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When chlorophyll tells a different story: Comparing ecotoxicological endpoints in Baltic microphytobenthos

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Short Report

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Abstract

Chlorophyll *a* concentration is widely used to assess microalgal responses to chemical exposure, but may diverge from structural and photophysiological measurements. We reanalysed chlorophyll *a*, community biovolume and maximum photosystem II quantum efficiency (F_v/F_m) measured in the same biological replicates of natural Baltic microphytobenthic assemblages exposed to 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) or the glyphosate-based formulation Roundup®. Responses were compared with same-day controls after 1, 3 and 7 days. On day 7, mean chlorophyll *a* concentrations under [BMIM]Cl exposure were approximately 113% higher than controls, whereas biovolume and F_v/F_m were 23.5–50.9% and 45.0–47.6% lower, respectively. Under Roundup® exposure, lower biovolume coincided with elevated or near-control chlorophyll *a*; F_v/F_m remained near the control at the lower concentration but was lower at the higher concentration. At the higher Roundup® concentration, all three endpoints were below controls initially, but chlorophyll *a* approached the control by day 7. These descriptive patterns show that endpoint agreement depends on exposure conditions and sampling time. Combined measurements distinguish changes in pigment content, biovolume and PSII efficiency that chlorophyll *a* alone does not capture.

1. Introduction

Microalgal toxicity studies assess hazards to aquatic primary producers, including microphytobenthos, which contributes to coastal production (Underwood and Kromkamp 1999). Standard freshwater algal tests assess growth inhibition through biomass or its proxies (OECD 2026). Cell abundance and biovolume quantify organism numbers and volume, chlorophyll *a* measures pigment content, and Fv/Fm describes maximum photosystem II (PSII) quantum efficiency (Hillebrand et al. 1999; Maxwell and Johnson 2000). These endpoints can respond differently to chemical exposure (Chao and Chen 2001).

Natural assemblages contain taxa differing in size, pigmentation, physiology and stress sensitivity. Changes in their relative abundance and physiological state can alter relationships between pigment content, biovolume and photosynthetic performance (Barranguet et al. 1998; MacIntyre et al. 2002). Differences between cyanobacterial and eukaryotic photosynthesis also affect community fluorescence measurements (Campbell et al. 1998).

Previous Baltic studies documented taxonomic, abundance and morphological responses to Roundup®, [BMIM]Cl and $CuCl_2$ (Sylwestrzak et al. 2021, 2022a, b; Sylwestrzak and Zgrundo 2023). Other community studies demonstrated divergent structural and functional responses (Feckler et al. 2018; Corcoll et al. 2019). Here, an exploratory reanalysis of a single experimental series compares chlorophyll *a*, biovolume and F_v/F_m within the same biological replicates across [BMIM]Cl and Roundup® concentrations and sampling times.

2. Materials and Methods

2.1. Experimental design and chemical exposure

This study presents an exploratory reanalysis of measurements from an experimental series conducted in 2015. Cell abundance, taxonomic composition and chloroplast damage were previously reported by Sylwestrzak et al. (2021, 2022a, b). Selected chlorophyll *a*, biovolume and chlorophyll fluorescence measurements from the Roundup® experiments were previously reported by Sylwestrzak et al. (2017), whereas the corresponding measurements for [BMIM]Cl are presented here for the first time. Natural microphytobenthic assemblages colonised glass slides (76 × 26 × 1 mm) deployed for 14 days near Sopot, Gulf of Gdańsk (southern Baltic Sea), approximately 300 m offshore at a depth of 1–2 m. Material was collected during the growing season at a salinity of 7.4. Colonised slides were transported to the laboratory, where the assemblages were scraped off, homogenised in filtered seawater and distributed among Erlenmeyer flasks.

Assemblages were acclimated for 72 h at 18°C, under 60 µmol photons m^{-2} s^{-1} and a 16:8 h light–dark cycle. These conditions were maintained throughout chemical exposure. Each flask contained 100 mL of suspension. Treatments comprised 1-butyl-3-methylimidazolium chloride ([BMIM]Cl; 0.00113 and 0.0175 g dm^{-3}) and Roundup® Ultra 170 SL, containing 170 g L^{-1} glyphosate [N-(phosphonomethyl)glycine] as its monoisopropylamine salt. Nominal glyphosate concentrations (0.042 and 8.5 g dm^{-3}) were calculated from the declared formulation content and the dilution used.

