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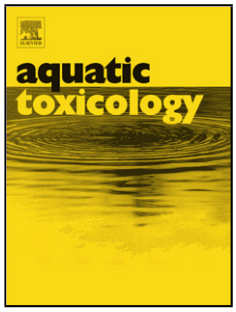

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Combined effects of nanoplastics and copper on the freshwater alga *Raphidocelis subcapitata*

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Highlights

- Interaction of carboxylated polystyrene nanoparticles with copper and algae
- No adsorption of copper ions observed on PS-COOH NPs
- Alteration of PS-COOH NPs surface charge and hydrodynamic diameter support eco-corona formation
- Ability of PS-COOH NPs to interact with algal exudates and algal cell walls
- PS-COOH food chain transfer plausible due to NPs adhesion to algal cell
- Additional endpoints and longer exposure scenarios recommended for nanoplastics risk assessment

Abstract

Nanoplastics are recognized as able to interact with other pollutants including heavy metals, and with natural organic matter, with implications for the potential risks to biota. We investigated the interaction of carboxylated polystyrene nanoparticles (PS–COOH NPs) with copper (Cu) and algal exudates (EPS) and how such interaction could affect Cu toxicity towards the freshwater microalga *Raphidocelis subcapitata*. PS–COOH NPs behavior in the presence of Cu and EPS was determined by dynamic light scattering (DLS), while PS–COOH NPs surface interaction with Cu ions and EPS was investigated by fluorimetric analysis. ICP-MS was used to test Cu ion

adsorption to PS–COOH NPs in the presence and absence of algae. The interaction between PS–COOH NPs and the algal cell wall was assessed by fluorescence microscopy. Short- and long-term toxicity tests were carried out in parallel to assess the impact of PS–COOH NPs on algal growth. Results showed altered nanoparticle surface charge and hydrodynamic diameter following algal EPS exposure, supporting the hypothesis of a protein corona formation. In contrast, no absorption of Cu ions was observed on PS–COOH NPs, either in the presence or absence of algae. No differences on algal growth inhibition were observed between exposure to Cu only, and to Cu in combination with PS–COOH NPs, in short-term as well as long-term tests. However, after 72 h of exposure, the adsorption of PS-COOH NPs to algal cell walls appeared to correspond to morphological alterations, revealing potential disturbances in the mitotic cycle. Our findings confirm the ability of PS–COOH NPs to interact with EPS as shown for other nanomaterials. Environmentally realistic exposure scenarios are thus needed for evaluating nanoplastic toxicity, as nanoparticles will not maintain their pristine nature once released into natural media. Prolonged exposure and use of different end-points such as cell morphological changes and EPS production seem more reliable for the investigation of nanoplastic/algal cell interactions which can drive food chain transfer of nanoplastics and ultimately toxicity.

Keywords: Polystyrene, Nanoplastic, Freshwater microalgae, Exopolymeric substances, Copper, Adsorption

1. Introduction

Nanoplastics are used in a variety of consumer products such as waterproof coatings, paints, lens cleaners, nanomedicine (e.g. drug delivery), biomedical products and medical diagnostics (Banerjee et al. 2016, Vance et al. 2015). They are also unintentionally produced in some processes, such as the thermal cutting of polystyrene (PS) foam (Zhang et al. 2012) and 3D printing (Stephens et al. 2013). Larger plastic objects undergo fragmentation into smaller particles, such as micro- (< 5 mm) and nanoplastics (< 100 nm), due to weathering (Wright &Kelly 2017, Wright et al. 2013). Laboratory studies have revealed the formation of nano-sized fragments from the weathering of various polymers such as PS, polylactic acid (PLA), polyethylene (PE), polypropylene (PP) and polyethylene terephthalate (PET) (Lambert & Wagner Gigault et al. 2016, 2016a, b). Recently, biological fragmentation by Antarctic krill has been reported (Dawson et al. 2018), whereby ingested microplastics (PE) was triturated and subsequently formed submicron fragments (150-500 nm). The occurrence of nanoplastics in natural waters was proven for the first time by Ter Halle et al. (2017) with the detection of submicron plastic fragments (100 – 1000 nm) within the water column of the North Atlantic subtropical gyre.

Given the continuous release of plastics to the environment, its persistence (Jambeck et al. 2015, Lebreton et al. 2017), and recent findings regarding formation of nanoplastics from micro- and macro-plastics (Dawson et al. 2018, Gigault et al. 2016, Lambert &Wagner 2016a, b), the concentrations of nanoplastics in the environment are

expected to increase.

Rivers are considered the primary route of plastic waste from land-based sources into the oceans (Williams & Simmons 1997). Based on a recent model study (Besseling et al. 2017) rivers also retain a portion of the released plastic fragments, leading to exposure of freshwater organisms.

PS is among the most commonly used plastics for packaging and disposable utensils (PlasticsEurope 2015); it is also frequently encountered as waste in aquatic environments (Wan et al. 2018). PS serves as a good proxy for environmental nano- and microplastics as its density is equal to that of the average microplastic present in the environment (Redondo-Hasselerharm et al. 2018).

PS NPs at relatively high concentrations (0.1-1 g/L) have been shown to cause severe damage to freshwater microalgae in terms of growth inhibition, decreased chlorophyll levels (Besseling et al. 2014), reduced photosynthetic activity and enhanced reactive oxygen species (ROS) production (Bhattacharya et al. 2010). Their adhesion onto algal surfaces has been observed (Bhattacharya et al. 2010, Chae et al. 2018, Nolte et al. 2017), in particular for the positively charged amino-modified PS NPs (PS–NH_2), and recognized to be the cause for the observed toxicity. Trophic transfer of PS NPs has additionally been documented in a sequential feeding study (Cedervall et al. 2012, Chae et al. 2018).

Toxic effects to microalgae have been reported mostly for positively-charged PS NPs such as amino-modified (PS–NH_2), while low toxicity has been observed for negatively-charged NPs such as carboxylated PS NPs (PS–COOH) (Bergami et al. 2017, Besseling et al. 2014, Nolte et al. 2017, Sjollema et al. 2016).

Surface characteristics such as chemical groups and consequent charge, more than the chemical type of NPs, appear to drive the behavior and consequent toxicity of PS NPs in aqueous media (Lowry et al. 2012). Virgin plastic has no surface charge; however, weathering and degradation processes like photo-oxidation from UV exposure, can lead to carbonyl group formation (Andrady 2017). Analysis of the surfaces of aged PE pellets showed a preponderance of ketone groups (C=O) and ester carbonyl groups (–COO–), resulting in a net negative surface charge (Fotopoulou &Karapanagioti 2012). However, it is difficult to predict surface characteristics of nanoplastics occurring in natural waters mainly because of the current lack of suitable analytical tools to track and separate them from water, sediment and biota. The high reactivity of nanomaterials makes them to behave dynamically, undergoing many transformations when in contact with natural matrices (Lowry et al. 2012). For example, NPs interact with natural organic matter (NOM) which modifies their aggregation capacity, surface charge and toxicity, and influences their behavior and interaction with surrounding media (Quigg et al. 2013). Exopolymeric substances (EPS), mainly constituted by polysaccharides and proteins and excreted by algae and bacteria, represent one of the main components of NOM in the aquatic environment (Verdugo et al. 2004). Proteins are known to form corona-like structures on NP surfaces (Monopoli et al. 2013). The presence of a protein corona was recently reported for titanium dioxide NPs incubated with EPS produced by the marine green alga *Dunaliella tertiolecta* (Morelli et al. 2018). Therefore, proteins present in EPS can interact with PS NPs in aquatic media and consequently change NP surface charge but also modify the NP surface–volume ratio by influencing their

aggregation (Quigg et al. 2013, Summers et al. 2018). Such changes in the external properties of a NP can consequently affect its capacity to adsorb pollutants and alter its potential role as a carrier, as already documented for other NPs (Velzeboer et al. 2014).

