	-0.11	-0.78	-0.16	0.11	-0.02	-0.21	-0.35	0.08	-0.45	-0.28	-0.39	1.00	-0.32	-0.07
4-celled	-0.12	-0.22	0.22	-0.33	0.28	0.53	-0.03	0.83	0.43	0.40	0.68	-0.17	-0.25	0.55	-0.12	-0.43	-0.13	0.48	-0.33	-0.32	1.00	-0.22
8-celled	-0.41	0.28	-0.28	-0.28	-0.30	0.12	0.21	-0.07	0.50	0.01	0.32	-0.40	0.34	-0.43	-0.25	-0.50	-0.42	-0.45	-0.30	-0.07	-0.22	1.00

NBT at EC50

Parameters	H ₂ O ₂	F ₀ /F _m	F _v /F _m	PI _{abs}	F _v /F ₀	ABS/RC	DI ₀ /RC	TR ₀ /RC	ψE ₀	φE ₀	ΔR ₀	RCM	MMP	Proline	Photosynthesis	Dark respiration	Chlorophyll _a	Chlorophyll _b	carotenoids	2-celled	4-celled	8-celled
H ₂ O ₂	1.00	0.02	-0.02	-0.18	-0.02	-0.03	0.02	-0.07	-0.56	-0.37	-0.40	0.27	-0.55	0.66	0.20	0.22	0.85	0.65	0.65	0.31	-0.58	0.49
F ₀ /F _m	0.02	1.00	-1.00	-0.92	-1.00	0.75	0.95	0.25	-0.48	-0.83	-0.28	-0.46	0.37	-0.42	-0.18	-0.33	-0.02	0.13	0.01	0.42	-0.48	0.28
F _v /F _m	-0.02	-1.00	1.00	0.92	1.00	-0.75	-0.95	-0.25	0.48	0.83	0.28	0.46	-0.37	0.42	0.18	0.33	0.02	-0.13	-0.01	-0.42	0.48	-0.28
PI _{abs}	-0.18	-0.92	0.92	1.00	0.92	-0.64	-0.92	-0.24	0.75	0.95	0.58	0.28	-0.04	0.31	0.30	0.28	-0.35	-0.16	-0.43	-0.51	0.73	-0.52
F _v /F ₀	-0.02	-1.00	1.00	0.92	1.00	-0.75	-0.95	-0.25	0.48	0.83	0.28	0.46	-0.37	0.42	0.18	0.33	0.02	-0.13	-0.01	-0.42	0.48	-0.28
ABS/RC	-0.03	0.75	-0.75	-0.64	-0.75	1.00	0.89	0.57	-0.13	-0.40	0.16	-0.73	0.52	-0.37	-0.19	-0.54	-0.29	0.31	-0.53	0.27	0.09	-0.38
DI ₀ /RC	0.02	0.95	-0.95	-0.92	-0.95	0.89	1.00	0.27	-0.48	-0.79	-0.25	-0.46	0.37	-0.38	-0.23	-0.40	0.00	0.26	-0.09	0.45	-0.36	0.08
TR ₀ /RC	-0.07	0.25	-0.25	-0.24	-0.25	0.57	0.27	1.00	-0.25	-0.13	0.18	-0.93	0.02	-0.28	-0.42	-0.65	-0.27	0.11	-0.35	0.07	-0.16	-0.13
ψE ₀	-0.56	-0.48	0.48	0.75	0.48	-0.13	-0.48	-0.25	1.00	0.87	0.81	0.31	0.55	-0.03	0.34	0.01	-0.49	-0.09	-0.63	-0.52	0.90	-0.72
φE ₀	-0.37	-0.83	0.83	0.95	0.83	-0.40	-0.79	-0.13	0.87	1.00	0.72	0.11	0.18	0.20	0.30	0.16	-0.42	-0.08	-0.58	-0.51	0.87	-0.70
ΔR ₀	-0.40	-0.28	0.28	0.58	0.28	0.16	-0.25	0.18	0.81	0.72	1.00	-0.28	0.60	0.05	0.30	0.05	-0.62	0.13	-0.85	-0.09	0.53	-0.64
RCM	0.27	-0.46	0.46	0.28	0.46	-0.73	-0.46	-0.93	0.31	0.11	-0.28	1.00	-0.40	0.38	0.38	0.80	0.46	-0.22	0.53	0.02	0.13	0.24
MMP	-0.55	0.37	-0.37	-0.04	-0.37	0.52	0.37	0.02	0.55	0.18	0.60	-0.40	1.00	-0.41	0.21	-0.25	-0.67	0.00	-0.83	-0.10	0.55	-0.73
Proline	0.66	-0.42	0.42	0.31	0.42	-0.37	-0.38	-0.28	-0.03	0.20	0.05	0.38	-0.41	1.00	0.56	0.56	0.82	0.73	0.52	-0.07	-0.05	-0.03
Photosynthesis	0.20	-0.18	0.18	0.30	0.18	-0.19	-0.23	-0.42	0.34	0.30	0.30	0.38	0.21	0.56	1.00	0.27	0.47	0.35	0.28	0.27	0.12	-0.40
Dark respiration	0.22	-0.33	0.33	0.28	0.33	-0.54	-0.40	-0.65	0.01	0.16	0.05	0.80	-0.25	0.56	0.27	1.00	0.15	-0.40	0.47	0.20	-0.38	0.38
Chlorophyll <i>a</i>	0.85	-0.02	0.02	-0.35	0.02	-0.29	0.00	-0.27	-0.49	-0.42	-0.62	0.46	-0.67	0.82	0.47	0.15	1.00	0.53	0.82	0.22	-0.33	0.28
Chlorophyll <i>b</i>	0.65	0.13	-0.13	-0.16	-0.13	0.31	0.26	0.11	-0.09	-0.08	0.13	-0.22	0.00	0.73	0.35	-0.40	0.53	1.00	-0.02	-0.10	0.07	-0.01
carotenoids	0.65	0.01	-0.01	-0.43	-0.01	-0.53	-0.09	-0.35	-0.63	-0.58	-0.85	0.53	-0.83	0.52	0.28	0.47	0.82	-0.02	1.00	0.29	-0.53	0.44
2-celled	0.31	0.42	-0.42	-0.51	-0.42	0.27	0.45	0.07	-0.52	-0.51	-0.09	0.02	-0.10	-0.07	0.27	0.20	0.22	-0.10	0.29	1.00	-0.68	0.29
4-celled	-0.58	-0.48	0.48	0.73	0.48	0.09	-0.36	-0.16	0.90	0.87	0.53	0.13	0.55	-0.05	0.12	-0.38	-0.33	0.07	-0.53	-0.68	1.00	-0.85
8-celled	0.49	0.28	-0.28	-0.52	-0.28	-0.38	0.08	-0.13	-0.72	-0.70	-0.64	0.24	-0.73	-0.03	-0.40	0.38	0.28	-0.01	0.44	0.29	-0.85	1.00

Control for NPX

Parameters	H ₂ O ₂	F ₀ /F _m	F _v /F _m	PI _{abs}	F _v /F ₀	ABS/RC	DI ₀ /RC	TR ₀ /RC	ψE ₀	φE ₀	ΔR ₀	RCM	MMP	Proline	Photosynthesis	Dark respiration	Chlorophyll _a	Chlorophyll _b	carotenoids	2-celled	4-celled	8-celled
H ₂ O ₂	1.00	0.33	-0.31	-0.40	-0.45	0.12	0.32	0.00	-0.72	-0.88	0.07	-0.28	0.48	-0.45	-0.10	-0.36	-0.38	0.85	-0.57	0.13	0.28	0.60
F ₀ /F _m	0.33	1.00	-1.00	-0.83	-0.98	0.87	0.98	-0.33	-0.18	-0.62	0.27	-0.97	0.19	-0.25	-0.31	0.60	0.10	0.18	-0.07	-0.02	-0.54	0.08
F _v /F _m	-0.31	-1.00	1.00	0.83	0.98	-0.86	-0.98	0.38	0.18	0.61	-0.25	0.95	-0.22	0.28	0.34	-0.59	-0.07	-0.16	0.08	0.03	0.52	-0.10
PI _{abs}	-0.40	-0.83	0.83	1.00	0.90	-0.64	-0.86	0.18	0.00	0.74	-0.48	0.83	-0.50	0.29	0.71	-0.21	0.07	-0.17	0.19	-0.67	0.51	-0.21
F _v /F ₀	-0.45	-0.98	0.98	0.90	1.00	-0.83	-0.97	0.33	0.23	0.72	-0.30	0.95	-0.26	0.28	0.36	-0.48	-0.03	-0.28	0.15	-0.03	0.49	-0.17
ABS/RC	0.12	0.87	-0.86	-0.64	-0.83	1.00	0.90	0.05	0.03	-0.43	0.43	-0.87	-0.07	0.13	-0.38	0.74	0.43	-0.07	0.32	0.03	-0.59	-0.28
DI ₀ /RC	0.32	0.98	-0.98	-0.86	-0.97	0.90	1.00	-0.26	-0.20	-0.63	0.30	-0.98	0.24	-0.27	-0.43	0.57	0.08	0.10	-0.08	0.10	-0.51	0.10
TR ₀ /RC	0.00	-0.33	0.38	0.18	0.33	0.05	-0.26	1.00	0.26	0.12	0.52	0.31	-0.61	0.67	-0.21	0.36	0.60	0.05	0.62	0.21	-0.01	-0.57
ψE ₀	-0.72	-0.18	0.18	0.00	0.23	0.03	-0.20	0.26	1.00	0.70	0.57	0.18	-0.36	0.60	-0.24	0.48	0.65	-0.37	0.68	-0.17	-0.69	-0.70
φE ₀	-0.88	-0.62	0.61	0.74	0.72	-0.43	-0.63	0.12	0.70	1.00	-0.08	0.58	-0.50	0.53	0.38	0.17	0.42	-0.58	0.57	-0.32	-0.11	-0.62
ΔR ₀	0.07	0.27	-0.25	-0.48	-0.30	0.43	0.30	0.52	0.57	-0.08	1.00	-0.28	0.14	0.27	-0.81	0.24	0.52	0.23	0.33	0.10	-0.73	-0.32
RCM	-0.28	-0.97	0.95	0.83	0.95	-0.87	-0.98	0.31	0.18	0.58	-0.28	1.00	-0.24	0.25	0.36	-0.52	-0.10	-0.07	0.10	-0.08	0.53	-0.08
MMP	0.48	0.19	-0.22	-0.50	-0.26	-0.07	0.24	-0.61	-0.36	-0.50	0.14	-0.24	1.00	-0.88	-0.71	-0.61	-0.71	0.33	-0.86	0.43	0.12	0.86
Proline	-0.45	-0.25	0.28	0.29	0.28	0.13	-0.27	0.67	0.60	0.53	0.27	0.25	-0.88	1.00	0.40	0.71	0.88	-0.27	0.92	-0.25	-0.30	-0.93
Photosynthesis	-0.10	-0.31	0.34	0.71	0.36	-0.38	-0.43	-0.21	-0.24	0.38	-0.81	0.36	-0.71	0.40	1.00	0.10	0.14	-0.14	0.24	-0.57	0.34	-0.31
Dark respiration	-0.36	0.60	-0.59	-0.21	-0.48	0.74	0.57	0.36	0.48	0.17	0.24	-0.52	-0.61	0.71	0.10	1.00	0.90	-0.36	0.93	-0.19	-0.65	-0.88
Chlorophyll _a	-0.38	0.10	-0.07	0.07	-0.03	0.43	0.08	0.60	0.65	0.42	0.52	-0.10	-0.71	0.88	0.14	0.90	1.00	-0.17	0.93	-0.33	-0.60	-0.95
Chlorophyll _b	0.85	0.18	-0.16	-0.17	-0.28	-0.07	0.10	0.05	-0.37	-0.58	0.23	-0.07	0.33	-0.27	-0.14	-0.36	-0.17	1.00	-0.35	-0.18	0.08	0.38
carotenoids	-0.57	-0.07	0.08	0.19	0.15	0.32	-0.08	0.62	0.68	0.57	0.33	0.10	-0.86	0.92	0.24	0.93	0.93	-0.35	1.00	-0.37	-0.45	-0.98
2-celled	0.13	-0.02	0.03	-0.67	-0.03	0.03	0.10	0.21	-0.17	-0.32	0.10	-0.08	0.43	-0.25	-0.57	-0.19	-0.33	-0.18	-0.37	1.00	0.03	0.38
4-celled	0.28	-0.54	0.52	0.51	0.49	-0.59	-0.51	-0.01	-0.69	-0.11	-0.73	0.53	0.12	-0.30	0.34	-0.65	-0.60	0.08	-0.45	0.03	1.00	0.47
8-celled	0.60	0.08	-0.10	-0.21	-0.17	-0.28	0.10	-0.57	-0.70	-0.62	-0.32	-0.08	0.86	-0.93	-0.31	-0.88	-0.95	0.38	-0.98	0.38	0.47	1.00

