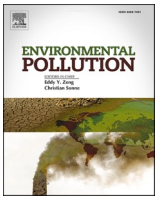
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Natural abundance $\delta^{13}C$ constraints on the detection of microplastic-derived carbon in freshwater environments☆

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ABSTRACT

Microplastics (MPs) are increasingly recognized as emerging pollutants in freshwater systems. Detecting and tracing MP-derived carbon in aquatic food webs, however, remains unresolved, limiting our understanding of ecological impacts. Here, we evaluate the potential and limitations of natural abundance stable carbon isotope measurements ($\delta^{13}C$) as a tool to identify MP signals in freshwater ecosystems. For this purpose, two freshwater algae, *Chlorella vulgaris* and *Chlamydomonas reinhardtii*, were exposed under controlled laboratory conditions to one non-biodegradable polymer, low-density polyethylene (LDPE), and two biodegradable polymers, polylactic acid (PLA) and polybutylene adipate-co-terephthalate (PBAT), to assess isotope composition and growth. Laboratory data were complemented by particulate organic carbon ($\delta^{13}C_{POC}$) measurements from seasonal Danube River campaigns (2023–2024) with modeled predictions based on dissolved organic carbon ($\delta^{13}C_{DIC}$).

MP exposure did not inhibit algae growth, but *C. vulgaris* exhibited significant ($p < 0.05$) $\delta^{13}C$ enrichment (+4 to +5 ‰), whereas *C. reinhardtii* showed no isotopic response. These shifts were unrelated to polymer isotope values and likely reflect indirect physiological stress rather than assimilation of polymer-derived carbon. Complementary binary mixing experiments further confirmed that measurable isotopic shifts occur only at unrealistically low algae-to-MP ratios (≤10:1), underscoring the limited sensitivity of isotope mass balances. Field surveys revealed pronounced seasonal $\delta^{13}C_{POC}$ variability in the Danube, spanning 7.4 ‰ annually. Yet deviations from modeled expectations were inconsistent with MP inputs and instead reflected natural drivers such as productivity and remineralization. Overall, while natural abundance $\delta^{13}C$ can capture subtle algae responses to MP exposure under laboratory conditions, its diagnostic power for tracing MP-derived carbon in complex freshwater systems appears limited.

1. Introduction

Freshwater ecosystems are pivotal sources of drinking water, yet highly susceptible to external disturbances and anthropogenic pollution (Bexeitova et al., 2024; Häder et al., 2020). Among these stressors, microplastics (MP) pose a growing risk, threatening biodiversity and ecological functioning. Particularly, the dynamic nature of freshwater not only disperses MPs vertically and horizontally, but also facilitates their transport across trophic levels and habitats (Elizalde-Velázquez & Gómez-Oliván, 2021; C. Li et al., 2020; Talbot & Chang, 2022). As a result, MPs are omnipresent, with numerous studies documenting their occurrence in aquatic systems across every continent (e.g., Anderson et al., 2017; Biginagwa et al., 2016; Gao et al., 2024).

MPs are defined as solid, water-insoluble plastic particles with at least one dimension between 1 μm and 1 mm, while those from 1 mm to 5 mm are classified as large MPs (ISO 24187, 2024). They can originate as primary MPs, intentionally manufactured at small sizes, or as secondary MPs, formed via degradation of larger debris (Cole et al., 2011; Wagner & Lambert, 2017). Common polymer types are polyethylene (PE), including high-density (HDPE) and low-density (LDPE) forms, polyethylene terephthalate (PET), polypropylene (PP), polystyrene (PS), polyurethane (PUR), and polyvinyl chloride (PVC) (Revel et al., 2018;

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Shah et al., 2008).

While numerous studies have documented MP impacts on aquatic organisms such as zooplankton and fish, their effects on potential disruptions at the base of the aquatic food webs, i.e., primary producers such as algae, remain less explored (de Sá et al., 2018; Garcia et al., 2021; Rani-Borges et al., 2021). Yet, they have critical functions in ecosystem processes, including carbon fixation, O_2 production, and overall nutrient cycling (Hitchcock, 2022; Podbielska & Szpyrka, 2023). Even subtle MP-induced changes in algae physiology could propagate along the food chains. However, the literature reports mixed results: Some studies show inhibition on growth, chlorophyll, or photosynthesis (Sjollema et al., 2016; Tunali et al., 2020; Q. Wang et al., 2020), while others find neutral or even stimulatory responses (Canniff & Hoang, 2018; Song et al., 2020; Yokota et al., 2017). These inconsistencies likely reflect differences in experimental design, polymer properties (e.g., concentration, type, size, aging state), and species-specific sensitivity. Moreover, mechanistic pathways for algae responses to MPs also remain unresolved - whether MPs are directly assimilated, adsorbed onto cell surfaces or act indirectly by altering metabolic balance. Thus, caution is advised, when interpreting potential MP-impacts on primary producers, particularly when extrapolating to ecosystem-level consequences.

Beyond biological impacts, even the reliable identification of MPs in natural environments introduces a complex set of methodological challenges. One of the most significant issues is the absence of standardized, universally accepted methods for their detection and classification (Hartmann et al., 2019). To overcome this limitation, a combination of visual inspections (e.g., microscopy) and chemical techniques (e.g., spectroscopy) is commonly applied (Araujo et al., 2018; Hidalgo-Ruz et al., 2012; Prata et al., 2019). However, these approaches are often time-consuming, labor-intensive, and prone to misclassification errors, where natural organic or mineral particles are mistaken for plastics or *vice versa* (Shim et al., 2017).

In an attempt to trace plastic-derived materials in the environment, more recent studies employed stable isotope techniques (Berto et al., 2017; Liu et al., 2024). Natural abundance $\delta^{13}C$ isotope measurements provide a non-invasive means of distinguishing among organic sources and can, in principle, identify MP-derived carbon without artificial labeling. As many polymers retain the isotopic signatures of their feedstocks, $\delta^{13}C$ values can differentiate plastics derived from plant-based versus petroleum-based sources. While such source-specific fingerprints allow to trace geographic or industrial origins (Birch et al., 2021), they provide limited insight into ecological processes and consequences. Specifically, natural abundance measurements cannot directly resolve how MP-derived carbon is transformed, remineralized, or assimilated into biomass in aquatic systems.

To resolve carbon turnover more directly, studies using ^{13}C-labeled MPs have shown that microbial communities can partially mineralize MP carbon and incorporate it into cellular biomass (Goudriaan et al., 2023; Taipale et al., 2019). These tracer experiments underscore the potential capacity of isotope tracers for resolving microbe-MP interactions at high resolution. However, despite their promise, ^{13}C-labeling techniques remain technically demanding, expensive, and vulnerable to interferences from background natural organic material, which can obscure the ^{13}C signal from labeled microplastics (Liu et al., 2024; Taipale et al., 2019). These limitations restrict a broad application of tracer-based methods, particularly in complex natural systems with heterogeneous organic components.

A promising complementary strategy is to examine natural abundance $\delta^{13}C$ systematics of freshwater particulate organic carbon (POC) to detect shifts associated with MP inputs. This approach relies on two well-established isotopic principles. First, many petroleum-derived plastic polymers exhibit $\delta^{13}C$ values that differ from those of natural organic matter. Second, microbial metabolism discriminates against ^{13}C, preferentially utilizing the lighter carbon isotopes (^{12}C) and thereby enriching the residual organic matter in ^{13}C (Fry, 2006). As a result, if MPs contribute carbon directly to the POC pool, or indirectly alter algal carbon processing through adsorption or toxicity, they could induce shifts in $\delta^{13}C$ even under natural abundance conditions. Although this possibility has not yet been systematically evaluated in aquatic environments, it offers a conceptual basis for testing whether $\delta^{13}C$ signatures can resolve *in situ* MP-derived carbon, forming the basis of the present study.