Plastic debris found in the aquatic environment is frequently associated with heavy metals (Ashton et al. 2010, Turner &Solman 2016, Vedolin et al. 2017), often as a result of adsorption processes (Wang et al. 2017). Weathered plastic is more likely to sorb trace metals than is virgin plastic (Brennecke et al. 2016, Holmes et al. 2012, 2014, Rochman et al. 2014, Turner &Holmes 2015), presumably due to surface modifications upon weathering (e.g. photo-oxidation by UV light). The risk to biota posed by nanoplastic exposure could thus increase as a consequence of adsorption of pollutants, as some nanoparticles attach to the exterior of organisms and some are capable of crossing cell membranes, causing physical damage and possible uptake and transfer through the food chain (Bergami et al. 2017, Cedervall et al. 2012, Nolte et al. 2017). Organisms in their natural habitat are generally not exposed to single chemicals but rather to mixtures; therefore, risk assessment studies must address realistic exposure scenarios.

To date, few studies have investigated the consequences of exposure to combined plastics and metals, such as adsorption and possible synergistic effects, suggesting a general lack of knowledge on occurring interactions, especially concerning nanoplastics (Barboza et al. 2018a, Barboza et al. 2018b, Davarpanah &Guilhermino 2015, Farhan et al. 2015, Khan et al. 2017, Kim et al. 2017, Luís et al. 2015).

Copper (Cu) is an essential micronutrient for both plants and animals at low concentrations but can become toxic when present in higher quantities. Cu concentrations can exceed natural levels (> 100 µg/L) in the aquatic environment as a consequence of anthropogenic activities (Hoang et al. 2009, Ma et al. 2003, Schuler et al. 2008). Concentrations exceeding natural levels have been reported to inhibit the growth of many algal species (Franklin et al. 2002, Franklin et al. 2001, Machado &Soares 2014, Soto et al. 2011, Wong &Chang 1991) and reduce photosynthetic activity (Juneau et al. 2002, Ouyang et al. 2012). To our knowledge, the combined toxicity of nanoplastic and Cu to microalgae has not yet been studied.

The aim of the present study was to assess whether the presence of PS–COOH NPs, and its interaction with EPS, might affect Cu toxicity to the freshwater microalga *Raphidocelis subcapitata*. We selected Cu concentrations that are relevant for contaminated freshwater environments (Hoang et al. 2009, Ma et al. 2003, Schuler et al. 2008). Although environmental nanoplastic concentrations are currently unknown (Lenz et al. 2016), they are expected to increase due to ongoing fragmentation of microplastics (Gigault et al. 2016, Ter Halle et al. 2017). Here we applied PS–COOH NPs concentrations that are far lower than those tested in previous studies (Besseling et al. 2014, Cedervall et al. 2012, Sjollema et al. 2016). Effects on algal growth were investigated using short-term (72 hours) and long-term (7 days) toxicity tests. PS–COOH NP characteristics and stability in algal medium in the presence of EPS were investigated as well as interactions between PS–COOH NPs and algal cells in terms of morphology and cell division.

2. Materials & Methods

A summary of all tests and analysis carried out in this study is available in supporting information, table S1.

2.1. Chemicals and NPs

Copper nitrate ($Cu(NO_3)2•2.5H_2O$, CHEM-LAB 99-102%) was used to prepare Cu solutions for algal toxicity tests. To avoid precipitation and adsorption to container walls the solution was acidified to pH < 2 by addition of 1N HCl. Glassware was rinsed with a 10% HCl solution and deionized water, before and after use.

Carboxylated polystyrene nanoparticles (PS–COOH NPs, subsequently referred to as PS NPs) were provided by the Physical Chemistry and Soft Matter Department in collaboration with the Food and Biobased Department of Wageningen University (The Netherlands). The original stock solution was 41.91% w/w of PS NPs containing 0.4% w/w of covalently bound dye (Rhodamine B methacrylate) and 1.1% w/w of sodium dodecyl sulfate (SDS). Stock was bubbled with clean air for 24 h to remove any traces of remaining styrene monomers and diluted with MilliQ water for preparation of test solutions. Each PS NP test solution was vortexed and bath sonicated for two minutes prior to use.

2.1. PS NPs characterization

Z-average (nm) and z-potential (mV) of PS NPs were determined by Dynamic Light Scattering (DLS, Malvern instruments), combined with the Zetasizer Nano Series software (version 7.02, Particular Sciences). Z-average is the average hydrodynamic diameter measured by the dynamic light scatter technique, while z-potential is the surface charge of particles measured by the electrophoretic mobility technique. PS NP suspensions were characterized in the following media: MilliQ water, as used for preparing PS NP stock solutions; WC test medium used for algal tests; and WC test medium plus increasing Cu concentrations (10, 50, 100, 200 µg Cu/L). In order to assess the potential effect of algal EPS on PS NP dispersion and suspension stability, a 250 mL culture of *R. subcapitata* at the end of the logarithmic growth phase (~ 2.5 x 10^6 cells/mL) was centrifuged (10,000 *g*, 10 min, 20° C) and filtered (1.2 µm). The resulting solution, containing algal EPS, was used to analyse PS NP dispersion at time (t) = 0, 72 and 168 h. At time 0 the same solution was also tested with increasing Cu concentrations (10, 50, 100, 200 µg Cu/L). Furthermore, PS NPs following 72 h algal exposure were analysed after a 0.45 µm filtration with cellulose nitrate filters (Sartorius).

PS NP suspensions were analysed for their fluorescence (expressed as Arbitrary Fluorescence Units, AFU) by means of a spectrophotofluorometer (Victor 3 1420 Multilabel Counter, PerkinElmer) combined with the Wallac software, using Rhodamine B Ex and Em wavelengths (λ_{ex} = 545 nm and λ_{em} = 575 nm), in order to evaluate PS NPs surface interaction with the medium. Fluorescence was measured at different PS NP concentrations (0.25,

0.5, 5, 10, 25 mg /L, data reported only for 10 mg/L) alone and in combination with Cu (50 µg Cu/L), at t = 0 and after 72 h incubation with extracted EPS and with *R. subcapitata*.

2.2. Cu measurement in aqueous media

In order to test the adsorption capacity of PS NPs towards Cu ions, a 20 mL subsample from each treatment of the 7-day toxicity test with *R. subcapitata* was sampled and filtered using a 50 nm cellulose nitrate membrane filter (Merck Millipore), at the end of the test. PS NPs were also incubated with Cu for 7 days without algae to compare data and filtrated as described above. Cu concentration was measured in the filtrates using an inductively coupled plasma – atomic emission spectrometer (nexION 350X ICP- MS, PerkinElmer).

2.3. Algal toxicity test

Algal toxicity tests were performed with *Selenastrum capricornutum* NIVA-CHL 1 (current taxonomic classification is *R. subcapitata*), as provided by the Aquatic Ecology and Water Quality Management group of Wageningen University & Research (The Netherlands). *R. subcapitata* is a widely used specie of freshwater microalga for toxicity testing and it's recommended in many standardized toxicity protocols (OECD 201, UNI EN ISO 8692:2012)

The algae were cultured in WC medium (Table S2, SI) and maintained in axenic exponential growth conditions in a growth chamber at 18 ± 1 °C and a 16:8 h light-dark cycle photoperiod.

