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SPECIAL ISSUE-LETTER

Plastic particles adsorb to the roots of freshwater vascular plant *Spirodela polyrhiza* but do not impair growth

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Scientific Significance Statement

Plastic particles constitute persistent environmental pollutants in aquatic ecosystems, where they accumulate in increasing concentrations and pose potential threats to aquatic life. The effects of nanoplastics (< 100 nm) and microplastics (> 100 nm, but < 5 mm) on vascular plants remain largely unknown, even though these plants have an important role in ecosystems. Results of this study show that the exposure of duckweed to nano- and microplastics does not significantly impact plant growth or chlorophyll production. Microscopy results clearly showed external attachment of nanoplastics on duckweed roots, which can potentially impact higher trophic levels in the food chain.

Abstract

We investigated the effect of nano- and microplastics on the freshwater duckweed species *Spirodela polyrhiza*, a vascular plant. *S. polyrhiza* was exposed for 120 h to concentrations ranging from 10^2 to 10^6 particles·mL^{-1}. We assessed effects on growth and chlorophyll production, and explored adsorption and absorption by way of confocal microscopy. For both nano- and microsized particles, no concentration-dependent effects on growth were found (expressed as fresh weight, frond, and root sizes). In addition, chlorophyll concentrations were not significantly affected. Confocal microscopy indicated that nanosized plastic particles adsorbed externally to the duckweed, especially to the roots. Internalized plastic particles could not be detected. Nevertheless, given their important role in ecosystems as a food source for a range of organisms, the adsorption of plastic particles to *S. polyrhiza* roots as detected in this study can result in the transfer of plastic particles to diverse herbivorous species within the ecosystem.

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Author Contribution Statement: L.C.D. and T.B. defined the research topic and together came up with the research question as well as the research design regarding the growth and chlorophyll aspects. L.C.D. performed and conducted the experiments, and the majority of the data collection, including the cultivation of test species, the experimental exposure of test species, and the measurement of growth and chlorophyll endpoints. T.B. assisted in performing the research by assisting the cleaning of plastic particles and preparation of exposure concentrations. B.W.B. introduced and conducted the methodology of microscopic imaging, with L.C.D. assisting. M.G.V. contributed to interpretation and discussion of the outcomes. All authors contributed to the data analyses and the completion of the manuscript.

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Plastic debris frequently enters the natural environment, where it accumulates and acts as an environmentally persistent contaminant (Horton et al. 2017). Smaller particles such as nanoplastics (< 100 nm) and microplastics (> 100 and < 5 mm) (Koelmans et al. 2015) have gained considerable attention, because they are potentially bioavailable to many organisms (Wright et al. 2013). The environmental concentrations of such small plastic particles < 100 μm are not well known, because standardized procedures for collection, fractionation, characterization, and quantification are lacking, which results in underestimation especially for smaller particles sizes (Huvet et al. 2016; SAPEA 2019). Concentrations are expected to increase with decreases in particle size, and predicted concentrations of 50 nm particles range between 10^3 and 10^{10} particles·mL^{-1} (Lenz et al. 2016). Accelerating production, deposition, and the bioinert character of plastics contribute to further growing environmental concentrations (Huvet et al. 2016; Horton et al. 2017; SAPEA 2019).

To date there are only a few studies that focus on the impact of plastic particles on primary producers (Yokota et al. 2017), of which only three focus on vascular plants. Kalčíková et al. (2017) reported that the exposure of duckweed (*Lemna minor*) to 30–600 μm plastic particles decreased root cell viability and growth. The two sediment-rooted macrophytes *Myriophyllum spicatum* and *Elodea sp*. exhibited reduced root to shoot ratios when exposed to 50–190 nm plastic particles, and *M. spicatum* also showed decreased shoot length for these nanoplastics and reduced main shoot length for 20–500 μm microplastics (van Weert et al. 2019). A study on cress (*Lepidium sativum*) found significant but transient effects of plastic particles on germination rates and root growth (Bosker et al. 2019).