To determine whether natural abundance measurements of stable carbon isotopes hold the potential to trace MP contributions in freshwater systems, we combined controlled algae exposure experiments with seasonal river monitoring. In the laboratory, two common freshwater algae species, namely *Chlorella vulgaris* and *Chlamydomonas reinhardtii*, were exposed to LDPE, a widely distributed non-biodegradable plastic, and PLA (polylactic acid) and PBAT (polybutylene adipate-co-terephthalate), two increasingly used biodegradable alternatives. Hereby, isotopic shifts and growth responses were evaluated to test whether natural abundance $\delta^{13}C$ can be used to detect polymer carbon or if indirect physiological effects occur. In parallel, POC was collected across five seasonal campaigns in the Danube River (2023–2024). The Danube, Europe's second-longest river, stretching 2857 km, represents a complex, high-discharge, and heavily utilized waterway in central and eastern Europe, with documented spatial and temporal variability in MP loads (Gao et al., 2024). Yet, reported findings remain inconsistent, particularly regarding polymer composition and distribution. Some studies identified PE as the predominant polymer (e.g., Kittner et al., 2022; Schrank et al., 2022; Van Der Wal et al., 2015), whereas others reported PET dominance (e.g., Kiefer et al., 2023; Pojar et al., 2021; Stanković et al., 2024). Such discrepancies, often linked to differences in sampling depths or methodological approaches, emphasize the heterogeneity of the system. Reported MP fluxes also vary by orders of magnitude, ranging from 0.0002 to 0.009 particles/L in Austria (Hohenblum et al., 2015; converted following Kiefer et al., 2023) to concentrations as high as 95 ±53 % particles/L in the delta region (Kiefer et al., 2023). This variability, further amplified by discharge conditions, complicates accurate detection and quantification.

In an attempt to link mechanistic sensitivity tests under controlled conditions to observations in large natural systems, natural abundance carbon isotopes measurements of $\delta^{13}C_{POC/DIC}$ were applied. We specifically investigated at what MP loads and under which algal responses $\delta^{13}C$ shifts become detectable at natural abundance and if $\delta^{13}C_{POC}$ values in the Danube deviate from autotrophic expectations in a manner consistent with MP inputs. Thereby, this study addresses a central knowledge gap regarding the detectability of MP-derived carbon under environmentally realistic conditions. By defining the sensitivity limits and diagnostic power of natural abundance $\delta^{13}C$ as a complementary tool in MP research, we provide a novel framework for its potential application in future freshwater monitoring.

2. Material and methods

2.1. Microplastics

Three polymer types were selected: A conventional petroleum-derived plastic LDPE, and two biodegradable plastics, i.e., PLA and PBAT. LDPE was included because it is among the most common polymers detected in rivers, while PLA and PBAT were tested as increasingly used biodegradable alternatives (Farah et al., 2016; Wei et al., 2015; Witt et al., 1999). All polymers were purchased from Zhangmu Huachuang Plastics Raw Materials (Guangdong, China). Their $\delta^{13}C$ values were −35.04 ±0.06 ‰ for LDPE, −27.07 ±0.01 ‰ for PBAT and −10,62 ±0.02 ‰ for PLA. The volume-weighted mean diameters of these particles ranged between 72.4 and 232.9 μm, and detailed particle characterization has previously been published by Zhang & Ruess (2025).

2.2. Microalgae experiment

2.2.1. Cultivation conditions

Axenic cultures of *Chlorella vulgaris* (CCAP 211/12) and *Chlamydomonas reinhardtii* (CCAP 11/32A) were obtained from the Culture Collection of Algae and Protozoa, Oban, Scotland. *C. vulgaris* and *C. reinhardtii* are both green microalgae, which are frequently used as model organisms and are known primary producers, ubiquitous in European freshwater systems (Hohenblum et al., 2015; Pencik et al., 2023; Prihanto et al., 2022). Cultures were grown in Bold's Basal Medium (Andersen et al., 2005) with 2.95 mM $NaNO_3$ (pH 7.2) at room temperature (21 °C) under natural light. For the passaging procedure of the pre-cultures, inocula (10 % v/v) from a pre-culture (7–9 days old) were transferred to new medium (500 mL Erlenmeyer flask, 100 mL medium), closed with cotton plugs and covered with aluminum foil. Culture acclimation to upscaled conditions was ensured by growing two sequential pre-cultures prior to each experiment under the same conditions. Culture maintenance, experiment setup and sampling were performed in sterile conditions under a SafeFAST classic clean bench (Carlo Erba, IT).

2.2.2. Biomass production and binary mixing

To test detection limits, bulk biomass of both algae was produced by cultivating three replicate flasks (1000 mL Erlenmeyer flask, 200 mL medium) per strain for 14 days. Cultures were pooled, transferred under sterile conditions to 50 mL Falcon tubes and centrifuged (20 min, 6729 rcf, Eppendorf 5804 R). Biomass pellets were washed by resuspension in fresh Bold's Basal Medium. After a final centrifugation step, the remaining medium was carefully decanted, and the Falcon tubes were kept at −20 °C overnight. The next day, samples were placed inside a lyophilizer (CHRIST ALPHA 1–4) and freeze-dried (vacuum <20 mbar, −54 °C) for 48–72 h. Defined masses of *C. reinhardtii* (5000 ±16 µg; $\delta^{13}C = -20.60 \pm 0.04$ ‰) were mixed with LDPE powder at ratios of 100:1, 20:1, 10:1, 7.5:1, and 5:1 (algae-to-MP by weight). Mixtures were suspended in 500 mL Milli-Q water, filtered, and analyzed identically to natural POC samples (see Section 2.4).

2.2.3. Microplastic incubation experiments

To test algal growth and isotopic shifts to MP additions, 12 Erlenmeyer flasks (500 mL, 100 mL medium) were prepared per species (9 treatments + 3 controls; n = 3). Cultures were inoculated with 10 % (v/v) of a 10-day-old pre-culture and kept on a rocking shaker (INFORS HT, Celltron, 70 rpm). For each polymer, triplicate batches were amended with ~56.03 ±4.17 mg/L (*C. vulgaris*; ~6–9 particles/L) or ~68.79 ±15.57 mg/L (*C. reinhardtii*; ~8–11 particles/L) LDPE, PBAT or PLA, respectively (Table S2 in Supplementary Material II (SM II)). For comparison, controls were grown in the absence of MPs.

Growth was tracked by optical density (NanoPhotometer, IMPLEN; λ = 540 nm and λ = 750 nm for *C. vulgaris*, *C. reinhardtii*, respectively) and cell counts. For the latter, 1 mL sample was transferred to a 2 mL Eppendorf tube, mixed with 5 % glutardialdehyde (1:2 v/v) and stored at 4 °C until counted using a Neubauer improved chamber and a Zeiss Axioplan Microscope (20x/40x). Aggregation of cells was common, leading to an underestimation of total counts; therefore, growth rates (µ) were calculated from ln(OD) during exponential growth (days 0–7 for *C. vulgaris* and days 0–6 for *C. reinhardtii*). Final biomass was harvested and processed as in section 2.2.2.