NPX at EC10

Parameters	H ₂ O ₂	F ₀ /F _m	F _v /F _m	PI _{abs}	F _v /F ₀	ABS/RC	DI ₀ /RC	TR ₀ /RC	ψE ₀	φE ₀	ΔR ₀	RCM	MMP	Proline	Photosynthesis	Dark respiration	Chlorophyll _a	Chlorophyll _b	carotenoids	2-celled	4-celled	8-celled
H ₂ O ₂	1.00	0.15	-0.15	-0.28	-0.15	0.12	0.17	0.10	-0.38	-0.50	-0.28	-0.15	0.03	-0.15	-0.18	-0.20	-0.48	-0.43	-0.37	0.11	0.45	0.53
F ₀ /F _m	0.15	1.00	-1.00	-0.92	-1.00	0.73	0.97	0.57	0.70	-0.62	-0.03	-0.93	0.32	-0.05	-0.43	-0.70	0.17	0.22	0.23	0.46	-0.27	0.28
F _v /F _m	-0.15	-1.00	1.00	0.92	1.00	-0.73	-0.97	-0.57	-0.70	0.62	0.03	0.93	-0.32	0.05	0.43	0.70	-0.17	-0.22	-0.23	-0.46	0.27	-0.28
PI _{abs}	-0.28	-0.92	0.92	1.00	0.92	-0.87	-0.95	-0.72	-0.52	0.80	-0.18	0.97	-0.17	0.28	0.48	0.85	0.20	0.13	0.10	-0.64	0.02	-0.50
F _v /F ₀	-0.15	-1.00	1.00	0.92	1.00	-0.73	-0.97	-0.57	-0.70	0.62	0.03	0.93	-0.32	0.05	0.43	0.70	-0.17	-0.22	-0.23	-0.46	0.27	-0.28
ABS/RC	0.12	0.73	-0.73	-0.87	-0.73	1.00	0.85	0.92	0.50	-0.70	0.12	-0.88	0.22	-0.08	-0.35	-0.63	-0.20	-0.12	-0.07	0.41	-0.27	0.33
DI ₀ /RC	0.17	0.97	-0.97	-0.95	-0.97	0.85	1.00	0.67	0.67	-0.65	0.00	-0.98	0.40	-0.03	-0.40	-0.68	0.10	0.17	0.20	0.43	-0.25	0.28
TR ₀ /RC	0.10	0.57	-0.57	-0.72	-0.57	0.92	0.67	1.00	0.47	-0.55	0.13	-0.68	0.03	-0.18	-0.45	-0.57	-0.28	-0.23	-0.17	0.31	-0.33	0.47
ψE ₀	-0.38	0.70	-0.70	-0.52	-0.70	0.50	0.67	0.47	1.00	0.03	-0.15	-0.62	0.23	0.00	-0.52	-0.37	0.52	0.47	0.43	0.08	-0.58	0.10
φE ₀	-0.50	-0.62	0.62	0.80	0.62	-0.70	-0.65	-0.55	0.03	1.00	-0.22	0.72	0.02	0.22	0.13	0.73	0.55	0.42	0.38	-0.69	-0.25	-0.42
ΔR ₀	-0.28	-0.03	0.03	-0.18	0.03	0.12	0.00	0.13	-0.15	-0.22	1.00	-0.07	-0.42	-0.63	-0.15	-0.43	-0.45	-0.48	-0.45	0.68	0.38	0.23
RCM	-0.15	-0.93	0.93	0.97	0.93	-0.88	-0.98	-0.68	-0.62	0.72	-0.07	1.00	-0.33	0.10	0.37	0.75	0.02	-0.07	-0.08	-0.51	0.15	-0.32
MMP	0.03	0.32	-0.32	-0.17	-0.32	0.22	0.40	0.03	0.23	0.02	-0.42	-0.33	1.00	0.70	0.43	0.22	0.60	0.73	0.78	-0.50	-0.38	-0.43
Proline	-0.15	-0.05	0.05	0.28	0.05	-0.08	-0.03	-0.18	0.00	0.22	-0.63	0.10	0.70	1.00	0.67	0.65	0.67	0.77	0.78	-0.72	-0.63	-0.82
Photosynthesis	-0.18	-0.43	0.43	0.48	0.43	-0.35	-0.40	-0.45	-0.52	0.13	-0.15	0.37	0.43	0.67	1.00	0.52	0.20	0.37	0.35	-0.45	-0.02	-0.72
Dark respiration	-0.20	-0.70	0.70	0.85	0.70	-0.63	-0.68	-0.57	-0.37	0.73	-0.43	0.75	0.22	0.65	0.52	1.00	0.47	0.42	0.43	-0.85	-0.32	-0.72
Chlorophyll <i>a</i>	-0.48	0.17	-0.17	0.20	-0.17	-0.20	0.10	-0.28	0.52	0.55	-0.45	0.02	0.60	0.67	0.20	0.47	1.00	0.97	0.95	-0.58	-0.70	-0.67
Chlorophyll <i>b</i>	-0.43	0.22	-0.22	0.13	-0.22	-0.12	0.17	-0.23	0.47	0.42	-0.48	-0.07	0.73	0.77	0.37	0.42	0.97	1.00	0.98	-0.59	-0.68	-0.70
carotenoids	-0.37	0.23	-0.23	0.10	-0.23	-0.07	0.20	-0.17	0.43	0.38	-0.45	-0.08	0.78	0.78	0.35	0.43	0.95	0.98	1.00	-0.60	-0.72	-0.68
2-celled	0.11	0.46	-0.46	-0.64	-0.46	0.41	0.43	0.31	0.08	-0.69	0.68	-0.51	-0.50	-0.72	-0.45	-0.85	-0.58	-0.59	-0.60	1.00	0.46	0.54
4-celled	0.45	-0.27	0.27	0.02	0.27	-0.27	-0.25	-0.33	-0.58	-0.25	0.38	0.15	-0.38	-0.63	-0.02	-0.32	-0.70	-0.68	-0.72	0.46	1.00	0.48
8-celled	0.53	0.28	-0.28	-0.50	-0.28	0.33	0.28	0.47	0.10	-0.42	0.23	-0.32	-0.43	-0.82	-0.72	-0.72	-0.67	-0.70	-0.68	0.54	0.48	1.00

NPX at EC50

Parameters	H ₂ O ₂	F ₀ /F _m	F _v /F _m	PI _{abs}	F _v /F ₀	ABS/RC	DI ₀ /RC	TR ₀ /RC	ψE ₀	φE ₀	ΔR ₀	RCM	MMP	Proline	Photosynthesis	Dark respiration	Chlorophyll _a	Chlorophyll _b	carotenoids	2-celled	4-celled	8-celled
H ₂ O ₂	1.00	0.20	-0.20	-0.25	-0.20	0.38	0.25	0.32	-0.05	-0.50	0.05	-0.12	0.21	-0.37	-0.21	-0.33	0.07	0.00	0.36	-0.17	0.38	0.07
F ₀ /F _m	0.20	1.00	-1.00	-0.98	-1.00	0.90	0.98	0.85	0.47	-0.63	0.53	-0.97	-0.11	-0.14	-0.48	-0.29	0.90	0.95	0.76	0.29	0.47	0.30
F _v /F _m	-0.20	-1.00	1.00	0.98	1.00	-0.90	-0.98	-0.85	-0.47	0.63	-0.53	0.97	0.11	0.14	0.48	0.29	-0.90	-0.95	-0.76	-0.29	-0.47	-0.30
PI _{abs}	-0.25	-0.98	0.98	1.00	0.98	-0.93	-1.00	-0.87	-0.45	0.70	-0.57	0.98	0.11	0.14	0.48	0.29	-0.90	-0.95	-0.76	-0.18	-0.57	-0.43
F _v /F ₀	-0.20	-1.00	1.00	0.98	1.00	-0.90	-0.98	-0.85	-0.47	0.63	-0.53	0.97	0.11	0.14	0.48	0.29	-0.90	-0.95	-0.76	-0.29	-0.47	-0.30
ABS/RC	0.38	0.90	-0.90	-0.93	-0.90	1.00	0.93	0.95	0.47	-0.68	0.53	-0.92	-0.32	-0.43	-0.50	-0.43	0.81	0.83	0.79	0.02	0.70	0.55
DI ₀ /RC	0.25	0.98	-0.98	-1.00	-0.98	0.93	1.00	0.87	0.45	-0.70	0.57	-0.98	-0.11	-0.14	-0.48	-0.29	0.90	0.95	0.76	0.18	0.57	0.43
TR ₀ /RC	0.32	0.85	-0.85	-0.87	-0.85	0.95	0.87	1.00	0.43	-0.67	0.42	-0.83	-0.54	-0.60	-0.26	-0.57	0.64	0.71	0.62	-0.09	0.75	0.55
ψE ₀	-0.05	0.47	-0.47	-0.45	-0.47	0.47	0.45	0.43	1.00	0.25	0.85	-0.47	-0.43	-0.31	-0.74	-0.10	0.67	0.60	0.81	0.23	-0.07	-0.08
φE ₀	-0.50	-0.63	0.63	0.70	0.63	-0.68	-0.70	-0.67	0.25	1.00	0.03	0.63	0.07	0.37	-0.12	0.52	-0.45	-0.55	-0.24	0.23	-0.82	-0.63
ΔR ₀	0.05	0.53	-0.53	-0.57	-0.53	0.53	0.57	0.42	0.85	0.03	1.00	-0.60	-0.18	-0.43	-0.88	0.05	0.86	0.76	0.90	0.17	0.05	0.10
RCM	-0.12	-0.97	0.97	0.98	0.97	-0.92	-0.98	-0.83	-0.47	0.63	-0.60	1.00	0.18	0.09	0.50	0.21	-0.93	-0.98	-0.74	-0.20	-0.53	-0.47
MMP	0.21	-0.11	0.11	0.11	0.11	-0.32	-0.11	-0.54	-0.43	0.07	-0.18	0.18	1.00	0.60	-0.25	0.68	-0.18	-0.18	-0.07	0.57	-0.57	-0.54
Proline	-0.37	-0.14	0.14	0.14	0.14	-0.43	-0.14	-0.60	-0.31	0.37	-0.43	0.09	0.60	1.00	0.09	0.71	-0.09	-0.09	-0.31	0.60	-0.77	-0.31
Photosynthesis	-0.21	-0.48	0.48	0.48	0.48	-0.50	-0.48	-0.26	-0.74	-0.12	-0.88	0.50	-0.25	0.09	1.00	-0.31	-0.71	-0.57	-0.88	-0.61	0.26	0.33
Dark respiration	-0.33	-0.29	0.29	0.29	0.29	-0.43	-0.29	-0.57	-0.10	0.52	0.05	0.21	0.68	0.71	-0.31	1.00	-0.10	-0.14	-0.12	0.85	-0.81	-0.50
Chlorophyll <i>a</i>	0.07	0.90	-0.90	-0.90	-0.90	0.81	0.90	0.64	0.67	-0.45	0.86	-0.93	-0.18	-0.09	-0.71	-0.10	1.00	0.98	0.88	0.32	0.29	0.29
Chlorophyll <i>b</i>	0.00	0.95	-0.95	-0.95	-0.95	0.83	0.95	0.71	0.60	-0.55	0.76	-0.98	-0.18	-0.09	-0.57	-0.14	0.98	1.00	0.79	0.29	0.38	0.43
carotenoids	0.36	0.76	-0.76	-0.76	-0.76	0.79	0.76	0.62	0.81	-0.24	0.90	-0.74	-0.07	-0.31	-0.88	-0.12	0.88	0.79	1.00	0.32	0.14	-0.05
2-celled	-0.17	0.29	-0.29	-0.18	-0.29	0.02	0.18	-0.09	0.23	0.23	0.17	-0.20	0.57	0.60	-0.61	0.85	0.32	0.29	0.32	1.00	-0.63	-0.51
4-celled	0.38	0.47	-0.47	-0.57	-0.47	0.70	0.57	0.75	-0.07	-0.82	0.05	-0.53	-0.57	-0.77	0.26	-0.81	0.29	0.38	0.14	-0.63	1.00	0.83
8-celled	0.07	0.30	-0.30	-0.43	-0.30	0.55	0.43	0.55	-0.08	-0.63	0.10	-0.47	-0.54	-0.31	0.33	-0.50	0.29	0.43	-0.05	-0.51	0.83	1.00

Gdańsk, 01.06.2026

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OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w manuskrypt:

*Kapuścińska D., Harshkova D., Shams M., Liakh I., Wielgomas B., Aksmann A.
Distinct biochemical effects of naproxen and nabumetone drive their differential toxicity in
Desmodemus armatus (2026)*

polegał na:

(i) przeprowadzeniu eksperymentów obejmujących hodowlę glonów, wyznaczanie wartości EC oraz oznaczanie liczebności komórek; (ii) wykonaniu analiz fotosyntetycznych, w tym intensywności fotosyntezy, analizy fluorescencji chlorofilu *a in vivo*, oznaczeniu zawartości barwników, oznaczeniu intensywności oddychania ciemniowego oraz oznaczeniu zawartości proliny; (iii) opracowaniu wyników; (iv) udziale w walidacji i interpretacji wyników; (v) przygotowaniu grafik i rycin przedstawiających wyniki badań; (vi) przygotowaniu pierwotnej wersji manuskryptu; oraz (vii) udziale w redagowaniu manuskryptu.

Kapuścińska Dominika

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Gdańsk, 01.06.2026

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Wita Stwosza 59, 80-308 Gdańsk

OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w manuskrypt:

*Kapuścińska D., Harshkova D., Shams M., Liakh I., Wielgomas B., Aksmann A.
Distinct biochemical effects of naproxen and nabumetone drive their differential toxicity in
Desmodemus armatus (2026)*

polegał na:

(i) udziale w przeprowadzeniu eksperymentów obejmujących hodowlę glonów oraz oznaczanie liczebności komórek; (ii) oznaczeniu mitochondrialnego potencjału błonowego (MMP) oraz produkcji nadtlenku wodoru; (iii) analizie statystycznej wyników; (iv) udziale w walidacji i interpretacji wyników; (v) współtworzeniu rozdziału dot. opisu uzyskanych wyników; (vi) udziale w redagowaniu manuskryptu.



PODPIS

Lodz, 01.06.2026

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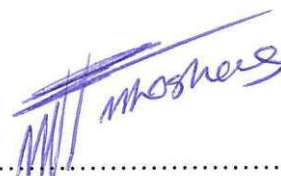
STATEMENT

I hereby declare that my contribution to the manuscript:

*Kapuścińska D., Harshkova D., Shams M., Liakh I., Wielgomas B., Aksmann A.
Distinct biochemical effects of naproxen and nabumetone drive their differential toxicity in
Desmodemus armatus (2026)*

included the participation in:

(i) conducting experiments involving enzyme activity analyses; (ii) performing formal analysis of the obtained data; and (iii) participating in the editing of the manuscript.



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SIGNATURE

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OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w manuskrypt:

*Kapuścińska D., Harshkova D., Shams M., Liakh I., Wielgomas B., Aksmann A.
Distinct biochemical effects of naproxen and nabumetone drive their differential toxicity in
Desmodemus armatus (2026)*

polegał na:

(i) oznaczeniu rzeczywistych stężeń badanych związków metodą HPLC; (ii) przeprowadzeniu formalnej analizy uzyskanych danych; oraz (iii) udziale w redagowaniu manuskryptu.

Ivan Liakh

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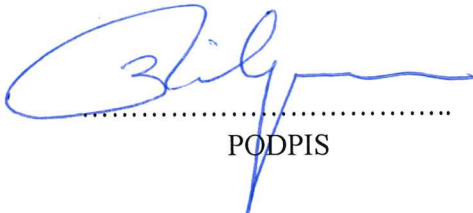
OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w manuskrypt:

Kapuścińska D., Harshkova D., Shams M., Liakh I., Wielgomas B., Aksmann A.
Distinct biochemical effects of naproxen and nabumetone drive their differential toxicity in
Desmodemus armatus (2026)

polegał na:

(i) udziale w opracowaniu koncepcji części analitycznej badań; (ii) sprawowaniu nadzoru nad poprawnością analiz wykonywanych metodą HPLC; (iii) udziale w opracowaniu i walidacji wyników uzyskanych metodą HPLC; (iv) udziale w redagowaniu manuskryptu.