The lack of research on vascular plants results in a major knowledge gap concerning the effects of plastic particles on ecosystem health (Eerkes-Medrano et al. 2015). For example, aquatic freshwater plants provide shelter for many organisms at higher trophic levels, and serve as food sources to herbivorous species in the water as well as in fringing ecosystems. To help address this knowledge gap, the objective of our study was to determine if plastic particles negatively impact the freshwater vascular plant *Spirodela polyrhiza*, a duckweed species. Therefore, we studied the effects of nanoplastics (50 nm) and microplastics (500 nm) on the growth of fronds, roots, and fresh weight, as well as the effects on chlorophyll content of *S. polyrhiza*. *S. polyrhiza* is a freshwater vascular plant at the base of aquatic food webs (Greenberg et al. 1992) and has commonly been used as an ecological indicator to assess the toxicity of substances because of its high sensitivity (Böcük et al. 2013). Additionally, to answer the question of potential transfer along the food web, we assessed adsorption and uptake of the nanoplastics.

Methods

Test materials

S. polyrhiza, a species of duckweed and a freshwater vascular plant, was obtained from a commercial source (MicroBioTests, Gent, Belgium). Spherical polystyrene fluorescent plastic particles (density 1.05 g·cm^{-3}) of 50 nm (red) and 500 nm (green) were used (Fluoro-Max Aqueous Fluorescent Particles; Thermo-Scientific, Waltham, MA, U.S.A.). To remove surfactants, plastic particles were cleaned prior to usage (*see* Supplementary Information).

Experimental design

Prior to the toxicity assessment, turions were germinated in a 48-well test plate with 1 mL of Steinberg growth medium for 72 h at 25°C with 6000 lux top illumination in an incubator (IPP110, Memmert GmbH, Schwabach, Germany). At the start of the experiment, plants were randomly placed in a 48-well plate, containing 1 mL the assigned treatment (n = 8 replicates/treatment; control, 10^2, 10^3, 10^4, 10^5, and 10^6 particles·mL^{-1}), and incubated for 120 h at conditions as previous described.

Endpoints assessed

Growth endpoints

Growth was assessed by measuring fresh weight, frond area and root length at 0 and 120 h. Before determining fresh weight, plants were carefully patted using Kim-Wipes. Total number of fronds and frond areas were determined by taking vertical photographs of test wells (Nikon D3100; 18–55 mm lens; Nikon, Miniato, Japan). To determine the total number of roots and root length, a photograph was taken using a digital microscope (AnMo Electronics Corporation, New Taipei City, Taiwan). Images were used to determine frond area and root length with Fiji software (v. 2.00-rc-67/1.52c) (Schindelin et al. 2012), and total number of fronds and roots were counted.

Average specific growth rates in fresh weight, frond area, and root length were calculated based on OECD protocol 221 (OECD 2006):

$$\mu_{i-j} = \frac{\ln(N_j) - \ln(N_i)}{t}$$

with μ_{i-j} average specific growth rate, N_i measurement of variable at t_0, and N_j measurement of variable at t_{120}. Subsequently, percentage inhibition of growth rate was calculated relative to the control:

$$\%I_r = \frac{(\mu C - \mu T)}{\mu C} \times 100$$

with $\%I_r$ percentage inhibition in average specific growth rate, μC mean value for μ in the control, μT mean value for μ in treatment group.

Chlorophyll content

The extraction of chlorophyll pigments was performed in dark rooms and samples were stored on ice during the operation in order to prevent the degradation of chlorophyll pigments, following established procedures (Porra and Thompson 1989). Fresh fronds with a weight of 0.03 g were transferred into a 1.5 mL Eppendorf together with 0.05 g of quartz sand and 100% methanol. The samples were

homogenized for 1 min at 30 Hz (Retsch Mixer Mill MM220, Retsch, Haan, Germany) and centrifuged for 1 min at 13,200 rpm (Eppendorf MicroCentrifuge 5415 D, Eppendorf, Hamburg, Germany). Of the supernatant fraction, chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*), and total chlorophyll were determined at 120 h for control, 10^2, 10^4, and 10^6 particles·mL^{-1}, according to established procedures (Lichtenthaler 1998) (for more details see Supplementary Information).