2.3. Danube field sampling

To examine whether natural abundance isotope measurements could reveal MP-related carbon signals in dynamic freshwater environments, we conducted five seasonal sampling campaigns (spring, summer, late summer, fall, winter 2023–2024), sampling at regular intervals along ~2800 km of the Danube River mainstream and its major tributaries (54–89 sites per campaign; details in Maier et al., 2025). A map of sampling sites during late summer is provided in SM II. Briefly, water samples were collected using a weighted 2-L polyethylene bottle that was submerged 1–2 m below the surface from the flowing section of the river to obtain well-mixed water and minimizing surface effects. During each campaign, triplicate samples were collected at a minimum of two sites to ensure reproducibility.

2.4. Stable isotope analyses

2.4.1. POC sampling and preparation

For POC analyses, unfiltered water was collected directly from the Danube River in 500 mL acid-washed HDPE Nalgene bottles. These were triple rinsed with sample water prior to collection. POC samples were filtered onto pre-weighted glass fiber filters (GF-5, pore size 0.4 µm; Macherey- Nagel, Düren, Germany) that had been pre-combusted at 400 °C for 4 h to eliminate any residual organic carbon. Filters were then freeze-dried for 60 min under vacuum conditions (<10 mbar) using a Lyovac GT 2 GT 2-E freeze-dryer (FinnAqua, Gemini BV, Apeldoorn, Netherlands). Dried filters were subsequently pulverized in a carbo-free mortar ball mill (CryoMill, Retsch, Verder, Vleuten, Netherlands), and subsequently dried and fumigated with a concentrated HCl in a desiccator for 24 h to remove potential remaining carbonate particles. In parallel, samples for algae-MP laboratory experiments were processed using the same protocol.

2.4.2. DIC/DOC sampling and preparation

For dissolved inorganic carbon (DIC) and dissolved organic carbon (DOC) $\delta^{13}C$ analyses, water was filtered (0.45 µm, surfactant-free cellulose acetate membrane) into 40 mL amber vials, poisoned with 20 µL of a saturated $HgCl_2$ solution to inhibit biological activity, sealed with a butyl rubber/PTFE septum and stored at 4 °C in the dark until further analyses. Although only $\delta^{13}C_{DIC}$ values were required for the present analyses, $\delta^{13}C_{DOC}$ was determined from the same vials and retained for calculations described later (see section 2.6).

2.4.3. Stable isotope measurements

All processed materials from algae-MP samples and Danube POC (section 2.2-2.4) were weighed into tin capsules (5 × 9 mm, IVA Analysentechnik GmbH & Co. KG, Meerbusch, Germany) and analyzed on an elemental analyzer (Costech ECS 4010, NC Technologies, Bussero, Italy) coupled in continuous flow mode to an IRMS (Delta V plus, ThermoFisher, Bremen, Germany).

Stable carbon isotope compositions of DIC and DOC were determined after passing samples through a TIC/TOC extraction unit (Aurora 1030 W, OI Analytical, College Station, Texas, USA) that applied a wet oxidation method with phosphoric acid and persulfate in two sequential steps. The extraction unit was operated in continuous-flow mode and coupled to an isotope ratio mass spectrometer (IRMS, Delta V plus, Thermo Scientific). Details of the analytical method are provided in St-Jean (2003) and van Geldern et al. (2013).

All isotope ratios are expressed against an international standard according to the δ notation with:

$$\delta^{13}C = \frac{{}^{13}/_{12}R_s}{{}^{13}/_{12}R_r} - 1 \quad (1)$$

where ${}^{13}_{12}R_s$ is the carbon isotope ratio of the heavy to light isotopes in the sample and ${}^{13}_{12}R_r$ is the ratio of the reference standard (Vienna Pee Dee Belemnite, VPDB, Coplen, 2011). All stable isotope values were multiplied by 1000 to express them in per mille (‰). The stable isotope data were corrected for instrumental drift and linearity after van Geldern et al. (2013). Standard variation (1σ) of repeat measurements was better than ±0.2 ‰. The analytical protocol aimed to minimize carbonate interference and ensure high precision, critical for detecting small isotopic shifts that might arise from MP assimilation.

2.5. Isotopic mass balance

To evaluate whether MP addition could produce detectable $\delta^{13}C$ shifts in organic matter, we conducted a series of controlled mixing experiments. Therefore a fixed amount of freeze-dried *C. reinhardtii* algal material (5000 ±16 µg; $\delta^{13}C_{POC}$ = −20.60 ±0.04 ‰) was combined with LDPE microplastic (<180 µm) ($\delta^{13}C_{POC}$ = −35.04 ±0.05 ‰) at weight ratios of approximately 100:1, 20:1, 10:1, 7.5:1, and 5:1. Mixtures were suspended in water, filtered and processed following the same procedure as natural POC samples (see section 2.4). Each mixture was measured in replicates (n = 5), and mean $\delta^{13}C_{POC}$ values and standard deviations were calculated. Expected $\delta^{13}C_{POC}$ values were calculated using a two-endmember mass balance:

$$\delta^{13}C_{expected} = \frac{\left[\left(\delta^{13}C_{Algae} \times m_{Algae}\right) + \left(\delta^{13}C_{LDPE} \times m_{LDPE}\right)\right]}{\left(m_{Algae} + m_{LDPE}\right)} \quad (2)$$

2.6. Isotopic modelling of POC sources

To investigate whether seasonal variation in $\delta^{13}C_{POC}$ reflects microplastic input or related biogeochemical shifts, we compared measured $\delta^{13}C_{POC}$ values with modeled expectations based on $\delta^{13}C_{DIC}$ and typical C_3 plant fractionation.

We adopted a conservative, autotrophic model by assuming that POC primarily originates from phytoplankton production. This assumption is supported by recent research (Maier et al., 2025), that documented strong photosynthetic impacts in the Danube. Accordingly, expected $\delta^{13}C_{POC}$ values were calculated from measured isotope values of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) using a typical isotope fractionation factor (ε) of 20 ‰ for C_3 photosynthesis (Ghashghaie et al., 2003):

$$\delta^{13}C_{POC(expected)} = \delta^{13}C_{DIC} - \varepsilon_{photosynthesis} \quad (3)$$

For comparison, we initially tried to apply a two-end-member isotope mass-balance mixing model, assuming that POC is a combination of dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC). In practice, this approach proves unsuitable, as POC constitutes a distinct carbon pool rather than a mixture of DOC and DIC. Consequently, the model frequently produced implausible estimates (i.e., DOC fractions >1 or negative DIC fractions; see section 1.1 in SM I (Eqs (1)–(3)).

Given that the two-end-member model was invalid, all related statistical analyses were based on the conservative autotrophic model. The deviation between measured and expected $\delta^{13}C_{POC}$ isotope signatures was calculated as:

$$\Delta = \delta^{13}C_{POC} - \delta^{13}C_{POC(expected)} \quad (4)$$

2.7. Statistical analyses

Normality of $\delta^{13}C_{POC}$ and $\delta^{13}C_{DIC}$ values was assessed using the Shapiro-Wilk tests (Shapiro & Wilk, 1965), which confirmed that data did not deviate significantly from normal distribution (p >0.05 for all cases). Subsequently, Student's *t*-tests were applied to evaluate (i) whether measured $\delta^{13}C_{POC}$ deviated from the expected autotrophic baseline (paired *t*-test, one sided), (ii) whether seasonal means (spring and late summer) differed significantly (two sample *t*-test, two sided), and (iii) whether growth rate of *C. vulgaris* and *C. reinhardtii* differed between MP treatments and controls (two-sample *t*-test, two sided). All statistical analyses were conducted in R (v. 4.3.2; R Core Team, 2023) using the shapiro.test and t.test functions.

