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Published in:
Environmental Science and Technology

Link to article, DOI:
10.1021/acs.est.6b01072

Publication date:
2016

Document Version
Peer reviewed version

TITLE

A Multimethod Approach for Investigating Algal Toxicity of Platinum Nanoparticles

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ABSTRACT

The ecotoxicity of platinum nanoparticles (PtNPs) widely used in for example automotive catalytic converters, is largely unknown. This study employs various characterization techniques and toxicity endpoints to investigate PtNP toxicity towards the green microalgae *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii*. Growth rate inhibition occurred in standard ISO tests (EC_{50} values of 15-200 mg Pt/L), but also in a double-vial setup, separating cells from PtNPs, thus demonstrating shading as an important artefact for PtNP toxicity. Negligible membrane damage, but substantial oxidative stress was detected at 0.1-80 mg Pt/L in both algal species using flow cytometry. PtNPs caused growth rate inhibition and oxidative stress in *P. subcapitata*, beyond what was accounted for by dissolved Pt, indicating NP-specific toxicity of PtNPs. Overall, *P. subcapitata* was found to be more sensitive towards PtNPs and higher body burdens were measured in this species, possibly due to a favored binding of Pt to the polysaccharide-rich cell wall of this algal species. This study highlights the importance of using multi-method approaches in nanoecotoxicological studies to elucidate toxicity mechanisms, influence of NP-interactions with media/organisms, and ultimately to identify artefacts and appropriate endpoints for NP-ecotoxicity testing.

INTRODUCTION

The aquatic fate and toxicity of various metal nanoparticles have been studied intensively in recent years,[1] but very few studies have focused on the effects of platinum nanoparticles (PtNPs) on aquatic organisms. This is somewhat surprising considering the extensive use of PtNPs in automotive catalytic converters during the past decades. In the three-way catalytic converter, Pt is wash-coated onto a ceramic carrier and deposited as NPs,[2] typically in the size range of 1-10 nm.[3] The well-known catalytic activity of Pt is improved for nanostructured particles, allowing for an increased specific surface area of the Pt.[4] During use, abrasion of the catalytic converter will cause emission of Pt to the environment, mainly as elemental nanocrystalline Pt attached to µm-sized alumina particles.[5] Automotive catalysts represent the largest use of Pt and one of the main sources for emissions into the environment.[6] Emitted particles will be spread in the environment via atmospheric transport and/or stormwater runoff into drainage systems. Thus, elevated Pt levels have been detected in roadside dust, river sediments, aquatic organisms[6,7] and even in Greenlandic snow isolated from heavy traffic.[8]

The ecotoxicological effects of PtNPs in the aquatic environment remains, however, largely unknown. Zebrafish embryos exposed to 3-10 nm PtNPs capped with polyvinyl alcohol, showed hatching delays, concentration-dependent drop in heart rate, touch response, and axis curvature.[9] Similarly, 10 nm PtNPs influenced the heart rate of zebrafish embryos, as well as hatching and morphology, while also causing mortality and cytotoxicity in *in vitro* assays.[10] More recently, PtNPs in the size range of 30-60 nm were shown to inhibit the growth of green algae with a 72 h mean effective concentration ($EC_{50,72h}$) of 17 mg Pt/L.[11]

Algal toxicity data are required in hazard assessments schemes for chemical classification and regulation,[12] but NPs comprise a challenge to aquatic toxicity testing, due to their heterogeneous and dynamic nature when suspended in aqueous media.[13] This results in varying exposure concentrations

during incubation which ultimately affect the test validity and reproducibility.[14–16] The issue of NP transformation during incubation is further magnified for algal growth inhibition tests, due to the exponential increase in algal cells as well as the presence of their exudates and metabolic products.[17] The presence of relatively high concentrations of NPs in algal growth inhibition tests may also restrict light from reaching the algae, thereby causing growth inhibition as a result of a physical shading effect and not as an effect of toxicity to the algae.[14,15,18]

When conducting algal toxicity testing for regulatory purposes, the tested substances, are considered hazardous to the aquatic environment when the mean effective concentration (EC_{50}) is $\leq$ 100 mg/L in tests with either algae, crustaceans or fish.[12] Consequently, the algal testing setup needs to be valid even at relative high NP-concentrations, compared to relevant environmental exposure concentrations. The algal test was originally developed for soluble chemicals, for which a high concentration is only problematic in the case of poorly soluble or very colored substances.[19] As NPs are not soluble chemicals, but rather particles suspended in the test medium, it is important to investigate testing artefacts, such as shading, to evaluate the appropriateness of the currently used standard tests.[20] Currently, the outcome of standard toxicity testing is applied in hazard identification and regulation of NPs, although the mechanisms behind the test outcome rarely are understood completely. A testing scheme involving various endpoints may contribute to a better understanding of potential NP-specific ecotoxicological effects and form a more solid foundation for NP regulation.

This study aims to investigate potential mechanisms involved in the growth rate inhibition caused by PtNPs in the standard algal test used for hazard identification purposes. A multi-method approach is applied to elucidate the role of: 1) Physical obstruction of light, referred to as shading, 2) Cellular effects including oxidative stress and membrane damage, 3) Dissolution of PtNPs, and 4) Association of PtNPs to algal cells, determined as measured body burdens. Different biological endpoints are

compared for PtNPs and dissolved Pt ($PtCl_4$) in two algal species *P. subcapitata* and *C. reinhardtii* and paralleled with the aggregation and dissolution behavior of PtNPs in the respective algal media.

MATERIALS AND METHODS

Test materials, chemical analysis and preparation of test suspensions

The PtNPs were synthesized as described by Engelbrekt and co-workers,[4] yielding an aqueous suspension of pH~4, containing residual amounts of starch (0.6% weight in total), 6 mM glucose, 4 mM gluconic acid, 10 mM 2-(N-morpholino)ethanesulfonic acid (MES), 9 mM K^+ and 12 mM Cl^-. The starch stabilized PtNPs have a primary metal core diameter of 1.7 ± 0.2 nm and an outer diameter (including the starch coating) of 5.8-6.0 nm as determined by transmission electron microscopy (TEM) and thermogravimetric analysis.[4] The nominal Pt concentration of 390 mg Pt/L in the synthesized suspension was confirmed by inductively coupled plasma – mass spectrometry (ICP-MS; Agilent 7700, Morges, Switzerland) upon *aqua regia* digestion, yielding an average recovery of 109 ± 1% (n=3). The two algal species *P. subcapitata* and *C. reinhardtii* were cultivated in ISO 8692 medium,[21] and four-fold diluted Tris-Acetate-Phosphate medium,[22] respectively (referred to hereafter as ISO and TAP4 media). Prior to all characterization and algal toxicity testing, a stock suspension was prepared from an aliquot of the synthesized suspension by adjusting the pH using 1 M NaOH and adding algal nutrients to match the two algal test media. These stock suspensions were then diluted further with algal medium to prepare the test concentrations. The Pt concentration in selected stock and diluted test suspensions of both $PtCl_4$ (0.1-400 mg Pt/L) and PtNPs (10-390 mg Pt/L) was measured by ICP-MS upon preparation. PtNPs were digested before ICP-MS by evaporating the media and re-dissolving the solid fraction in *aqua regia*. The average recovery was 85 ± 15% (n=66). A series of studies on abiotic ROS generation was carried out with a second batch of PtNP synthesized as outlined above. For this batch the average recovery was 68 ± 9.6% (n=6) in media suspensions of 1-200 mg Pt/L. Platinum (IV) chloride ($PtCl_4$, 96%) was purchased from Sigma-Aldrich and included as a soluble Pt material. Other

reagents were analytical grade and all suspensions were prepared with Ultrapure Milli-Q water (> 18.2 Ω Milli-Q Direct system, Merck Millipore, Darmstadt, Germany).

Characterization of PtNPs suspended in algal media

The size distributions and zeta potentials of PtNPs in algal media were determined by Dynamic Light Scattering (DLS) using a Malvern ZetaSizer Nano ZS (Malvern Instruments, Malvern, UK). Measurements were conducted 1, 24 and 48 h after preparation of the PtNP suspensions of 30 mg Pt/L in TAP4 and ISO medium, respectively. The size distributions of PtNPs suspended in both media were also determined by Asymmetric Flow Field-Flow Fractionation (AsFlFFF) using an AF2000 (Postnova Analytics, Landsberg, Germany) immediately upon preparation and after 1, 24 and 48 h. For the elemental detection, the AsFlFFF system was coupled to an ICP-MS (Agilent 7700, Morges, Switzerland) monitoring the ^{195}Pt signal. The outflow of the AsFlFFF system was connected directly to the nebulizer of the ICP-MS.

The PtNP agglomeration and sedimentation behavior during 48 h in the two media were investigated respectively by nanoparticle tracking analysis (NTA) with a NanoSight LM10 (Malvern Instrument, Malvern, UK) and spectrophotometry (Agilent 8453, Agilent Technologies, USA). The PtNP suspensions (80 mg Pt/L) were prepared as for toxicity testing, and stored at 4 °C between measurements, with TAP4 and ISO media as blank references. The measurements were conducted 1, 24 and 48 h after preparation of suspensions. The size and number of agglomerates (> ≈ 50 nm) present in the suspensions were determined using NTA 3.1 with automated settings, camera level 16 and a detection threshold of 5. For each measurement, three videos of 60 s were recorded and the sample advanced before each video. Sedimentation was investigated by recording the absorbance of suspensions at wavelengths ranging from 190 to 1100 nm.

The concentration of dissolved Pt in the stock suspensions of PtNPs and dilutions in algal media was determined by ultracentrifugation (Beckman L8-60M) using a swinging bucket rotor (SW 41 Ti; Beckman). PtNPs were suspended at 68 mg Pt/L in Milli-Q, ISO and TAP4 media. Immediately upon suspension, and after 48 h incubation under algal testing conditions, samples of 10 mL (n=2) were centrifuged for 16 h at 3×10^4 rpm (68000 × g) to ensure settling of particles ≥ 5.7 nm. The supernatant (5 mL) was removed, acidified with nitric acid and the Pt content was measured by ICP-MS.

The abiotic generation of reactive oxygen species (ROS) by PtNPs and $PtCl_4$ suspended in algal media (without algae present) was determined using the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA, Sigma Aldrich) as described by Ivask and co-workers.[23] Specific details are given the Supporting Information (SI).

Algal growth rate inhibition and ^{14}C-assimilation tests

Tests were performed in accordance with the ISO 8692 algal growth inhibition test protocol[21] with modifications as described below, and 48 h incubation.[24] Tested concentrations (n=3) and controls (n=6) were inoculated with algae (10^4 cells/mL) yielding average control growth rates of 1.0-1.3 d^{-1} for *P. subcapitata* and 1.7-1.8 d^{-1} for *C. reinhardtii*. A maximum pH change of 1.7 units occurred in controls as well as exposed algae during the 48 h incubation. The quantity of algal pigments was quantified at 0, 24 and 48 h by acetone extraction[25] followed by fluorescence spectrophotometry (Hitachi F-7000) at 430 and 670 nm excitation and emission wavelengths, respectively. The ^{14}C-incorporation was performed as described in previous work[16] (details are included in SI). A maximum change in pH of 1.5 units was measured during the 2 h incubation.