Nanoplastic particle localization

A separate experiment was conducted to explore potential adsorption and internalization of plastic particles. Briefly, *S. polyrhiza* was exposed to 10^{14} particles·mL^{-1} of 50 nm red fluorescent nanoplastics for 120 h under conditions as previously described. Plants were placed on a glass slide and imaged employing an inverted LSM 880 microscope (Zeiss, Oberkochem, Germany) equipped with EC Plan-Neofluar 10×/0.30 M27 objective. Plastic particles were excited with a 543 nm helium-neon laser and detected using a 620–700 BP filter. Transmitted light was detected in a separate channel. In order to distinguish potential adsorption and internalization of plastic particles, z-stacks were obtained comprising 2.27-μm thick optical slices. In order to obtain an overview along the entire root length, we applied the tile scan option of ZEN microscope software (Zeiss, Oberkochem, Germany), stitching eight acquired scans of 642.86 × 642.86 μm into an 8 × 1 panoramic tile. The software Fiji was used to process the images.

Statistical analysis

All data are recorded and deposited in Dryad (Dovidat 2019). Statistical analyses were performed using the RStudio software (v. 1.1.456). ANOVA was used to assess differences among treatments. Normality and homogeneity of the data was tested using Shapiro–Wilk and Levene's test, respectively. When assumptions failed, statistical analyses were continued due to the robustness of ANOVA, but results were interpreted with caution if p was close to alpha. Interaction effects between concentration and particle size were assessed using two-way ANOVA, and concentration-dependent effects using one-way ANOVA. The significance level (α) was set at 0.05. When statistically significant differences were detected, a Dunnett's post hoc test was conducted. All test statistics are provided in Table S1.

Results

Growth

There were no statistically significant interaction effects between size and concentration of plastic particles affecting fresh weight, single largest frond area, total frond area, frond number, single longest root, total root length, or root number (Table 1). The observed percent inhibition of these growth endpoints was not concentration dependent (Table S1). Only the 50 nm plastic particles significantly inhibited the growth of the total frond area by 5.81% for concentrations of 10^4 and 10^5 particles·mL^{-1} (Table S2). However, the assumption of homogeneity of variance was violated, and as the p-value is close to 0.05, these results need to be interpreted with caution. For all other growth endpoints, differences in growth inhibition were observed, but these were not statistically significant and did not follow a dose-dependent pattern (Table S2).

Chlorophyll concentrations

There was no statistically significant interaction between size and particle exposure concentration when comparing different exposure treatments for any of the measured chlorophyll

Table 1. The effect of 50 and 500 nm plastic particles fresh weight, fronds, and roots of *Spirodela polyrhiza* after 120 h of exposure (n = 8 ± SEM). Statistically significant differences in comparison to the control, which are determined using Dunnett's post hoc test, are indicated with *(0.01 < p < 0.05).

