Algal bioaccumulation and toxicity of platinum are increased in the presence of humic acids

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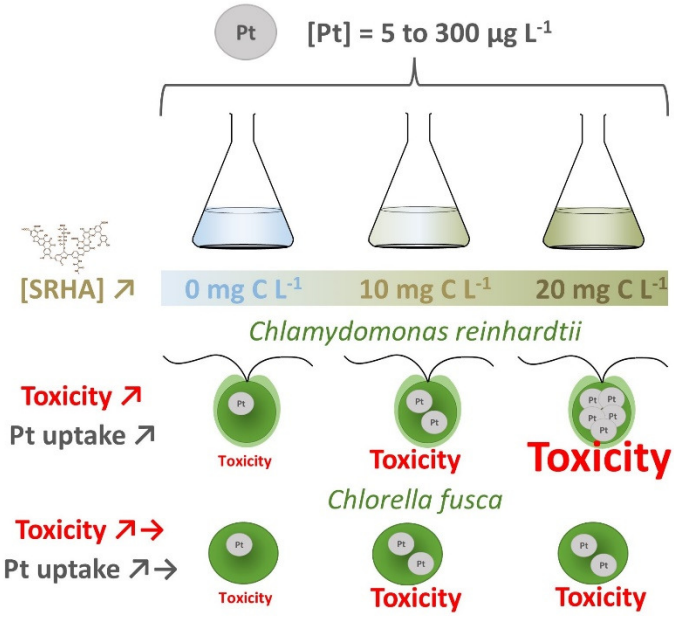
Graphical abstract



Author Contribution statement

Océane Hourtané: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. **Geneviève Rioux:** Formal analysis, Investigation, Methodology, Writing – original draft. **Peter G.C. Campbell:** Conceptualization, Supervision, Writing – review & editing. **Claude Fortin:** Conceptualization, Formal Analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Visualization, Writing – review & editing.

Environmental context

The growth in demand for platinum has led to an increase in the presence of this metal in the environment but little is known about its toxicity to aquatic organisms. The presence of organic matter should contribute to decrease metal bioavailability but the opposite was found for platinum. How ubiquitous natural organic matter can alter the accumulation and effects of platinum group elements remains to be fully elucidated.

ABSTRACT

Rationale: There is a growing interest for platinum in ecotoxicology, mainly because of its use in automobile exhaust catalysts. When it reaches aquatic ecosystems, platinum can interact with ligands such as natural organic matter. According to the Biotic Ligand Model, the formation of such complexes should reduce metal bioavailability. As a consequence, toxicity should decrease in the presence of organic matter. Methodology: This study focused on the uptake of platinum by two microalgae species (*Chlorella fusca* and *Chlamydomonas reinhardtii*) and its subsequent inhibitory effects on growth (96 h). Cells were exposed to platinum (5 to 300 µg L^{-1}) at three concentrations (0, 10 and 20 mg C L^{-1}) of standard Suwannee River Humic Acid. Platinum bound to humic acid was determined experimentally using partial ultrafiltration to relate metal uptake and toxicity to speciation. Results: Unexpectedly, results show that platinum toxicity, expressed as ultrafiltrable Pt (not bound to humic acid) and total Pt concentrations, is enhanced in the presence of humic acid for both algae. For *C. fusca*, the EC_{50} values decreased from 93 to 37 and 35 µg L^{-1} of ultrafiltrable Pt in the presence of respectively 0, 10 and 20 mg C L^{-1} and from 89 to 36 and 0.31 µg L^{-1} for *C. reinhardtii*. Discussion: In contradiction with the Biotic Ligand Model, the results show that the presence of SRHA can significantly and importantly increase platinum uptake and toxicity as determined in two unicellular green algae, *C. reinhardtii* and *C. fusca*. The present work raises the issue of the impact of platinum on microalgae under realistic environmental conditions (ubiquitous presence of organic matter), primary producers being of great ecological importance.