The influence of PtNPs' shading on algal growth rates and ^{14}C-assimilation inhibition was studied under the same conditions as described above, but using a double-vial test setup. Algae in media (2

156 mL) were kept in a small inner-vial, and physically separated from the PtNP suspension (6 mL) placed
157 in the larger outer-vial (Figure S1). The control growth rates were in the range given for the regular
158 setup. Finally, the potential photochemical efficiency was monitored over 48 h in algae exposed to 0, 2
159 and 80 mg Pt/L, as described in the SI.
160
161 **Algal cell damage and oxidative stress**
162 Test suspensions were prepared in volumetric flasks, inoculated to 10^5 cells/mL and distributed (25
163 mL, n=3) to 100 mL Erlenmeyer flasks incubated as described in the SI. Tests with $PtCl_4$ were
164 conducted using the setup for growth inhibition tests. A maximum variation of 0.4 (PtNPs) and 1.3
165 ($PtCl_4$) pH-units was found before testing and after 48 h incubation in controls and the highest test
166 concentrations. After 2, 24 and 48 h incubation, algae were sampled from each concentration and
167 controls, and incubated with fluorescent dyes for 30 min in the dark. CellROX Green (Life
168 Technologies Europe B.V., Zug, Switzerland) was employed as intracellular oxidative stress indicator
169 (5 µM), and propidium iodide (Sigma-Aldrich, Buchs, Switzerland) was used to determine membrane
170 permeability alteration (7 µM), as previously described in details for *C. reinhardtii*.[26,27] Unexposed
171 algae were used as negative controls, whereas the positive controls prior to staining, were incubated
172 with 10 mM H_2O_2 (30 min in the dark) and in a 90°C water bath (10 min) for CellROX Green and
173 propidium iodide, respectively. Flow cytometry was conducted using a BD Accuri C6 flow cytometer
174 (BD Biosciences, San Jose, CA, USA) with an argon-ion excitation laser (488 nm) and FL1 green
175 channel (530 ± 15 nm), FL2 orange channel (585 ± 20 nm) and FL3 red channel (670 ± 25 nm). For
176 tests with $PtCl_4$, results were analyzed using a BD FACSCanto II flow cytometer (BD Biosciences, San
177 Jose, CA, USA). Gating strategies were applied to discriminate positively stained cells from the

negative control (Figure S2-4). Data analysis was conducted using BD Accuri C6 software 264.15 and FlowJo V10 for the two flow cytometers, respectively.

Algal body burden of PtNPs

Algae were exposed to PtNPs (2 and 80 mg Pt/L) in triplicate 250 mL flasks with 75 mL suspension inoculated to10^5 cells/mL. A sample of 20 mL suspension from each replicate was taken after 2, 24 and 48 h incubation and filtered through a 3.0 µm nitrocellulose filter (Merck Millipore). The algal cells retained by the filter were gently washed with 20 mL medium before filters were digested in Teflon tubes (1 mL *aqua regia* at 90°C for 2 h). The Pt content was determined by ICP-MS analysis (Agilent 7700, Morges, Switzerland) after dilution with 5% (v/v) HCl (Baker, instar grade). The cell number for each replicate suspension was determined after 0, 2, 24 and 48 h incubation on a Coulter Multisizer III particle counter (Beckman-Coulter, Switzerland). Suspensions of PtNPs in media (80 mg Pt/L) without algae were applied as background controls, and treated as described above. The particle counts and Pt content of digested filters were all background corrected using data from these controls.

Atomic force microscopy imaging

For atomic force microscopy (AFM) both algal species were exposed to PtNPs (10 mg Pt/L) under the same conditions as described for growth inhibition testing. After 48 h incubation, a drop of each suspension was placed on sliced silicon wafers and allowed to dry. To remove dry salt particles, the wafer pieces were carefully washed with distilled water and dried again with nitrogen gas. Atomic Force Microscope (AFM NX20, Park Systems) images were taken of the two samples using non-contact mode, an amplitude of 1.67×10^6 nm and a scan rate of 1 Hz.

Statistical analysis and data interpretation

Mean effective concentrations (EC_{50}) and corresponding 95% confidence intervals for the inhibition of algal growth rates and carbon assimilation were estimated using the statistical program LOG457, which applies the log-logistic model for nonlinear regression analysis of responses versus concentration, minimizing the sum of squares between calculated and measured inhibitions.[28] Nominal concentrations were used, as the average Pt recovery from ICP-MS analyses was 84 ±15% in selected stock and test suspensions of $PtCl_4$ and PtNPs (n=72). Comparison of growth rate inhibition data is based on EC_{50}-values and their variability provided by corresponding 95% confidence intervals.

RESULTS AND DISCUSSION

Characterization of PtNPs in algal media

The size distributions of PtNPs suspended in algal media were determined after 1, 24 and 48 h by AsFlFFF (4 mg Pt/L), DLS (30 mg Pt/L) and NTA (80 mg Pt/L), see Figure 1. A size peak of 10 nm was identified by AsFlFFF and DLS. For NTA the size detection limit is higher than 10 nm, but NTA measurements contribute with information about agglomeration of PtNPs in algal media. As shown in Figure 1C, the number of PtNP agglomerates (> 50 nm) increased almost three orders of magnitude in the TAP4 medium, whereas PtNPs in the ISO medium remained within the same order of magnitude over the 48 h period. The agglomerates formed were in the size range of 50-400 nm for both media; this finding is supported by the DLS measurements, showing hydrodynamic diameters within this range at all times measured (Figure 1B). Moreover, the measured zeta potentials of PtNP suspensions (20-25 mg Pt/L) after 1 and 48 hours indicated higher stability of PtNPs in ISO (-28 ± 0.3 mV) than in TAP4 medium (-15 ± 0.9 mV). Besides the increasing agglomerate number, agglomerate sizes increased with time according to NTA (Figure 1C). Although the PtNPs agglomerated substantially, especially in the TAP4 medium, the UV-VIS absorbance did not change during the 48 h, indicating that the PtNPs remained suspended, and did not settle in the suspensions (Figure S5). The ICP-MS analyses of samples fractionated using AsFlFFF showed constant Pt recoveries in ISO medium over time (around 80%), whereas recoveries in TAP4 decreased from 80% at 0 h to 62% and 63% at 24 and 48 h, respectively (Table S2). This decrease may be due to agglomeration, as larger agglomerates will not elute from the channel and hence not be detected by ICP-MS.

The concentration of dissolved Pt in PtNPs suspensions (total concentration 68 mg Pt/L) prepared in Milli-Q water, ISO and TAP4 media and incubated for 48 h under algal testing conditions were in the ranges of 2.3-2.4 mg Pt/L (Milli-Q water suspensions), 2.0-2.2 mg Pt/L (ISO medium suspension) and

233 2.0-2.5 mg Pt/L (TAP4 medium suspension). The concentrations increased only slightly during the 48
234 h incubation. This shows that dissolved Pt (corresponding to about 3% of the total Pt content) in the
235 test suspensions mainly was non-reacted Pt from the particle synthesis.
236 For the abiotic ROS generation analyses of PtNPs and $PtCl_4$ in both algal media (Figure S6) a relatively
237 high, and varying, background DCF fluorescence was measured. As a consequence, the abiotic ROS
238 was determined concomitantly for both PtNPs and $PtCl_4$ in the two media within one test run, to allow
239 for a relative comparison between the ROS generation of the two forms of Pt. It should be noted that
240 these analyses were made using another batch of PtNPs than otherwise used in this study. The PtNPs in
241 this new batch were synthesized as described in the Materials and Methods and identical primary
242 diameters were obtained. The algal-free assay measuring abiotic ROS generation revealed increasing
243 DCF fluorescence relative to the backgrounds in a concentration-dependent manner for PtNPs in both
244 media. A slightly higher response was detected in the ISO medium compared to TAP4, especially after
245 48 h. Conversely for $PtCl_4$, the relative DCF fluorescence was greater in TAP4 than in ISO medium.
246 Collectively, these data suggest that abiotic ROS generation is influenced by the media and that abiotic
247 ROS measured in the PtNP suspension cannot be solely ascribed to dissolved Pt. The abiotic ROS
248 generation activity by PtNPs of various shapes has been reported as low, based also on a cell-free DCF
249 assay.[29] Comparison of results is however challenged by differences in methods and media applied.
250
251 **Effects of PtNPs on algal photosynthesis, carbon assimilation and growth rate**
252 Exposure to PtNPs resulted in decreased growth rates of both *P. subcapitata* and *C. reinhardtii* in
253 standard ISO tests, with $EC_{50,48h}$ values (95% confidence intervals in brackets) of 15 [13-16] and 201
254 [173-235] mg Pt/L, respectively. Based on the results from tests with *P. subcapitata*, the PtNPs would
255 be classified as "harmful" to algae in accordance with the CLP regulation.[12] These results are generally

256 in agreement with the reported $EC_{50,72h}$ value of 17 mg Pt/L for growth inhibition in *P. subcapitata*
257 exposed to PtNPs.[11] Due to the dark color of the suspensions, we hypothesized that PtNPs limited the
258 available light for algal growth causing shading, thus inhibiting growth physically rather than by a toxic
259 action of the PtNPs to the algal cells. As growth rate inhibition also occurred in the double-vial setup
260 with no contact between algae and PtNPs, we cannot falsify this hypothesis. The growth rate inhibition
261 found using the double-vial setup was however slightly lower that in the standard test setup with
262 $EC_{50,48h}$ = 45 [30-68] and 373 [167-838] mg Pt/L for *P. subcapitata* and *C. reinhardtii,* respectively
263 (Figure 2A, C). These results suggest that physical shading from PtNPs lowered the algal growth rates,
264 but also indicate that PtNPs inhibit algal growth rates by other means than shading, possibly by direct
265 toxic effects. However, the higher response in the regular setup could also arise from PtNPs adhesion to
266 the algal surface, potentially causing "localized" shading and/or interference with the membrane,
267 nutrient uptake and other cellular processes involving the cell surface.[14] As described by Hjorth and co-
268 workes [30] :"Shading and toxicity are not additive effects. The impact of shading cannot be eliminated
269 by simply subtracting the effect observed in the shading test from the actual test. Deducting the effect
270 of shading is more complicated for NPs as the exact mode of action is unknown and the observed
271 effects are potentially multicausal." Also, shading can mask or limit potential toxicity, because slowly
272 growing algae under low light intensity are less sensitive to toxicants than faster growing algae.[30]
273 In agreement with our results, shading effects have been reported to markedly influence growth rate
274 inhibition in green algae exposed to gold NPs[31] and carbon nanotubes,[32] while studies with ZnO, CuO
275 and TiO_2 have found shading negligible.[14,33] It is likely that exposure concentration, suspension color,
276 and NP adhesion to algal surfaces are influencing factors on shading. Consequently, growth rate
277 inhibition alone is not an appropriate endpoint for disclosing PtNP toxicity, as it does not allow for
278 discrimination between direct toxic effects and indirect physical effects. For this reason, ^{14}C-

assimilation was included as an alternative endpoint to quantify the toxicity of PtNPs towards the two algal species (Figure 2B, D). Comparable EC_{50} values were obtained for *P. subcapitata* and *C. reinhardtii* of 47 [43-50] and 37 [31-46] mg Pt/L, respectively, in the regular setup, and 32 [16-65] and 32 [18-56] mg Pt/L in the double-vial setup. The slopes of the concentration response curves are however different between data from the shading and the regular test with *C. reinhardtii* (Figure 2B). As the EC_{50} values does not differ between the regular and the double-vial setup, the 2 h ^{14}C-assimilation inhibition in both algal species may be solely ascribed to physical shading effects of PtNPs. Thus, the endpoint of 2 h carbon assimilation is even more sensitive to shading and/or less applicable for testing PtNP toxicity than the standard 48 h growth rate inhibition test.