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PODPIS

Gdańsk, 01.06.2026

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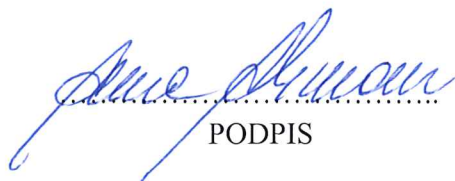
OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w manuskrypt:

*Kapuścińska D., Harshkova D., Shams M., Liakh I., Wielgomas B., Aksmann A. Distinct
biochemical effects of naproxen and nabumetone drive their differential toxicity in
Desmodemus armatus (2026)*

polegał na:

(i) przygotowaniu koncepcji badań; (ii) udziale w walidacji i interpretacji wyników; (iii) nadzorze nad realizacją badań; (iv) pozyskaniu finansowania; (v) udziale w przygotowaniu pierwotnej wersji manuskryptu; oraz (vi) udziale w redagowaniu manuskryptu.



PODPIS

14. Praca nr 5.

NSAID-induced stress triggers physiological and morphological plasticity in *Desmodemus armatus*: a multi-biomarker approach

Kapuścińska D., Harshkova D., Shams M., Liakh I., Wielgomas B., Aksmann A.
(2026)

NSAID-induced stress triggers physiological and morphological plasticity in *Desmodesmus armatus*: a multi-biomarker approach

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Highlights

Multi-biomarker profiling revealed compound-specific NSAID toxicity.
FFA promoted metabolic reprogramming despite strong growth inhibition.
IBU elicited buffered photochemical adjustment with limited oxidative stress.
DCF caused oxidative photosynthetic collapse and pigment degradation.
Morphological plasticity may indicate resilience to pharmaceutical pollution.

Abstract:

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most frequently detected pharmaceutical contaminants in aquatic ecosystems; however, the mechanisms underlying microalgal acclimation to NSAID-induced stress remain poorly understood. Here, we applied an integrated multi-biomarker approach to investigate physiological and morphological responses of the freshwater green microalga *Desmodesmus armatus* exposed for 24 h to diclofenac (DCF), ibuprofen (IBU), and flufenamic acid (FFA) at sublethal effect concentrations (EC10 and EC50). Endpoints included photosynthetic performance, pigment

content, oxygen metabolism, oxidative stress markers, antioxidant enzymes, proline accumulation, and morphological structure of population. Although all NSAIDs tested inhibited population growth, they induced distinct stress-response strategies. FFA was the most growth-inhibiting compound and triggered an energetically costly acclimation response characterized by enhanced dark respiration, oxidative stress, increased ascorbate peroxidase activity, proline accumulation, and a pronounced shift toward 4-celled coenobia, while overall photosynthetic performance was maintained. IBU triggered a buffered photochemical adjustment, causing moderate alterations in PSII function and increased proline accumulation with limited effects on oxidative balance and cellular metabolism. In contrast, DCF induced an oxidative photosynthetic collapse marked by severe inhibition of PSII photochemistry and electron transport, pigment depletion, elevated hydrogen peroxide production, catalase inhibition, and disruption of cellular bioenergetics. Multivariate analyses confirmed compound-specific physiological fingerprints. Our findings demonstrate that NSAIDs producing comparable growth inhibition can activate different physiological pathways in *D. armatus*. The results highlight morphological plasticity and metabolic reprogramming as important components of microalgal resistance to pharmaceutical contamination and emphasize the value of multi-biomarker approaches for detecting sublethal ecotoxicological effects.

Keywords

pharmaceutical contaminants; *Desmodesmus armatus*; multi-biomarker approach; physiological plasticity; morphological plasticity.

1. Introduction

Unicellular photosynthetic microalgae are increasingly recognized as sensitive yet highly adaptable organisms that respond rapidly to environmental stressors through coordinated physiological and biochemical adjustments. These responses include modifications in photosynthetic performance, such as alterations in energy flow within photosystems, activation of antioxidant defense systems to scavenge reactive oxygen species (ROS), and accumulation of protective osmolytes (Barera & Forlani, 2023; Koletti et al., 2025; Singh et al., 2024). Such physiological plasticity enables microalgae to maintain cellular homeostasis under adverse conditions and underpins their ecological success. Consequently, microalgae are considered valuable bioindicators of aquatic contamination and promising tools for biotechnological applications, including bioremediation and biomass production (Alqahtani &

Altoaimi, 2026; Barkia et al., 2019; Gauthier et al., 2020; Hejna et al., 2022; Parmar et al., 2016; Wu et al., 2017).

Among emerging aquatic contaminants, pharmaceuticals have attracted growing attention due to their increasing consumption, continuous environmental release, and chemical diversity. Non-steroidal anti-inflammatory drugs (NSAIDs) are particularly relevant in ecotoxicological studies owing to their frequent detection in aquatic ecosystems and their potential to induce chronic effects in primary producers even at low concentrations (Hejna et al., 2022). Beyond their direct toxic effects, NSAIDs may act as drivers of adaptive plasticity by disturbing redox homeostasis and triggering complex stress responses. Diclofenac (DCF) and ibuprofen (IBU) are among the most widely used NSAIDs and are commonly detected in surface waters, while flufenamic acid (FFA), although less frequently studied, has also been reported in aquatic environments and warrants ecotoxicological evaluation (Kapuścińska et al., 2024, 2026; Świacka et al., 2021).

The environmental relevance of these compounds is highlighted by their occurrence in wastewater and surface waters. For instance, Alygizakis et al., (2019) reported the presence of over 100 pharmaceuticals in sewage samples, with cumulative concentrations reaching up to 17,220 ng/L. Diclofenac was detected at all sampling sites (36.1–1356.3 ng/L), while ibuprofen reached concentrations up to 1046.3 ng/L. Flufenamic acid, although present at lower concentrations (1.0–42.8 ng/L), contributes to the overall pharmaceutical burden. Despite occurring at relatively low levels, long-term exposure to such compounds, often in mixtures, may lead to subtle yet biologically significant alterations in aquatic organisms.

The ability to acclimate to environmental stressors is a characteristic feature of many microalgae and depends on both species-specific traits and the nature of the contaminant (Lin et al., 2020). Species of the genus *Desmodesmus* are considered highly resilient and adaptable, exhibiting pronounced physiological and morphological plasticity under fluctuating environmental conditions, including anthropogenic pollution (Peña-Castro et al., 2004). A key manifestation of this plasticity is the ability to transition between unicellular forms and multicellular coenobia. This morphological shift is increasingly recognized as an adaptive strategy that may enhance resistance to stress by improving structural protection and reducing vulnerability to external factors. Although coenobia formation has been linked to biotic and abiotic stressors such as grazing pressure, bacterial interactions, and heavy metal exposure (Peña-Castro et al., 2004; Trainor & Egan, 1991), its role in response to pharmaceutical contaminants remains largely unexplored.

In addition to morphological changes, *Desmodesmus* species possess effective physiological defense mechanisms, including a robust antioxidant system and structural cell wall adaptations. Their characteristic two-layered cell wall, composed of cellulose-polysaccharide and glycoprotein-rich layers, provides enhanced protection against environmental stressors (Staehelin & Pickett-Heaps, 1975; Voigt et al., 2014). Furthermore, these microalgae exhibit efficient ROS detoxification through antioxidant enzymes and non-enzymatic metabolites such as proline and carotenoids (Bouzidi et al., 2022; Javed et al., 2024; Piotrowska-Niczyporuk et al., 2024). Under stress conditions, they can modulate photosynthetic processes and redox balance, reflecting a high degree of cellular plasticity (Pokora et al., 2014; Singh et al., 2024; Ware et al., 2020).

Among the species within this genus, *Desmodesmus armatus* remains relatively underexplored in the context of pharmaceutical ecotoxicology, despite its considerable adaptive potential (Cao et al., 2020; Lima et al., 2025). This species exhibits strong antioxidant capacity, including pronounced free radical scavenging activity associated with its polysaccharide fractions (Cao et al., 2020). Its ability to regulate antioxidant defenses and accumulate protective metabolites under stress conditions makes it a suitable model for studying adaptive responses to pharmaceutical exposure (El-Enany & Issa, 2001). However, to date, no study has comprehensively evaluated both the physiological and morphological plasticity of *D. armatus* under NSAID exposure using an integrative multi-biomarker framework.

Given the complexity of cellular responses to pharmaceutical pollutants, single-parameter assessments may not adequately capture the full spectrum of stress-induced alterations. Therefore, a multi-biomarker approach that integrates physiological, biochemical, and morphological responses is essential for understanding the mechanisms underlying acclimation and tolerance in microalgae.

We hypothesize that exposure to NSAIDs induces both physiological and morphological plasticity in *Desmodesmus armatus*, manifested as coordinated changes in photosynthetic performance, redox homeostasis, antioxidant defense, metabolite accumulation, and colony organization. To test this hypothesis, we conducted a comprehensive analysis of the effects of diclofenac, ibuprofen, and flufenamic acid on *D. armatus*. The study integrates multiple biomarkers, including photosynthetic performance (OJIP parameters, chlorophyll content, oxygen exchange), oxidative stress indicators (H_2O_2 levels), protective metabolite accumulation (proline, carotenoids), antioxidant enzyme activity (CAT, APX), and morphological responses, particularly coenobia formation and structural reorganization.

2. Materials and methods

2.1 Algae culture

Desmodesmus armatus 276-4d (SAG, Germany) was maintained on slants before transfer to liquid HSM medium (Harris, 2009). Algae were grown at 30 ± 1 °C using glass test tubes with continuous bubbling with sterilized air enriched in 2.5% CO₂ (v/v) passed through a bacteriological filter (0.2 mm PTFE; Sartorius, Germany), under white fluorescent light (125 ± 5 μmol photons m⁻² s⁻¹). Toxicity testing was conducted using mid-logarithmic phase cultures with an initial density of 0.5×10^6 cells/mL.

2.2. Assessment of NSAIDs phytotoxicity

The NSAIDs selected for this study were flufenamic acid (FFA, Alfa Aesar, Ward Hill, Massachusetts, USA), diclofenac (DCF, ABRC, Niederzenz, Germany), and ibuprofen (IBU, Alfa Aesar, Ward Hill, Massachusetts, USA). The key characteristics of the studied NSAIDs are summarized in Tab. S1. The stock solution of FFA was prepared in DMSO (dimethyl sulfoxide, analytical grade, Acros Organics, USA), while DCF and IBU were dissolved in ddH₂O, and then added to HSM medium to achieve the required concentrations. The concentration of DMSO in all experimental variants was maintained at 0.1% (v/v), which our previous studies demonstrated had no significant effect on physiological parameters in several algal species, including *D. armatus* (Aksmann et al., 2016; Aksmann & Tukaj, 2004; Majewska et al., 2018; Tukaj & Aksmann, 2007). The experiment was carried out in two steps. Initially, concentration-response curves were generated based on 24-hour growth inhibition data (See Fig. 1A-C; Tab. S2). The tested concentrations were as follows: 6.3, 12.5, 25.1, 50.1, 75.2 mg/L for DCF, 30, 60, 90, 120, 150 mg/L for IBU, and 0.5, 1, 2, 4, 8 mg/L for FFA. In the second stage, physiological analyses were conducted by exposing algal cultures to the determined EC10 and EC50 values of DCF (7.4 and 41.7 mg/L), IBU (42.3 and 81.2 mg/L), and FFA (0.56 and 2.34 mg/L). Actual exposure concentrations were verified by HPLC analysis (detailed methodology in Text S1, Tab. S3). Algal cultures without NSAID exposure were used as controls throughout the experiment. After 24 hours, cultures were sampled for further analyses described in subsequent sections.

2.3 Endpoints

2.3.1 Population density

The population density of *D. armatus* was determined microscopically using a Bürker chamber (Andersen, 2005). Counting was performed at 200x magnification using light microscope (Leica, Germany), and results were expressed as a cell number per mL of culture.

2.3.2. Chlorophyll *a* fluorescence *in vivo*

Chlorophyll *a* fluorescence *in vivo* was measured using a Handy PEA fluorometer (Hansatech Ltd., UK) with dedicated software, following (Pokora et al., 2017). Cell suspension was diluted to $OD_{680} = 0.2 \pm 0.01$ immediately after collection from the cultures, dark-adapted for 15 min, and glass vials with 2 mL of algae suspension were placed in the measuring chamber. Measurements were at room temperature with a saturating light pulse of $3000 \mu\text{mol photons} \times \text{m}^2 \times \text{s}^{-1}$. The parameters used in this analysis are described in Suppl Tab. S5.

2.3.3. Oxygen evolution and consumption.

A Clark-type oxygen electrode system (Hansatech Ltd., UK) supported by Oxyview software was used to determine oxygen consumption and evolution, following Harshkova et al., (2021). Algal suspension (1 mL) was placed in the stirred measurement chamber at 30°C. Oxygen fluxes were recorded under light conditions similar to culture illumination and in darkness. The obtained values were expressed as nmol O_2 per million cells $\times \text{min}^{-1}$.

2.3.4. Mitochondrial Membrane Potential (MMP)

The mitochondrial membrane potential of *D. armatus* cells was estimated using the fluorescent dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide, Sigma-Aldrich), as described by Harshkova et al., (2019). Following centrifugation at $460 \times g$ for 5 min, cell pellets were suspended in HSM medium and adjusted to a density of $1 \text{ mln cells} \times \text{mL}^{-1}$. JC1-stained cells were incubated for 20 min in black 98-well plates. Fluorescence was determined using a Varioskan Flash Microplate Reader (Thermo Fisher Scientific, USA), with excitation at 488 nm and emission monitored at 538 and 596 nm (monomers/oligomers). MMP was expressed as the red/green fluorescence ratio (a.u).

2.3.5. Photosynthetic Pigments Quantification

To determine the photosynthetic pigment content, biomass from 15 mL of algal culture was first pelleted by centrifugation ($2000 \times g$, 10 min, RT), homogenized with glass beads using a FastPrep-24 homogenizer (5 m/s, 1 min; MP Biomedicals, USA), and extracted using 80% acetone (Kapuścińska et al., 2024). Pigment concentrations in the extract were determined spectrophotometrically using a Varioskan Flash Multimode Microplate Reader (Thermo Fisher Scientific, USA) by absorbance measurement at 447, 647, 664, and 740 nm. Chlorophyll *a*, chlorophyll *b*, and carotenoids concentration were calculated based on equations proposed by Jeffrey & Humphrey, (1975) and Lichtenthaler & Wellburn, (1983) with path

length correction applied according to Warren, (2008). Finally, the pigment content was expressed in pg per million cells.