3. Results

3.1. Binary mixing experiment

Binary mixtures of freeze-dried *C. reinhardtii* and LDPE demonstrated that detectable isotopic shifts only occur at high MP loadings. LDPE, with $\delta^{13}C$ of −35.04 ±0.06 ‰, was selected for these experiments due to its pronounced isotopic contrast to *C. reinhardtii* (−20.60 ±0.04 ‰) (Fig. 1; Table S1, SM II). At low MP loadings, corresponding to higher algae-to-MP mixing ratios (100:1 and 20:1), $\delta^{13}C_{POC}$ values remained close to the original algae endmember, indicating minimal or no accumulation of MP in algal tissue. Shifts became more apparent at lower admixture levels (10:1, 7.5:1, 5:1), with the largest $\delta^{13}C_{POC}$ deviation reaching 2.17 ±0.14 ‰ relative to the algae baseline. While this trend is consistent with a two-component mixing model, the magnitude of $\delta^{13}C_{POC}$ remained limited, even at MP concentrations unlikely to occur in natural environments (10:1, 7.5:1, 5:1). Minor deviations between measured $\delta^{13}C_{POC}$ values and those predicted by the ideal binary mixing model were observed across all ratios, but most were within analytical uncertainty. These findings indicate that the isotopic shift becomes detectable by natural abundance analyses only when substantial MP additions are present.

3.2. Microalgae responses to microplastics

Overall, isotopic responses to the presence of MPs during algal growth differed between species. In *C. vulgaris*, exposure to all three MPs led to significant increases in $\delta^{13}C_{POC}$ values of approximately 4–5 ‰ relative to the control (p <0.05; Fig. 2). Yet, albeit the originally different $\delta^{13}C$ signatures of the MPs supplied, measured $\delta^{13}C_{POC}$ values of treated algae did not correspond linearly to the isotopic signatures of individual MP types (PLA: −10.62 ±0.02 ‰, PBAT: −27.07 ±0.01 ‰, and LDPE: −35.04 ±0.06 ‰). Notably, the LDPE treatment resulted in $\delta^{13}C_{POC}$ values of −32.90 ±0.04 ‰, which were more positive than the $\delta^{13}C$ of the LDPE itself (Fig. 2, Table S3 in SM II). Contrarily, in *C. reinhardtii*, $\delta^{13}C_{POC}$ values across all treatments remained close to the control value (−26.8 ±0.87 ‰), with no pronounced differences between treatments (Fig. 2; Table S3 in SM II).

In addition to isotopic composition ($\delta^{13}C_{POC}$), cell densities ($OD_{540/}$

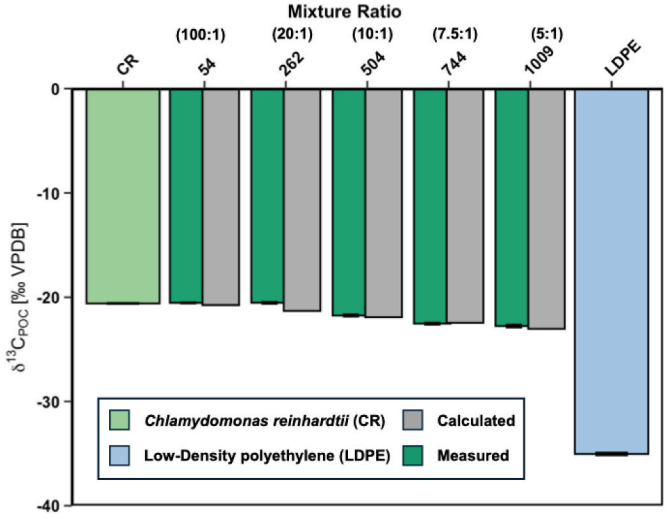


Fig. 1. Comparison between measured and mass-balance-calculated $\delta^{13}C$ values in algal-microplastic mixtures. $\delta^{13}C$ values (‰ VPDB) of freeze-dried *Chlamydomonas reinhardtii* (CR) biomass (−20,60 ± 0.04 ‰) and low-density polyethylene microplastic (LDPE; −35,04 ± 0.06 ‰), defined as isotope endmembers. Bars display measured $\delta^{13}C_{POC}$ values for mixtures with constant CR biomass (5000 µg ± 16 µg) and progressively increasing microplastic proportions, corresponding to algae-to-MP ratios of 100:1, 20:1, 10:1, 7.5:1, and 5:1. Green bars represent measured data; gray bars show expected values calculated from the mass balance (Eq. (2)). Error bars indicate standard deviations from replicates (n = 5). See Table S1 in SM II for the full dataset. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

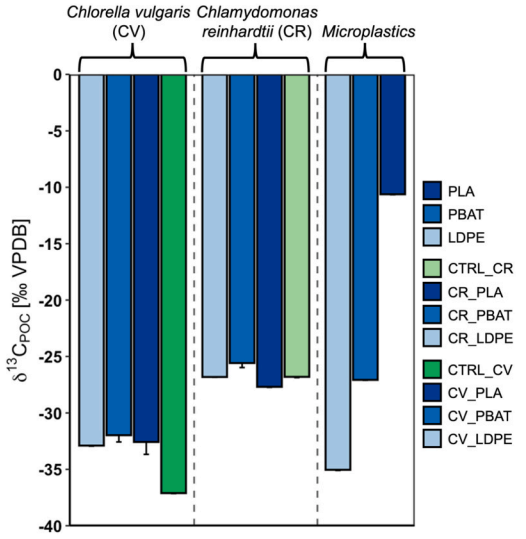


Fig. 2. $\delta^{13}C_{POC}$ values of *Chlorella vulgaris* (CV) and *Chlamydomonas reinhardtii* (CR) following exposure to various microplastic (MP) types. $\delta^{13}C_{POC}$ values (‰ VPDB) from growth experiments with CV and CR exposed to different MP types. Treatments include controls without MPs (CTRL_CV, CTRL_CR) and exposures to polylactic acid (PLA; CV_PLA, CR_PLA), polybutylene adipate-co-terephthalate (PBAT; CV_PBAT, CR_PBAT), and low-density polyethylene (LDPE; CV_LDPE, CR_LDPE). Pure PLA, PBAT, and LDPE MPs are shown for reference on the right. Values represent means ± SD (n = 5). See Table S3 in SM II for the full dataset.

$_{750}$, counts) and growth rates (μ_7/μ_6, d^{-1}) were determined to assess general growth performance and to evaluate potential MP-related effects under controlled laboratory conditions. However, no inhibitory effect induced by the presence of MPs was discernible.

Cell densities of *C. vulgaris* increased over an 11-day cultivation period across all MP treatments, including PLA, PBAT, LDPE, and the control (Fig. 3A). While cultures treated with PLA and LDPE exhibited slight deviations, the overall growth trend was quite similar, with exponential increase up to day 7 followed by a plateau phase. However, this plateau phase becomes more evident upon looking at the log-transformed absorbance values (ln[OD_{540}]), revealing comparable temporal dynamics between treatments (Fig. 3B). Surprisingly, control values usually fell below those of MP-treated samples, which also became more evident when calculating growth rates over the first 7 days. Here, MP-treated samples exhibited growth rates >0.3 d^{-1}, whereas the control was <0.3 d^{-1} but differences were not statistically significant (p >0.05; Fig. 3C; Table S4 in SM II).