Using the ultracentrifugation results, concentration–response data for PtNPs were recalculated based on the dissolved Pt concentration rather than the total nominal concentration (Figure 2A, C). For *C. reinhardtii* these data aligned closely with the $PtCl_4$ data, as also seen by the overlapping 95% confidence intervals of the EC_{50} values. Thus, the PtNP toxicity to this algal species may be caused by the dissolved Pt. For *P. subcapitata* however, data based on dissolved Pt showed greater inhibition than $PtCl_4$, suggesting a possible NP-specific effect.

Taken together, the 48 h growth inhibition data (Figure 2A, C) demonstrate that *P. subcapitata* is more sensitive to the toxic effects of both $PtCl_4$ and PtNPs than *C. reinhardtii*. Furthermore, the results from the double-vial setups indicate that *P. subcapitata* is more affected by shading than *C. reinhardtii*. It may be, that *P. subcapitata* is less efficient in adapting to light conditions over time, and thus more affected by this physical effect than *C. reinhardtii*. The potential photochemical efficiency monitored over 48 h in algae exposed to 0, 2 and 80 mg Pt/L (Figure S7 was indeed slightly lower for *P. subcapitata* than *C. reinhardtii*, both for controls and exposed algae. This agrees with the difference in

301 growth rate measured for the two algal species controls in growth rate inhibition tests (app. 1.1 vs. 1.8
302 d^{-1} for *P. subcapitata* and *C. reinhardtii*, respectively).
303 Overall, the results demonstrate that shading from PtNPs does occur and affects the growth rates
304 measured in a standard guideline test. If the double-vial setup had not been applied, comparing the
305 results for $PtCl_4$ and PtNPs in Figure 2 could easily be misinterpreted and lead to faulty conclusions.
306 Due to the influence of shading, neither growth rate inhibition nor ^{14}C-assimliation can be considered
307 appropriate endpoints to test algal toxicity of PtNPs for hazard identification purposes. As the PtNP
308 toxicity may be attributed to dissolved Pt for *C. reinhardtii*, but not entirely for *P. subcapitata*, the NP-
309 specific effect(s) found could be algal species specific though the behavior of PtNPs in the two
310 different algal media used, may also affects the toxicity.
311
312 **Cellular effects of PtNPs in algae: Oxidative stress and membrane damage**
313 Extensive oxidative stress was observed for both algal species upon PtNP exposure, as demonstrated by
314 the increasing percentage of stained cells (Figure 3A, B). *C. reinhardtii* was highly stressed after 2 h
315 exposure to PtNPs, even at the lowest exposure concentration (0.1 mg Pt/L). However, the algal
316 population recovered over time for all PtNP-concentrations up to 10 mg Pt/L. Some indication of
317 recovery over time was also seen for *P. subcapitata*, although much less pronounced than for *C.*
318 *reinhardtii*. After exposure to $PtCl_4$, oxidative stress was only detected in *C. reinhardtii* with no
319 indication of recovery over time as it was seen after exposure to PtNPs (Figure 3D). Interestingly, no
320 signs of oxidative stress were detected in *P. subcapitata* upon $PtCl_4$ exposure (Figure 3C), indicating
321 the oxidative stress from PtNP exposure is not related to the dissolved Pt. Despite the substantial
322 percentage of cells with oxidative stress caused by PtNPs, the percentage of cells with membrane
323 damage were < 2% for *P. subcapitata* and < 22% for *C. reinhardtii* (supporting data, Figure S8). This

suggests that the antioxidant systems of both algal species were able to cope with the oxidative stress induced by PtNPs, thereby preventing its progression to membrane damage. In vitro studies using human cell lines similarly report that PtNPs do not affect the membrane integrity.[10,34,35] Whether or not PtNPs induce oxidative stress in human cells is more ambiguous, with biotic ROS and oxidative stress from PtNPs being both confirmed[36] and rejected,[29,35] and even detoxification of ROS has been suggested.[34]

Algal body burden of PtNPs

Although exposed to similar PtNP concentrations in suspension, the Pt body burden differed greatly for the two algal species (Figure 4A, B). In general, the body burdens were higher for *P. subcapitata*, especially at the highest tested concentration of 80 mg Pt/L. This may explain the more pronounced 48 h growth rate inhibition found for this algal species since higher attachment of PtNPs to the algal surface is likely to cause a higher (local) shading effect and/or toxicity. For *C. reinhardtii* the body burden decreased significantly over 48 h for 2 mg Pt/L, but increased slightly at 80 mg Pt/L. This observation correlates well with the oxidative stress pattern showing recovery at 2 mg Pt/L, but not at 80 mg Pt/L (Figure 3). Conversely, *P. subcapitata* recovered slightly from oxidative stress after 48 h at the highest concentration of 80 mg Pt/L and the body burden also decreased with time at this concentration (Figure 3 and 4A). The differences in body burdens in the two algal species may relate to the different composition of their cell walls. The cell wall of *P. subcapitata* contains cellulose and polysaccharides, whereas the cell wall of *C. reinhardtii* does not,[37] but rather consists of several layers of glycoproteins.[22] It has been proposed, that while Pt (II) has higher affinity for amino acids and proteins, Pt (IV) may preferentially bind to a polysaccharide matrix.[38] This may explain why higher growth rate inhibition was found for *P. subcapitata* than *C. reinhardtii* upon $PtCl_4$, and PtNP exposure.

347 Another factor influencing the body burden is the higher growth rate of *C. reinhardtii* compared to *P.*
348 *subcapitata*. This causes the ratio of PtNPs to algal cells to decrease faster in *C. reinhardtii*, and thus
349 yield a lower body burden after 48 h exposure to PtNPs.
350 In a separate series of tests, the two algal species were examined by AFM after 48 h exposure to PtNP,
351 providing some indication of PtNP-agglomerates on the algal cell surface (Figure S9). Several studies
352 have demonstrated how various NPs attach to the surface of algae.[14,15,33] Attachment of NPs to algae is
353 most likely a dynamic process, changing the NP body burden over time depending on algal physiology
354 and NP properties. This is an area that needs more investigation and could prove very useful for the
355 interpretation of data from aquatic toxicity testing of NPs.
356
357 **PtNP behavior in test media and related biological effects**
358 Overall, the characterization of PtNPs in algal media showed a higher degree of agglomeration and
359 dissolution of PtNPs in the TAP4 medium, whereas slightly more abiotic ROS was generated in the
360 ISO medium. The implications of these findings are discussed below, along with the possible
361 connection between the toxicity endpoints, and the difference in toxicity of PtNPs vs. dissolved Pt.
362 According to NTA, a significantly higher number of agglomerates was formed during 48 h incubation
363 in the TAP4 medium (*C. reinhardtii*) than in the ISO medium (*P. subcapitata*). The lower PtNP body
364 burden in *C. reinhardtii* cells may be linked to the agglomeration behavior in the TAP4 medium, as
365 smaller particle sizes theoretically favor greater adhesion to the algal surface, due to the increased
366 number of particles available for contact with the algae. The lower Pt body burden in *C. reinhardtii*
367 may in turn explain why this species was less affected in the growth inhibition test with PtNPs, as less
368 contact between algal cells and PtNPs also reduces any localized shading and/or physical effects.
369

Both AsFlFFF and ultracentrifugation data showed higher dissolution of PtNPs in the TAP4 medium (*C. reinhardtii*) than in the ISO medium (*P. subcapitata*). The growth rate inhibition of *C. reinhardtii* could be explained by the dissolved fraction of Pt in the medium. For *P. subcapitata* however, the PtNPs caused higher growth rate inhibition than explained by the measured dissolved Pt. Furthermore, the oxidative stress responses in the two algal species were not governed by dissolved Pt. For *P. subcapitata* all cells were affected by PtNPs, but none by $PtCl_4$ (Figure 3). *C. reinhardtii* cells were affected by both PtNPs and $PtCl_4$, but the presence of dissolved Pt cannot fully account for the level of oxidative stress nor the recovery observed when cells were exposed to PtNPs (Figure 3). To fully understand the role of dissolved Pt in algal toxicity, knowledge on speciation and binding to media components is crucial. Unfortunately, speciation data such as solubility constants is limited[39] and not included in speciation models such as MINTEQ. The main differences between the two media are the pH, buffer types and the content of chloride and organic components. The ISO medium (pH 8) contains sodium bicarbonate buffer, whereas TAP4 (pH 7) contains TRIS, sodium acetate and roughly half the chloride amount of the ISO medium. The speciation and solubility of Pt is influenced by chloride species, pH, and organic ligands such as citric acid.[39–41] However, determining the exact Pt speciation in the actual media is challenged by the different scopes and variables of available speciation studies, as well as the obscure number of chemical species found even in a simple system of Pt, chloride and water.[41]

Abiotic ROS, generated by PtNPs when suspended in the two algal media, may have caused or contributed to the oxidative stress detected in both algal species. Abiotic ROS was however, also generated by PtNPs in Milli-Q water (data not shown) suggesting that the ROS generation from PtNPs may occur on the surface of PtNPs. This is further supported by the results of the positive controls in the tests of abiotic ROS (Figure S6) and oxidative stress (Figure S3 and S4) as no abiotic ROS or

393 oxidative stress occurred from the positive control (H_2O_2) or the dissolved reference ($PtCl_4$) in the ISO
394 medium, whereas PtNPs caused very clear responses in both these tests. Similarly, it has been
395 suggested by other publications that NPs may induce elevated intracellular ROS by direct
396 physical/chemical interactions with biomolecules.[42,43] In this case, the algal species with the highest Pt
397 body burden, i.e. *P. subcapitata,* would be expected to exhibit most oxidative stress. However, most
398 oxidative stress was found in *C. reinhardtii* cells. In contrast, *P. subcapitata* was the most affected
399 species in the growth inhibition tests. Thus, oxidative stress and growth rate inhibition appear
400 unrelated. Generally, ROS generation and oxidative stress has been suggested as likely mechanisms
401 related to NP toxicity in algae and other aquatic microorganisms, although the causal link between
402 particle properties and ROS generation or effects is not yet established.[42] The formation of extra- or
403 intracellular ROS can trigger a cascade of cellular events that may cause toxicity.[42] The reverse may
404 also occur, i.e. that NPs induce toxicity by another mechanism, such as DNA lesions, leading to
405 cellular stress and accumulation of intracellular ROS.[43] DNA damage is a known effect of platinum
406 compounds and is also confirmed for PtNPs in human cells.[35,36] However, whether DNA damage
407 results in cytotoxicity strongly depends on the nature of formed DNA adducts, as documented for the
408 stereoisomers cis- and transplatin in their toxicity towards cancer cells.[35] Least oxidative stress was
409 found for *P. subcapitata* even though higher toxicity occurred for this species and more abiotic ROS
410 was produced in the medium of this species (ISO medium). The many pathways interlinking
411 abiotic/biotic ROS, oxidative stress, DNA damage and cellular toxicity challenge the establishment of
412 causality.
413 Our results demonstrate that shading is an important artefact in standard algal growth rate inhibition
414 testing of PtNPs. If not taken into account, the standard method is not applicable for regulatory hazard
415 identification purposes. The shading issue will be relevant for other NPs as well, especially those with

416 EC_{50} values in the higher end of the classification range (10-100 mg/L) and those adhering strongly to
417 the algal surface. While the environmental relevance of toxicity testing of NPs at such high
418 concentration levels is questionable, it is of high regulatory relevance for toxicity identification and
419 ranking as well as classification, and labeling of NPs within the current regulatory framework. The
420 cellular toxicity quantified by flow cytometry revealed no marked membrane damage, but significant
421 oxidative stress in both algal species. This may be linked with abiotic ROS generated by the PtNPs. For
422 *P. subcapitata*, PtNPs caused both growth rate inhibition and oxidative stress in higher levels than what
423 could be accounted for by dissolved Pt. This indicates a NP-specific effect possibly related to the
424 catalytic properties of PtNPs and/or their adhesion to algal cells. Overall, *P. subcapitata* was more
425 sensitive to the effects of PtNPs than *C. reinhardtii*. Furthermore, higher body burdens were measured
426 for *P. subcapitata*, most likely due to favored binding of Pt to the polysaccharide containing cell wall
427 of this algal species. The multi-method approach in this study provided insight into the possible
428 underlying mechanisms behind the observed PtNP-cell interaction and toxicity. Until more knowledge
429 on NP-specific toxicity mechanisms becomes available, it is crucial to investigate and account for
430 artefacts and NP interactions with organisms and media. Generally, a broader and more exploratory
431 approach to aquatic toxicity testing, employing various endpoints and testing methods, may assist to
432 avoid false negative as well as false positive test results and advance the understanding within the field
433 of nanoecotoxicology.