Each treatment comprised three independent replicate flasks. A single set of three untreated control flasks was shared across both substances and all concentrations. The same treatment and control flasks were sampled on days 1, 3 and 7, with separate aliquots used for chlorophyll *a*, biovolume and fluorescence measurements.

2.2. Chlorophyll *a* determination

Suspension aliquots were filtered through glass-fibre filters, and pigments were extracted in 2 mL of 90% acetone. Absorbance was measured at 630, 647, 664 and 750 nm using a Beckman spectrophotometer. Extract chlorophyll *a* concentration was calculated following Jeffrey and Humphrey (1975):

$$\text{Chl a} = 11.85(A_{664} - A_{750}) - 1.54(A_{647} - A_{750}) - 0.08(A_{630} - A_{750}).$$

Concentrations were corrected for extraction and filtered suspension volumes and expressed as µg chlorophyll *a* mL^{-1} of the original suspension.

2.3. Community biovolume determination

Organisms in suspension samples were identified and counted using a Nikon Eclipse 80i microscope equipped with a Nikon DSU2 camera. Biovolume was estimated following Kruk-Dowgiałło et al. (2010), using assigned taxon-specific cell volumes. These were adjusted when observed cell dimensions differed from the assumed values, using geometric approximations appropriate to cell shape (Hillebrand

et al. 1999). For filamentous taxa, biovolume estimates incorporated filament length and the assigned filament width, using the corresponding geometric shape.

Community biovolume was the sum of taxon-specific contributions. For taxa quantified by cell counts, contributions were $N_i \times V_i \times 10^{-9}$ ($mm^3\ mL^{-1}$), where N_i is abundance ($cells\ mL^{-1}$) and V_i is cell volume ($\mu m^3\ cell^{-1}$).

2.4. Chlorophyll fluorescence measurements

Chlorophyll fluorescence was measured using a Hansatech FSM-1 pulse-amplitude-modulated fluorometer. For each measurement, a 5-mL aliquot of culture suspension was filtered through a Whatman GF/C glass-fibre filter. Filters were covered with a thin layer of the corresponding culture suspension and placed in a thermostatted DW2/2 chamber (Sylwestrzak et al. 2017). Following 15 min of dark adaptation, minimum fluorescence (F_o) was recorded under weak measuring light and maximum fluorescence (F_m) during a saturating pulse. Maximum PSII quantum efficiency was calculated as $F_v/F_m = (F_m - F_o)/F_m$ (Maxwell and Johnson 2000).

2.5. Data analysis

Only treatments with all three endpoints measured at each sampling time were included. Percentage responses were calculated as 100 × (treatment mean / same-day control mean − 1). Temporal patterns were assessed descriptively using $\log_2$ treatment-to-control mean ratios; individual day-7 observations were plotted relative to the shared control mean. Shared controls were not counted as additional independent replicates. Day-7 within-flask chlorophyll *a*-to-biovolume and chlorophyll *a*-to-F_v/F_m ratios were natural-log transformed. Exponentiated treatment–control differences in mean log ratios yielded ratios of geometric means. Two-sided exact permutation tests enumerated all 20 allocations of three observations per group (minimum attainable $p = 0.10$) and formed the primary inferential analysis. Welch's *t*-tests with Benjamini–Hochberg adjustment across eight contrasts served as exploratory sensitivity analyses.

3. Results

3.1. Temporal variability in endpoint responses

Under [BMIM]Cl exposure, chlorophyll *a* was near control levels on day 1, while biovolume was lower at the lower concentration and higher at the higher concentration (Fig. 1). On day 3, both endpoints were below controls, with larger relative reductions in biovolume; F_v/F_m was slightly lower at the lower concentration and near the control at the higher concentration. By day 7, chlorophyll *a* exceeded controls, whereas biovolume and F_v/F_m were lower.

At the lower Roundup® concentration, biovolume remained below controls, chlorophyll *a* shifted from below-control values on days 1 and 3 to above-control values on day 7, and F_v/F_m remained near

controls. At the higher concentration, all endpoints were below controls on days 1 and 3; by day 7, chlorophyll *a* approached the control, while biovolume and F_v/F_m remained lower.