2.3.1. Standard 72 h growth inhibition tests and longer exposure test (7 day)

Toxicity tests (72 h) were carried out in modified WC medium, composed only of the principal components (subsequently referred to as 'WC test medium'), to avoid the introduction of chelating agents (i.e. EDTA). The use of chelating agents is not recommended when performing heavy metals toxicity testing (Leal et al. 2016, Resgalla Jr et al. 2012). Tests were carried out in PS single-use sterile multiwell with 2 mL capacity for each well, as plastic is more suitable for metals testing (Leal et al. 2016). Algae from a stock culture were inoculated in WC test medium 72 h before every test and maintained under the following conditions: 22 ± 2 °C, pH = 7.5 ± 0.3. Initial algal concentration in toxicity tests was $1x10^5$ cells/mL and a 16:8 light-dark photoperiod was used in order to mimic natural conditions. Algae were manually aerated every 24 h using a pipette with sterile tips.

In order to evaluate single and combined effects of Cu and PS NPs on *R. subcapitata*, algae were exposed to Cu, PS NPs and to a range of PS NPs and Cu concentrations as follows: Cu (1, 5, 10, 15, 20, 25, 50, 100, 200 µg Cu/L); PS NPs (0.5, 1, 2.5, 5, 10, 50 mg PS/L); combination: PS plus increasing Cu (0.5 mg PS/L + 1, 5, 10, 15, 20, 25, 50, 100, 200 µg Cu/L) and Cu plus increasing PS (35 µg Cu/L + 0.5, 1, 2.5, 5, 10, 50 mg PS/L).

Three replicates were prepared for each concentration and each experiment was repeated three times. Potassium dichromate ($K_2Cr_2O_7$) was used as a reference toxicant and a growth inhibition curve was run as positive control.

Furthermore, in order to isolate any possible role of SDS present in the PS NP batch, an SDS growth inhibition test was run. After 72 h the algae were fixed in a 1:1 lugol:ethanol solution and cell density was determined by counting under an optical microscope (Olympus, BX51, 40X) using a Neubauer chamber.

In order to assess long-term effects, a 7-day test was performed at the following exposure concentrations: 50 µg Cu/L, 0.5 mg PS/L, 50 µg Cu/L + 0.5 mg PS/L. Test conditions were the same as described above for the 72 h toxicity test except that it was carried out in 50 mL PS flasks. There were three replicates of each concentration, and the experiment was repeated three times. At the end of 7 days the content of the three replicates was combined and used as follows: 2 mL were collected for algal density calculation, 20 mL for measuring Cu levels in the media and the remainder for EPS extraction and protein quantification.

2.3.2. Sub-lethal effects

For assessment of algal morphology, images were recorded during cell counting with an optical microscope (Olympus BX51 coupled with Olympus DP-software).

In order to evaluate the interaction between cell wall and PS NPs, algae from the 72 h exposure tests (50 mg PS/L) were observed under an optical fluorescent microscope AXIO IMAGER Z1 using the Apotome system (Zeiss). Images were taken with an Axio CamMRm camera at 63X using Axio Vision Software.

EPS extraction for protein quantification and analysis was performed as follows: the remaining volume of the 7-day test was centrifuged (10,000 *g*, 15 min, 20°C) and the supernatant filtered with a glass microfiber filter (0.7 µm, Whatman, UK). An initial 12 mL aliquot and six successive 10 mL aliquots of the filtrate (72 mL) were centrifuged (5000 *g*, 35 min, 20 °C) by using a centrifugal filter device with a 3 kDa cut-off (Amicon Ultra-15 mL, Millipore, USA). With each centrifugation, 10 mL of the resulting filtrate were discarded; the final aliquot was centrifuged for 80 min until the sample was reduced to a final volume of approx. 420 µL. Using this protocol, the organic components were concentrated about 170 times. A colorimetric method according to the 2D-Quant Kit (GE Healthcare, USA) was used for protein content analysis of EPS. The absorbance of the samples was read at 480 nm with a spectrophotometer (PerkinElmer Lambda 25 UV/VIS Spectrometer) and the protein concentration calculated by comparison to a standard curve.

2.4. Statistical analysis

All data obtained were processed using Statistica software 6.0. and growth inhibition curve and EC_{50} data were calculated with the Graphpad Prism 5 program using a non-linear regression method in a dose-response model, normalized compared to the control (0-100%).

3. Results & Discussion

3.1. NP behavior in freshwater media with algal EPS and Cu

DLS measurements showed a good dispersion of PS NPs in all tested media (MilliQ water, WC test medium, WC test medium + Cu, WC test medium + Cu + EPS) with no discernible aggregation (Table 1 and S3, SI). These findings are in agreement with previous studies reporting a good dispersion of PS NPs in freshwater medium (Nolte et al. 2017).

PS NP hydrodynamic diameter was near constant in all tested media, ranging from 87.17 nm to 106 nm. Z-potential indicated a negative surface charge, from -50.4 to -16.7 mV (Table 1 and S3, SI). Incubation with extracted EPS (Table 1) did not affect PS NP z-average and z-potential at 0 and 72 h. Furthermore, no change in z-average or z-potential was observed in the presence of Cu. The only exception observed was for z-potential at the highest Cu concentration, which increased significantly (p<0.001) from -33.6 to -16.7 mV (Table S3, SI), with no apparent correlation with other results. We hypothesise that no adsorption of Cu ions onto PS NP surface occurred, which was further supported by ICP-MS results showing similar Cu levels in aqueous solution before and after incubation with PS NPs (Table 3). Our data are in agreement with previous findings reported by Kim *et al.* (2017) for nickel (Ni) and PS NPs in freshwater media, and raise questions about the lack of interaction, since metals are known to sorb onto plastic in both natural and experimental conditions. Based on the charges of both cationic Cu and anionic COO–, an interaction to some extent was expected. Plastic-metal interaction is highly dependent on the level of weathering of the polymer, which affects the functional groups to which metals would bind to, and affects the attachment of organic matter (Turner &Holmes 2015). We used –COOH functionalized NPs as a proxy for aged nanoplastics (van Weert et al. 2019). Furthermore, Cu adsorption experiment with algae was performed in order to account for the presence of EPS, thus mimicking natural conditions. No adsorption of Cu to PS NPs was observed. We propose two hypotheses to explain the lack of adsorption of Cu to PS NPs: (1) Cu has no affinity for PS, since most studies on plastic-metal interactions involve PE (Ashton et al. 2010, Holmes et al. 2012, 2014, Turner &Holmes 2015); and (2) the freshwater medium plays a role in preventing PS-metal interaction. Holmes *et al.* (2014) observed changes in the adsorption capacity of micro-PE along a salinity gradient, with the lowest Cu adsorption in the freshwater end of the gradient. Therefore, adsorption in freshwater could be negligible. Brennecke *et al.* (2016) reported the adsorption of Cu onto micro-PS beads in seawater, with concentrations as high as 800 times that of the surrounding medium.

Regarding the impact of EPS on PS NPs behavior (z-average and z-potential), incubation with *R. subcapitata* for 72 h resulted in an increase of both parameters (Table 1). This result is attributed to the adsorption of EPS onto PS NPs. The production of EPS is considered a defensive response by microalgae subject to stress (Koukal et al. 2007, Quigg et al. 2013, Zhou et al. 2016), and has been observed upon exposure to metals (Miao et al. 2009, Paquet et al. 2015, Zhang et al. 2013) and to other NPs (Chiu et al. 2017). The observed increase in z-average and z-potential

following 72 h incubation with *R. subcapitata* could therefore be the result of EPS adsorption to PS NPs, produced by the algae as a reaction to NP exposure.