ACKNOWLEDGEMENTS

The authors thank Signe Qualmann (DTU Environment), Giulia Cheloni, Nadia von Moos, Séverine Le Faucheur, Coralie Suscillon, Rebecca Flück, Sophie Moisset and Sonia Blanco-Amijeiras (University of Geneva) for assistance with technical and practical matters. This work is part of the project ENVNANO (Environmental Effects and Risk Evaluation of Engineered Nanoparticles) supported by the European Research Council (grant no. 281579). Javier Jiménez-Lamana acknowledges the European Commission for receiving funding from the European Union's Horizon 2020 research and innovation program under the Marie Sklodowska-Curie grant agreement No. 660590.

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SUPPORTING INFORMATION

Additional experimental details regarding size distribution measurements by AsFlFFF, abiotic ROS formation by PtNPs and $PtCl_4$, algal culturing, algal shading and ^{14}C-assimilation tests, gating strategies for flow cytometry, as well as determination of the potential photochemical efficiency of PSII in algae. Results and data for sedimentation of PtNPs in algal media, abiotic ROS generation by PtNPs and $PtCl_4$ in algal media, potential PSII photochemical efficiency and membrane damage in algae, and atomic force microscopy images of algal cells exposed to PtNPs.

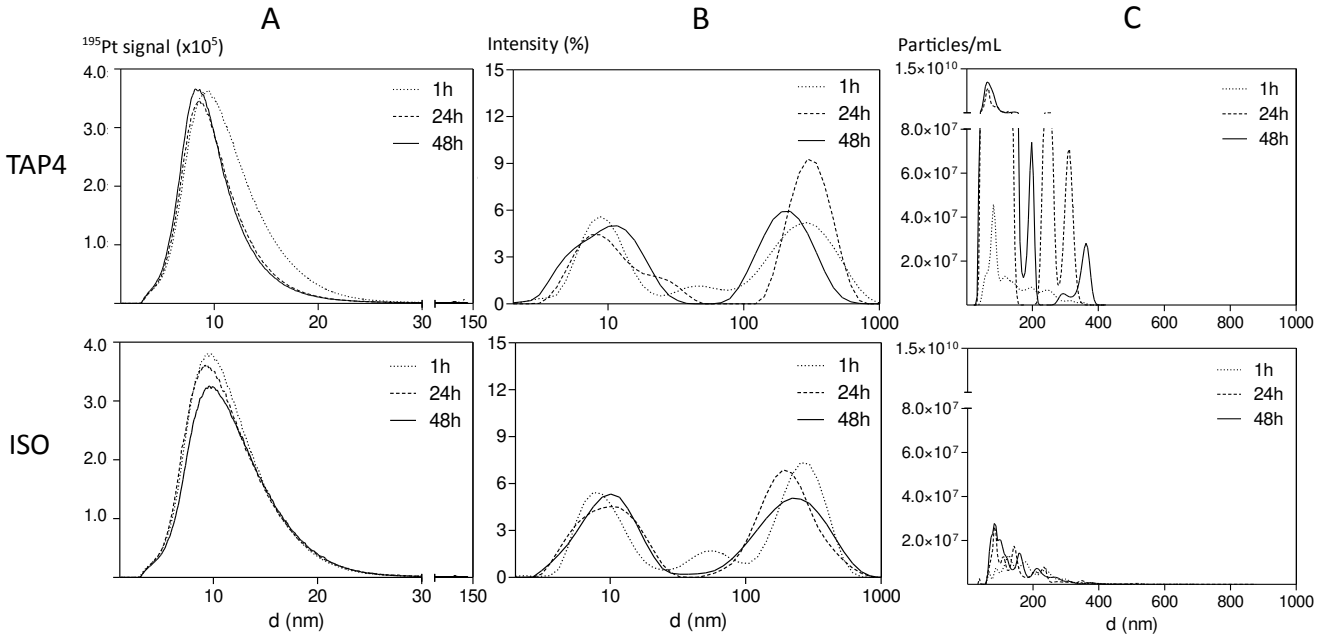


Figure 1. Size distributions after different incubation periods (1-48h) for PtNPs suspended in TAP4 medium (top row) and ISO medium (bottom row) determined by different methods. Column A) Suspensions of 4 mg Pt/L analyzed by Asymmetric Flow Field-Flow Fractionation (AsFlFFF); Column B) Suspensions of 30 mg Pt/L analyzed by Dynamic Light Scattering (DLS); Column C) Suspensions of 80 mg Pt/L analyzed by Nanoparticle Tracking Analysis (NTA).

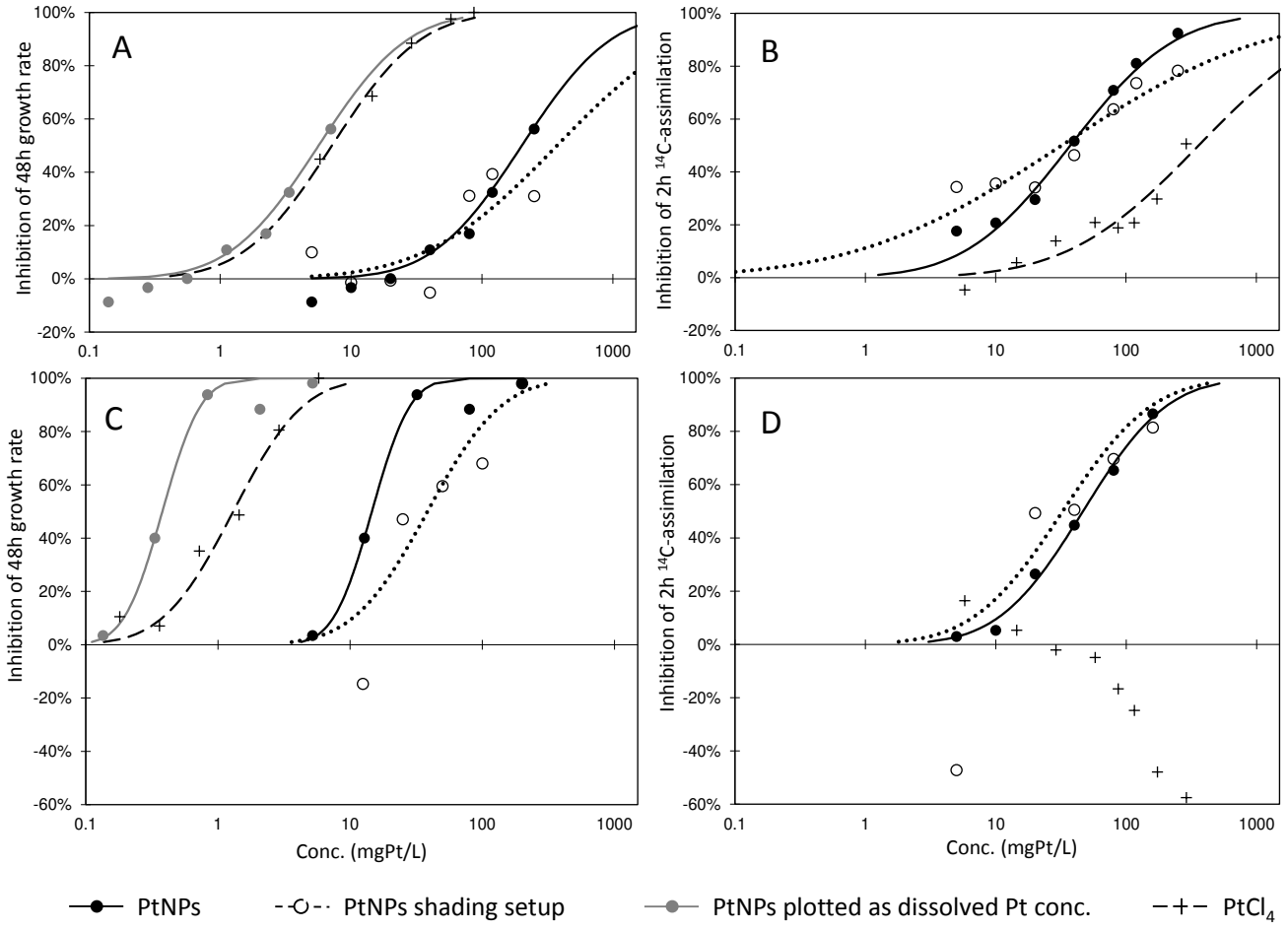
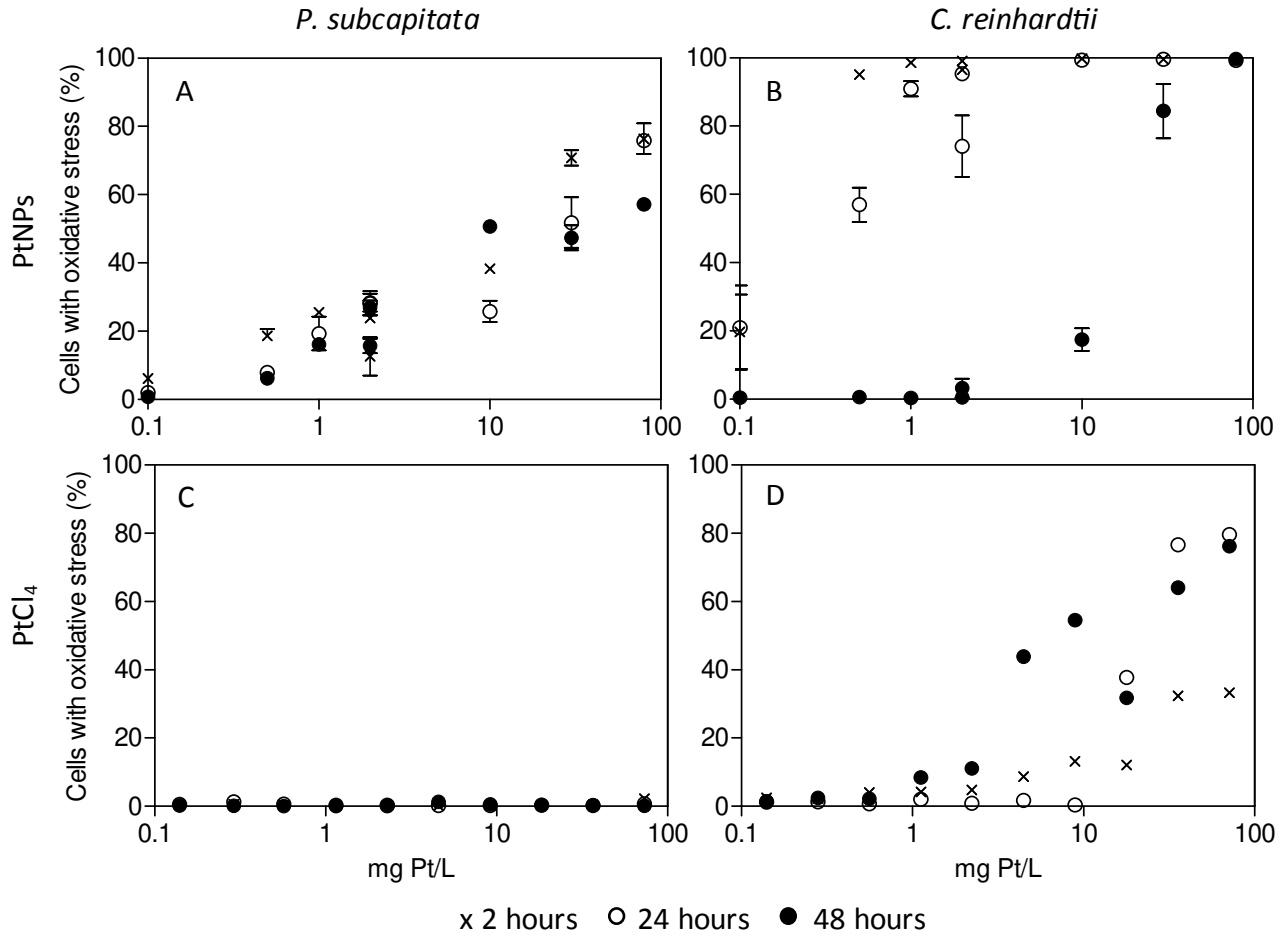
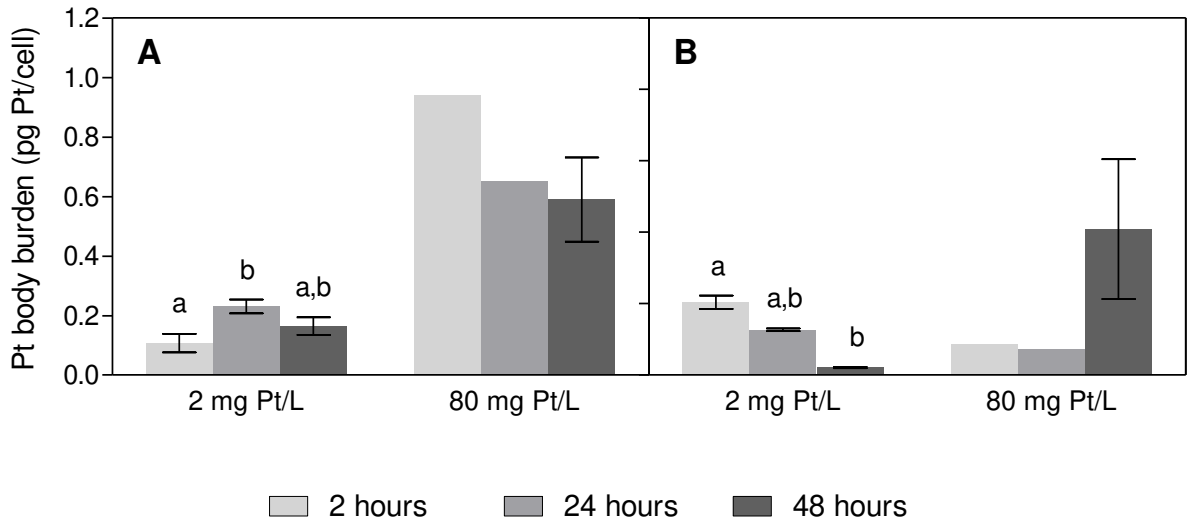