3.2. Divergent pigment, biovolume and PSII responses after seven days

On day 7, mean chlorophyll *a* concentrations at 0.00113 and 0.0175 g dm^{-3} [BMIM]Cl were 112.6% and 113.9% higher than the corresponding control, respectively, whereas community biovolume was 23.5% and 50.9% lower and F_v/F_m was 45.0% and 47.6% lower (Fig. 2). At 0.042 g glyphosate dm^{-3}, Roundup® responses relative to the control were + 24.8% for chlorophyll *a*, − 36.5% for biovolume and − 1.6% for F_v/F_m. At 8.5 g glyphosate dm^{-3}, the corresponding responses were + 5.6%, − 62.6% and − 39.3%.

Geometric mean chlorophyll *a*-to-biovolume and chlorophyll *a*-to-Fv/Fm ratios exceeded controls in all treatments, with treatment-to-control fold changes of 1.96–4.80 and 1.28–4.13, respectively (Table S1). No exact permutation test reached significance: $p = 0.10$ for seven contrasts and $p = 0.20$ for chlorophyll *a*-to-Fv/Fm at the lower Roundup® concentration. Exploratory Welch tests yielded BH-adjusted $q < 0.05$ for five contrasts, without providing confirmatory evidence of significance.

4. Discussion

After seven days of [BMIM]Cl exposure, higher chlorophyll *a* coincided with lower biovolume and Fv/Fm (Fig. 2). Pigment plasticity may contribute: chlorophyll content varies with environment, acclimation and taxonomy. Geider et al. (1996) described light-dependent chlorophyll-to-carbon ratios, MacIntyre et al. (2002) linked pigment regulation to photosynthetic characteristics, and Begouen Demeaux et al. (2025) reported higher chlorophyll-to-estimated-carbon ratios under lower irradiance. Here, higher pigment content per unit biovolume could reflect altered cellular pigmentation or taxonomic composition.

Samorì et al. (2011) assessed growth, photosynthetic efficiency and cellular components in *Skeletonema marino*i and *Phaeodactylum tricornutum* exposed to imidazolium ionic liquids. Latała et al. (2010) demonstrated salinity-dependent toxicity to Baltic microalgae and a cyanobacterium. Here, pigment, biovolume and photochemical responses were compared in a natural assemblage.

Periphyton studies support combining structural and functional measurements. Diuron exposure altered biomass, algal class composition and tolerance (McClellan et al. 2008); increased tolerance accompanied changes in pigment composition, lower biomass and lower production-to-respiration ratios (Lips et al. 2022). Greater copper tolerance likewise coincided with lower chlorophyll concentrations and photosynthetic activity in marine biofilms (Corcoll et al. 2019). During diflufenican exposure and recovery, increased chlorophyll fluorescence masked adverse structural responses (Feckler et al. 2018). Although fluorescence differs from extracted pigment concentration, these findings support independent structural measurements.

Differences in taxon sensitivity may help explain the divergent Roundup® responses. Wang et al. (2016) found growth inhibition, no effect or stimulation across 14 marine phytoplankton species exposed to glyphosate, depending on species, concentration and phosphorus availability; some species used glyphosate as their sole phosphorus source. Such variation could favour shifts among taxa differing in size and physiology. Under Roundup® exposure, Pérez et al. (2007) reported reduced micro- and nanophytoplankton abundance but increased picocyanobacterial abundance and primary production, alongside higher periphyton dead-to-live ratios and cyanobacterial abundance. Vera et al. (2010) observed delayed colonisation, lower dry mass, ash-free dry mass and chlorophyll, and predominantly diatom mortality alongside cyanobacterial development. Unlike the concordant biomass and pigment responses reported by Vera et al. (2010), chlorophyll here remained near or above control values on day 7, despite lower biovolume.

Endpoint agreement changed during Roundup® exposure, highlighting the importance of sampling time. Previous periphyton studies also demonstrate effects of exposure duration and history (McClellan et al. 2008; Tlili et al. 2011). Under [BMIM]Cl, biovolume was below controls at both concentrations by day 3, whereas Fv/Fm reductions were more pronounced on day 7. Fluorescence was therefore not consistently the earliest indicator. Endpoint sensitivity also differed between cadmium and cobalt in *Raphidocelis subcapitata* (dos Reis et al. 2021). Despite its widespread use in rapid ecotoxicological assays (Ralph et al. 2007; Suresh Kumar et al. 2014), dark-adapted Fv/Fm describes maximum PSII quantum efficiency, not organism quantity or pigment content (Maxwell and Johnson 2000).