			Fronds			Roots		
Size	Concentration (particles·mL^{-1})	Fresh weight (g)	Largest area (mm^2)	Total area (mm^2)	Number (count)	Longest root (mm)	Total roots (mm)	Number (count)
50 nm	Control	1.22±0.05	1.43±0.10	1.48±0.03	0.94±0.10	0.57±0.10	1.49±0.17	1.22±0.11
	10^2	1.12±0.06	1.52±0.05	1.44±0.02	0.86±0.07	0.64±0.09	1.64±0.12	1.36±0.10
	10^3	1.18±0.04	1.60±0.06	1.41±0.01	0.75±0.06	0.52±0.07	1.45±0.08	1.22±0.09
	10^4	1.09±0.03	1.43±0.09	1.40±0.04*	0.73±0.08	0.44±0.10	1.52±0.15	1.35±0.11
	10^5	1.19±0.08	1.45±0.11	1.40±0.03*	0.78±0.08	0.52±0.07	1.60±0.15	1.44±0.12
	10^6	1.29±0.06	1.58±0.06	1.51±0.02	0.93±0.06	0.81±0.10	1.81±0.12	1.36±0.11
500 nm	Control	1.09±0.06	1.55±0.05	1.53±0.02	0.88±0.08	1.03±0.18	1.97±0.18	1.43±0.09
	10^2	0.91±0.09	1.37±0.05	1.40±0.03	0.89±0.08	0.68±0.11	1.71±0.10	1.37±0.07
	10^3	1.08±0.11	1.37±0.05	1.46±0.05	1.01±0.04	0.81±0.14	1.86±0.16	1.44±0.09
	10^4	1.17±0.10	1.48±0.08	1.51±0.05	0.88±0.08	0.79±0.12	1.71±0.08	1.45±0.12
	10^5	1.20±0.09	1.30±0.09	1.37±0.07	0.93±0.06	0.50±0.20	1.56±0.24	1.27±0.17
	10^6	1.15±0.07	1.34±0.07	1.42±0.05	0.98±0.04	0.72±0.12	1.74±0.08	1.43±0.11

Table 2. The effects of 50 and 500 nm plastic particles on the chlorophyll concentrations of *Spirodela polyrhiza* after 120 h of exposure. The values for each endpoint are reported as means of the concentration groups (n = 3) ± SEM.

Size	Concentration (particles·mL^{-1})	Chlorophyll *a* (mg·g^{-1})	Chlorophyll *b* (mg·g^{-1})	Total chlorophyll (mg·g^{-1})
	Control	324.21±12.52	86.98±4.67	396.15±16.60
50 nm	10^2	336.74±10.09	80.66±2.97	401.90±9.28
	10^4	214.66±106.34	56.19±26.06	260.91±127.38
	10^6	329.75±3.19	88.64±8.78	403.08±9.64
	Control	324.21±12.52	86.98±4.67	396.15±16.60
500 nm	10^2	333.01±8.91	89.16±1.55	406.72±8.83
	10^4	324.93±7.04	75.92±2.10	385.91±8.61
	10^6	354.19±31.70	87.79±6.62	425.63±36.85

concentrations (Table 2). Differences in the measured chlorophyll concentrations between exposure treatments were small. Only the plants exposed to 50 nm particles in a treatment of 10^4 particles·mL^{-1} exhibited large, but nonstatistically significant reductions in Chl *b* concentration up to 35% (Table 2).

Nanoplastic particle localization

Confocal microscopy indicated that the 50 nm nanoplastics adsorb externally on to *S. polyrhiza*, as demonstrated by red fluorescence. Particle densities were higher on the root shafts and tips (Fig. 1a) than on the frond lower epidermis (Fig. 1b). The fluorescence displays irregular patterns of larger sizes than the 50 nm nanoplastics, which suggests clustering of the particles. In orthogonal projections, nanoplastic particles were detected surrounding the entire roots surface (Fig. 1c). No internalized particles could be detected.

Discussion

Here, we provide results on the impact of nano- and microplastics on a vascular plant, an area of research that is