Further support for the hypothesis of EPS adsorption to PS NPs was provided by fluorimetric analysis, which showed a distinct reduction in fluorescence of PS NPs incubated for 72 h with *R. subcapitata*, compared to a negligible fluorescence reduction for PS alone and with EPS (Table 2).

EPS interactions with NPs can result in different possible outcomes depending on EPS composition, NP surface properties and characteristics of the media (Adeleye &Keller 2016, Chen et al. 2011, Kroll et al. 2014). Interactions are often driven by the proteins present in EPS, as proteins are known to adsorb to suspended NPs. Chen *et al.* (2011) studied the assembly of EPS microgels and observed that PS NPs accelerated, or even induced this process, acting as a bridge between protein molecules. In our previous studies on marine algae, EPS produced by *Dunaliella tertiolecta* formed a protein corona around TiO_2 NPs thus affecting their behavior and stability (Morelli et al. 2018). Based on our data, therefore, the formation of a protein corona around PS NPs incubated with *R. subcapitata* could be hypothesized. The lack of such evidence in PS NPs incubated with EPS may be explained by the low protein content of *R. subcapitata* EPS (Koukal et al. 2007), while PS NP exposure to algae could have enhanced EPS production and produced the observed effects of higher z-average (106 ± 0.66 nm), z-potential (-22.4 mV) and lower fluorescence (994 ± 107 AFU), compared to PS NP incubation with EPS without algae (84.7 ± 0.56 nm, -34 ± 0.72 mV and 1784 ± 45 AFU, respectively).

Further support for the protein corona hypothesis is derived from the protein content measured in EPS obtained after the 7-day toxicity test (Table 4). As an induced protective mechanism (Maršálek &Rojíčková 1996), Cu can stimulate the production of EPS of *R. subcapitata*, which results in a high protein value. On the other hand, the 3-fold lower protein content measured upon exposure to the combination of Cu and PS NPs could be explained by the adsorption of proteins to the NP surface, reducing the quantity measured in solution.

3.2. Growth inhibition and sub-lethal effects on *R. subcapitata*

A similar growth inhibition was observed after 72 h exposure to Cu (EC_{50} = 84.29 µg Cu/L, r^2= 0.910) and to Cu in combination with PS NPs (EC_{50} = 86.28 µg Cu/L, R^2 = 0.877) (Fig. 1a), showing no influence of PS NPs on Cu toxicity. The same result was observed in the 7-day toxicity test and in the test with fixed Cu concentration (35 µg Cu/L) and increasing PS NP concentrations (Fig. S1, SI). These data are consistent with DLS data and equal Cu concentrations detected in aqueous solution both in the presence and absence of PS NPs, confirming the lack of Cu sorption to PS NPs.

Our findings are in agreement with previous studies reporting no additional effect of micro-PE both on growth inhibition of the marine alga *Tetraselmis chuii* exposed to Cu (Davarpanah & Guilhermino 2015) and on intestinal uptake of silver in rainbow trout (Khan et al. 2017). Other studies, however, reported that the interaction of metals with micro- and nanoplastics (PE, PS) led to differences in toxicity and uptake of metals (Barboza et al. 2018a,

Barboza et al. 2018b, Farhan et al. 2015, Kim et al. 2017, Luís et al. 2015). Not all mechanisms involved are understood, in particular in the case of nanoplastics. Kim *et al*. (2017) observed differences in the toxic effect of combined Ni and PS NPs (both virgin and functionalized with –COOH) compared to Ni alone, although no adsorption of Ni onto PS NPs is documented. It is possible that other unaccounted factors have influenced the experimental outcome. This likely results from the complex behavior of nanoscale particles in a dynamic environment where organisms, organic matter and multivalent ion mixtures interact (Quigg et al. 2013).

PS NPs were associated with low inhibition of algal growth rate (Fig. 1b) which was found statistically significant ($p < 0.001$) only at the highest exposure concentrations, confirming previous findings on the lack of effects of PS NPs on algal growth below 10 mg/L (Bergami et al. 2017, Besseling et al. 2014, Nolte et al. 2017, Sjollema et al. 2016). However, data from PS NP exposure were highly variable, probably due to the unequal distribution of PS NPs in the test media, as is also shown by the fluorescence images (Fig. 2). This lack of homogeneity could be due to particle behavior itself, i.e. not behaving as a soluble chemical in a standardized toxicity test. This is a challenging topic since nanomaterials are documented to experience many processes and transformations including agglomeration, settling and/or dissolution in a test medium, which make their behavior different from that of dissolved chemicals (Peijnenburg et al. 2015). This phenomenon must be considered in toxicity assessments of NPs as such processes can significantly alter NP exposure levels, resulting in erroneous assessments of potential hazard (Handy et al. 2012, Petersen et al. 2015). Moreover, standard algal growth inhibition tests use an incubation period of three days and consider only lethal endpoints (e.g., growth inhibition) to assess the effects of a toxicant. Additional effects such as alteration of cell morphology and production of exudates, and an extended test duration, will offer more detailed and more ecologically relevant information about the effects of NPs on algae.

We therefore investigated the interaction between PS NPs and the cell surface by fluorescence microscopy, detecting fluorescent cluster-like structures, probably hetero-aggregates of PS NPs and EPS, surrounding the algae (Fig. 2). This was evident only at the highest PS concentrations (50 mg/L) due to the weak fluorescent signal at lower concentrations. These findings again support the hypothesis of interaction between PS NPs and EPS. It is observed that, besides being associated with the cells, the particles occur in large aggregates probably composed of EPS and NPs (Fig. 2). In Figure 2b (see arrow) this is particularly evident as a large fluorescent aggregate is shown, which is not associated with the cell wall, but embedded within a polymeric matrix, probably EPS.

Particle adhesion to the algal surface has been observed before (Bergami et al. 2017, Bhattacharya et al. 2010, Chae et al. 2018, Nolte et al. 2017). Besides having the potential to disturb cells in various ways, natural predators of algae such as zooplanktonic species may be affected, therefore causing indirect effects to the food web by nanoplastic transfer (Cedervall et al. 2012). Geitner and co-authors (2016) showed that surface affinity of NPs is a reliable predictive tool of trophic transfer through predation. Laboratory studies (Cedervall et al. 2012, Chae et al. 2018) demonstrated the transfer of PS NPs through a food chain, with eventual toxic effects to the ultimate consumers (i.e., fish). Hence, our results confirm that PS NPs have the potential to enter the food web by means of a ‘trojan horse’ mechanism, starting from among the lowest trophic levels.

Morphology can provide useful information regarding health status of cells. In Figure S2a algae from the control reveal good dispersion and semi-circular shape, typical for healthy *R. subcapitata*. In contrast, algae exposed to 35 µg Cu/L appear slightly deformed with uneven and grainy surfaces (Fig. S2b). There is an obvious effect on algae exposed to 10 mg PS/L (Fig. S2c): average volume exceeds that of the control and some algae appear clearly enlarged and turgid. Co-exposure to 35 µg Cu/L and 10 mg PS/L resulted in greater morphological changes, additional agglomeration and loss of the original shape of the cell (Fig. S2 d; Fig. 3g, h).