Biovolume accounts for organism size and shape but remains distinct from measured carbon biomass (Hillebrand et al. 1999). Chao and Chen (2001) reported that cell division can be more strongly inhibited than photosynthesis at low toxicant concentrations, with increased cell volume partly offsetting reduced abundance. Here, biovolume provided a quantitative measure derived independently of pigment measurements. This comparison of endpoints within matched biological replicates over time extends earlier Baltic microphytobenthos research (Sylwestrzak and Zgrundo 2014).

5. Conclusions

Assessment of natural Baltic microphytobenthos depended on endpoint choice and sampling time. Maintained or increased chlorophyll *a* did not necessarily indicate preserved biovolume or PSII efficiency, while near-control F_v/F_m could coexist with lower biovolume. Measuring these endpoints across sampling times distinguishes pigment, biovolume and photochemical responses: no single endpoint captures the full pattern of community change.

Declarations

Competing interests

The author declares no financial or non-financial competing interests.

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Author Contribution

Z.S. conceived the present study, analysed and interpreted the data, prepared the figures and tables, and wrote and revised the manuscript. Z.S. approved the final version and takes responsibility for all aspects of the work.

Data Availability

The data supporting the findings of this study, including replicate-level measurements and detailed statistical results, are available from the corresponding author upon reasonable request.