Figure 2. Concentration-response data and fitted curves from 48 h growth rate inhibition tests (A and C) and 2 h ^{14}C-assimilation tests (B and D) with *C. reinhardtii* (A and B) and *P. subcapitata* (C and D) for PtNPs and $PtCl_4$. For PtNPs two setups were applied in accordance with Figure S1: A regular setup and a double-vial setup for investigation of shading effects. Furthermore, the concentration-response data and curves for PtNPs was recalculated based on the dissolved Pt fraction, and plotted for A and C to reflect the toxicity of the dissolved Pt in the PtNP suspension.



585

586 **Figure 3.** Oxidative stress in *P. subcapitata* and *C. reinhardtii* upon 2, 24 and 48 h exposure to $PtCl_4$,
587 in single concentrations (0.14-73 mg Pt/L) and PtNPs in two parallel tests with triplicate low
588 concentrations (0.1-2 mg Pt/L) and high (2-80 mg Pt/L), respectively. Data for *C. reinhardtii* exposed
589 to $PtCl_4$ for 2 h are based on very low cells numbers. The error bars represent standard deviations.



590

591 **Figure 4.** Body burdens of platinum for *P. subcapitata* (A) and *C. reinhardtii* (B) after 2, 24 and 48 h
592 exposure to PtNPs at 2 and 80 mg Pt/L. The error bars represent standard deviations. For body burdens
593 determined at 80 mg Pt/L after 2 and 24 hours n=2 or n=1. For all other data n=3. The letters "a" and
594 "b" denotes statistically significant differences in medians ($p < 0.05$) according to Kruskal-Wallis and
595 Dunn's multiple comparison tests, over time within each algal species and exposure concentration
596 treatment.

Supporting information

A Multimethod Approach for Investigating Algal Toxicity of Platinum Nanoparticles

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This supporting information contains:

16 Pages

2 Tables

9 Figures

Table of contents

S1 Materials and methods - additional details, including references.

Size distribution measured by AsFlFFF

Suspensions of 4 mg Pt/L were injected in the separation system and characterized immediately upon preparation and after 1, 24 and 48 h. A spacer of 350 µm thickness, a 20 µL loop and a regenerated cellulose (RC) membrane with a cut-off of 1 kDa were used in all the experiments. 0.01% sodium dodecyl sulfate (SDS) in 1 mM NH_4NO_3 (Sigma-Aldrich) solution at pH 8 was used as carrier. The optimal separation program found is detailed in Table S1. In order to characterize the sample, the system was first calibrated against polystyrene standards of known size (22, 58 and 97 nm). The following linear relationship between the logarithm of the retention ratio R (defined as elution time corresponding to the void volume divided by the retention time for a given particle) and the logarithm of the diameter (d) in nanometers was experimentally found:

$$\mathrm{Log}(R) = 0.4939 - 0.9539 \times \mathrm{Log}(d); \; r = 0.9958 \qquad (1)$$

Fractograms obtained as a function of time for each sample were converted into size distributions according to equation (1).

Abiotic ROS formation

H_2DCF-DA was dissolved in ethanol (1.3 mM) and deacetylated to H_2DCF by letting 1 mL react with 4 mL 0.01 M NaOH for 30 min in the dark. The mixture was added 20 mL sodium phosphate buffer (25 mM, pH 7.4) and the resulting 52 µM H_2DCF solution was placed on ice in the dark until use. PtNPs and $PtCl_4$ were added to TAP4 and ISO media in the concentration range 0.001-390 mg Pt/L. After 2 h and 48 h incubation in media, under the same conditions as for growth inhibition testing, 100 µL of the PtNP suspensions was each mixed with 100 µL H_2DCF solution in the wells of a 96-well black microplate (three replicates of each concentration). After 1 h incubation in the dark, the fluorescence of DCF was

measured (readings, n=3) (excitation/emission at 485/527 nm) using a fluorescence plate reader (Biotek Synergy Mx plate reader). As positive control, H_2O_2 was diluted in ISO and TAP4 media in the concentration range 0.16–20% (w/w). The ROS level in samples was calculated in relative fluorescence units (RFUs) by dividing the fluorescence of samples (PtNPs in media incubated with H_2DCF) by fluorescence of background (media incubated with H_2DCF).

Algal culturing

P. subcapitata was obtained from the Norwegian Institute for Water Research, Oslo, Norway (NIVA) and cultivated in ISO 8692 medium[1] and the *C. reinhardtii* strain CPC11 was obtained from the Canadian Phycological Culture Center (CPCC, Department of Biology, University of Waterloo, Canada) and grown in four times diluted Tris-Acetate-Phosphate medium[2]. The two respective media are hereafter referred to as ISO and TAP4 media. For all toxicity testing, the algae were exposed in their respective cultivation medium and testing was conducted under the same incubation conditions as for the culture. For cellular toxicity, photosynthesis efficiency and body burden studies 250 mL Erlenmeyer flasks were fitted with permeable stoppers, containing 50 mL algal suspension and incubated (Infors, Bottmingen, Switzerland) at 20 ± 2°C with continuous agitation (100 rpm) and illumination from above (110 ± 10 µmol/m^2/s). For growth and carbon assimilation inhibition tests 20 mL glass vials with perforated screw cap lids, containing 5 mL suspension were kept at 20 ± 2°C, continuous shaking (300 rpm) and illuminated from below by fluorescent tubes (30W/33; Philips, The Netherlands) at a light intensity of 100 ± 20 µmol/m^2/s. The algal cultures were re-inoculated in fresh media every second to third day, to ensure an exponentially growing culture.

Algal ^{14}C-assimilation tests

Preparation of test concentrations, control and replicates was similar to the growth rate inhibition tests, but the initial algal density was 10^5 cells/mL. Immediately before incubation, 50 µL of $NaH^{14}CO_3$ solution (specific activity: 20 µCi/mL; obtained from DHI, Hoersholm, Denmark) was added to all vials which were then closed with airtight screw caps. The tests were terminated after 2 h incubation by adding 0.2 mL 10% HCl to each vial (yielding pH < 2). The vials were left open overnight in a fume hood and 10 mL scintillation liquid (Optiphase "Hisafe" 3, Perkin Elmer, Waltham, MA, USA) was added to each vial. After thorough mixing, they were left in the dark for 8 h and submitted to liquid scintillation counting (Hidex 300 SL). $H^{14}CO_3$ solution was also added to three replicates of medium only, as controls to confirm that all added ^{14}C that had not incorporated into biomass, was being converted into $^{14}CO_2$ and removed in the evaporation step.

Potential photochemical efficiency of PSII

Algae exposed to PtNPs at 2 and 80 mg Pt/L, along with a control, were incubated for 48 h in triplicate 100 mL flasks of 25 mL suspension. After 2, 24 and 48 h incubation, samples of 3.5 mL were drawn from all replicates, and upon 1 h of dark acclimation, fluorescence variables were measured by Fast Repetition Rate Fluorometry (FRRF) using a FastOcean FRR plus FastAct fluorometer (Chelsea Technologies Group Ltd). Six acquisitions were run per sample and each acquisition comprised of 36 sequence repeats with saturation/relaxation phases of 100/40 flashlets per sequence and a 2/50 µs pitch. By use of the program FastPro8 © (Version 1.0.50, Kevin Oxborough, Chelsea Technologies Group Ltd), the potential photochemical efficiency of PSII (Fv/Fm) was obtained, reflective of changes in the photochemical energy conversion efficiency.[3] Specific blank corrections with pure medium and PtNPs suspension of 80 mg Pt/L in medium were carried out to rule out a possible direct increase of the fluorescence signal due to the presence of PtNPs. Fluorescence blank readings were lower than 10% of the sample fluorescence (22% for 2 h measurements).

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(2) Harris, E. H. Chlamydomonas in the laboratory. *The Chlamydomonas Sourcebook. Introduction to Chlamydomonas and its laboratory use*, 2nd ed.; Academic Press, San Diego, California, USA, 2009.

(3) Greene, R. M.; Kolber, Z. S.; Swift, D. G.; Tindale, N. W.; and Falkowski, P. G. Physiological Limitation of Phytoplankton Photosynthesis in the Eastern Equatorial Pacific Determined from Variability in the Quantum Yield of Fluorescence. *Limnol. Oceanogr.* **1994**, *39*, 1061–1074.