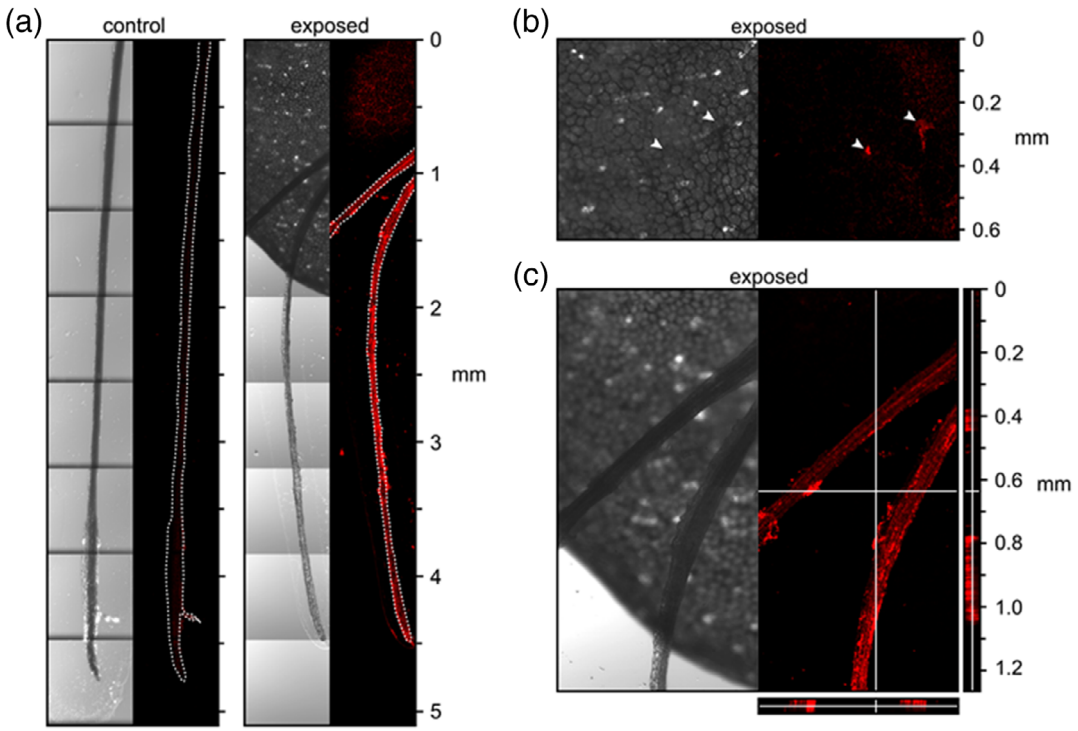

Fig. 1. Localization of 50 nm plastic particles on *Spirodela polyrhiza* after 120 h of exposure to 10^{14} particles·mL^{-1}. Transmitted light images are shown to the left of the fluorescence signal (red). (a) Maximum intensity projection of a tile scan of adsorbed particles (red) along the entire root shaft of control (left) and exposed (right) plants. Dashed lines indicate the outlines of root shafts. (b) Clusters of adsorbed plastic particles identified at the lower epidermis of exposed fronds, indicated by white arrows. (c) Orthogonal projections of adsorbed plastic particles surrounding exposed root shafts.

Table 3. Reported effects of plastic particles on primary producers (plants and algae) in the peer-reviewed literature, summarized per response variable.

Response variable	Effect	Test organism	Size and concentration of plastic particle	Characteristics of plastic particles	Source
Photosynthesis and content of photosynthetic pigments	Reduced	*Chlorella* (single celled), *Scenedemus* (multi-celled)	0.2 μm polystyrene beads at 1.6–40 mg·mL^{-1}	Positively and negatively charged	Bhattacharya et al. (2010)
	Reduced	*Scenedesmus obliquus*	0.07 μm polystyrene beads at 44–1100 mg·L^{-1}		Besseling et al. (2014)
	Reduced (1 μm), no effect (1000 μm)	*Skeletonema costatum*	1 μm, 100 μm PVC beads at 20, 100, and 200 mg·L^{-1}		Zhang et al. (2017)
	No effect	*Dunaliella tertiolecta*, *Chlorella vulgaris*, *Thalassiosira pseudonana*	0.05, 0.5, and 6 μm polystyrene beads at 25 and 250 mg·L^{-1}	Uncharged, positively and negatively charged	Sjollema et al. (2016)
	No effect	*Lemna minor*	30–600 μm plastics beads extracted from exfoliating products at 10, 50, 100 mg·L^{-1}	Sharp and smooth surface structures	Kalčíková et al. (2017)
	No effect	*Lepidium sativum*	50, 500, and 4800 nm at 10^3–10^7 particles·mL^{-1}		Bosker et al. (2019)
Growth rate	Reduced. Adverse effects increased with decreases in particle size.	*D. tertiolecta*, *C. vulgaris*, *T. pseudonana*	0.05, 0.5, and 6 μm polystyrene beads at 25 and 250 mg·L^{-1}	Uncharged, positively and negatively charged	Sjollema et al. (2016)
	Reduced	*S. obliquus*	0.07 μm polystyrene beads at 44–1100 mg·L^{-1}		Besseling et al. (2014)
	Reduced (1 μm), no effect (1000 μm)	*S. costatum*	1 μm, 1000 μm PVC beads at 20, 100, and 200 mg·L^{-1}		Zhang et al. (2017)
	Reduced (50 and 500 nm), no effect (4800 nm)	*L. sativum*	50, 500, and 4800 nm at 10^3–10^7 particles·mL^{-1}		
	Reduced main shoot length (20–500 μm), reduced shoot to root ratios and main shoot length (50–190 nm), no effect on shoot biomass	*Myriophyllum spicatum*, *Elodea sp.*	50–190 nm and 20–500 μm polystyrene particles at 0.1, 0.3, 1, 3 (50–190 nm and 20–500 μm) and 10% (20–500 μm) of sediment dry weight	Irregularly shaped, negatively charged (50–190 nm)	van Weert et al. (2019)