2.3.6. Hydrogen Peroxide Production

Hydrogen peroxide production was quantified fluorometrically in a reaction mixture containing Amplex UltraRed reagent (10 mM; Molecular Probes, USA) and horseradish peroxidase (10 U/mL) prepared in phosphate buffer (0.1M, pH 7.0) (Hejna et al., 2024). After centrifugation 10 mL of cells suspension ($291 \times g$, 5 min, RT), 50 μ L of the supernatant was mixed with 50 μ L of the reaction mixture, incubated for 25 min at 25 °C, and fluorescence was measured at λ_{ex} = 560 nm and λ_{em} = 585 nm using a Varioskan Flash Multimode Microplate Reader (Thermo Fisher Scientific, USA). For quantification, standard additions of H₂O₂ (to a final concentration of 3 nmol/mL) were used for all treatment samples. The results were expressed as nmol H₂O₂ produced per million cells of microalgae.

2.3.7. Protein extraction and Enzyme activities Assays

Proteins were extracted from 50 mL of algal culture. Biomass was centrifuged ($3000 \times g$, 5 min, RT) and resuspended in dedicated extraction buffer: 0.05 M potassium phosphate buffer (pH 7.0) for CAT, and 0.05 M phosphate buffer (pH 7.6) containing 1 mM ascorbic acid, 0.1 mM EDTA, 10% sorbitol, and 0.1 % PVP for APX (Pokora et al., 2014). Cells were disrupted with glass beads using a FastPrep-24 homogenizer (5 m/s, 1 min; MP Biomedicals, USA), followed by centrifugation ($20000 \times g$, 20 min, 4 °C). Supernatants were used for enzyme activity assays.

APX activity was measured using a spectrophotometric microplate-based method adapted from Nakano & Asada, (1981) with protocol optimizations for microalgal extracts (Aksmann et al., 2016). The assay mixture consisted of 50 mM phosphate buffer (pH 7.0), 18 mM pyrogallol, and 0.1 mM hydrogen peroxide. The enzymatic reaction was started by adding 50 μ L of supernatant to 150 μ L of the reaction mixture and monitored at 430 nm for 5 min at 25 °C using a Varioskan Flash Multimode Microplate Reader (Thermo Fisher Scientific, USA). Enzyme activity was calculated using $\epsilon = 2.47 \text{ mM}^{-1} \text{ cm}^{-1}$.

CAT activity was measured using a UV-Vis spectrophotometer (Hitachi U-2900, Japan) by monitoring the decrease in absorbance at 240 nm ($\epsilon = 43.6 \text{ M}^{-1} \text{ cm}^{-1}$) upon H₂O₂ decomposition (Aebi, 1984). The assay was conducted in a quartz cuvette containing 2500 μ L of reaction mixture consisting of 50 mM sodium phosphate buffer (pH 7.0) and 10 mM H₂O₂.

The reaction was initiated by the addition of 500 μ L of supernatant, and the decrease in absorbance was recorded. Results of APX and CAT were normalized to a million cells.

2.3.8. Proline content

Proline content in algae cells was determined using a modified acid ninhydrin assay, according to the method of Bates et al., (1973), with adaptation introduced by Carillo et al., (2011). Briefly, after centrifugation of 15 mL of algal suspension ($2000 \times g$, 10 min, RT), the cell samples were homogenized with 70% ethanol using glass beads and FastPrep-24 homogenizer (5 m/s, 1 min; MP Biomedicals, USA). The homogenate was transferred to Eppendorf tubes and incubated at 70 °C for 20 min. After centrifugation ($12000 \times g$, 5 min, temp 20 °C), the supernatant was transferred to new tubes. Next, 150 μ L of supernatant was mixed with 300 μ L of freshly prepared acidic ninhydrin reagent (Chempur, Poland), vortexed, and incubated at 95 °C for 20 minutes. The samples were then centrifuged ($3000 \times g$, 10 s), and 100 μ L were used for absorbance measurements at 520 nm using Varioskan Flash Multimode Microplate Reader (Thermo Fisher Scientific, USA). Proline concentration was calculated based on a standard calibration curve prepared using L-proline, and the results were expressed as μ mol per million cells.

2.3.9. Statistical analysis

All statistical analyses were conducted using Statistica 13.1 (StatSoft Europe, Germany) and Origin Pro 10.2 (OriginLab Corp., USA) software. Data are presented as means $\pm$ standard error (SE) from at least three independent experiments. Outliers were identified and removed based on the interquartile range (IQR). Normality of the data distribution was assessed using the Shapiro-Wilk test, and homogeneity of variances was verified with Levene's test. Parametric data were analyzed using one-way ANOVA with Tukey's post hoc test, while non-parametric data were evaluated using the Kruskal-Wallis test followed by Dunn's test. Canonical discriminant analysis and Spearman's rank correlation matrices were applied to identify relationships among variables and to evaluate their contribution to group separation.

3. Results

3.1. Effects of FFA exposure

Exposure to FFA resulted in population growth inhibition with EC10 and EC50 values of 0.56 and 2.34 mg/L, respectively (referred to further as EC10_{FFA} and EC50_{FFA}). (Fig. 1A,

Tab. S2). The ECs were calculated in a standard way, namely on the basis of nominal FFA concentration at time zero (0 h), verified by real concentrations analysis in the media (Fig 1A; Tab. S3). What is important, the real FFA concentrations in the media after 24 h were markedly reduced relative to the nominal 0 h concentrations (Tab. S3).

At EC10_{FFA}, a shift in morphological structure of the population was observed, with a larger percentage of four-celled coenobia than in the control. The trend was even stronger at EC50_{FFA}, where 4-celled coenobia accounted for 72.5% and 8-celled forms declined significantly to 21.5% of the total population (Fig. 1D, Tab. S4).

FFA exposure affected both oxidative balance and photosynthetic efficiency of *D. armatus* cells. Changes in OJIP-derived parameters were observed particularly at EC50_{FFA}, while EC10_{FFA} caused only minor alterations (Fig. 2E). Compared with control cells, the lower FFA concentration significantly reduced electron transport efficiency (RE_0/RC) to 95% as compared with untreated algae, whereas most of the other PSII-related parameters remained unchanged. In contrast, EC50_{FFA} caused a clear shift in photosynthetic performance, with significant increases in PI_{inst} (142%), PSI acceptors efficiency (ΔR_0 to 152%; ϕR_0 to 166%), and electron transport efficiency (RE_0/RC to 153%) relative to control cells (Fig. 2E). The number of active reaction centers (RCM) in the EC50_{FFA}-treated cells also increased to 122% of the value noted in the untreated cells. Photosynthetic oxygen evolution rate had not changed significantly, neither at EC10_{FFA} nor at EC50_{FFA} (Fig. 2E), while dark oxygen consumption (respiration rate) increased at EC50_{FFA} to 169% of control (Fig. 3A).

At EC50_{FFA}, H₂O₂ production was significantly enhanced, reaching about 537% of the control value, indicating persistent oxidative stress, which was accompanied by increased proline accumulation to approximately 133% of the control (Fig. 4A). APX activity increased with increasing FFA concentration and was significantly higher at EC50_{FFA}, reaching 230% of control (Fig. 4D).

The Spearman correlation analysis generally showed strong negative correlations between PSII efficiency parameters (e.g., F_v/F_m , F_v/F_0 , ϕP_0 , PI_{inst}) and energy dissipation-related parameters (e.g., DI_0/RC , ABS/RC) (Fig. 5). At EC10_{FFA}, the correlation structure especially stood out as strong negative correlations between PSII efficiency indicators and photosynthetic pigments content. The pigments content (chlorophyll *a*, chlorophyll *b*, carotenoids) formed a strong negative correlation (r_s frequently > -0.85) with MMP efficiency (Fig. 5A). Proline concentration was strongly positively correlated with pigment content and negatively associated with MMP efficiency (Fig. 5B).

Discriminant analysis was calculated to highlight the differences between the control, EC10_{FFA}, and EC50_{FFA} groups based on respiration efficiency and oxidative stress markers. Among these variables, proline exhibited the highest positive standardized coefficient, whereas dark respiration and carotenoid content showed the strongest negative contributions, indicating their major role in group differentiation (Fig. 8A; Tab. S6).

3.2. Effects of IBU exposure

IBU inhibited *D. armatus* growth, leading to a concentration-dependent decrease in population density (Fig. 1C). Analogous to FFA, the ECs for IBU were calculated in a standard way, namely on the basis of nominal IBU concentration at time zero (0 h), verified by real concentrations analysis in the medium (Fig 1A; Tab. S3). In NSAID-treated cultures, the EC10 and EC50 values calculated based on nominal IBU concentrations were 42.3 and 81.2 mg/L, respectively (referred to further as EC10_{IBU} and EC50_{IBU}). What is more, after 24 h the real concentrations further decreased, ranging between $\approx 13\%$ and $\approx 84\%$ of nominal 0 h concentrations Tab. S3).

The effect of IBU on the morphological structure of the population was significant at EC50_{IBU}, leading to a lower abundance of 8-celled coenobia (Fig. 1D; Tab. S4) as compared with the control.

The intensity of photosynthesis, expressed as the rate of oxygen evolution (Fig. 2A), remained unchanged, despite the tendency for oxygen evolution stimulation have been observed. On the other hand, the OJIP analysis revealed concentration-dependent changes in PSII functioning, which became clearly visible at EC50_{IBU} (Fig. 2A; Fig. 2E). At this concentration, F_0/F_m increased to 139% of the control, while PI_{inst} decreased to 56% of the control, indicating a reduction in PSII efficiency. The energy absorption per active reaction center (ABS/RC) increased to 122% as compared with control (Fig. 2E). Further, energy dissipation per active reaction center (DI_0/RC), energy trapping (TR_0/RC), and energy transport (ET_0/RC) increased to 172%, 106%, and 112% of the control, respectively (Fig. 2E). RE_0/RC increased to 170%, and ET_0/RC to 112%, while the reduction to 60% of control in the active reaction center (RCM) fraction was observed (Fig. 2E). The photosynthetic pigments content was not strongly affected at EC50_{IBU}, and the observed decrease in chlorophyll *a*, chlorophyll *b*, and carotenoids content was not statistically significant (Fig. 2B-D).

Analysis of dark respiration parameters revealed that neither oxygen uptake nor mitochondrial membrane potential (MMP) values were significantly affected in IBU-treated cells (Fig. 3A, B).

IBU treatment seemed not to induce strong oxidative stress in the algal cells. No statistically significant differences in the H_2O_2 production or activity of antioxidant enzymes were observed (Fig. 4A, C, D). In contrast, proline content in the cells was significantly higher in both EC10_{IBU} (105% of control) and EC50_{IBU} (130% of control)(Fig. 4B).

At both EC10_{IBU} and EC50_{IBU} exposure, very strong positive correlations were observed among PSII efficiency parameters, including ϕP_0 and PI_{inst} , indicating tight functional coupling of photochemical performance variables (Fig. 6). In contrast, parameters associated with energy fluxes per reactive centers, such as ABS/RC, DI₀/RC, RE₀/RC showed strong negative correlations with PSII efficiency parameters (Fig. 6). Proline content exhibited strong positive associations with all pigment contents (Fig. 6).

According to discriminant analysis, the first canonical function was primarily defined by positive contributions of carotenoid content, while proline content showed a negative loading (Fig. 8B; Tab. S6). Overall, in the case of IBU treatment, we did not observe strong discrimination between the test groups compared to the control parameters.

3.3 Effects of DCF exposure

DCF showed concentration-dependent inhibition of *D. armatus* population growth, with EC10 and EC50 values of 7.4 and 41.7 mg/L, respectively. The EC values (referred to further as EC10_{DCF} and EC50_{DCF}) were calculated from growth inhibition curves, based on nominal DCF concentrations (Fig. 1B, Tab. S2). No clear shifts in morphological structure of the population were observed under diclofenac exposure, neither at EC10_{DCF} nor at EC50_{DCF} (Fig. 1D; Tab. S4).

Photosynthetic oxygen evolution was inhibited to 35% of control at EC50_{DCF}, and the OJIP-derived parameters confirmed a severe decline in quantum efficiency of primary photochemistry (jP_0) to 18% of control, a quantum efficiency of electron transport (ϕE_0 , ψE_0) to 5 and 29% of control, respectively, and a decrease in fraction of active PS II reaction centers (RCM) to 5% of control. As a consequence, the performance index (PI_{inst}) dropped to nearly zero. At the same time, energy processing by one active reaction center changed, since energy absorption, trapping, and dissipation (ABS/RC, TR₀/RC, DI₀/RC) increased (143 – 3053% of control) while electron transport (ET₀/RC, RE₀/RC) decreased to 45 and 37% of control, respectively (Fig. 2E). The dark oxygen consumption at gressive loss of PSII functionality from EC10_{DCF} to EC50_{DCF} (Fig. 2A; Fig 2E). At EC10_{DCF}, chlorophyll *a*, chlorophyll *b*, and carotenoid content remained essentially unchanged (Fig. 2B-D). However, a pronounced decline at EC50_{DCF} was seen, where chlorophyll *a*, chlorophyll *b*, and carotenoids decreased

to 32%, 37%, and 37% of the control, respectively, indicating substantial loss of pigment integrity under DFC-induced stress (Fig. 2B-D). Photosynthetic performance was already significantly impaired at EC10, with a marked decrease in ϕP_0 to 97% and PI_{inst} to 70% of control, accompanied by a slight increase in energy flux parameters per reaction center (ABS/RC, DI_0/RC , RE_0/RC to 106%, 115%, and 88% of control, respectively)(Fig. 2E). At EC50_{DCF}, PSII functionality collapsed dramatically, as indicated EC50_{DCF} was severely disturbed, shifting from oxygen uptake to oxygen release (Fig. 3A). No change in mitochondrial membrane potential was seen (Fig. 3B).

Production of H₂O₂ was significantly elevated at both EC10_{DCF} (741% of control) and EC50_{DCF} (359% of control) treatments (Fig. 4A). The antioxidant enzyme responses were divergent, with unchanged APX activity and a complete inhibition of CAT activity (Fig. 4C-D).

Under DCF exposure (EC10_{DCF} and EC50_{DCF}), the correlation structure was dominated by strong and highly consistent interrelationships among chlorophyll fluorescence parameters (Fig. 7). In both treatments, F_0/F_m was perfectly negatively correlated with ϕP_0 and F_v/F_0 ($\rho = -1.00$), while ϕP_0 showed very strong positive correlations with PI_{inst} , and F_v/F_0 (Fig. 7).