S2 Settings and results for AsFlFFF measurements

Table S1. Separation program used for AsFlFFF measurements with an outflow of 1.0 mL/min.

	Time [s]		Crossflow [mL/min]
Injection/focusing	300	Injection flow 0.2 mL/min	2
Crossflow	1200	constant	1
	300	linear decay	0
	300	constant	0

Table S2. Diameters and widths of the different size distributions and Pt recoveries obtained by AsFlFFF.

	ISO 8692 medium			TAP4 medium		
Time for PtNPs in media (h)	Size (nm)	Width (nm)	Recovery (%)	Size (nm)	Width (nm)	Recovery (%)
0	9.5	7.2	78 ± 4	9.5	7.1	80 ± 4
1	9.4	7.1	82 ± 4	9.5	6.9	80 ± 4
5	9.5	7.1	87 ± 4	8.4	6.1	75 ± 4
24	9.2	7.4	83 ± 4	8.6	5.4	62 ± 3
48	9.6	7.6	77 ± 4	8.2	5.1	63 ± 3

S3 Experimental setup for shading experiments

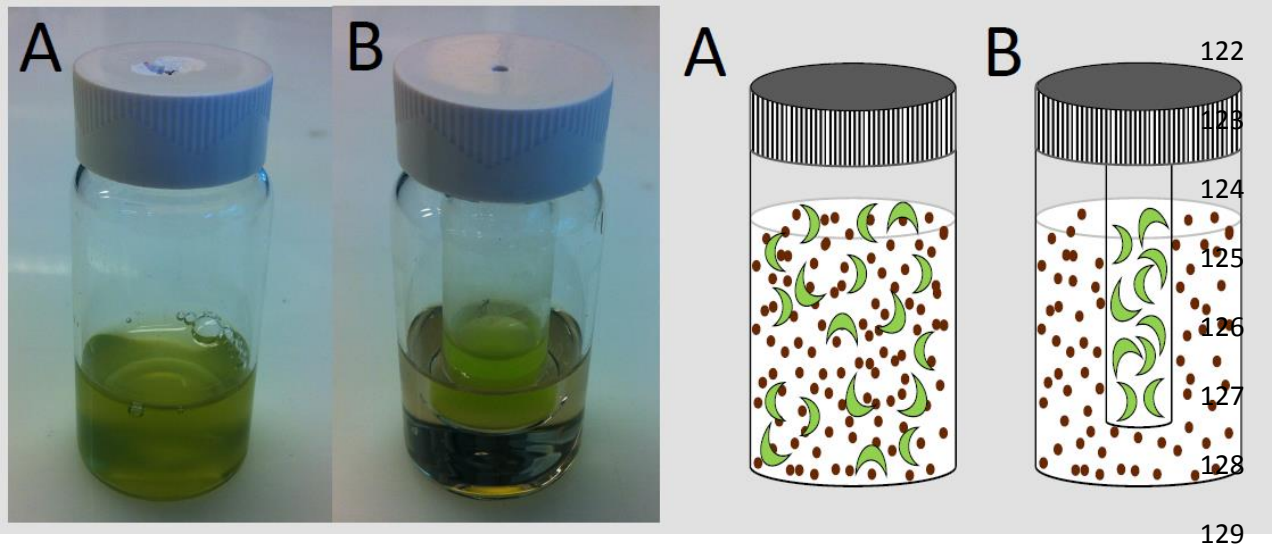


Figure S1. Photos and illustrations of the test setup used for determining shading effects in the algal growth inhibition and ^{14}C-assimilation tests. A) The setup used for standard testing and B) The double-vial setup, allowing for separation of algal cells in the smaller inner-vial from the surrounding PtNP suspension in the larger outer-vial.

S4 Flow cytometry gating strategies

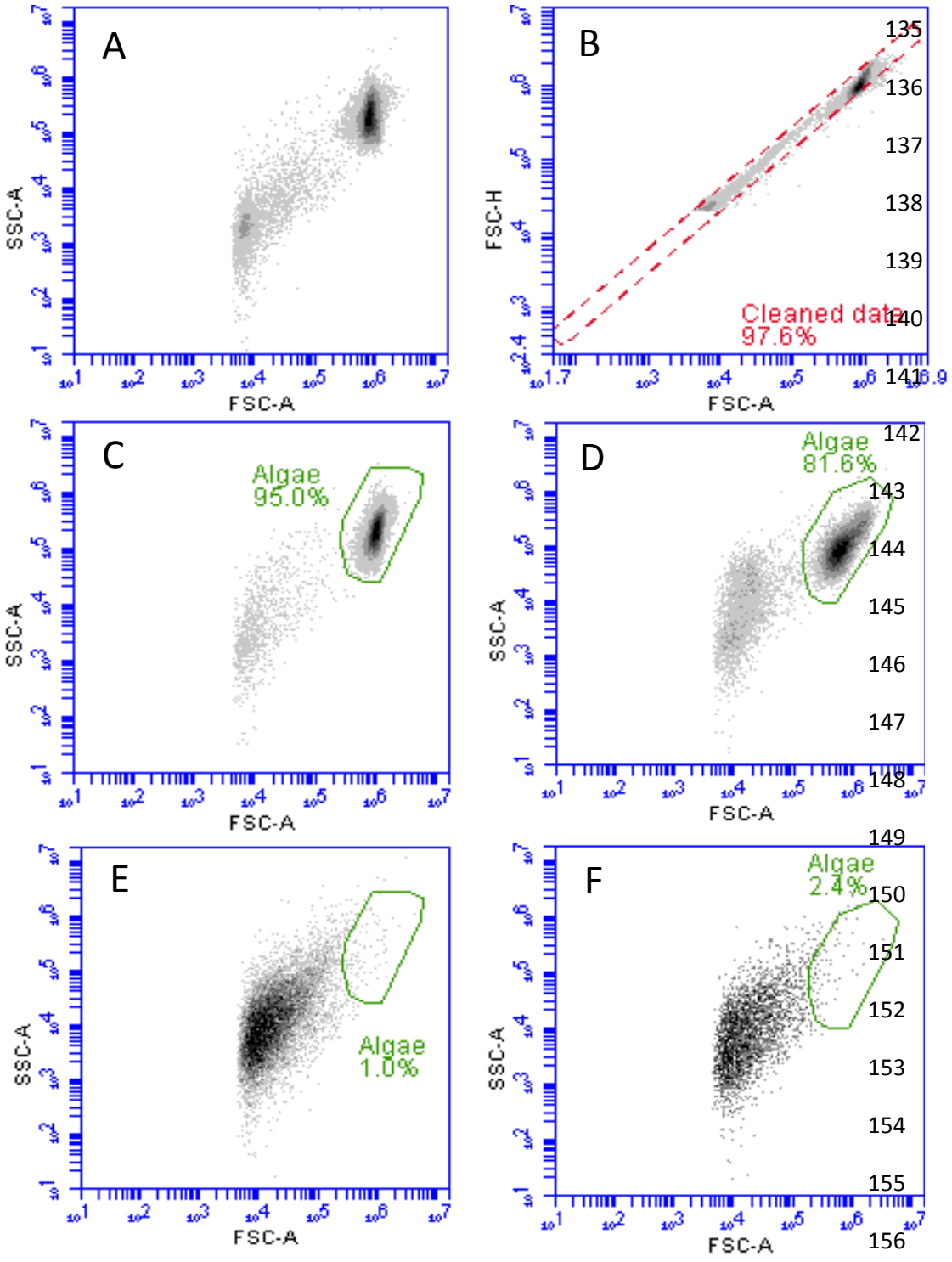


Figure S2. Flow cytometry gating strategy. A) Raw data for *C. reinhardtii* upon 48 h exposure to PtNPs (80 mg PtL), B) Removing doublets with "cleaned data" gate, C) Algal gate for unexposed *C. reinhardtii*, D) Algal gate for unexposed *P. subcapitata*, E) PtNPs in TAP4 medium, and F) PtNPs in ISO medium. FSC = Forward scatter, SSC = Side scatter, A = Area, H = Height.

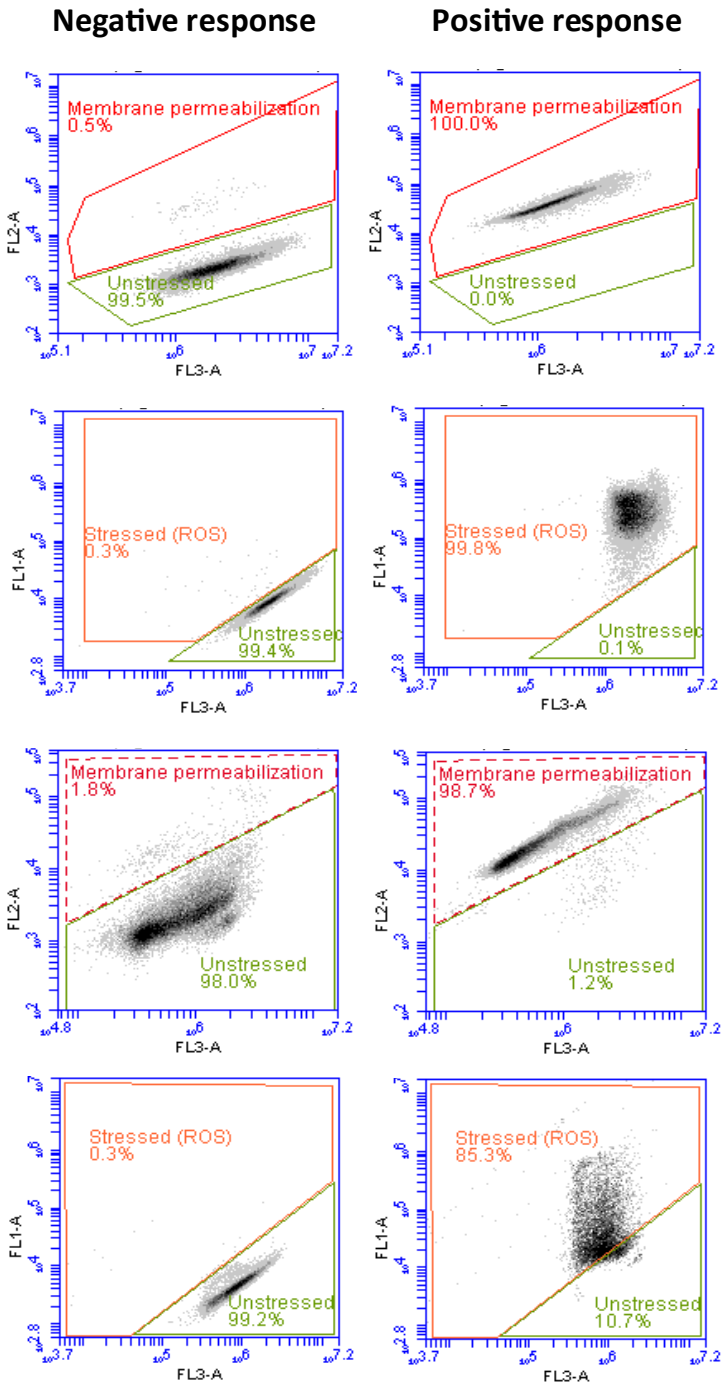


Figure S3. Flow cytometry gating strategy for biological end points using flow cytometer BD Accuri C6. The four graphs on top apply to *C. reinhardtii* and the four below, to *P. subcapitata*. The autofluorescence is measured in the fluorescence channel 3 (FL3), membrane permeabilization with the fluorescent probe propidium iodide in fluorescense channel 2 (FL2) and oxidative stress with the fluorescent probe CellROX green in fluorescense channel 1 (FL1). The gates are determined based on the negative and positive controls, except for CellROX in *P. subcapitata*, as H_2O_2 was not a usable postitive control for this alga/medium. The experiments are considered valid, as a very clear response was obtained from algae exposed to PtNPs.