Yamagishi *et al.* (2017) observed three different reproductive patterns in *R. subcapitata*, which can produce two, four or eight daughter cells in the same mitotic cycle. The frequency of each pattern could depend on culture conditions, but it has also been observed that chemicals like potassium dichromate ($K_2Cr_2O_7$), 3,5-dichlorophenol (3,5-DCP) and Cu, can interfere with the cell cycle, changing the ratio between these patterns (Yamagishi et al. 2017). Our findings are in agreement with these studies, since Cu-exposed algae experienced some disturbances in the mitotic process (Fig. 3 a-c, see arrows). In fact, cells are frequently observed during the mitotic stage, probably while forming four cells, in contrast with control cells which are rarely seen during mitosis. This could be related to a delay in the mitotic process caused by metal exposure, as noted by Machado and Soares (2014), making the process slower and therefore easier to observe.

In PS NP-exposed algae, the altered cell volume could also be a result of an abnormality in the mitotic process. A high number of structures representing the eight-cell reproductive pattern is observed (Fig. 3, d-f, see arrows), which is in agreement with Yamagishi *et al.* (2017). Such anomalies may be linked, as the larger size may be attributed to formation of multinucleated cells, a preliminary step anticipating cell division. Also in this case, as well as for Cu exposure, the mitotic process has been slowed since numerous cells are observed during the replication process. Although this pattern is observed upon exposure to high concentrations (10 mg PS/L), PS NPs at lower concentrations may also cause a change in the reproductive pattern of *R. subcapitata*, as also described for $K_2Cr_2O_7$, 3,5-DCP and Cu (Machado &Soares 2014, Yamagishi et al. 2017).

It cannot be excluded that SDS from the PS NPs stock solution played a role in the observed morphological abnormalities, even if present at low concentrations (final SDS concentration in the 10 mg PS/L exposure: 250-300 µg/L). Lürling and Beekman (2002) reported morphological changes (induced colony formation) in the freshwater green alga *Scenedesmus obliquus* exposed to 200 µg/L of SDS. The current experiments showed increased cell volume but no evidence of an altered reproductive cycle upon exposure to SDS, with only a few cells during mitosis and no cells displaying the eight-cell reproductive pattern observed in PS NP-exposed algae (Fig. S3, SI). Therefore, the observed morphology could be associated with PS NP exposure and further investigation is needed in order to clearly elucidate this cellular response and potential detrimental effect.

Despite minimal or no observed effect on algal growth, algae could still be encountering stress upon exposure to PS NPs. Bhattacharya *et al.* (2010) reported that particles adsorbed to algal surfaces hinder gaseous exchanges and interfere with the normal cell metabolism. We hypothesize that an interference in the mitotic process is occurring

as a consequence of PS NPs exposure, as was previously observed in algae for chemicals exposure (Machado &Soares 2014, Yamagishi et al. 2017).

4. Conclusions

We investigated the interaction between Cu and PS NPs and their single and combined effects on the freshwater alga *R. subcapitata*. Our findings show that Cu ions are not likely to adsorb to –COOH functionalized PS NPs in freshwater media. As a consequence, they do not alter Cu toxicity to algae. PS NPs do not appear to constitute a threat to *R. subcapitata* growth, up to 50 mg PS/L exposure, even though toxicity data are quite variable. The observation of altered algal morphology, however, highlights that PS NPs may interfere with the algal replication process, as already recorded for Cu, but further investigations are needed to clarify potential detrimental effects. Food chain transfer is plausible due to the adhesion of PS NPs to the algal cell surface. Such bio-interaction is probably driven by the presence of EPS which might form a bio-corona around PS NPs as shown by changes in their dimension and surface charge. Our data emphasize the need to develop *ad hoc* strategies for nanoplastics testing in aquatic environments by establishing new endpoints and applying longer exposure times. This could further validate the assessment of the potential risk posed by nanoplastics, including considerations of potential trophic transfer in the food chain.

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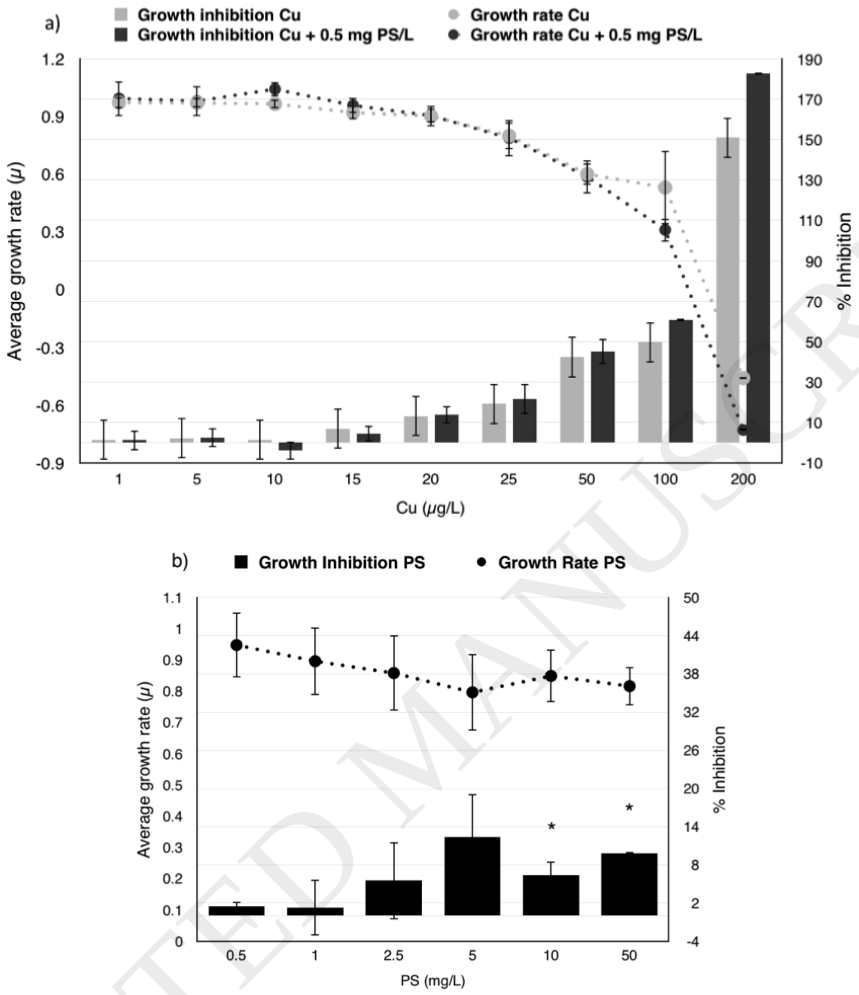


Figure 1. Percentage growth inhibition compared to control (bars) and average growth rate (circles) of 72 h algal toxicity test of *R. subcapitata* exposed to (a) Cu (light gray) and Cu + 0.5 mg PS/L (dark gray), and to (b) PS NPs, in WC test medium. Data shown as mean ± standard deviation (columns marked with * are statistically different from the control with $p<0.001$). (1 column fitting image)