Cell densities of *C. reinhardtii* steadily increased over an 18-day cultivation period across all MP treatments, including PLA, PBAT, LDPE, and the control (Fig. 4A). All cultures followed a similar growth trajectory, with exponential increase up to day ~9, followed by a slight plateau phase. Log-transformed absorbance values confirmed previous observations by showing comparable temporal dynamics between treatments (Fig. 4B). This trend was further confirmed by growth rates calculated over the first 6 days (μ_6), which did not differ substantially across all conditions (p >0.05), with mean values of 0.34 ±0.01 in the control and 0.35–0.37 ±0.01 d^{-1} in the MP treatment (Fig. 4C; Table S5 in SM II).

3.3. Seasonal variability of $\delta^{13}C_{POC}$ in the Danube River

Strong seasonal variability of $\delta^{13}C_{POC}$ values was observed in the Danube River, ranging from −31.7 ‰ to −24.3 ‰ across the five sampling campaigns (Fig. 5). The highest mean values occurred in late summer (−25.8 ±0.7 ‰) and were significantly higher (p <0.05) than the lowest values in spring (−29.6 ±0.7 ‰). Summer samples showed the greatest variability (−29.0 ±0.9 ‰), with three pronounced $\delta^{13}C_{POC}$ minima in the upper (−31.0 ‰), middle (−30.8 ‰), and lower Danube (−30.7 ‰). In contrast, the other seasons showed less pronounced variability and lacked distinct $\delta^{13}C_{POC}$ minima or maxima.

Comparisons with the autotrophic baseline ($\delta^{13}C_{DIC}$ - ε_p) revealed consistent deviations. Therefore, ΔPOC, as the difference between measured and expected values across all seasons, was calculated. Recognizing that algae growth and their isotope values vary seasonally, $\delta^{13}C_{POC}$ was measured in samples collected along the Danube River and several of its major tributaries during five campaigns in spring, summer, late summer, fall, and winter. This approach allowed for a comprehensive assessment of both seasonal and spatial variability.

Measured $\delta^{13}C_{POC}$ consistently exceeded model expectations and resulted in positive mean ΔPOC across all seasons (Fig. 6, Table S6 in SM II). These ranged from 0.28 ‰ in spring 2024 to 5.26 ‰ in late summer 2024. One-sided t-tests for isotopic depletion (H_1: ΔPOC <0) were not significant (p >0.05). In contrast, the complementary test for isotopic

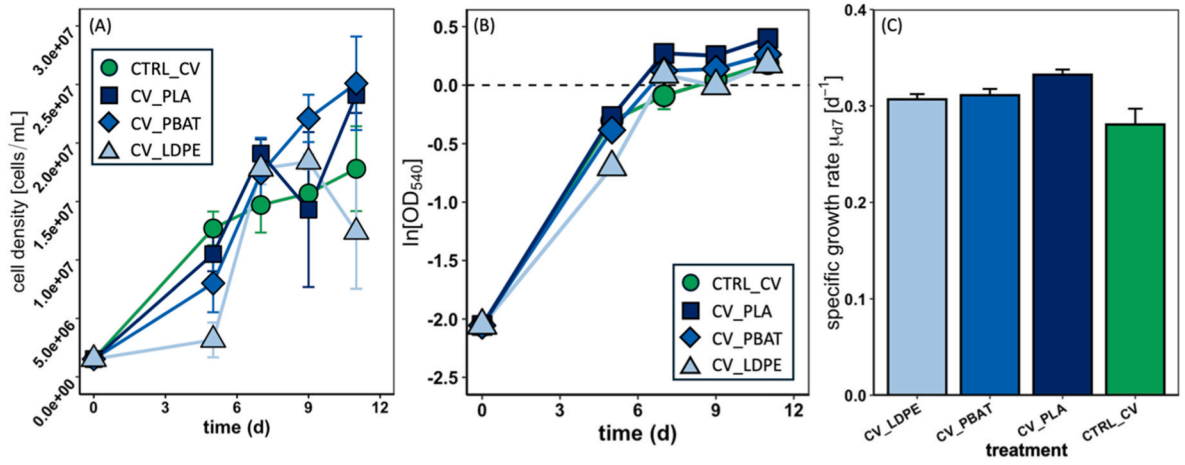


Fig. 3. Growth performance of *Chlorella vulgaris* (CV) in response to microplastic (MP) exposure and a control treatment without MPs (CTRL_CV, green). (A) Cell density [cells/mL] over time following exposure to polylactic acid (CV_PLA, dark blue), polybutylene adipate-co-terephthalate (CV_PBAT, blue), and low-density polyethylene (CV_LDPE, light blue). (B) Natural logarithm of optical density at 540 nm (ln[OD_{540}]) over the experimental period for the same treatments as in (A). (C) Specific growth rate (μd_7; [d^{-1}]), calculated as the slope of ln[OD_{540}] during the first 7 days of exposure. Error bars indicate standard error. See Table S4 in SM II for the full dataset. Values represent means ± SD (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

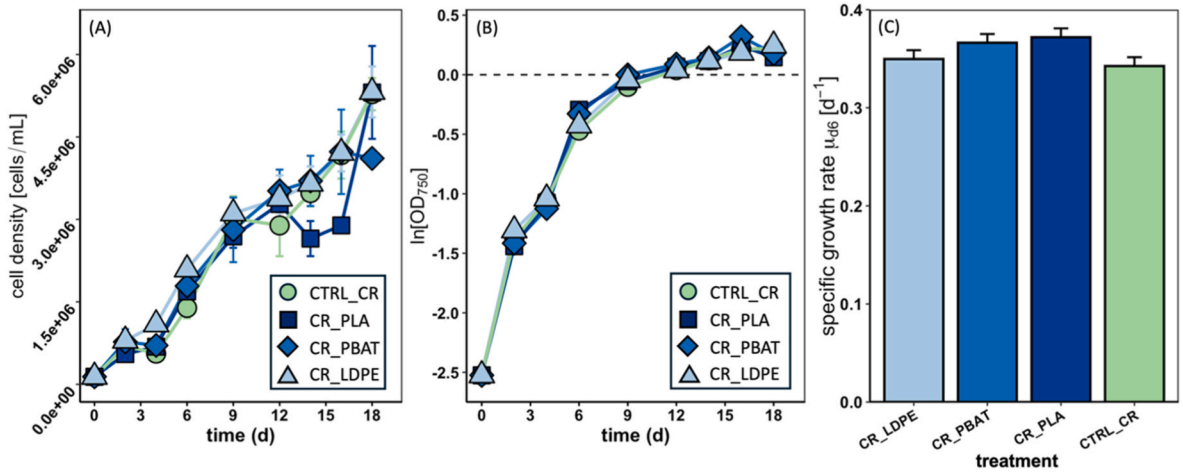


Fig. 4. Growth performance of *Chlamydomonas reinhardtii* (CR) in response to microplastic exposure and a control treatment without MPs (CTRL_CR, green). (A) Cell density [cells/mL] over time following exposure to polylactic acid (CR_PLA, dark blue), polybutylene adipate-co-terephthalate (CR_PBAT, blue), and low-density polyethylene (CR_LDPE, light blue). (B) Natural logarithm of optical density at 750 nm (ln[OD750]) over the experimental period for the same treatments as in (A). (C) Specific growth rate (μd_6; [d^{-1}]), calculated as the slope of ln[OD750] during the first 6 days of exposure. Error bars represent standard error. See Table S5 in SM II for the full dataset. Values represent means ± SD (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