(Continues)

Table 3. Continued

Response variable	Effect	Test organism	Size and concentration of plastic particle	Characteristics of plastic particles	Source
	No effects (leaves), reduced (root)	*L. minor*	30–600 μm plastics beads extracted from exfoliating products at 10, 50, 100 mg·L^{-1}	Sharp and smooth surface structures	Kalčíková et al. (2017)
	No effect	*Tetraselmis chuii*	1–5 μm polystyrene beads at 0.046 to 1.472 mg·L^{-1}		Davarpanah and Guilhermino (2015)
	No effect	*Chlamydomas reinhardtii*	400–1000 μm milled propylene fragments at 400 mg·L^{-1}		Lagarde et al. (2016)
Cell viability	Reduced root cell viability (sharp particles)	*L. minor*	30–600 μm plastics beads extracted from exfoliating products at 10, 50, 100 mg·L^{-1}	Sharp and smooth surface structures	Kalčíková et al. (2017)
Adsorption	Increased adsorption of positively, negligible adsorption of negatively charged particles.	*Chlorella* (single celled), *Scenedemus* (multi-celled)	0,2 μm polystyrene beads at 1.6–40 mg·mL^{-1}	Positively and negatively charged	Bhattacharya et al. (2010)
	Increased concentration in algae aggregates compared to the background concentration	*Rhodomonas salina*, *Chaetoceros neogracile*	2 μm polystyrene beads at 10^4 particles·mL^{-1}		Long et al. (2015)
	Adsorption of neutral and positively charged particles	*Pseudokirchneriella subcapitata*	0.02 μm, 0.11 μm polystyrene beads at 400–88,900 mg·L^{-1}	Carboxyl- and amidine-modified, non-functionalized particles	Nolte et al. (2017)
	Accumulation of the 4800 nm plastic particles on the root hairs	*L. sativum*	50, 500, and 4800 nm at 10^3–10^7 particles·mL^{-1}		Bosker et al. (2019)
Uptake	Internalization and accumulation of 20 and 40 nm, no uptake of 100 nm particles	BY-2 cells of *Zea mays L.*	20, 40, and 100 nm beads		Bandmann et al. (2012)