At both concentrations, parameters describing energy fluxes within PSII remained tightly interrelated. ABS/RC and DI_0/RC were strongly or perfectly positively correlated, and both were negatively associated with F_v/F_m (Fig. 7). Similarly, ψE_0 and ϕE_0 exhibited strong positive correlations with ϕP_0 and PI_{inst} , indicating a coordinated response of primary photochemistry and electron transport efficiency (Fig. 7). Pigment-related variables (chlorophyll *a*, chlorophyll *b*, and carotenoids) were strongly and consistently positively correlated with each other at both EC10_{DCF} and EC50_{DCF}, showing near-perfect co-variation (Fig. 7). Intensity of photosynthesis was strongly positively correlated with chlorophyll *a* and chlorophyll *b*, particularly under EC10 conditions. PI_{inst} was strongly positively correlated with RCM and MMP at both exposure levels, while under EC10_{DCF} it showed strong negative correlations with chlorophyll *a*, chlorophyll *b*, and carotenoids (Fig. 7). Proline content under EC10_{DCF} displayed strong positive correlations with photosynthesis and pigment content (Fig. 7). Overall, both DCF concentrations were characterized by strong coupling among fluorescence-derived parameters and pigment-related variables, with EC10_{DCF} additionally showing pronounced associations between oxidative stress-related parameters and pigment content (Fig. 7).

Discriminant analysis performed for DCF revealed strong negative standardized coefficients for carotenoid, as well as proline, and positive contributions were observed

for H₂O₂ and 2-cells coenobia count in the parameter groups separation analysis (Fig. 8C; Tab. S6).

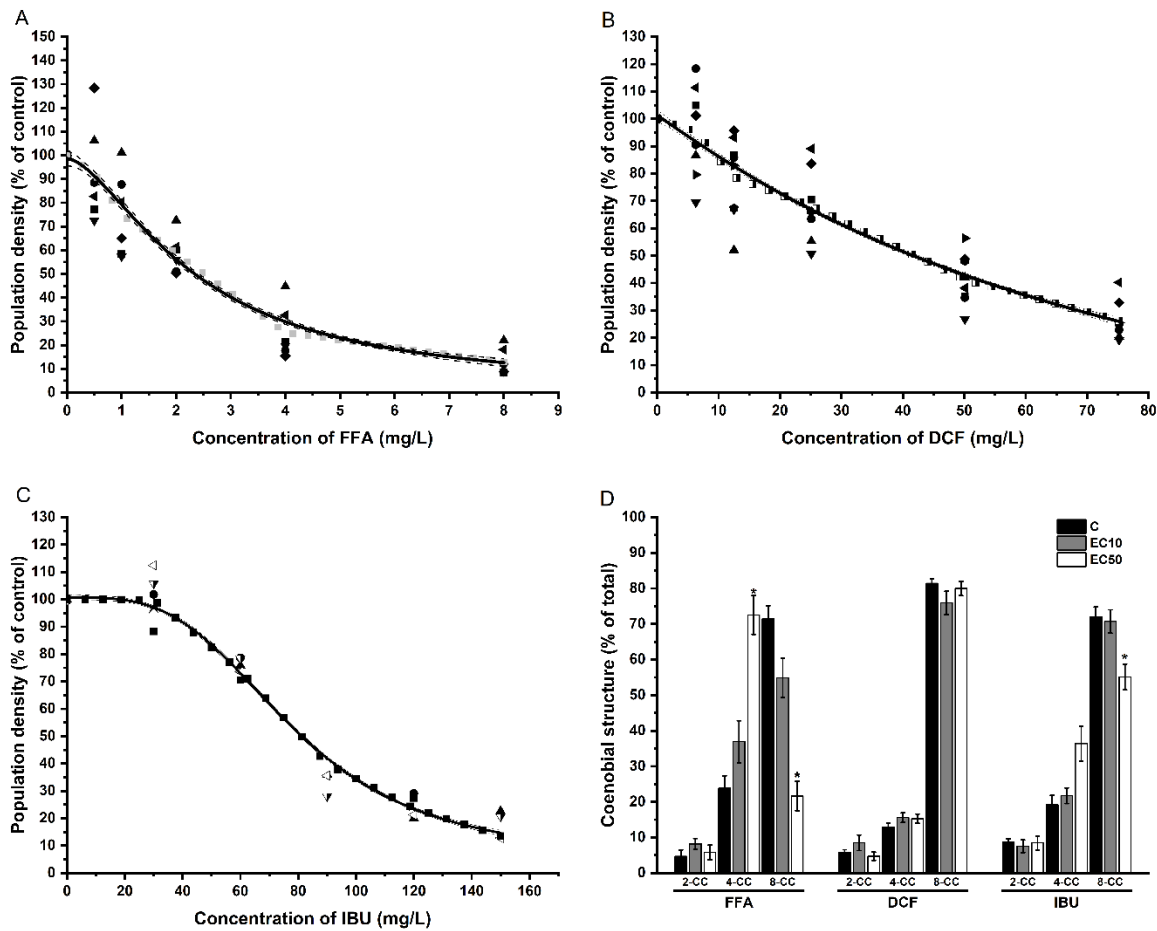


Fig. 1. Effects of flufenamic acid (FFA), diclofenac (DCF), and ibuprofen (IBU) on population density and morphological population structure of *D. armatus* after 24 h exposure to the pharmaceuticals. (A-C) Concentration-response curves for population density expressed as % of control. (D) Distribution of 2-, 4-, and 8-celled coenobia (2-CC, 4-CC, 8-CC, respectively), expressed as the percentage of total coenobia count in each treatment at concentrations corresponding to EC10 and EC50. Data are presented as means of at least three biological replicates. Asterisks indicate significant differences compared to the control ($p \leq 0.05$; Kruskal-Wallis test followed by Dunn's post hoc test).

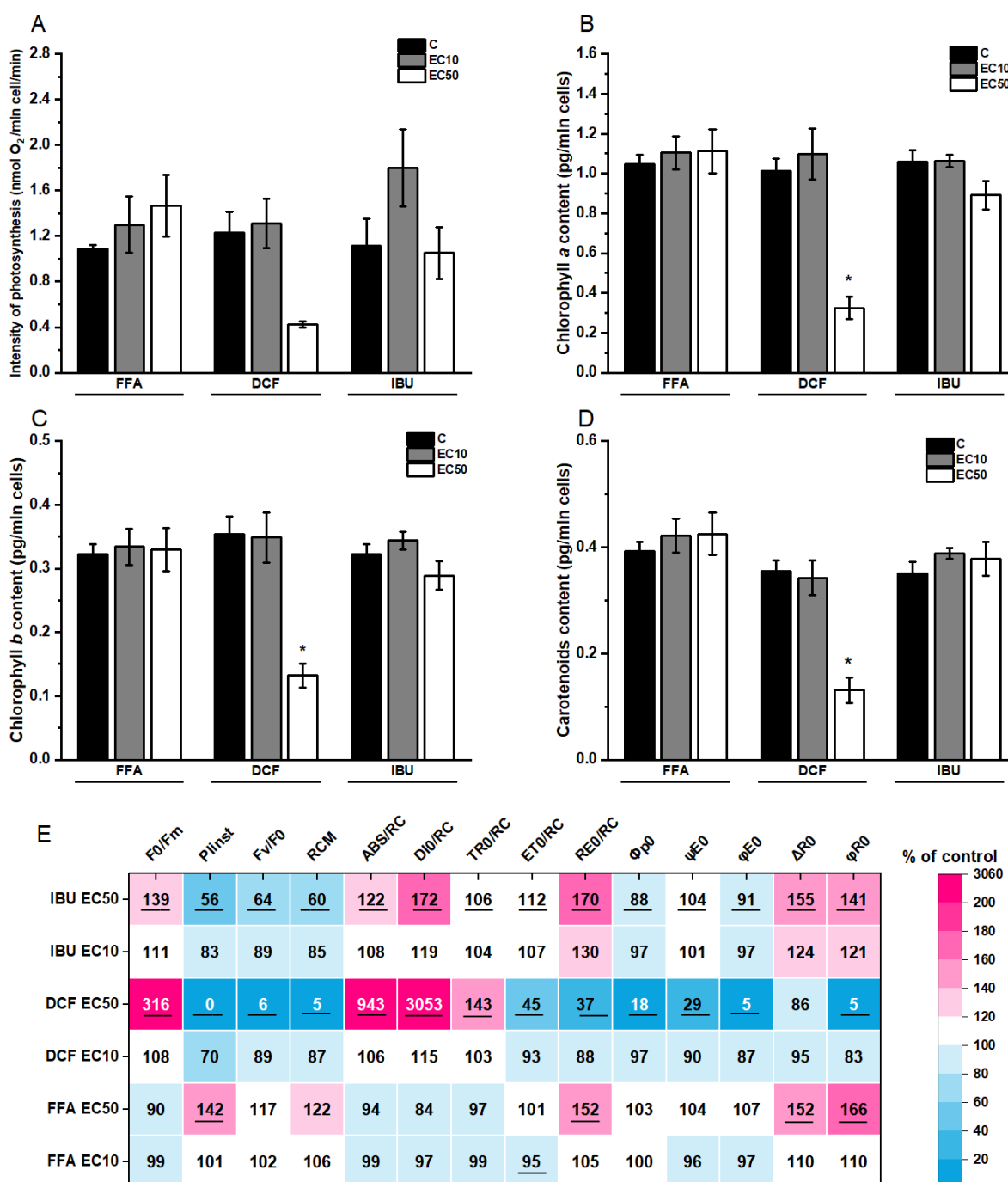


Fig. 2. Effects of FFA, DCF, and IBU applied at EC10 and EC50 concentrations on photosynthesis-related endpoints in *D. armatus* after 24h exposure. (A) Intensity of photosynthesis; (B-D) Chlorophyll *a*, chlorophyll *b*, and carotenoid content; (E) Heatmap of OJIP parameters expressed as a % of control. Data are presented as means $\pm$ SE of at least three biological replicates. Asterisks and underlined values indicate significant differences compared to the control ($p \leq 0.05$; Kruskal-Wallis test followed by Dunn's post hoc test).

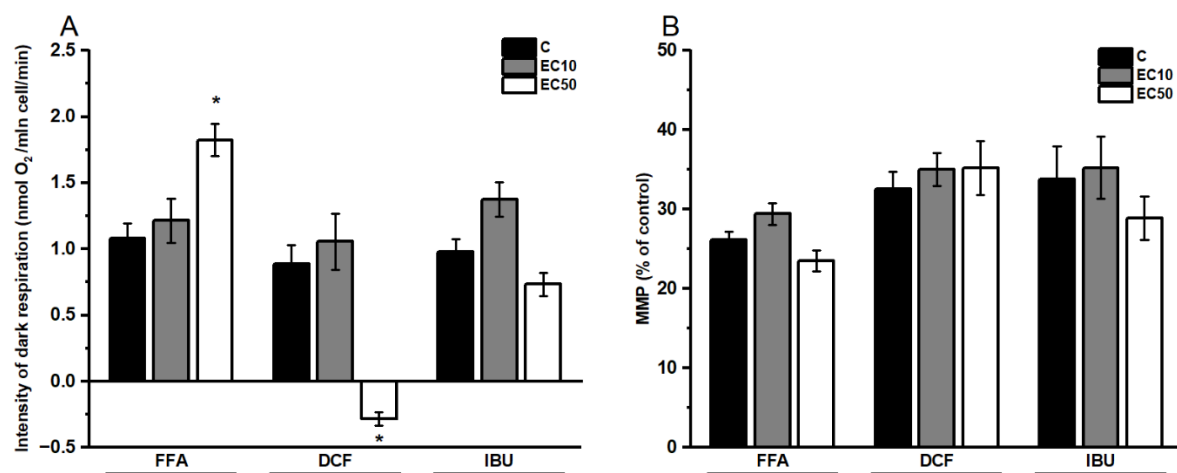


Fig. 3. Effects of FFA, DCF, and IBU applied at EC10 and EC50 concentrations on mitochondrial function endpoints in *D. armatus* after 24h exposure. (A) Dark respiration intensity; (B) Mitochondrial membrane potential (MMP) assessed with JC-1 and calculated as the ratio of JC-1 oligomers fluorescence ($\lambda=596$ nm) to JC-1 monomers fluorescence ($\lambda=538$ nm). Data are presented as means $\pm$ SE of at least three biological replicates. Asterisks indicate significant differences compared to the control ($p \leq 0.05$; Kruskal-Wallis test followed by Dunn's post hoc test).