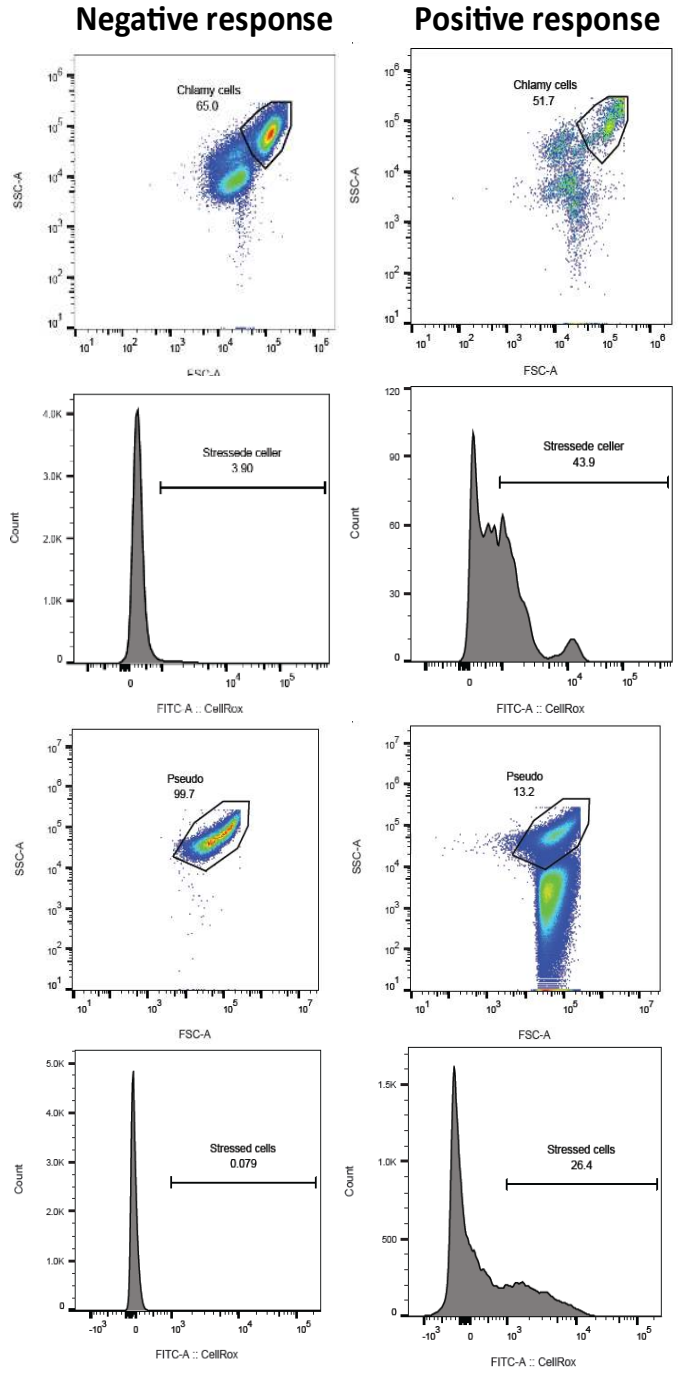


Figure S4. Flow cytrometry gating strategy for biological end points using flow cytometer BD FACSCanto II. The four top graphs apply to *C. reinhardtii* and the four below, to *P. subcapitata*. The oxidative stress response is determined by the fluorescense of CellROX green in the FITC channel 1 of algal cells in the assigned gates. For *C. reinhardtii* a likely contamination was observed, and algal cells were distinguished by autoflourescence of algal pigments at 690 nm. The gates are determined based on negative and positive controls, except for CellROX in *P. subcapitata*, as H_2O_2 was not a usable positive control for this alga/medium. The experiments are considered valid, as a very clear response was obtained from algae exposed to PtNPs.

S5 Sedimentation of PtNPs in algal media

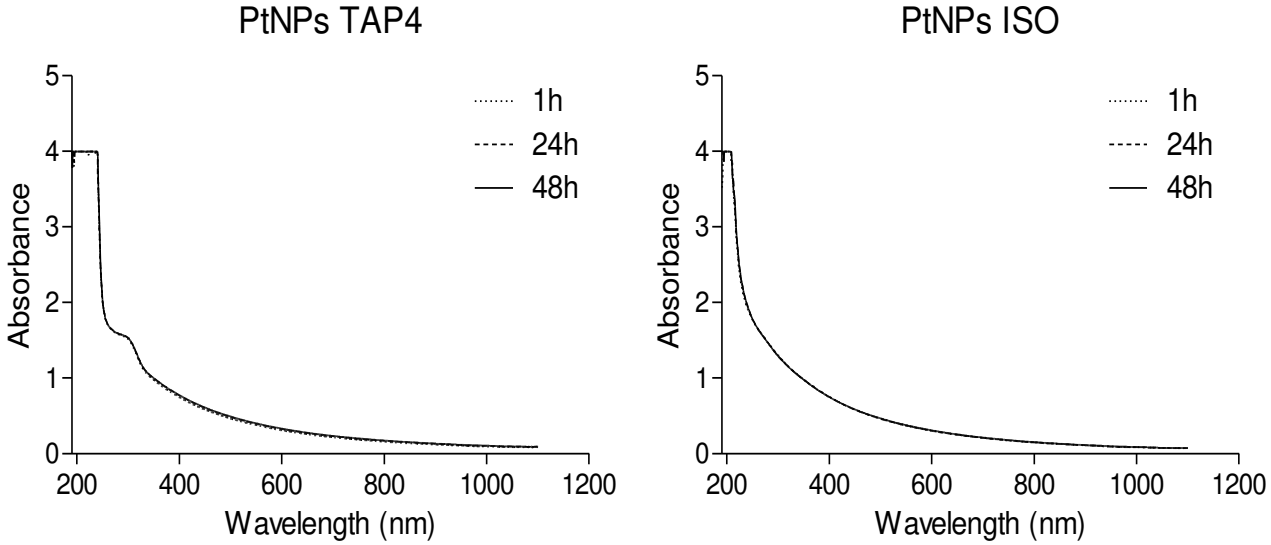


Figure S5. Absorbance spectra for PtNPs (80 mg Pt/L) suspended in ISO and TAP4 algal media after 1, 24, and 48 h.

S6 Abiotic ROS generation by PtNPs and PtC_{l4} in algal media

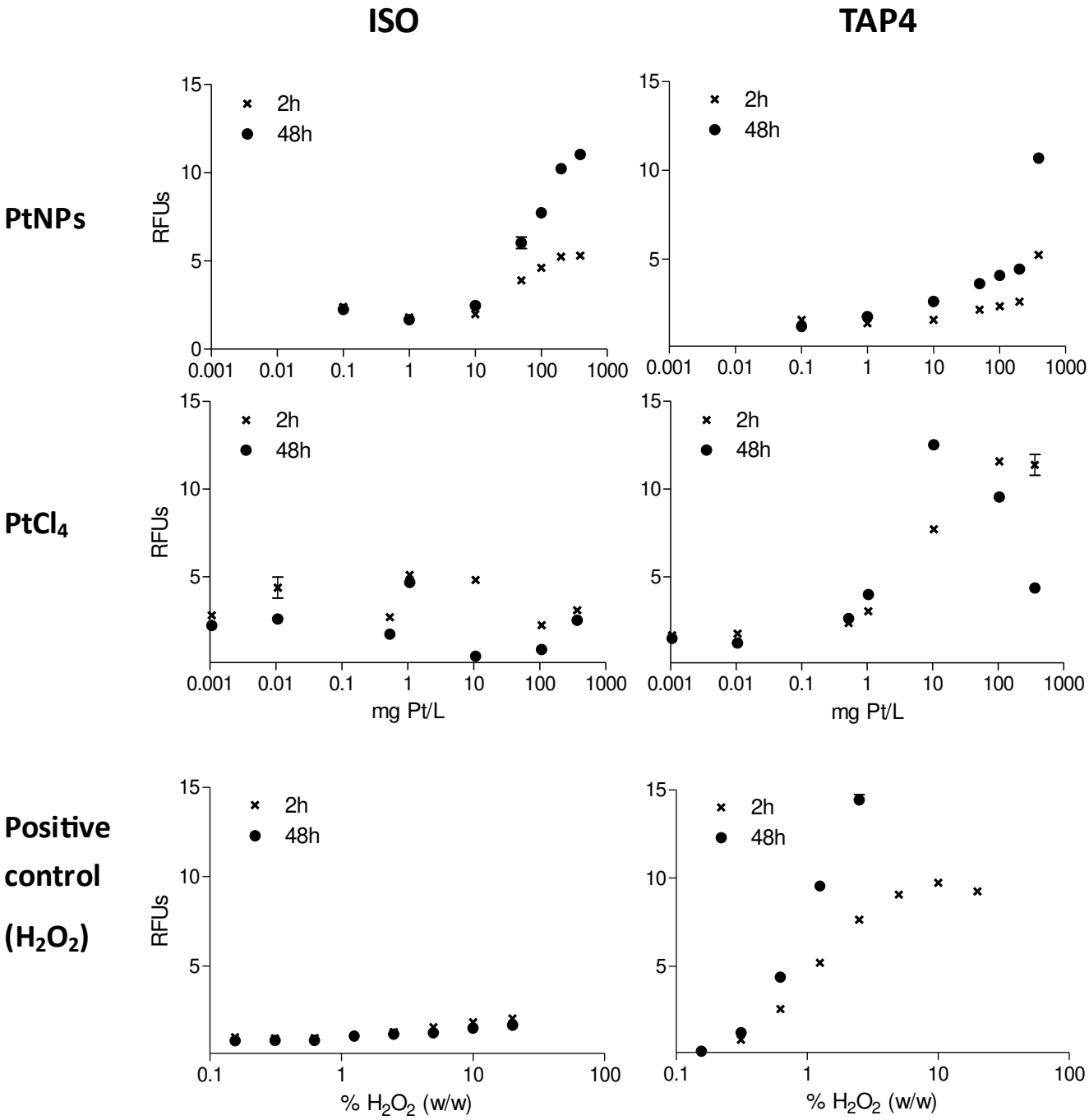
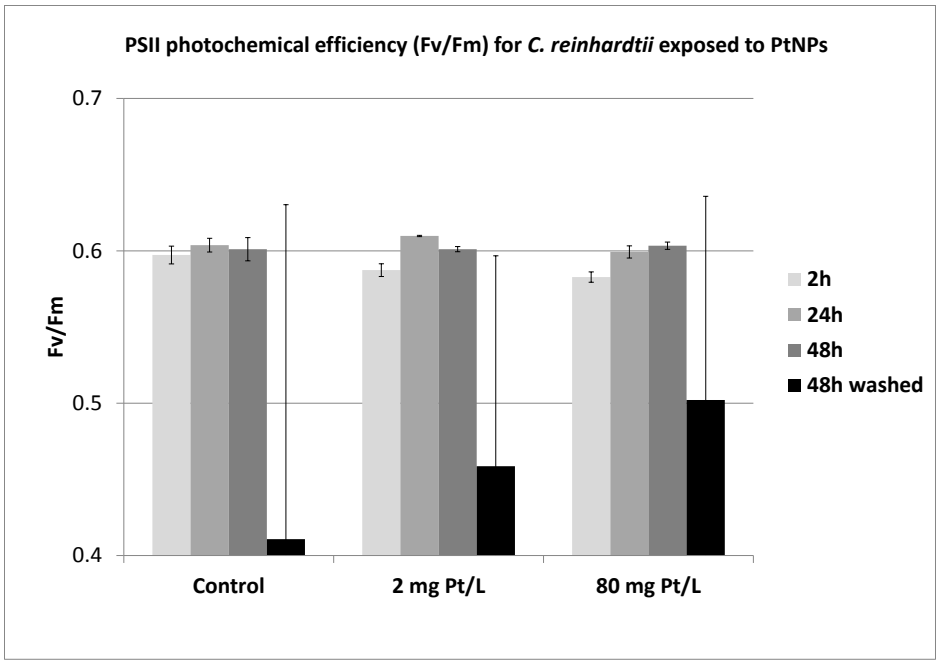


Figure S6. Abiotic ROS generation of PtNPs, $PtCl_4$ and the positive control (H_2O_2) upon 2 and 48 h suspension in ISO and TAP4 algal media, given as relative fluorescence units (RFUs) determined by the fluorescence of DCF from the tested suspension, relative to the fluorescence of the background (DCF in the respective media). The error bars represent standard deviations (n=3). The fluorescence exceeded the detection range in the positive controls of 5-20%w/w H_2O_2 in TAP4 medium for 48 h measurements.