understudied. We investigated the question if plastic particles negatively impact the growth and chlorophyll concentrations of the freshwater vascular plant duckweed. Additionally, we examined uptake and adsorption to provide indications for potential trophic transfer. Our results indicate no significant adverse effects of nano- and microplastics on *S. polyrhiza*, even when exposed to high concentrations. The absence of effects on duckweed growth, as observed in the current study, differs from a study on a closely related species of lesser duckweed (*L. minor*), in which significant adverse effects on root growth, but no effects on frond growth (Kalčíková et al. 2017). Importantly, the plastic particles used by Kalčíková et al. (2017) were approximately 1000 times larger than the particles used in our study, and the exposure duration was 48 h longer. Research on other organisms has found that toxicity is further complicated by plastic particles with modified shape or function (Dris et al. 2015). In another study in our laboratory using the same 50 and 500 nm particles, we found significant effects on root growth of cress (*L. sativum*), although these effects were short-lived and transient (Bosker et al. 2019). A study on macrophytes found that 20–500 μm plastic particles only impacted the main shoot length of *M. spicatum* with clear dose-dependent relationships, whereas 50–190 nm plastic particles reduced shoot to root ratios of *M. spicatum* and *Elodea sp.* (van Weert et al. 2019).

In order to compare our results with other studies, Table 3 provides a summary of available studies on the impact of plastic particles on primary producers (Table 3). Research on algae has resulted in mixed outcomes, with several studies reporting no effects on the growth of algae (Davarpanah and Guilhermino 2015; Lagarde et al. 2016) while others observed significant growth inhibition (Besseling et al. 2014; Sjollema et al. 2016; Zhang et al. 2017) (Table 3). This demonstrates the heterogeneity of findings, limiting the ability to make generalizable conclusions (Burns and Boxall 2018).

Effects of plastic particles on photosynthesis are similarly equivocal (Table 3). For example, Kalčíková et al. (2017) and Bosker et al. (2019) conclude that the exposure to plastic particles does not negatively impact photosynthesis, supporting the findings of our study. However, several studies on algae detected reduced concentrations of photosynthetic pigments (Bhattacharya et al. 2010; Besseling et al. 2014; Zhang et al. 2017). Only Sjollema et al. (2016) reported no effects of plastic particles on the photosynthesis of algae (Table 3).

There is little evidence for plastic particle uptake by vascular plants (Ng et al. 2018), with only one study known to us that found accumulation on the root hairs of the cress *L. sativum* (Bosker et al. 2019). In research on algae, however, the particle sizes used range from 20 nm (Nolte et al. 2017) to 2000 nm (Long et al. 2015). These two studies found adsorption without negative impacts, such as external adsorption of plastic particles to the plant tissue of the microalga *Pseudokirchneriella subcapitata* (Nolte et al. 2017) and increased accumulations of microplastics in algae aggregates compared to background levels (Long et al. 2015). Bhattacharya et al. (2010) observed that adsorption of positively charged, 200-nm sized plastic particles to algae reduced photosynthesis due to the physical blockage of light. The confocal microscopy in this study indicates external attachment of 50 nm plastic particles to the root tips and shafts of *S. polyrhiza*, but this could not be related to adverse effects, which is potentially due to different mechanisms of photosynthetic pigment reduction between algae and vascular plants. A second explanation could be that the photosynthetic pigments are not located in the roots but in the fronds/leafs, and the particles mainly adsorbed to the roots. In addition, most studies conducted to date on plants and algae are short-term acute exposures, highlighting the need to investigate the impact of chronic exposure of nano- and microplastics on plants. Furthermore, we could have missed potentially internalized particles due to limited penetration of the fluorescence signal through the root tissue. Nevertheless, our observed adsorption of plastic particles to the plant is important as adsorption might still contribute to biomagnification along the food web (Nolte et al. 2017). In addition, transfer to herbivorous species (both aquatic as well as terrestrial species feeding on aquatic plants) can occur, as we demonstrated that particles can attach and hence concentrate around the roots of duckweed.

To conclude, here, we present novel research on the effects of plastic particles on a freshwater vascular plant, and the first study to include nanoplastics. The results indicate that plastic particles of 50 and 500 nm do not negatively affect the growth and chlorophyll production of *S. polyrhiza*. Fluorescent imaging suggests, however, that the 50 nm nanoplastics adsorb externally. This study contributes to our understanding on the effects of microplastics on plants, an area which is currently understudied (Burns and Boxall 2018).

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