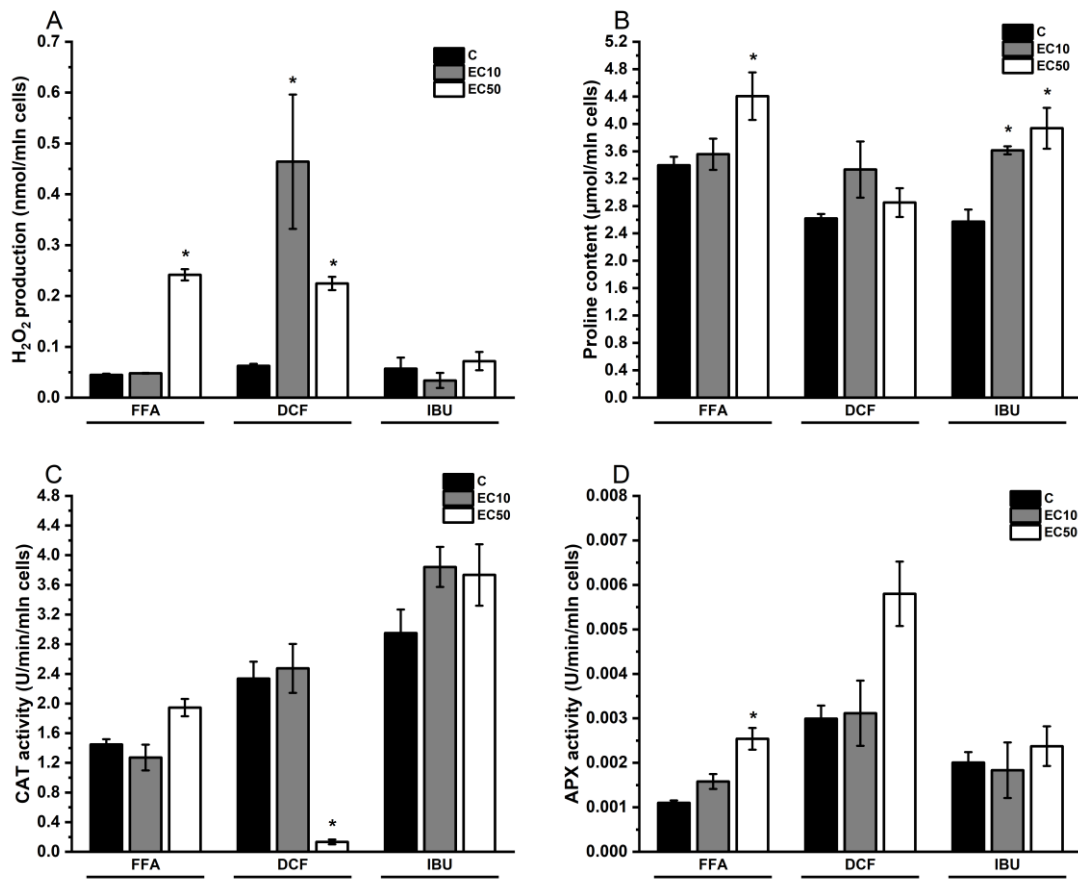
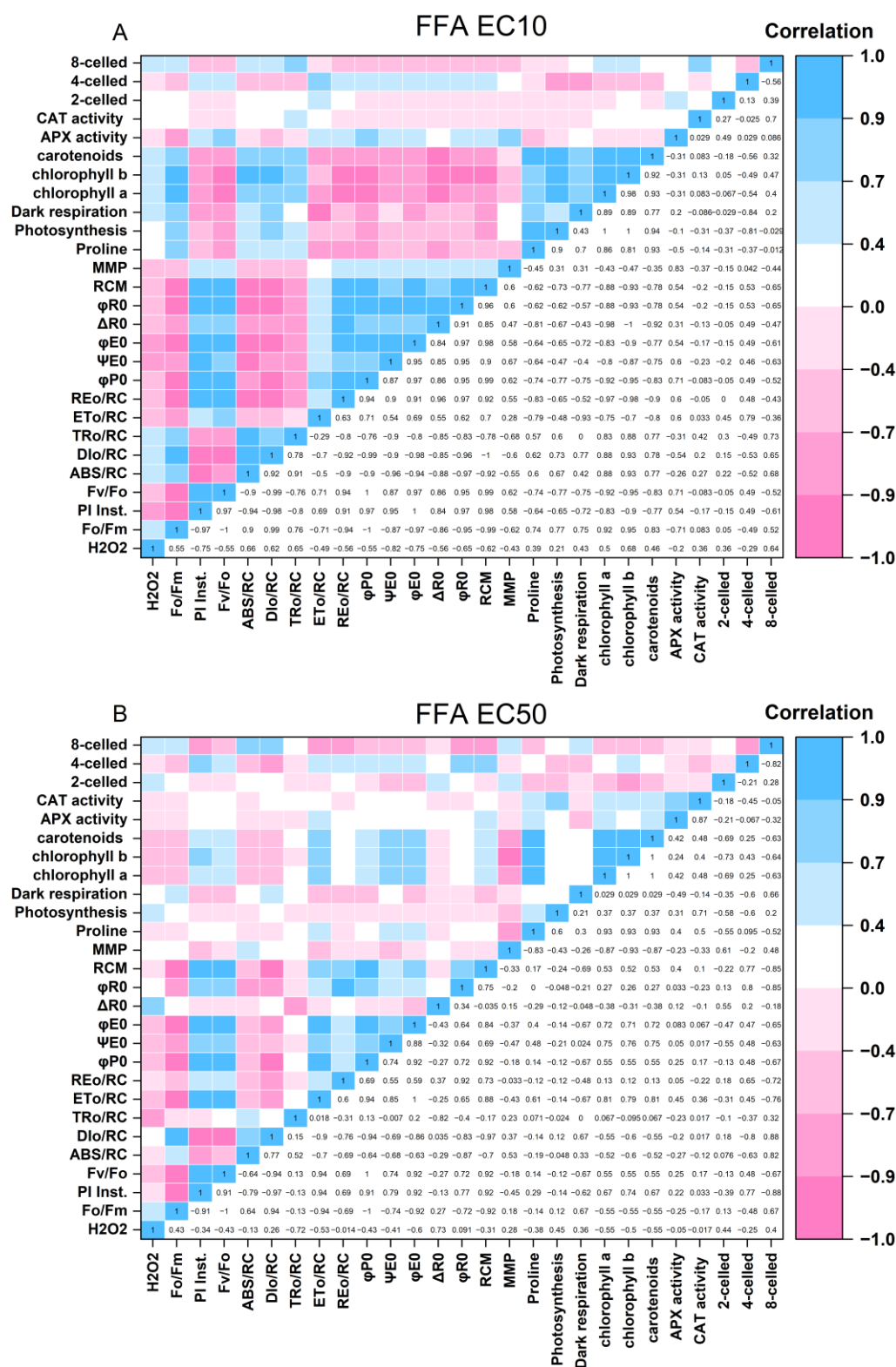


Fig. 4. Effects of FFA, DCF, and IBU applied at EC10 and EC50 concentrations on oxidative stress markers and antioxidant response in *D. armatus* after 24h exposure. (A) Hydrogen peroxide production; (B) Proline content; (C) Catalase (CAT) activity; (D) Ascorbate peroxidase (APX) activity. Data are presented as means $\pm$ SE of at least three biological replicates. Asterisks indicate significant differences compared to the control ($p \leq 0.05$; Kruskal-Wallis test followed by Dunn's post hoc test).



427

428 **Fig. 5.** Multivariate analyses summarizing relationships among the measured parameters under

429 FFA exposure of *D. armatus*. Spearman correlation matrices are shown for EC10_{FFA}, and

430 EC50_{FFA} groups (A-B); colors indicate correlation coefficient (blue = positive, pink = negative).

431 Control data are provided in Fig. S1.

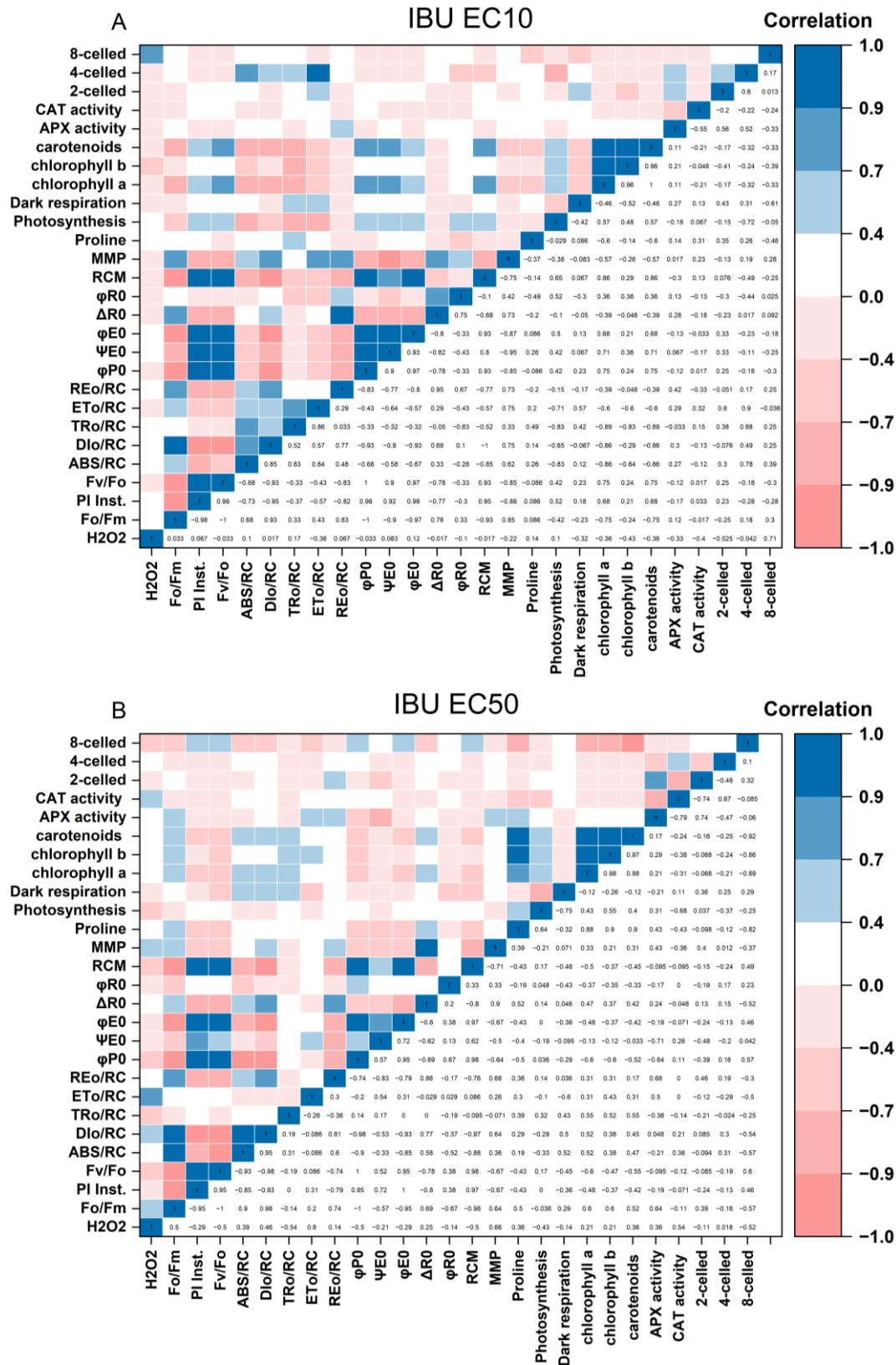


Fig. 6. Multivariate analyses summarizing relationships among the measured parameters under IBU exposure of *D. armatus*. Spearman correlation matrices are shown for EC10_{IBU} and EC50_{IBU} groups (A-B); colors indicate correlation coefficients (blue = positive, orange = negative). Control data are provided in Fig. S1.

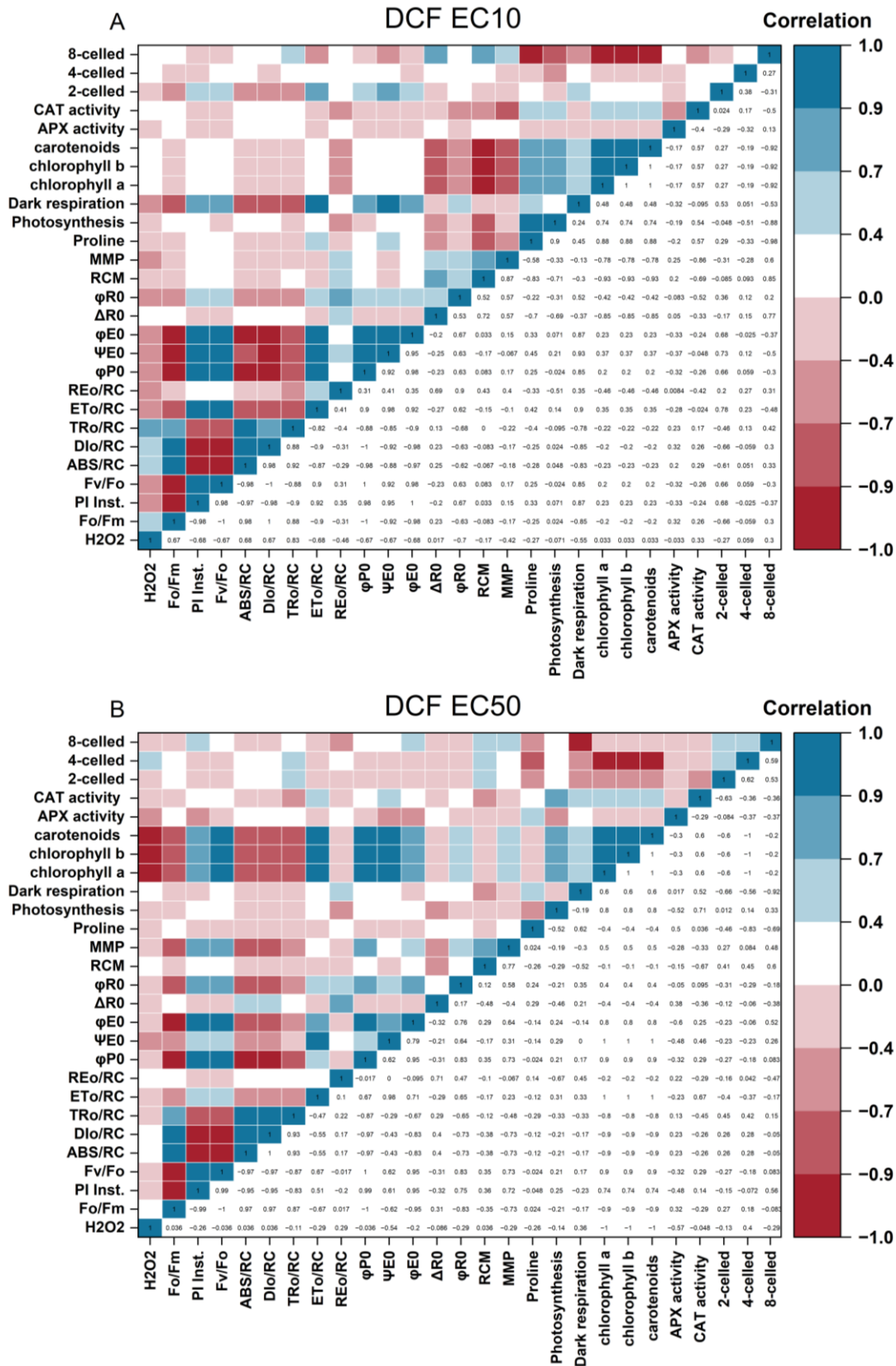


Fig. 7. Multivariate analyses summarizing relationships among the measured parameters under DCF exposure of *D. armatus*. Spearman correlation matrices are shown for EC10_{DCF}, and EC50_{DCF} groups (A-B); colors indicate correlation coefficient (blue = positive, red = negative). Control data are provided in Fig. S1.

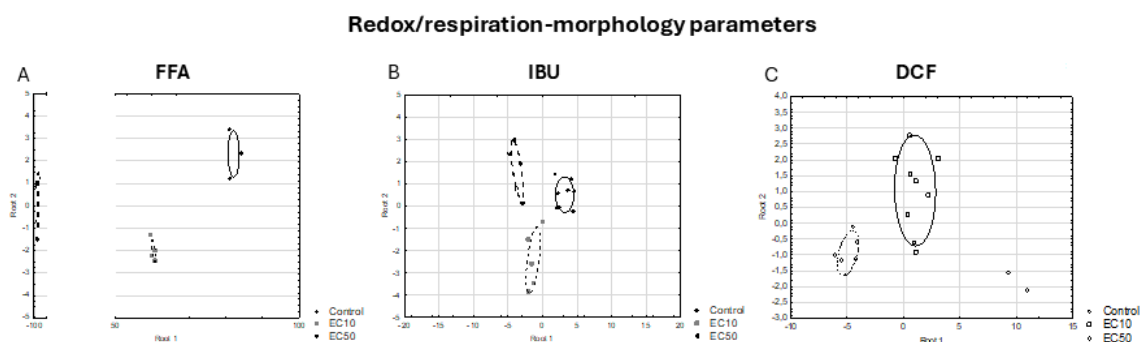


Fig. 8. Canonical discriminant score plots for Control, EC10, and EC50 groups exposed to (A) FFA, (B) IBU, and (C) DCF based on redox/respiration-morphology parameters (MMP; H₂O₂, proline, dark respiration, carotenoids, and proportions of 2- 4-celled coenobia). The ellipses in the figure show the data distribution with a range of coefficients of 0.95.