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Figures

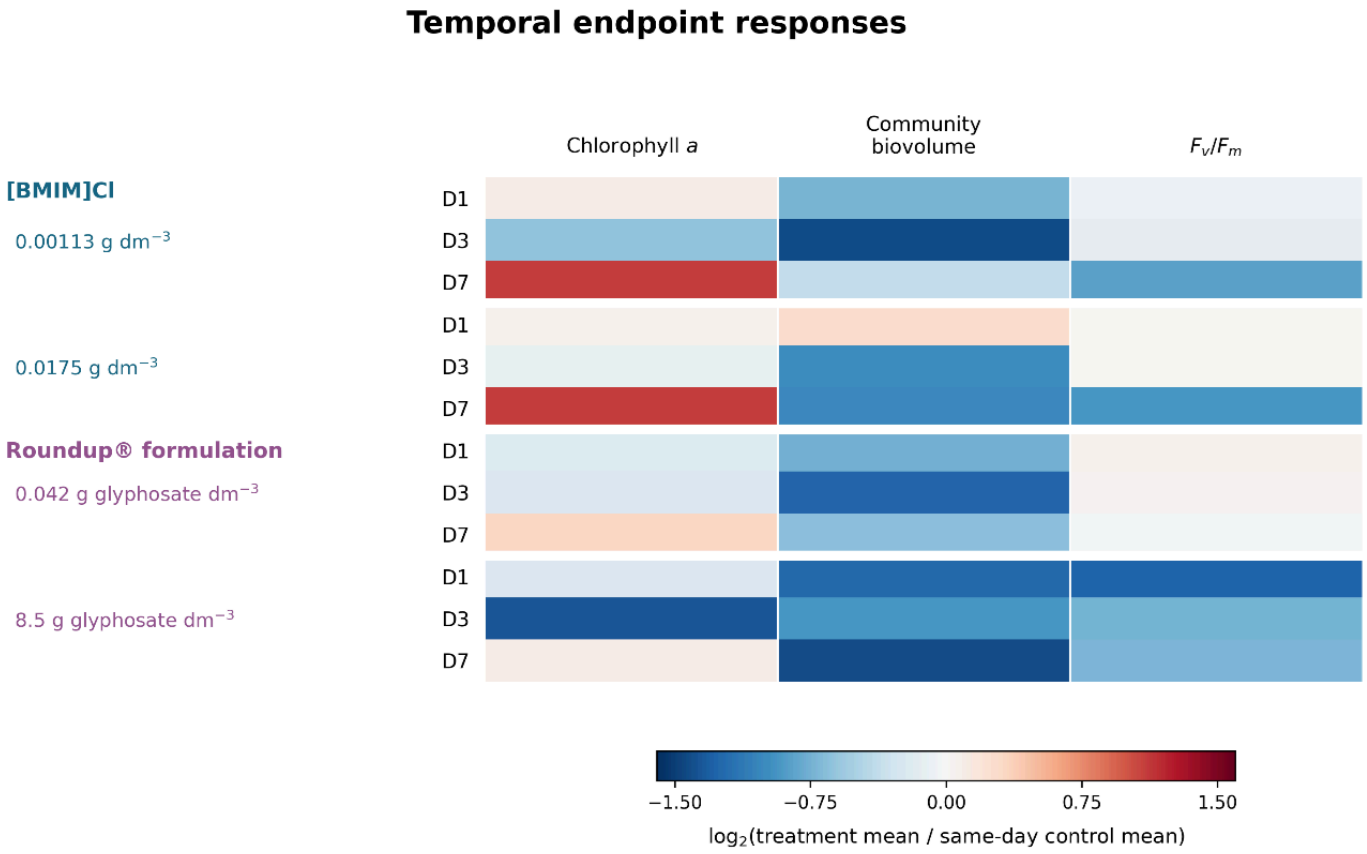

Figure 1

Temporal responses of chlorophyll *a*, biovolume and Fv/Fm, expressed as $\log_2$(treatment mean / same-day control mean) on days 1, 3 and 7. Blue and red indicate lower and higher values; colour intensity denotes effect magnitude, not significance. Concentrations are in g dm^{-3}, referring to glyphosate for Roundup®.

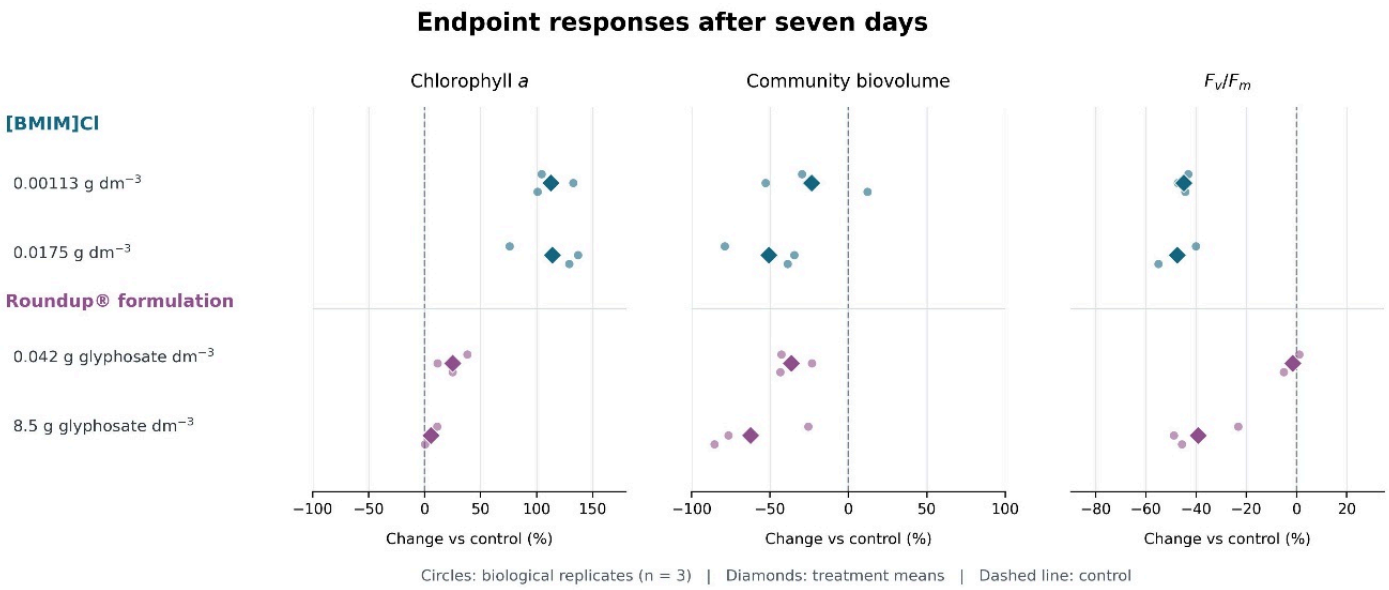

Figure 2

Day-7 endpoint differences relative to the control mean (%). Circles represent individual flasks (n = 3 per treatment), diamonds treatment means, and the dashed line the mean of three control flasks. Concentrations are in g dm⁻³, referring to glyphosate for Roundup®. Horizontal axis ranges differ among panels.

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