INTRODUCTION

Since the late 50's, there has been an important increase in demand for platinum group elements (PGEs). These rare metals are of prime importance as they have valuable characteristics, including catalytic properties and resistance to oxidation even at very high temperatures (Heck *et al.* 2009). As a consequence, PGEs are used in a variety of industrial fields, which has led to an increase in their overall geochemical mobility and a growing concern about the impacts of these elements on ecosystems (Zereini and Wiseman 2015). Automobile catalytic converters correspond to the most important demand for PGEs and are the main source of emissions to the environment. These devices allow a reduction of the emissions of atmospheric pollutants in exhaust fumes. The presence of platinum and palladium favors the oxidation of carbon monoxide and non-combusted hydrocarbons, and rhodium allows the reduction of nitrogen oxides. Due to mechanical abrasion of the catalysts during their use, platinum is progressively released into the environment (Zereini and Wiseman 2015). The total amount lost during the lifetime of a catalytic converter is estimated to be less than 5% (Hagelüken *et al.* 2005), which is coherent with engine test bench experiments conducted by Artelt *et al.* (2000) with emissions ranging between 9 and 124 ng Pt km^{-1} as determined for medium-powered gasoline engines. Following its release within exhaust fumes, Pt can reach aquatic environments and be found in fresh waters and sediments (Rauch and Peucker-Ehrenbrink 2015). Hospital effluents are also a non-negligible source of release into aquatic systems because of the use of platinum complexes in cancer treatments (Kümmerer and Helmers 1997). For example, concentrations of up to 35 µg Pt L^{-1} and 150 µg Pt L^{-1} have been measured respectively in river waters (Odiyo *et al.* 2006) and hospital effluents (Lenz *et al.* 2005), whereas it is usually below analytical detection limits in pristine environments.

Once present in aquatic environments, metals can interact with compounds naturally present in surface waters such as natural organic matter (NOM). This NOM is a heterogeneous mixture of macromolecules originating from the decomposition of living organisms, mostly plants. Its soluble fraction is mainly composed of humic (HA) and fulvic acids (FA), which are operationally differentiated on the basis of their solubility (humic acids tend to aggregate at low pH). HA are generally larger in size (≥ 2000 Da) and contain more aromatic constituents than FA, which have a lower molecular weight (800 to 2000 Da) and are more aliphatic. Measured NOM concentrations in rivers and lakes typically range from 0.5 to 30 mg C L^{-1} (Thurman 1985). In aquatic environments, NOM can influence the mobility, partitioning and toxicity of numerous solutes, especially metals (Tipping 2002). The current paradigm in metal ecotoxicology is that binding of metals by ligands (including NOM) reduces their bioavailability to living organisms. This is the underlying premise of the Biotic Ligand Model (BLM) (Mebane *et al.* 2020). A large body of evidence has shown that metal uptake and toxicity depend on the free ion concentration in the exposure medium, other factors being constant (e.g., pH, hardness). However, some studies of metal bioavailability in the presence of NOM show contradictory results. The examples presented in Table 1 are apparent failures of the BLM with unicellular algae, where the algal response could not be predicted by free metal ion concentrations in the presence of NOM.

This variation in the effects of NOM on metal bioavailability is potentially a consequence of its heterogeneous nature. For instance, the molecular weight, elemental composition and functionalization of NOM depend greatly on its origin (Mueller *et al.* 2012). The modification of metal toxicity could therefore be variable depending on NOM composition and source, as shown for Cu by Macoustra *et al.* (2019). In the case of platinum, it is known that the presence of HA and FA changes its mobility and bioavailability (Dubiella-Jackowska *et al.* 2009). Furthermore,

contradictory results concerning the effect of NOM on Pt toxicity are documented in the literature. Diehl and Gagnon (2007) reported an increase in Pt bioaccumulation for the aquatic plant *Elodea canadensis* with the addition of humic acid whereas a decrease was observed for the terrestrial plant *Peltandra virginica*. Rauch *et al.* (2004) showed a decreased uptake of platinum by a natural biofilm in stream water containing NOM. However, work on zebra mussels demonstrated increased bioaccumulation of Pt in the presence of NOM (Sures and Zimmermann 2007). In order to better understand the role of complexation in platinum toxicity, determination of its speciation in the presence of NOM is fundamental. However, in the case of platinum, we are faced with a lack of knowledge and contradictory published data on thermodynamic constants, which lead to difficulties in predicting platinum speciation in the presence of inorganic and organic ligands through thermodynamic modelling (Azaroual *et al.* 2001, Azaroual *et al.* 2003, Byrne 2003, Colombo *et al.* 2008). As a consequence, the ability of the BLM to predict platinum toxicity in the presence of NOM is compromised.