4. Discussion

The research presented in this paper is consistent with the currently prevailing belief among ecotoxicologists that growth inhibition tests alone do not adequately describe chemicals' phytotoxicity (Hejna et al., 2022). Because microalgal sensitivity to NSAIDs is often highly species-dependent, growth-based endpoints can simplify acclimatory responses and ecological outcomes, particularly for more stress-tolerant and plastic organisms. In this context, *Desmodesmus armatus* is a good model of acclimatory potential, representing relatively high stress tolerance, physiological flexibility, and plasticity in population morphological structure. These features make *D. armatus* suitable for studying the physiological mechanisms of metabolic homeostasis in response to NSAID exposure. Our findings show that even in relatively stress-tolerant species, NSAIDs induced a coordinated, multilevel response. Changes in population morphological structure occurred alongside with changes in photosynthetic apparatus function, symptoms of mitochondrial dysfunction, and activation of selected antioxidant defense mechanisms. Importantly, although all three NSAIDs tested here reduced *D. armatus* population growth, the physiological pathways leading to this common endpoint differed markedly among compounds. Integration of different physiological and biochemical endpoints, further supplemented by appropriate multivariate analyses, indicates the existence of three distinct stress-response strategies *D. armatus*. The first strategy, which could be called "energetically costly acclimation," emerged in the FFA-treated population. The second one, which we have called "buffered photochemical adjustment," could be seen under IBU exposure, and the third one, "oxidative photosynthetic collapse," was characteristic of the DCF-treated population.

From the three abovementioned NSAIDs, FFA triggered the most intriguing pattern of algal response. Despite exhibiting the lowest EC50 value and therefore the highest toxicity toward growth (Fig. 1A-C), FFA did not suppress photosynthetic oxygen evolution (Fig. 2A). Instead, cells maintained photosynthetic activity while showing increased dark oxygen consumption (Fig. 3A), elevated H₂O₂ production (Fig. 4A), enhanced APX activity (Fig. 4D), proline accumulation (Fig. 4B), and restructuring of population morphology (Fig. 1D). Together with increases in several OJIP-derived parameters (Fig. 2E), these results suggest that FFA toxicity was expressed primarily through complex metabolic reorganization leading to acclimation to stress conditions. Since the effect of FFA on green algae remains poorly characterized, the high toxicity observed in the current study and in our previous work on *C. reinhardtii* (Kapuścińska et al., 2024, 2026) makes our investigations interesting and environmentally important. Our attention was especially drawn to the observation that at EC50_{FFA} concentration, the increase in ΔR_0 , ϕR_0 , and RE₀/RC, together with alterations in PSII-related parameters and PI_{inst}, points to a reorganization of energy fluxes between PSII and PSI (Fig. 2E). The marked increase in the fraction of active reaction centers may be explained by their reactivation or enhanced functional efficiency rather than *de novo* formation. The above results were somewhat surprising, being in opposition to our previous findings that in *C. reinhardtii* FFA caused inhibition of photosynthesis and dark respiration, increased dissipation of energy, and reduced fraction of active reaction centers (Kapuścińska et al., 2024). A probable explanation was provided by the works of Ware et al., (2020) and Milrad et al., (2024), suggesting that *D. armatus* depends strongly on alternative electron transport (AET) with minor contribution from non-photochemical quenching, and exhibits a rapid PSII repair cycle through efficient D1 turnover. The alga therefore seems to have a unique and efficient photoprotective strategy. However, this apparent stability was linked to increased dark respiration (Fig. 3A) and activation of stress-protective mechanisms (Fig. 4), suggesting a high metabolic cost to maintain cellular homeostasis. Consequently, growth inhibition likely reflects a diversion of cellular resources from biomass production to metabolic maintenance, molecular repair, and antioxidant defense. The increase in H₂O₂ production, along with increased APX activity at EC10_{FFA} concentrations and proline accumulation, as well as a slight increase in CAT activity at EC50_{FFA} (Fig. 4 A-D), suggests that the initial enzymatic defense was supported by non-enzymatic mechanisms, both of which are energetically costly. This is consistent with the complexity of the antioxidative defense system in algae and the significant role of proline as a protective metabolite involved in ROS scavenging, structural stabilization, and redox homeostasis (Hayat et al., 2012;

Rezayian et al., 2019). The discriminant analysis strongly supports this interpretation because group separation was driven predominantly by proline, dark respiration-related variables, and carotenoids (Fig. 8A; Tab. S6), identifying metabolic cost and stress mitigation as the principal components of the FFA response. The population's morphological response provides an additional component of this acclimation process. In EC50_{FFA}-treated cells, a significant increase in the 4-celled coenobia and a reduction in 8-celled ones were observed, suggesting stress-induced structural reorganization of the population (Fig. 1D). It seems that such modifications may optimize the balance between surface-to-volume ratio and reduce energetic costs necessary to maintain larger coenobia under stress. Such structural plasticity is consistent with well-documented ability of *Dedmodesmus sp.* to modify population' organization in response to environmental stress (Lüring, 2003; Tukaj & Bohdanowicz, 1995).

In contrast to FFA, IBU induced a much milder physiological response. Although population growth was inhibited (Fig. 1A-C), oxidative stress markers and antioxidant enzymes remained largely unchanged (Fig. 4A-C). Physiological changes were centered mainly on PSII photochemistry, where moderate alterations in fluorescence-derived parameters occurred without substantial impairment of photosynthetic oxygen evolution. At EC10_{IBU}, effects were minor and limited to a small reduction in PI_{inst} and shifts in energy fluxes (Fig. 2E). At EC50_{IBU}, a decrease in ϕP_0 , RCM, and PI_{inst} , along with increased ABS/RC, DI_0/RC , and RE_0/RC , points to partial inactivation or overexcitation of PSII reaction centers and enhanced energy dissipation (Fig. 2E) to prevent photodamage. This is consistent with other studies indicating concentration-dependent effects of IBU on chlorophyll fluorescence parameters in microalgae reflecting mild-to-strong disturbances in photosynthesis (Wang et al., 2020). Here, accumulation of proline (Fig. 4B) in the absence of marked oxidative stress suggests that non-enzymatic protective mechanisms were sufficient to buffer cellular disturbance. This interpretation is further supported by the weak discrimination (Fig. 8B) among treatment groups observed in the canonical discriminant analysis, indicating that IBU exposure caused only moderate deviations from the physiological state of untreated cells. Strong coupling among PSII efficiency parameters and a negative correlation between photochemical efficiency and energy fluxes per reaction center indicate that changes in these parameters were coordinated (Fig. 6). The strong positive associations between proline and pigment contents suggest that proline accumulation was necessary to maintain stability of the photosynthetic apparatus. These observations suggest that IBU toxicity in *D. armatus* is defined primarily by the buffering potential of the antioxidative system and photochemical adjustment.

At the population level, the number of 8-cell coenobia decreased significantly at EC50_{IBU}, while 2- and 4-celled coenobia remained stable (Fig. 1D). This suggests that coenobia size adjustment may be a part of the acclimatory response of *D. armatus*, as it was observed under FFA treatment and as it was reported to be a stress acclimation strategy of this organism (Chung et al., 2018; Peña-Castro et al., 2004).

DCF occupied the opposite end of the stress-response scale. Unlike FFA, growth inhibition was accompanied by severe deterioration of photosynthetic performance. Strong declines in PSII efficiency, electron transport capacity, active reaction center availability (Fig. 2A; Fig. 2E) and pigment content (Fig 2B-D), together with increased H₂O₂ production (Fig. 4A), indicate failure of cellular compensatory mechanisms. Noteworthy, significant photosynthesis alterations were already observed at EC10_{DCF}, where a decrease in PI_{inst}, ψE_0 , ϕP_0 , and ϕE_0 , along with an increase in ABS/RC and DI₀/RC, point to reduced photochemical efficiency and enhanced energy dissipation (Fig. 2E). At EC50_{DCF}, these effects were more pronounced, with clear inactivation of PSII reaction centers. The increase in TR₀/RC despite a decline in ET₀/RC and RE₀/RC suggests that excitation energy was still trapped by the remaining active centers but could not be efficiently transferred beyond Q_A, leading to overloading of the remaining active centers and oxidative imbalance (Fig. 2E). The results are consistent with our previous studies, indicating that DCF disrupts photosynthesis in *C. reinhardtii*, reducing the efficiency of this process and electron transport and causing the formation of silent reaction centers, dissipating energy (Majewska et al., 2018). In isolated spinach chloroplasts and thylakoids, DCF appears to interfere with photosynthetic membranes in a non-specific way (Majewska et al., 2024). The membrane-disruptive mode of action proposed for DCF may also explain the abnormal oxygen exchange observed in darkness. As shown in *C. reinhardtii*, DCF can destabilize mitochondrial membranes, resulting in altered respiration and uncoupling of oxidative phosphorylation (Harshkova et al., 2024). Accordingly, dark oxygen evolution likely reflects disrupted chloroplast-mitochondrial coordination, suggesting that DCF induces broader bioenergetic disturbance linked to membrane damage (Fig. 3A). Because chloroplasts and mitochondria are major organelles responsible for electron transport and ATP production, their dysfunction may enhance electron leakage to oxygen, leading to ROS formation, as reflected by increased H₂O₂ levels in this study (Fig. 4A) (Chadee et al., 2021; Møller, 2001; Raghavendra & Padmasree, 2003). Significant decrease in carotenoid content and CAT activity, along with a slight increase in APX activity, indicate that both primary photoprotective and enzymatic detoxification systems were insufficient to maintain cellular

homeostasis (Fig. 2D; Fig. 4C-D). Importantly, the increase in H₂O₂ at EC10_{DCF} suggests that ROS accumulation is triggered already at low DCF concentrations. The multivariate relationships further support the view that DCF caused a broad disturbance of photosynthetic and cellular energy balance (Fig. 7; Fig. 8C; Tab. S6). Taken together, these results point to severe impairment of PSII function, oxidative stress, and reduced photoprotective capacity. The discriminant analysis corroborated this interpretation by identifying H₂O₂, carotenoids, and proline as major contributors to treatment discrimination, emphasizing oxidative stress and loss of photoprotective capacity as key determinants of DCF toxicity. Consequently, DCF toxicity appears to result from widespread bioenergetic disturbance and oxidative imbalance rather than adaptive reorganization.

An important implication of our findings is that morphological plasticity appears closely associated with the energetic status of the cells. Significant shifts toward smaller coenobial forms occurred under FFA and IBU exposure, where compensatory responses remained functional, whereas DCF failed to induce comparable restructuring despite causing strong physiological disturbance. This suggests that coenobial reorganization is not merely a symptom of stress, but an active acclimatory strategy deployable only when sufficient metabolic resources remain available. Morphological plasticity may therefore represent an important indicator of resistance in *Desmodesmus* populations exposed to pharmaceutical contaminants.

Conclusions and Perspectives

Our study demonstrates that growth inhibition alone is insufficient to explain the toxicity of NSAIDs towards microalgae, as compounds producing similar effects on population growth can trigger fundamentally different physiological responses. In the stress-tolerant microalga *Desmodesmus armatus*, FFA, IBU, and DCF elicited three distinct response strategies, ranging from energetically costly acclimation and buffered photochemical adjustment to oxidative photosynthetic collapse. The integration of physiological, biochemical, and morphological endpoints revealed that maintenance of metabolic homeostasis under pharmaceutical stress depends on the balance between photoprotective capacity, antioxidant defenses, and cellular energy allocation. Our findings further suggest that morphological plasticity of *D. armatus* populations is closely linked to physiological resilience and may serve as a sensitive indicator of acclimatory potential in contaminated environments. From an ecotoxicological perspective, these results highlight the need to complement conventional growth-based assays with biomarkers that capture sublethal effects and stress-response pathways. Future studies should examine the persistence and reversibility of these acclimatory responses under chronic exposure

scenarios, investigate their consequences for interspecific competition and population morphological structure, and assess whether similar response strategies occur under environmentally realistic pharmaceuticals concentrations. Such approaches will improve the ecological relevance of algal ecotoxicity assessments and enhance our understanding of the mechanisms governing microalgal resistance to anthropogenic pollutants.

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CRedit authorship contribution statement

Dominika Kapuścińska: Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing, Funding acquisition. Darya Harshkova: Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. Mostafakamal Shams: Investigation, Writing - review & editing. Ivan Liakh: Investigation, Formal analysis. Bartosz Wielgomas: Conceptualization, Supervision, Data curation. Anna Aksmann: Conceptualization, Validation, Writing - original draft, Writing - review & editing, Supervision, Data curation, Project administration, Funding acquisition.

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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Supplementary material for:
NSAID-induced stress triggers physiological and morphological plasticity in
Desmodesmus armatus: a multi-biomarker approach

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Table S1. Characteristics of pharmaceuticals used in the study.

NSAID	Abbreviaiton	Chemical name (IUPAC)	CAS numer	Solvent	Purity
Flufenamic acid	FFA	N-(3-[Trifluoromethyl]phenyl) anthranilic acid	530-78-9	DMSO	97%
Diclofenac	DCF	2-[(2,6-dichlorophenyl)amino]benzeneacetic acid sodium salt	15307-79-6	H ₂ O	98%
Ibuprofen	IBU	4-Isobutyl- α -methylphenylacetic acid	15687-27-1	H ₂ O	99%

23

24 **Table S2.** Parameters of the logistic model ($y = A2 + (A1-A2)/(1 + (x/x0)^p)$) used to fit
 25 concentration-response curves for FFA, DCF, and IBU after 24h exposure. Values are estimates
 26 $\pm$ SE.

NSAID	A1	A2	x0	p	Reduced Chi-Sqr	R-Square (COD)	Adj. R-Square
FFA	98.63891 $\pm$ 1.49708	0.84734 $\pm$ 2.8548	2.35337 $\pm$ 0.09824	1.6267 $\pm$ 0.10748	3.73529	0.99547	0.99494
DCF	101.1794 $\pm$ 1.19599	-63.45574 $\pm$ 27.82196	88.59161 $\pm$ 25.61003	1.0563 $\pm$ 0.09136	1.92861	0.99666	0.99627
IBU	100.69583 $\pm$ 0.41749	4.98169 $\pm$ 1.30383	78.42398 $\pm$ 0.81754	3.36186 $\pm$ 0.09502	0.81502	0.99934	0.99925

27

28 **Text S1.** HPLC method for the analysis of real NSAID concentrations in culture medium.