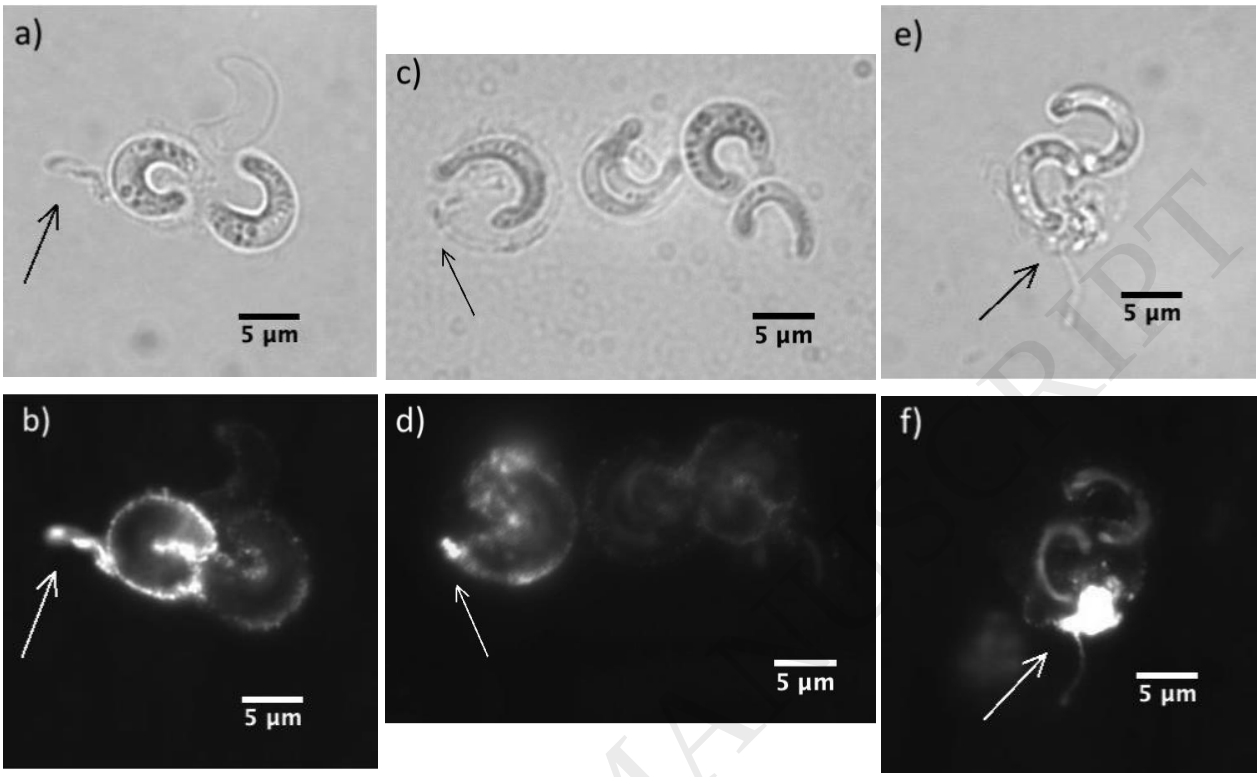


Figure 2. Light microscopy (a, c, e) and corresponding fluorescent (λ_{ex} = 545 nm and λ_{em} = 575 nm) images (b, d, f) of *R. subcapitata* after 72 h exposure to 50 mg/L of PS NPs. Arrows point at aggregates of PS NPs and EPS. (1.5 column fitting image)

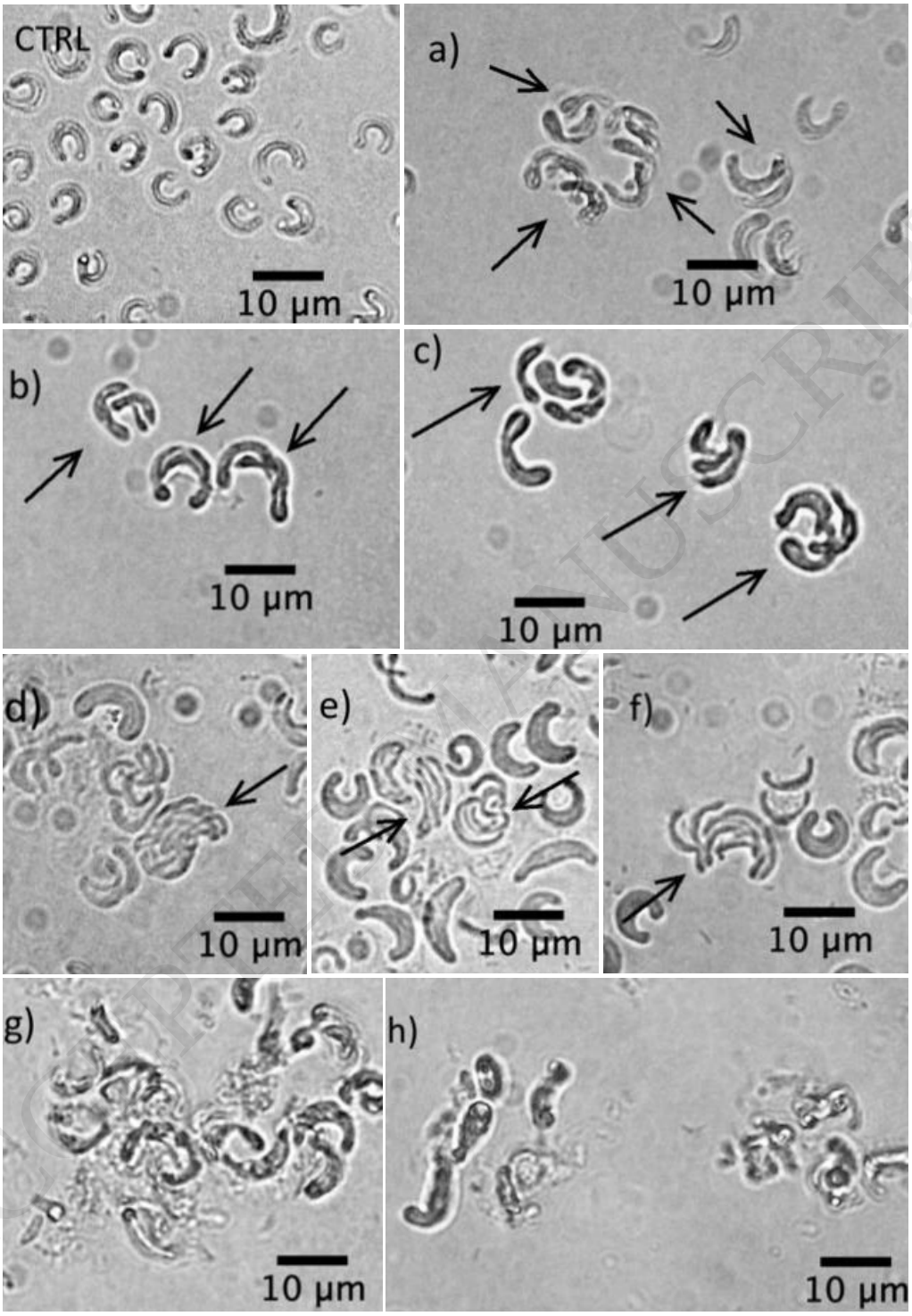


Figure 3. Details of optical microscope images of *R. subcapitata* exposed to 35 µg Cu/L (a-c), 10 mg PS/L (d-f) and 35 µg Cu/L + 10 mg PS/L (g, h). CTRL = control group. Arrows point at cells during mitosis, mostly observed in exposed algae compared to control group. (1.5 column fitting image)

Supporting information:

Table S1. Summary of all tests and analysis carried out in the present study. v.c.= various concentrations; f.c.= fixed concentration.