In this work, we used ultrafiltration to determine the partitioning of Pt in the presence of NOM (Guo and Santschi 2006) and to test the influence of NOM complexation on Pt uptake and toxicity using two unicellular green algal species. We hypothesized that Pt complexation by HA would decrease its bioavailability as predicted by the BLM and that its uptake and toxicity could be predicted by the concentration of Pt that is not bound to HA. The aims of this project were 1) to determine the extent of Pt binding to SRHA in exposure media using a partial ultrafiltration method, and, 2) to compare platinum accumulation and toxicity for two algal species at different SRHA concentrations.

EXPERIMENTAL

Labware and solutions

Algal exposures were carried out in 250 mL polycarbonate (PC) Erlenmeyer flasks to minimize platinum adsorption on the container walls. For the same reason, polysulfone filtration units and PC filter membranes were used unless mentioned otherwise. All solutions were prepared with ultrapure (milliQ) water (≥ 18 MΩ cm). All labware used was washed with deionized water, then soaked in a 10% (volume/volume) nitric acid solution for 24 hours and rinsed with deionized water three times and with ultrapure water five times. When needed, labware was sterilized at 121 °C for 20 minutes in an autoclave. Platinum was added to the exposure media from a 1000 µg mL^{-1} stock solution (10% HCl; Plasma CAL, SCP Science) originally prepared from $(NH_4)_2PtCl_6$ salts. We hypothesized that equilibrium was reached within the 72-h equilibration period respected before the beginning of exposure experiments. The addition NH_4^+ from the stock solution was considered negligible in comparison to the background NH_4^+ concentration in the growth medium used (2 to 3 orders of magnitude difference). Similarly, the addition of Cl^- should not modify Pt inorganic speciation which was predicted to be > 99.99% in the form of $Pt(OH)_2$ in our exposure medium, based on MINEQL calculations (Schecher and McAvoy 2001). Also, according to the work of Colombo *et al.* (2008), under our experimental conditions, Pt should be mainly present under the form $Pt^{(II)}(OH)_2$.

Suwannee River humic acid (SRHA) was purchased as a dry powder from the *International Humic Substances Society* (IHSS): catalogue numbers 2S101H and 3S101H. Using the protocol described in Leguay *et al.* (2016), the powder was dissolved in a 10^{-2} M NaOH (Fisher) solution in an opaque container and agitated for 24 h and then filtered through a polyethersulfone (PES) membrane (0.45 µm porosity; Pall Corporation) and stored at 4°C in the dark. Dissolved organic carbon

concentrations of SRHA stock solutions were determined analytically (Shimadzu VCPH Total Organic Carbon Analyzer).

Algal cultures

Algal strains were obtained from the *Canadian Phycological Culture Centre* (CPCC) at the University of Waterloo (ON, Canada). Axenic cultures of *Chlamydomonas reinhardtii* (CPCC#11) and *Chlorella fusca* (CPCC#89) were maintained in the same growth medium: MHSM-1 (Modified High Salt Medium), modified from Macfie *et al.* (1994) and adjusted to pH = 6.00 ± 0.05 : $MgSO_4 \cdot 7\ H_2O$ (20.0 mg L^{-1}), $Ca(NO_3)_2 \cdot 4\ H_2O$ (161 mg L^{-1}), NH_4NO_3 (75 mg L^{-1}), KH_2PO_4 (7.4 mg L^{-1}), K_2HPO_4 (14.4 mg L^{-1}), KNO_3 (404 mg L^{-1}), H_3BO_3 (186 µg L^{-1}), $MnCl_2 \cdot 4\ H_2O$ (415 µg L^{-1}), $FeCl_3 \cdot 6\ H_2O$ (160 µg L^{-1}), $Na_2EDTA \cdot 2\ H_2O$ (300 µg L^{-1}), Zn (1.59 µg L^{-1}) , Co (0.64 µg L^{-1}), Mo (2.88 µg L^{-1}), Cu (4.47 ng L^{-1}). A 2-(N-morpholino)ethanesulfonic acid (MES) buffer was used (10^{-2} M) to ensure pH stability. The cultures were always manipulated under sterile conditions (autoclave, laminar flow hood and Bunsen burner) and grown in an incubation chamber (Conviron; CMP 4030) at 20.0 °C ± 0.5 with a measured continuous light of 73 ± 13 µE m^{-2} s^{-1} and with gentle agitation (50 to 100 rpm). Continuous light allowed for smooth growth over 96 h and for asynchronous cellular division.