S7 Potential PSII photochemical efficiency in algae



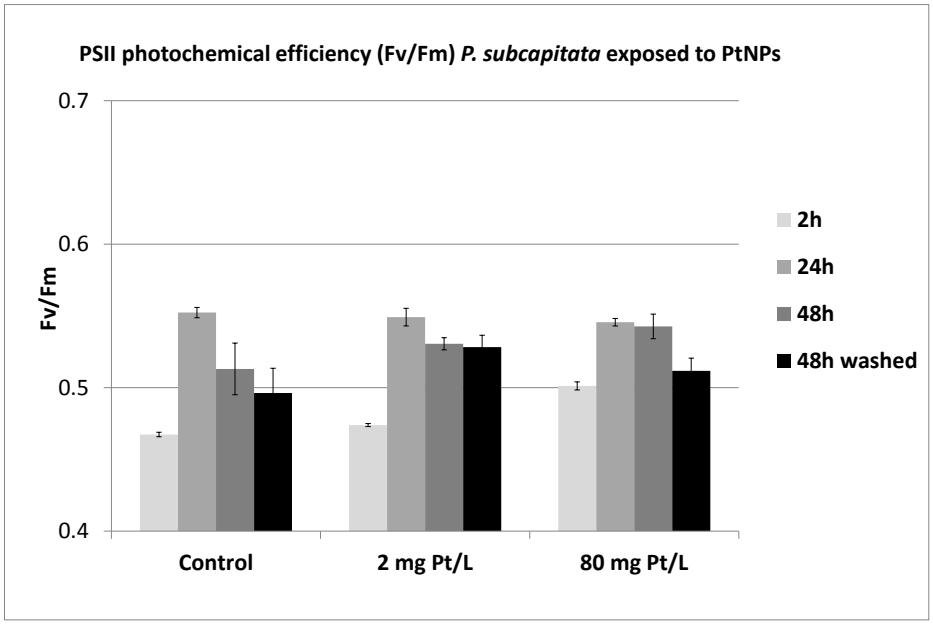


Figure S7. Fast Repetition Rate Fluorometry (FRRF) measurements of the potential PSII photochemical efficiency (Fv/Fm) for *C. reinhardtii* and *P. subcapitata* upon 2, 24 and 48 h exposure to 0, 2 or 80 mg Pt/L. Measurements were conducted for the algae in the PtNP suspensions, and for 48 h measurements also for algal cells washed with medium through a filter (48 h washed). The error bars represent standard deviations (n=3).

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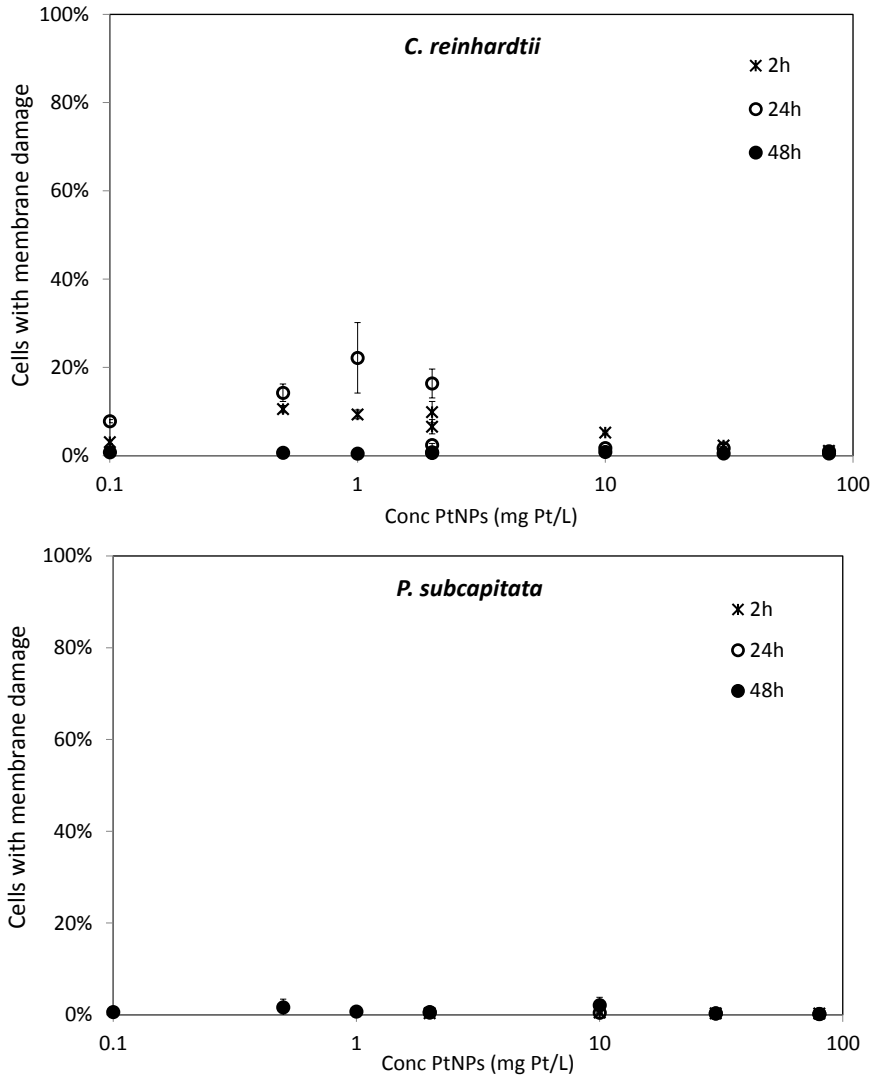
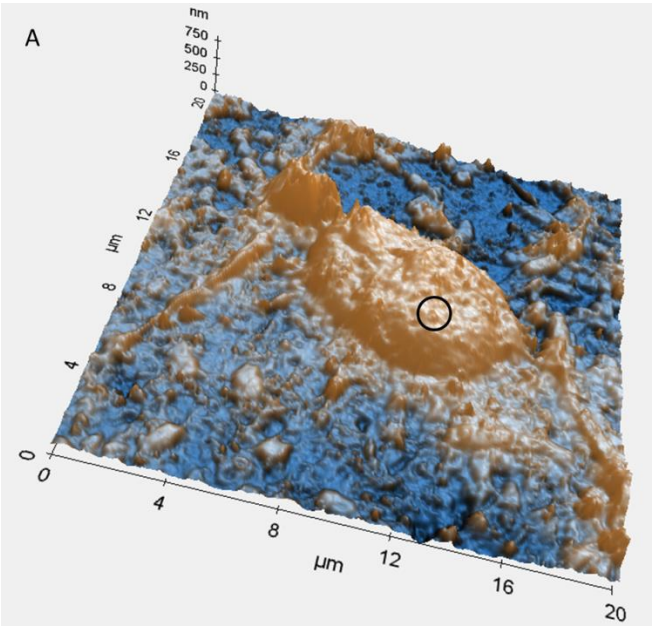
S8 Membrane damage in algal cells



200

201 **Figure S8.** Membrane damage in *C. reinhardtii* and *P. subcapitata* upon 2, 24 and 48 h exposure to PtNPs
202 in two parallel tests with low concentrations (0.1; 0.5; 1 and 2 mg Pt/L nominal) and high concentrations
203 (2, 10, 30 and 80 mg Pt/L), respectively. The error bars represent standard deviations (n=3).

S9 Atomic force microscopy images of algal cells



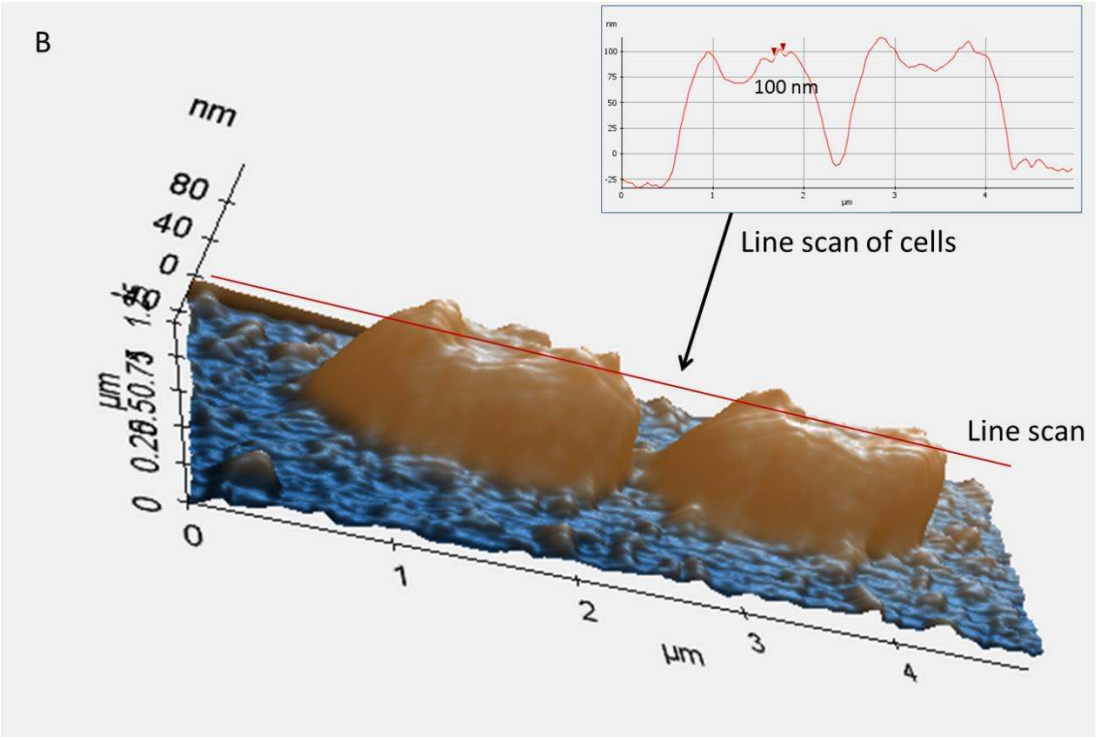


Figure S9. AFM visualizations of algal cells after 48 h incubation with PtNPs showing a single cell of *C. reinhardtii* (A) and two cells of *P. subcapitata* (B). The circle shown on the image of *C. reinhardtii* (A) shows the likely presence of PtNP agglomerates. According to the line scan completed for *P. subcapitata* (B), structures of app. 100 nm are identified on the cell surface, which may likely be PtNPs agglomerates.