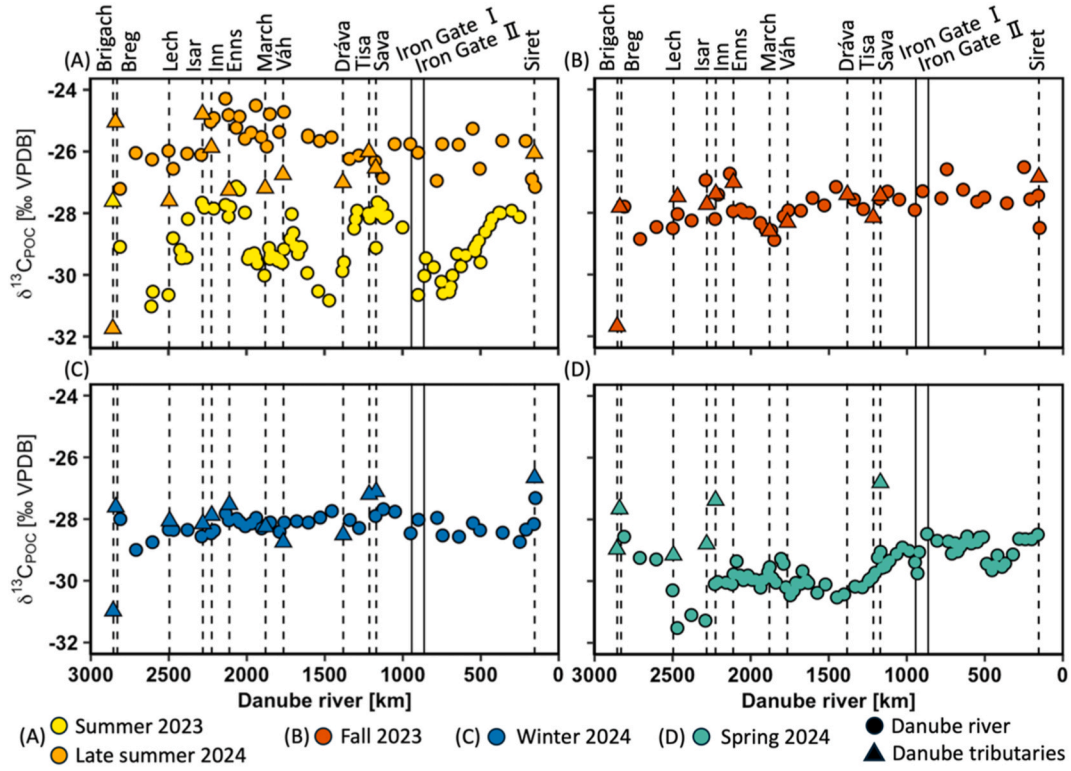


Fig. 5. Stable carbon isotopes of particular organic carbon ($\delta^{13}C_{POC}$) along the Danube River and its major tributaries. Measured $\delta^{13}C_{POC}$ illustrates variations with distance from the river mouth across different seasons, highlighting spatial trends and seasonal shifts in isotopic composition. Circles represent Danube River samples, and triangles denote tributary samples. Dashed lines indicate the river km where tributaries enter, and vertical solid lines represent the Iron Gate I and II dams. Standard error bars are within the symbol size.

enrichment (H_1: ΔPOC >0) yielded significant results (p <0.05), demonstrating that $\delta^{13}C_{POC}$ was consistently enriched in ^{13}C relative to the modeled phytoplankton-derived carbon signature across all seasons.

4. Discussion

4.1. Sensitivity limits of isotopic detection

To assess potential isotopic shifts caused by MPs, we first conducted binary mixing experiments using freeze-dried *C. reinhardtii* biomass (−20.60 ±0.04 ‰) mixed with varying amounts of LDPE (−35.04

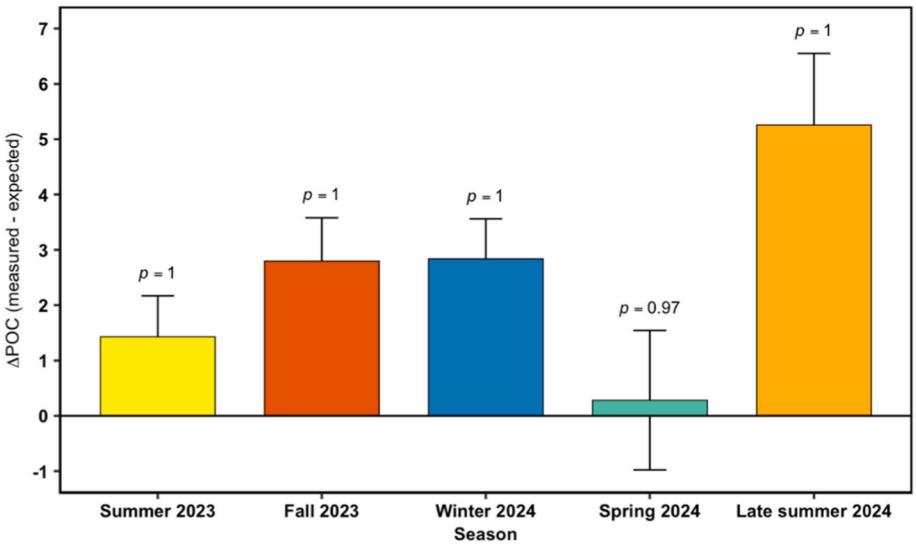


Fig. 6. Seasonal variation in $\delta^{13}C_{POC}$ deviations (ΔPOC = measured − expected) across five time periods. Expected $\delta^{13}C_{POC}$ values were derived from $\delta^{13}C_{DIC} - \varepsilon$, with a fractionation factor $\varepsilon = 20$ ‰, representative for C_3 plant photosynthesis. Bars shows mean ΔPOC values; error bars indicate standard deviations. One-sided *t*-tests (H_0: ΔPOC ≥0; H1: ΔPOC <0; $\alpha = 0.05$) were used to assess significant isotopic depletion in each season. Corresponding p-values are displayed above each bar. See Table S6 in SM II for the full dataset.

±0.02 ‰) (Fig. 1; Table S1 in SM II). Isotopic shifts became increasingly apparent at lower algae-to-MP ratios (10:1, 7.5:1, 5:1), although the overall magnitude of change remained moderate. However, these MP ratios are far above concentrations typically reported in natural waters, with a minor fraction of total POC ~0.1–5 % (Zhao et al., 2025). Thus, the mixing experiment does not mirror realistic MP pollution scenarios but provides a controlled test to confirm the validity of our binary two-component mass-balance model (Eq. (2)). Furthermore, small but consistent deviations between measured and theoretical values were observed. We attribute these to non-ideal mixing behavior, likely resulting from incomplete homogenization due to particle size, shape, or density differences between algal detritus and MP fragments. Such heterogeneity can influence the effective carbon contribution of each component and lead to measurable discrepancies from simple linear predictions. Hence, this observation apparently underlines the practical limitation of isotope mixing approaches since even under controlled laboratory conditions, the physical properties of MPs may complicate isotopic quantification.