Exposure conditions

Exposure experiments were carried out in triplicate with a volume of 100 mL each, over 96 h. Each experiment included a negative control for reference and between 5 and 7 Pt concentrations. For each of the experiments with SRHA, the controls contained no Pt, but the same SRHA concentrations as the other conditions. Cell growth was compared between controls containing SRHA or not and was not significantly different. The exposure medium composition was the same as for the cultures except for the addition of platinum and SRHA. The range of platinum

concentrations tested was between 5 and 300 µg Pt L^{-1} to capture sufficient growth inhibition data to calculate effective concentration values (EC_x). This range includes maximum environmental measured concentration in rivers and effluents that are respectively 35 and 150 µg Pt L^{-1} (Lenz *et al.* 2005, Odiyo *et al.* 2006). Exposures were performed for three SRHA concentration conditions (0, 10 and 20 mg C L^{-1}). The pH of each exposure medium was adjusted to 6.00; this choice was made to reflect the slightly acidic pH usually observed in highly humic surface waters. The exposure media were left to equilibrate for 72 h in the dark prior to use. This equilibration period was designed to favor chemical equilibrium between Pt and dissolved ligands as well as with adsorption sites of the flask surface, thus minimizing potential changes in Pt speciation and concentration during the exposure period. At t = 0, exposure media were inoculated with cells in exponential growth phase at an initial cell density between 10,000 and 15,000 cells mL^{-1}. The pH of each exposure medium was measured at the beginning and the end of the exposure (0 and 96 h). Algal cell numbers were measured every 24 h for 96 h with a Coulter particle counter (Multisizer 3) using Isoton II solution (Beckman Coulter) and the final cell yield was used as the toxicity endpoint. For some exposure conditions, more than one experiment was carried out in order to determine the EC_x value. Since control exposures result in slightly different culture yields from one batch to another, the relative culture yield (average cell number/average control cell number) was used to establish dose-response curves. Propagation of error calculations were performed to integrate the uncertainty of all parameters. The Toxicity Relationship Analysis Program (TRAP) software (Erickson 2015) was used to determine EC_{50}, EC_{20} and EC_{10} values (the effective concentrations for which there was 50, 20 or 10% growth inhibition). Software parameters used were: non-linear regression analysis type; threshold sigmoid model shape; two parameters; logarithm as the exposure variable transform; and fixed Y_0=1.

Ultrafiltrable Pt concentrations were determined as described below at 0 and 96 h, respectively before adding and after removal of the algae by filtration. Total platinum concentrations and NOM concentrations were measured every 24 hours. At 24, 48 and 72 h algae were separated from the exposure medium by centrifugation. The SRHA concentrations were estimated by spectrofluorimetry the same day and subsamples for Pt quantification were stored in HCl (5%, volume/volume) before ICP-MS analysis (see the *Monitoring of exposure parameters* section for more detailed information). After 96 h of exposure, based on the measured cell density, a known number of algae was collected for each replication on a 2-µm porosity PC filter membrane (Millipore Sigma) and mineralized to determine the dose of internalized Pt. Before digestion the filters were rinsed with a solution containing 10^{-5} mol L^{-1} of EDTA (ethylenediaminetetraacetic acid) to remove the platinum adsorbed on the cell walls so that the actual intracellular platinum could be quantified (Hassler *et al.* 2004). For the digestion step, filters containing the exposed algae were dried (70°C, 48 h), after which 2.5 mL of concentrated hydrochloric acid HCl (37%, TraceMetal Grade; Fisher) were added. After 48 h, the tubes were filled to a total volume of 50 mL with ultrapure water and Pt was quantified in that final solution.