29 To check the real concentrations of the NSAIDs in the culture medium during the experiment,
 30 high-performance liquid chromatography (HPLC) analysis was performed using a Dionex
 31 UltiMate 3000 HPLC system (Germering, Germany) equipped with UV detector set at 225 nm.
 32 Separation was achieved on an Agilent Zorbax Extend-C18 column (150 mm $\times$ 4.6 mm, 3.5
 33 μ m; Agilent Technologies, Santa Clara, CA, USA) maintained at 40 °C. The mobile phase
 34 consisted of (A) water acidified with 0.1% (v/v) formic acid and (B) acetonitrile. A linear
 35 gradient elution program was applied as follows: 40% B held for 1 min, then increased linearly
 36 to 70% B from 1 to 17 min, maintained at 70% B from 17 to 18 min, returned to 40% B in 0.1
 37 min (18–18.1 min), and finally equilibrated at 40% B for 3,9 min (total run time: 22 min). The
 38 flow rate was 1.0 mL/min, and the injection volume was 5 μ L. Data acquisition and processing
 39 were performed using Chromeleon software (version 7.2.10, Thermo Fisher Scientific).

40

41 **Table S3.** Nominal and measured concentrations used to determine EC values, including EC10
 42 and EC50 for FFA, DCF, and IBU in *D. armatus* culture. Data are presented as means $\pm$ SE of
 43 at least three biological replicates.

Nominal concentration (mg/L)	Real concentration at 0 h (mg/L)	Real concentration at 24h (mg/L)	Concentration remaining after 24 h (% of nominal)
Flufenamic acid			
0.5	0.52 $\pm$ 0.02	0.07 $\pm$ 0.03	14.8 $\pm$ 5.90
1	0.97 $\pm$ 0.03	0.11 $\pm$ 0.05	10.7 $\pm$ 4.89
2	1.73 $\pm$ 0.03	0.23 $\pm$ 0.10	11.6 $\pm$ 4.85
4	3.40 $\pm$ 0.06	1.98 $\pm$ 0.32	49.6 $\pm$ 7.99

8	7.13 ± 0.16	5.03 ± 0.32	63.5 ± 3.96
EC10 = 0.56	0.724 ± 0.024	0.022 ± 0.002	3.9 ± 0.39
EC50 = 2.34	2.395 ± 0.111	0.166 ± 0.044	7.1 ± 1.88
Ibuprofen			
30	19.7 ± 6.18	7.07 ± 1.45	23.6 ± 4.82
60	18.7 ± 0.68	7.73 ± 0.18	12.9 ± 0.30
90	77.1 ± 2.96	62.0 ± 0.20	68.9 ± 0.23
120	110 ± 3.99	85.1 ± 4.05	70.9 ± 3.38
150	140 ± 1.98	99.1 ± 3.06	66.1 ± 1.44
EC10 = 42.3	41.5 ± 0.30	33.1 ± 0.51	78.1 ± 1.21
EC50 = 81.2	77.9 ± 0.56	68.5 ± 0.97	84.3 ± 1.19
Diclofenac			
6.3	6.25 ± 0.57	3.99 ± 0.07	63.3 ± 1.15
12.5	10.9 ± 0.47	7.94 ± 0.11	63.6 ± 0.87
25.1	21.3 ± 3.47	16.1 ± 0.13	64.2 ± 0.53
50.1	32.5 ± 1.78	24.2 ± 2.33	48.2 ± 4.66
75.2	46.7 ± 7.30	50.4 ± 1.76	67.0 ± 2.34
EC10 = 7.4	6.5 ± 0.28	3.8 ± 0.03	51.5 ± 0.37
EC50 = 41.7	38.6 ± 0.46	35.9 ± 0.32	86 ± 0.76

Table S4. Relative abundance of 2-, 4-, and 8-celled coenobia in NSAID-treated cultures. Data are presented as means of at least three biological replicates.

NSAID	Concentration	Relative abundance of coenobia (%)		
		2-celled coenobia	4-celled coenobia	8-celled coenobia
FFA	C	4.7	23.8	71.5
	EC10	8.2	36.9	54.9
	EC50	5.8	72.5	21.7
DCF	C	5.7	12.9	81.4
	EC10	8.5	15.6	75.9
	EC50	4.7	15.3	80.0
IBU	C	8.7	19.2	72.1
	EC10	7.6	21.7	70.7
	EC50	8.5	36.4	55.1

51 **Table S5.** Definitions of OJIP-derived parameters.

Parameter	Definition
PI_{inst.}	the performance index
F_v/F₀	the activity of the water-splitting complex on the donor side of PSII
RCM	fraction density of active reaction centers referring to time when maximal fluorescence is reached
φP₀	maximum yield of primary photochemistry
ψE₀	efficiency/probability with which electron trapped in PSII is transferred from Q _A to Q _B
φE₀	the maximum yield of the electron transport quantum yield of the electron transport flux from Q _A to Q _B
ΔR₀	efficiency/ probability that an electron from the intersystem electron carriers is transferred to reduce final electron acceptors at the PSI acceptor side
φR₀	quantum yield for the reduction of end electron acceptors at the PSI acceptor side
ABS/RC	energy absorption per active PSII reaction center
DI₀/RC	energy dissipation per active PSII reaction center
TR₀/RC	energy trapping per active PSII reaction center
ET₀/RC	efficiency of electron transport per active PSII reaction center
RE₀/RC	reduction of final electron acceptors in PSI calculated per active PSII reaction center.

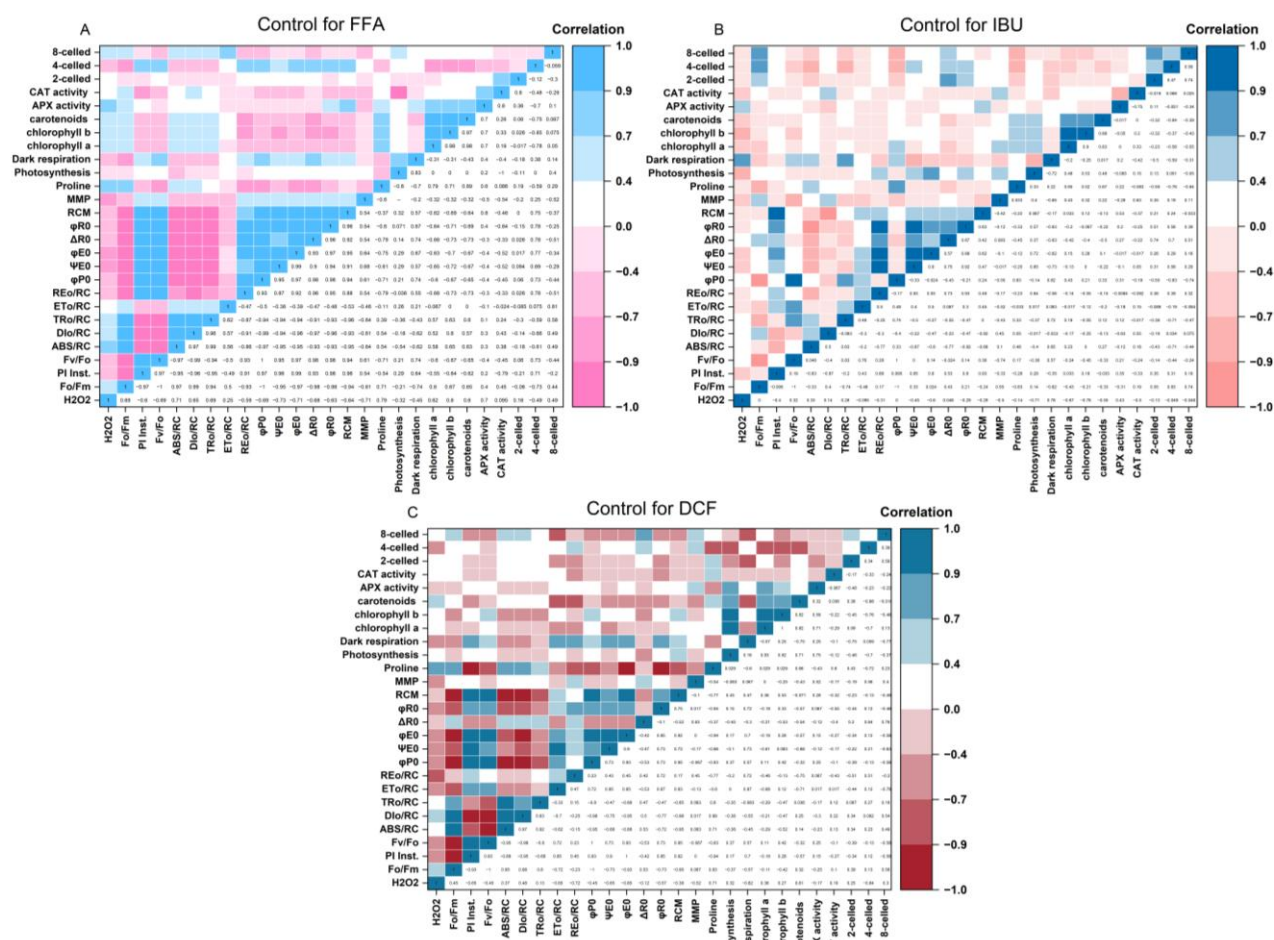
52

53 **Table S6.** Standardized coefficients for selected canonical variables.

Variables	IBU		FFA		DCF	
	Root 1	Root 2	Root 1	Root 2	Root 1	Root 2
H₂O₂	0.60594	0.97720	-8.416	0.41574	1.00333	1.38594
MMP	0.02148	-0.13169	3.121	-0.15696	-0.28883	0.65627
Proline	-2.25705	-1.35222	23.149	3.04258	-1.33719	-1.64433
Dark respiration	0.18667	-0.84875	-13.330	-2.28793	0.19578	1.23249
Carotenoids	2.22383	0.59208	-3.198	-4.14450	-2.66651	0.54978
Total number of cells per mL	0.50581	-1.54172	13.884	-0.58244	-3.48969	-0.93354
2-celled coenobia	0.27394	0.54695	2.926	-0.88791	0.91187	0.73597
4-celled coenobia	-0.38904	0.73018	-14.061	-2.63719	-0.40963	-0.49136

54

Fig. S1. Spearman correlation matrices of the control groups from the (A) FFA, (B) IBU, and (C) DCF experiments.



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OŚWIADCZENIE

Niniejszym oświadczam, iż mój wkład w manuskrypt:

*Kapuścińska D., Harshkova D., Shams M., Liakh I., Wielgomas B., Aksmann A.
NSAID-induced stress triggers physiological and morphological plasticity in *Desmodemus armatus*: a multi-biomarker approach (2026)*

polegał na:

(i) przeprowadzeniu eksperymentów obejmujących hodowlę glonów, wyznaczanie wartości EC oraz oznaczanie liczebności komórek; (ii) wykonaniu analiz fotosyntetycznych, w tym intensywności fotosyntezy, analizy fluorescencji chlorofilu *a in vivo*, oznaczeniu zawartości barwników, oznaczeniu intensywności oddychania ciemniowego oraz oznaczeniu zawartości proliny; (ii) opracowaniu wyników; (iii) udziale w walidacji i interpretacji wyników; (iv) przygotowaniu grafik i rycin przedstawiających wyniki badań; (v) przygotowaniu pierwotnej wersji manuskryptu; oraz (vi) udziale w redagowaniu manuskryptu.

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polegał na:

(i) udziale w przeprowadzeniu eksperymentów obejmujących hodowlę glonów oraz oznaczanie liczebności komórek; (ii) oznaczeniu mitochondrialnego potencjału błonowego (MMP) oraz produkcji nadtlenku wodoru; (iii) analizie statystycznej wyników; (iv) udziale w walidacji i interpretacji wyników; (iv) współtworzeniu rozdziału dotyczącego opisu uzyskanych wyników; oraz (v) udziale w redagowaniu manuskryptu.


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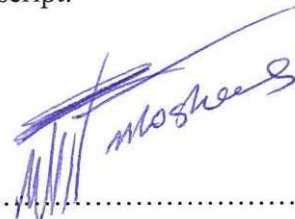
STATEMENT

I hereby declare that my contribution to the manuscript:

*Kapuścińska D., Harshkova D., Shams M., Liakh I., Wielgomas B., Aksmann A.
NSAID-induced stress triggers physiological and morphological plasticity in *Desmodemus
armatus*: a multi-biomarker approach (2026)*

included the participation in:

(i) conducting experiments involving enzyme activity analyses; (ii) performing formal analysis of the obtained data; and (iii) participating in the editing of the manuscript.



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polegał na:

(i) oznaczeniu rzeczywistych stężeń badanych związków metodą HPLC; (ii) przeprowadzeniu formalnej analizy uzyskanych danych; oraz (iii) udziale w redagowaniu manuskryptu.

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NSAID-induced stress triggers physiological and morphological plasticity in Desmodium
armatum: a multi-biomarker approach (2026)*

polegał na:

(i) udziale w opracowaniu koncepcji części analitycznej badań; (ii) sprawowaniu nadzoru nad poprawnością analiz wykonywanych za pomocą HPLC; (iii) udziale w opracowaniu i walidacji wyników uzyskanych za pomocą HPLC; oraz (iv) udziale w redagowaniu manuskryptu.



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armatus*: a multi-biomarker approach (2026)*

polegał na:

(i) przygotowaniu koncepcji badań; (ii) udziale w walidacji i interpretacji wyników; (iii) nadzorze nad realizacją badań; (iv) pozyskaniu finansowania; (v) udziale w przygotowaniu pierwotnej wersji manuskryptu; oraz (vi) udziale w redagowaniu manuskryptu.



PODPIS

15. Pozostała aktywność naukowa

Publikacje naukowe

1. Hejna M., **Kapuścińska D.**, Aksmann A. Pharmaceuticals in the Aquatic Environment: A Review on Eco-Toxicology and the Remediation Potential of Algae. *Int. J. Environ. Res. Public Health*. 2022, 19(13):7717. DOI: 10.3390/ijerph19137717
2. Wilmowicz E., Kućko A., Tranbarger TJ., Ostrowski M., Niedojadło J., Karwaszewski J., **Kapuścińska D.**, Panek K. EPIP as an abscission promoting agent in the phytohormonal pathway. *Plant Physiol Biochem.* 2022, 1;178:137-145. DOI: 10.1016/j.plaphy.2022.03.008
3. Wilmowicz E., Kućko A., Golińska P., Burchardt S., Przywieczerski T., Świdziński M., Brzozowska P., **Kapuścińska D.** Absciscic acid and ethylene in the control of nodule-specific response on drought in yellow lupine. *Environmental and Experimental Botany*. 2020, 169, 103900, DOI:10.1016/j.envexpbot.2019.103900

Wystąpienia na konferencjach naukowych

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