Building on this proof-of-principle, we next applied the natural abundance stable isotope approach to living algal biomass to test whether MP exposure could induce measurable shifts in $\delta^{13}C$ during growth. For this purpose, two freshwater algae species — *C. vulgaris* (−37.11 ±0.03 ‰) and *C. reinhardtii* (−26.80 ±0.07 ‰) — both widely distributed in European freshwater systems, were chosen (e.g., Safi et al., 2014; Sasso et al., 2018). Each species was exposed to three polymer types spanning a wide isotopic range: PLA (−10.62 ±0.02 ‰), PBAT (−27.07 ±0.01 ‰), and LDPE (−35.04 ±0.02 ‰). These polymers represent commonly detected plastics (i.e., LDPE) and also less frequently encountered plastics (e.g., PLA) in freshwater habitats (Fig. 2; Table S3 in SM II). Notably, the $\delta^{13}C$ signatures of the algae cultures partially overlapped with those of the tested polymers, particularly LDPE and PBAT. The applied exposure concentrations (~68.79 ±15.57 mg/L for *C. vulgaris* and 56.03 ±4.17 mg/L for *C. reinhardtii*) exceed typical environmental levels reported in most freshwater systems, including the Danube River (e.g., Gao et al., 2024; Lechner et al., 2014). However, such concentration can serve as a conservative upper-end scenario and may still be relevant for heavily polluted or localized hotspots. Employing them under controlled laboratory conditions increased the likelihood of detecting subtle isotope effects that would otherwise be obscured at environmentally realistic concentrations.

Overall, MP exposure did not cause pronounced isotopic changes in either species. However, *C. vulgaris* consistently exhibited a significant enrichment in $\delta^{13}C_{POC}$ by approximately +4 to +5 ‰ across all three MP treatments ($p < 0.05$). Notably, this enrichment was not proportional to the isotopic offset between the algae and the respective MP types. For instance, PLA had the largest isotopic difference from algal biomass, yet did not induce stronger isotopic enrichment than PBAT or LDPE. In fact, an inverse trend emerged, where exposure to the more $\delta^{13}C$-depleted LDPE also led to higher $\delta^{13}C$ values in *C. vulgaris*. Such a pattern indicates that direct incorporation of MP-derived carbon into algal biomass is not the dominant mechanism, suggesting indirect physiological effects. Surface adsorption of MPs onto algae cell surfaces is well documented (Wang et al., 2023; Xie et al., 2024), and thus could indirectly influence cellular metabolism. *C. vulgaris* is also known to possess a very thick cell wall (100–200 nm) that provides resistance to chemical, physical, and mechanical stress (Weber et al., 2022). Thus, the observed enrichment in ^{13}C most likely stems from indirect physiological responses, such as stress-induced changes in photosynthetic fractionation via MP adsorption, rather than direct MP assimilation. However, evidence from higher plants shows that micro- and nanoplastics can interfere with the photosynthetic system (e.g., Jia et al., 2023; Sun et al., 2024), suggesting that such metabolic perturbations are mechanistically possible. Nevertheless, in terms of the algae tests here, such metabolic effects appear less likely. Instead, we consider physical mechanisms, such as shading or particle adsorption (Sun et al., 2023), to be the dominant drivers, while a direct impact on metabolic effects and carbon incorporation cannot be entirely ruled out.

In contrast, *C. reinhardtii* displayed no measurable isotopic enrichment following MP exposure, with $\delta^{13}C_{POC}$ values remaining near the control (−26.8 ±0.87 ‰). This stability suggests that neither carbon pool composition nor fractionation during photosynthesis was substantially altered, indicating either resilience or reduced interaction with MPs. While both species possess relatively thick cell walls, differences in wall composition and surface properties may influence MP interactions, potentially limiting adsorption and associated physiological responses in *C. reinhardtii* (Inwood et al., 2008; Svensen et al., 2007). Accordingly, direct incorporation of MP-derived carbon into biomass is also unlikely, although adsorption processes may still occur. Nonetheless, ^{13}C-labeling studies demonstrate that some degree of assimilation and even mineralization of plastic-derived carbon can occur under certain conditions

(Goudriaan et al., 2023; Taipale et al., 2019), however, they also emphasize that such processes remain inconclusive and highly context-dependent and are species- and polymer-specific. Additionally, the lower MP exposure concentration used for *C. reinhardtii* (ca. 56 mg/L versus 68 mg/L for *C. vulgaris*) may have reduced the likelihood of detectable isotopic impacts. The absence of $\delta^{13}C$ shifts in *C. reinhardtii,* therefore highlights species-specific susceptibility in algal species related to MP.

Taken together, these results highlight distinct taxon-dependent isotopic responses to MP exposure. While $\delta^{13}C$ values in *C. vulgaris* were consistently enriched, *C. reinhardtii* remained isotopically stable. This contrast reveals that isotopic responses likely reflect subtle, taxon-specific metabolic stress rather than generalizable assimilation of MP-derived carbon. Consequently, natural abundance $\delta^{13}C$ signals in algae should be interpreted cautiously, as they may serve as early-warning indicators of physiological perturbation, but not as straightforward tracers of plastic carbon incorporation.

4.2. Algae responses to MP exposure

Exposure to MPs did not result in notable inhibitory effects on growth in either of the freshwater primary producers. Hence, growth curves and final biomasses of *C. vulgaris* and *C. reinhardtii* were comparable between treatments and controls, regardless of the polymer type amended (Figs. 3 and 4; Tables S4 and S5 in SM II). In fact, *C. vulgaris* and *C. reinhardtii* exhibited slightly enhanced growth in some treatments, though differences were not statistically significant ($p > 0.05$), consistent with previous studies reporting neutral or even stimulatory MP effects on algal biomass production (Canniff & Hoang, 2018; Rani-Borges et al., 2021).

Nevertheless, reports for *C. vulgaris* vary widely. For instance, growth inhibition and impaired photosynthesis have often been linked to PS and PVC, reflecting their comparatively higher toxicity (Fu et al., 2019; Hazeem et al., 2020; Xie et al., 2024), whereas PE and PET showed generally less detrimental impacts under similar conditions (Pencik et al., 2023; Wang et al., 2023). Furthermore, strong growth inhibition has mainly been observed at higher concentrations (>50–1000 mg/L; Sjollema et al., 2016; Tunali et al., 2020) and smaller particle sizes (<6 μm; Sjollema et al., 2016). Similar context dependence emerges for *C. reinhardtii*, where PVC and PS have been linked to impaired growth at higher concentrations (>100 mg/L; Li et al., 2020; Q. Wang et al., 2020; Yan et al., 2021), PET reduced growth only at very small particles (<7 μm; Pencik et al., 2023), while larger particles of HDPE and PP (>400 μm) produced no detectable effects or only delayed responses (Lagarde et al., 2016). This underpins that plastic type is an essential factor in the adverse influence of MPs on biota (e.g., Ji et al., 2021). In soil ecosystems, the greatest differences in effect were reported between petroleum-based and biodegradable MPs (Fan et al., 2022; Liu et al., 2023), plastic types, which were also used in this study. However, no difference in the response of algae to conventional and biodegradable MP could be detected.