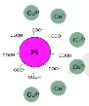
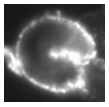
				Outcome
PS Characterization	72 h	•  Hydrodynamic diameter and z- potential (DLS)	- MilliQ water 0 h - WC test medium 0 h - WC test medium + Cu v.c. 0 h - EPS 0 h - EPS + Cu v.c. 0 h - EPS 72 h - *R. subcapitata* 72 h	Increase in z-average and z-potential after 72 h incubation with *R. subcapitata*
		•  Fluorescence (0 - 72 h) (Spectrophotofluorometer)	- WC test medium - *R. subcapitata* - PS NPs - PS NPs + EPS - PS NPs + *R. subcapitata* - PS NPs + Cu - PS NPs + *R. subcapitata* + Cu	Decrease of fluorescence after 72 h incubation with *R. subcapitata*
	7 d	• Cu adsorption on PS NPs (ICP-MS) 	- PS NPs + Cu - PS NPs + Cu + *R. subcapitata*	No decrease in Cu levels
Toxicity Tests		•  Growth Inhibition	- PS NPs v.c. - Cu v.c. - PS NPs f.c. + Cu v.c. - Cu f.c. + PS NPs v.c.	Similar levels of growth inhibition between Cu and PS NPs + Cu exposure
	72 h	• Cell/PS NPs interaction (Fluorescent microscopy) 	- *R. subcapitata* + PS NPs	PS NPs attached to *R. subcapitata* surface; aggregates of PS NPs and probably EPS
		• Cells Morphology (Optical microscopy) 	- *R. subcapitata* + Cu - *R. subcapitata* + PS NPs - *R. subcapitata* + Cu + PS NPs	Cu, PS NPs and Cu + PS NPs caused morphological anomalies in *R. subcapitata*
	7 d	•  Growth Inhibition	- PS NPs f.c. + Cu f.c.	Similar levels of growth inhibition between Cu and PS NPs + Cu exposure
		•  Protein content of EPS	- CTRL - PS NPs - Cu - PS NPs + Cu	Protein content of PS NPs + Cu treatment is 3-fold lower compared to Cu treatment

Table S2. WC medium components

Components	Concentration
Principal components	
TES	85.0 mg/L
$CaCl_2 \cdot 2H_2O$	36.76 mg/L
$MgSO_4 \cdot 7H_2O$	36.97 mg/L
$NaHCO_3$	12.60 mg/L
K_2HPO_4	8.71 mg/L
$NaNO_3$	85.01 mg/L
$Na_2SiO_3 \cdot 9H_2O$	28.42 mg/L
H_3BO_3	24.0 mg/L
Trace elements	
$Na_2 \cdot EDTA$	4.36 mg/L
$FeCl_3 \cdot 6H_2O$	1.00 mg/L
$MnCl_2 \cdot 4H_2O$	0.18 mg/L
$CuSO_4 \cdot 5H_2O$	0.001 mg/L
$ZnSO_4 \cdot 7H_2O$	0.022 mg/L
$CoCl_2 \cdot 6H_2O$	0.012 mg/L
$Na_2MoO_4 \cdot 2H_2O$	0.022 mg/L
H_2SeO_3	0.0016 mg/L
Na_3VO_4	0.0018 mg/L
Vitamins	
Thiamin·HCl	0.1 mg/L
Biotin	0.52 µg/L
B_{12}	0.56 µg/L

Table S3. DLS measurement of hydrodynamic diameter (z-average) and surface charge (z-potential) of PS NPs in MilliQ water, WC test medium and various Cu concentrations, at 25°C in WC test medium at 0 h.

	0 h					
	MilliQ water	WC test medium	10 µg Cu/L	50 µg Cu/L	100 µg Cu/L	200 µg Cu/L
Z-average(nm)	96.31 ± 3.3 [a]	88.47 ± 2.28 [a]	87.17 ± 1.99 [b]	88.92 ± 1.16	89.49 ± 1.49	94.14 ± 1.92 [b]
Z-potential(mV)	-50.4 [c]	-33.6 [c]	-28.9 [d]	-31.5	-30.9	-16.7 [d]
PDI	0.047 ± 0.01	0.120 ± 0.02	0.139 ± 0.02	0.097 ± 0.03	0.100 ± 0.01	0.184 ± 0.02

Data with the same letter are statistically different with $p<0.001$ (data shown as mean ± standard deviation)

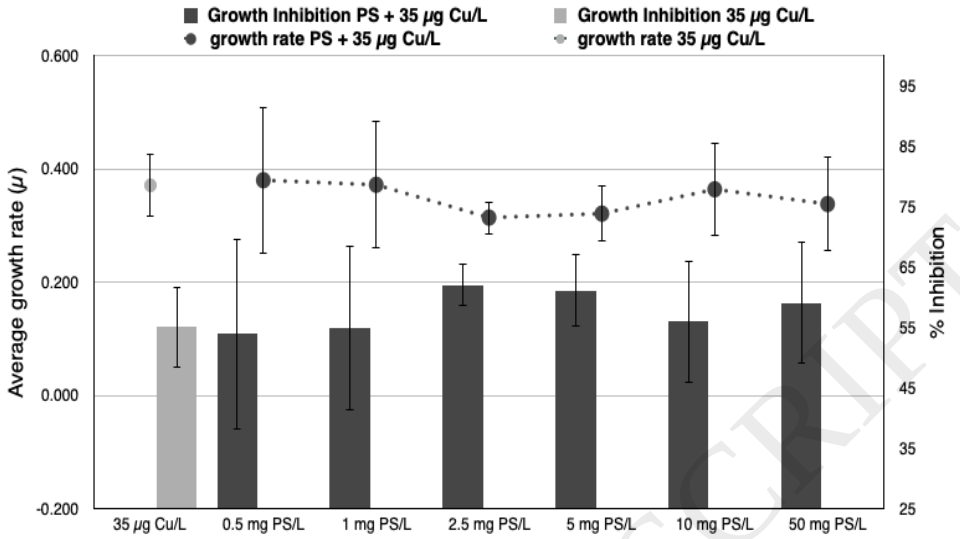


Figure S1: Percentage of growth inhibition compared to control (bars) and average growth rate (circles) of 72 h algal toxicity test of *R. subcapitata* exposed to Cu (light gray) and Cu (35 µg/L) with increasing PS NPs concentration (dark gray). Data shown as mean ± standard deviation**.**

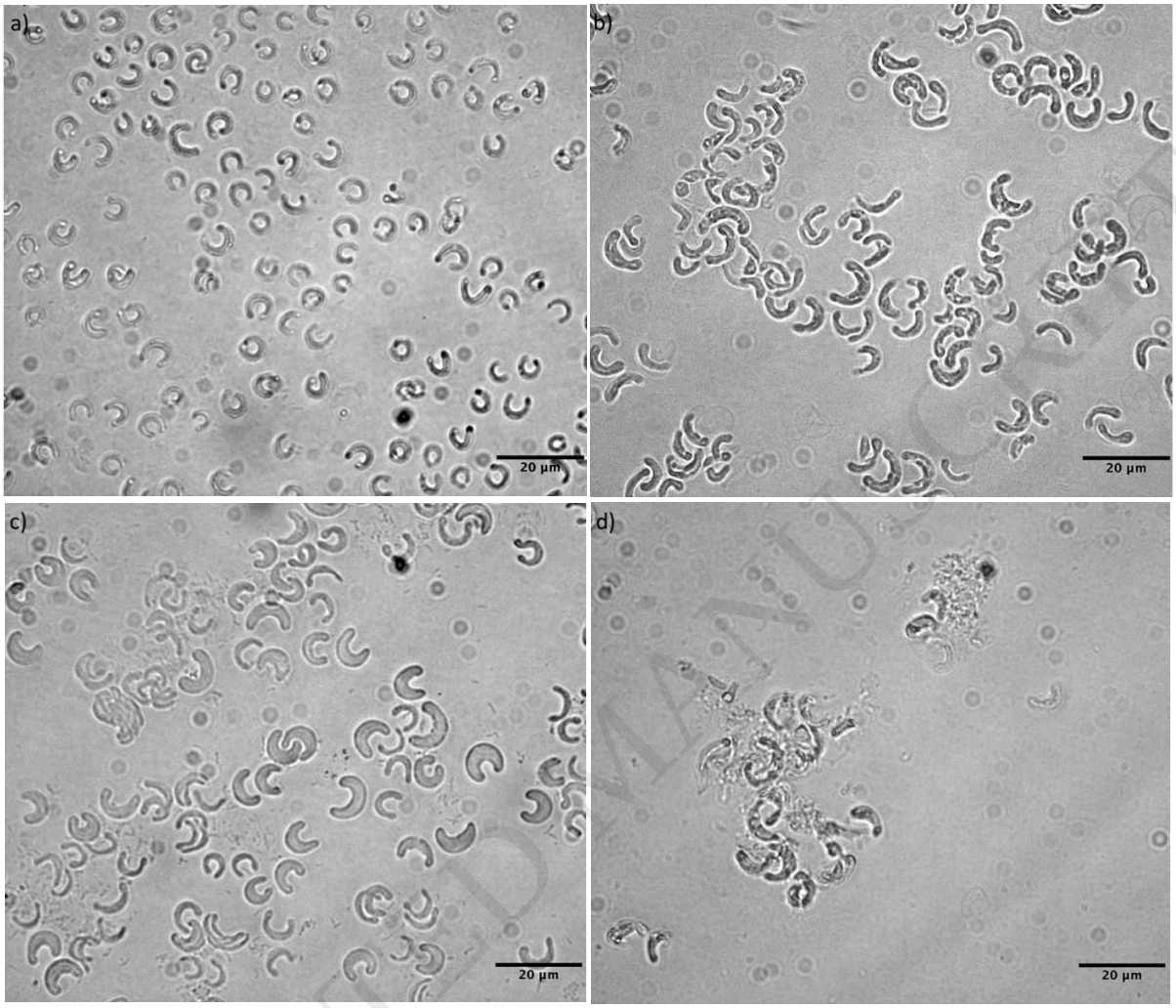


Figure S2. Optical microscope images of *R. subcapitata* at the end of 72 h toxicity test: a) control treatment; b) *R. subcapitata* exposed to 35 µg Cu/L, altered morphology; c) *R. subcapitata* exposed to 10 mg PS/L, altered volume; d) *R. subcapitata* co-exposed to 35 µg Cu/L + 10 mg PS/L, growth rate is strongly inhibited and cells morphology is substantially altered .

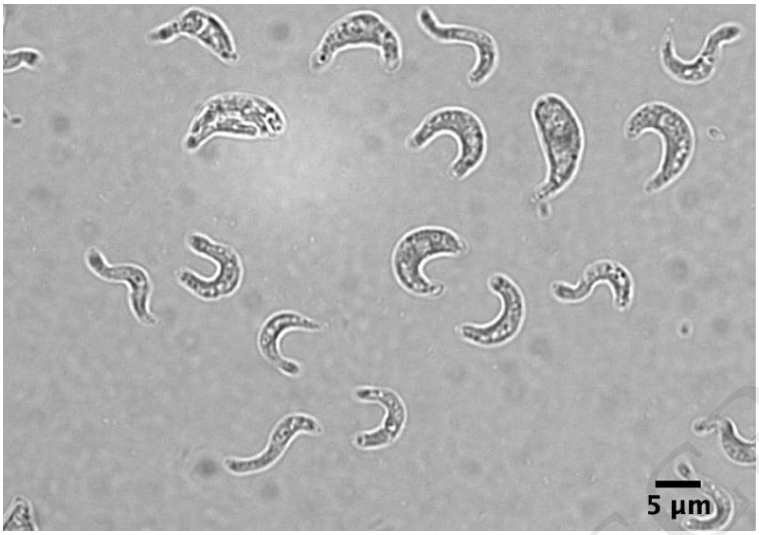


Figure S3: Optical microscope images of *R. subcapitata* exposed to 300 µg SDS/L. Algal growth was not affected, but some alterations in cell volume and replication processes were observed.

Tables and Figures:

Table 1. DLS measurement of hydrodynamic diameter (z-average) and surface charge (z-potential) of PS NPs incubated with Cu, EPS and *R. subcapitata* (algae were filtered out with a 0.45 µm filter before measurements), at 0 h and after 72 h, at 25°C in WC test medium.

	0 h					72 h	
	EPS	EPS + 10 µg Cu/L	EPS + 50 µg Cu/L	EPS + 100 µg Cu/L	EPS + 200 µg Cu/L	EPS	Algae
Z-average(nm)	88.63 ± 0.83[a]	91.7 ± 1.78	89.1 ± 1.35	91.5 ± 1.48	93.5 ± 0.24	84.7 ± 0.56[b]	106 ± 0.66[ab]
Z-potential(mV)	- 36.05	-31.5	-31.6	-32.5	-33.6	-34	-22.4
PDI	0.138 ± 0.01	0.114 ± 0.04	0.172 ± 0.02	0.091 ± 0.02	0.057 ± 0.03	0.23 ± 0.016	0.203 ± 0.02

Data with the same letter are statistically different with $p<0.001$ (data shown as mean ± standard deviation).

Table 2. Fluorescence (expressed as Arbitrary Unit of Fluorescence) of PS NPs incubated with *R. subcapitata*, EPS and/or Cu at 0 h and after 72 h, at 25°C in WC test medium.

	WC test medium	Algae	10 mg PS/L	10 mg PS/L + EPS	10 mg PS/L + Algae	10 mg PS/L + 50 µg Cu/L	10 mg PS/L + Algae + 50 µg Cu/L
0 h	278 ± 17	232 ± 32	2207 ± 142^{a}	2430 ± 72	2025 ± 113^{b}	1782 ± 89	1671 ± 51^{c}
72 h	249 ± 39	169 ± 14	1850 ± 221^{ade}	1784 ± 45	994 ± 107^{bd}	1552 ± 146	956 ± 99^{ce}

Data with the same letter are statistically different with $p<0.005$ (data shown as mean ± standard deviation).

Table 3. Dissolved Cu levels measured at the end of the 7-day toxicity test, with and without *R. subcapitata* (algae and PS NPs were filtered out with a 50 nm filter before measurements).

	Cu (µg/L)	
	No Algae	Algae
CTRL	0.26 ± 0.13	1.32 ± 0.44
PS (0.5 mg PS/L)	0.36 ± 0.32	0.67 ± 0.06
Cu (50 µg/L)	26.21 ± 0.4	21.64 ± 3.04
Cu (50 µg/L) + PS (0.5 mg/L)	25.43 ± 0.13	21.47 ± 3.74

Data shown as mean ± standard deviation

Table 4. Protein concentration extracted from *R. subcapitata* culture at the end of the 7-day toxicity test.

	Protein Concentration (mg/mL)	Cell Density (cells/mL)	Proteins (mg)/cells (10^5cells)
CTRL	$3.67*10^{-4}$	$3.99*10^{6}$	$9.2*10^{-6}$
Cu	$3.16*10^{-4}$	$0.55*10^{6}$	$56.9*10^{-6}$
PS	$3.36*10^{-4}$	$4.09*10^{6}$	$8.21*10^{-6}$
Cu + PS	$0.901*10^{-4}$	$0.49*10^{6}$	$18.2*10^{-6}$