Taken together, our findings indicate that algal responses to MP exposure were mainly species dependent, while previous work suggests that polymer type, particle size and concentration may also play a role. Although *C. vulgaris* showed consistently $\delta^{13}C$ enrichment, neither species displayed signs of growth inhibition under the tested MP exposures. The apparent absence of toxicity under tested conditions likely reflects a combination of factors, including the exclusion of highly toxic polymers (PS and PVC), the use of moderate, environmentally relevant MP concentrations (~50–70 mg/L; well below the >100 mg/L typically associated with toxicity), relatively large particle sizes (72–233 μm; exceeding the <7 μm where adverse effects are generally observed) and the short exposure duration (~2 weeks), which may have limited the manifestation of detectable adverse effects. Consistent with this finding, we further did not observe microscopically visible shading effects due to e.g., adsorption of MP particles onto cell surfaces in our experiments (Figures S2 A-D and S3 A-D in SMII), a result that may be attributable to factors outlined above, despite prior reports describing such phenomena (Schwab et al., 2011; Wang et al., 2023; Xie et al., 2024). Based on these findings, future studies should prioritize investigating size-dependent responses across a broader range of MPs and algal taxa under environmentally realistic scenarios.

4.3. Field application and seasonal dynamics of POC measurements in the Danube River

The binary mixing and the MP exposure experiments established both the detection thresholds and the isotopic response patterns of freshwater algae under MP exposure. Although effects were generally small, species-specific, and detectable only at concentrations above environmental relevance, these insights provided the basis to examine whether comparable signals could be resolved in the Danube River, a large and dynamic freshwater system that is known to contain MPs (e.g., Gao et al., 2024; Kiefer et al., 2023; Kittner et al., 2022), and additionally exhibits strong natural variability in carbon cycling (e.g., Maier et al., 2021). This approach allowed us to evaluate whether natural abundance $\delta^{13}C$ can serve as a diagnostic tool under environmental conditions, or whether the isotopic effects of MPs are masked by other processes.

Across the five sampling campaigns, $\delta^{13}C_{POC}$ values in the Danube exhibited pronounced seasonal variability, ranging from −31.7 ‰ in spring to −24.3 ‰ in late summer (Fig. 5), reflecting an amplitude of 7.4 ‰ over the annual cycle. Such variability is expected in a large river system and reflects the dynamic interplay between autochthonous primary production, terrestrial inputs, and hydrological forcing in shaping POC isotope composition (Stumm & Morgan, 1995; Wang et al., 2012; Wetzel, 2011). Winter $\delta^{13}C_{POC}$ values were comparatively stable, reflecting reduced in-river primary production and greater terrestrial contributions during high-discharge periods (Aramaki et al., 2010; Maier et al., 2025; Reddy et al., 2021). In contrast, summer values were more variable and enriched, coinciding with peak algal productivity under warmer, light-rich conditions when autochthonous inputs dominate the POC pool. Transitional seasons in spring, fall, and late summer revealed a progression from the lightest isotopic signals associated with freshly produced, minimally processed organic matter in spring, to increasingly enriched values later in the year as new biomass inputs declined and microbial decomposition preferentially removed lighter carbon (Fry, 2006). This seasonal trajectory culminated in the highest $\delta^{13}C_{POC}$ values by late summer, when respiration and remineralization processes left the residual POC pool enriched in heavier isotopes (DeNicola, 1996; Maier et al., 2025; Uehlinger et al., 2000).

Yet, measured POC values diverged systematically from model predictions (Fig. 6). Neither our DIC/DOC two-end-member model (Eqs. (1)–(3) in SM I) nor calculations based on a fractionation (ε_p) of 20 ‰ for C_3 photosynthesis (Eq. (3)) were able to reproduce observed values across all seasons. Accounting for realistic variability in ε_p (±5 ‰ depending on CO_2 supply, light, and growth) slightly reduced the positive residuals but did not eliminate them, indicating that the observed enrichment represents a genuine, real seasonal signal rather than a modeling artefact. Hence, this persistent mismatch suggests that $\delta^{13}C_{POC}$ dynamics in the Danube are shaped by additional biogeochemical processes that extend beyond the scope of simple end-member approaches.

However, in the context of MP exposure in freshwater systems, these dynamics are particularly revealing. The isotopic impact of biomass production and its turnover processes is sufficiently strong to effectively mask any subtle MP-related signals in a large, productive river system. Our binary mixing experiments reinforce this interpretation and show that only unrealistically high MP concentrations produce measurable isotopic shifts. These levels far exceed those found in freshwater environments. As a result, the likelihood to resolve an MP signature *in situ* is therefore limited, except perhaps during narrow seasonal periods when biomass contributions are minimal. Spring season may represent such a

period, as our calculated ΔPOC offset is minimal following the complete degradation of overwintered organic material and before new production reaches its peak. During this period, the relative contribution of alternative carbon sources—including MPs—could become more apparent, making the calculation of potential plastic fraction theoretically possible. Even then, the overwhelming dominance of fresh biomass production during bloom onset, combined with relatively low concentrations of MPs in the system, makes it unlikely that an isotopic fingerprint of MPs could be resolved with confidence. Overall, while our results outline a conceptual framework for when and how MPs might influence $\delta^{13}C_{POC}$, they also highlight a key limitation: in highly productive freshwater systems like the Danube, MP-derived isotopic effects remain largely obscured by the natural biomass signal.

5. Conclusions

This study combined controlled laboratory experiments, binary mixing tests, and seasonal monitoring of a large river system to assess whether MP exposure could induce detectable shifts in natural abundance $\delta^{13}C_{POC}$ as a potential tool for detecting MP-derived carbon in freshwater systems. Across all approaches, a consistent picture emerged. Laboratory exposures of *C. vulgaris* and *C. reinhardtii* to three MP polymers (LDPE, PLA, PBAT) revealed species-specific responses: neither species showed growth inhibition, and only *C. vulgaris* exhibited subtle but significant $\delta^{13}C$ increases, independent of the isotope values of added polymers. Complementary binary mixing experiments reinforced this interpretation, demonstrating that isotope shifts only become measurable at unrealistically low algae-to-MP ratios ($\leq$10:1), which underscores the fact that MP contributions are generally too small to alter $\delta^{13}C$ signals. Consistently, field monitoring of the Danube River revealed pronounced seasonal $\delta^{13}C_{POC}$ variability driven by primary production and its remineralization, as well as terrestrial inputs and hydrological forcings, yet no consistent isotope attributed to MPs was discernible. Overall, our results clearly demonstrate that the $\delta^{13}C$ method exhibited very low environmental detection sensitivity, because natural biomass signals dominate the $\delta^{13}C$ composition. These backgrounds therefore mask any potential contribution from realistic MP loads, a finding confirmed by laboratory experiments, $\delta^{13}C_{DIC}$ modeling, and field observations.

Additionally, determining toxic effects of MPs on microalgae remains particularly challenging, as current evidence is still inconclusive, and the extent to which plastics are assimilated into biomass remains unclear. Although the $\delta^{13}C$ enrichment observed in *C. vulgaris* may suggest indirect physiological stress, changes in natural abundance isotope ratios alone are insufficient to draw definitive conclusions. Future research should therefore focus on defining toxicity in algae-MP interactions and differentiate between physiological stress, physical interference, and pathological effects. Future complementary approaches will be essential to close these knowledge gaps and to assess the ecological significance of MP for the carbon cycle and primary production in freshwater systems.

CRediT authorship contribution statement

Jan Maier: Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization. **Johannes A.C. Barth:** Writing – review & editing, Resources, Project administration, Methodology, Funding acquisition. **Liliane Ruess:** Writing – review & editing, Visualization, Resources, Methodology. **Kathrin Castiglione:** Writing – review & editing, Visualization, Resources, Methodology. **Patrik Blenk:** Visualization, Resources, Methodology. **Anna-Neva Visser:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Data curation, Conceptualization.

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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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2025.127435.

Data availability

Data will be made available on request.

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