Determination of Pt speciation by partial ultrafiltration (PUF)

A partial ultrafiltration method was used in order to determine the concentration of Pt that was not bound to HA (ultrafiltrable Pt) and the concentration bound to HA (non-ultrafiltrable Pt). This PUF measurement was performed at the beginning and the end (96 h) of every exposure experiment in which SRHA was present. A volume of 20 mL of exposure media was added to a PC ultrafiltration tube (Vivaspin; 20 mL; Sartorius) fitted with a polyethersulfone (PES) membrane (3 kDa molecular cutoff) and centrifuged at 4,300 g for 8 minutes. The PES membranes were pre-conditioned to ensure that the glycerine protecting the membrane was not a source of carbon in the samples that

were analyzed afterwards. The preconditioning method was a 9-day-long alternation of soaking in 20 mL of ultrapure water and rinses with approximately 15 mL of ultrapure water passing through the membrane (in the order: 96 h soaking, 3 rinses; 48 h soaking, 4 rinses; 48 h soaking, 3 rinses the same day the ultrafiltration of the samples was performed). During and after conditioning, the membranes were maintained submerged in ultrapure water at all times to prevent any drying that could alter their efficiency. During sample centrifugation, substances with a molecular weight lower than 3 kDa such as free Pt ions, inorganic and small organic Pt complexes, can pass through the membrane (ultrafiltrate) whereas soluble or colloidal molecules with a molecular weight higher than 3 kDa, such as most HA-Pt complexes, remain in the upper part of the tube (retentate). The partial ultrafiltration (PUF) method yields two solutions: the ultrafiltrate, a very small volume (about 2 mL) containing the operationally defined ultrafiltrable platinum, and the retentate, which corresponds to the exposure medium. The word "partial" in PUF refers to the partial recovery of the initial small volume to minimize changes in metal speciation during the course of the centrifugation. Indeed, as the solution volume in the upper part of the tube decreases, the HA concentration increases, potentially leading to greater metal complexation. We minimized this bias by collecting less than 15% of the initial volume. The determination of the proportion of total platinum concentration that is ultrafiltrable was determined by linear regressions using R software and t tests were performed in order to compare the results for the different SRHA conditions.

Monitoring of exposure parameters

In all samples, platinum was quantified by ICP-MS (Thermo instrument, Xseries2). The instrument was calibrated over a concentration range between 0.25 and 200 µg Pt L^{-1}, the detection limit was always below 0.04 µg L^{-1} and the average standard addition recovery was 83 ± 14 %. Between each measurement, a rinse solution composed of diluted aqua regia (5% volume) was injected. A Re/Rh internal standard was used to validate every run: a variation of less than ± 20% was accepted.

In addition, a series of blanks and quality controls (0.75 and 25 µg L^{-1}) prepared from S409 solution (C00-061-409, PlasmaCAL) was analyzed every 10 to 15 samples to correct for ICP-MS drift if needed (if above ± 5%). Platinum was measured in exposure samples, PUF samples (ultrafiltrate and retentate) in the presence of SRHA and in the digested samples (algae obtained after 96-hour exposures). Digestion efficiency was verified using a certified reference sample (IAEA-450, a unicellular microalga, *Scenedesmus obliquus*) and the platinum recovery was 80 ± 9% (n = 8). Before analysis, all samples were acidified with concentrated HCl to a final concentration of 5% (volume/volume) in polypropylene tubes.

Molecules of HA are known for their optical properties, and were quantified in exposure media sampled every 24 h as well as in the ultrafiltrate and retentate from partial ultrafiltration by a spectrofluorometric technique. Since a total organic carbon determination could not be performed for SRHA quantification due to the presence of other organic compounds in solution (e.g., the pH buffer, essential metal buffer and algal exudates), a spectrofluorimeter (Varian Cary Eclipse) was calibrated beforehand and used to estimate the SRHA concentration in mg C L^{-1}. Excitation and emission wavelengths were respectively 350 and 463 nm and the calibration range was between 0.25 and 25 mg C L^{-1}. Calibration solutions were prepared in MHSM-1 culture medium at pH = 6.0 with the SRHA stock solution. The total organic carbon concentration of the calibration solutions was confirmed (Shimadzu VCPH) prior to the addition of the MES (2-(N-morpholino)ethanesulfonic acid) buffer (10^{-2} mol L^{-1}).

Statistical analysis:

The data from the partitioning of Pt (ultrafiltrable platinum concentration as a function of the total platinum concentration) as well as from its bioaccumulation (platinum accumulated by the algal cells as a function of the ultrafiltrable Pt exposure concentrations) were analyzed by linear

regression with R software. For Pt speciation, the intercept was fixed at the origin and the slopes were expressed as the % of the total platinum that is ultrafiltrable ± error for the different conditions in the *Results and Discussion* section. The regressions were followed by t-tests on the slopes obtained in order to compare among the different SRHA concentrations tested. The residuals were not always normally distributed, a result that was attributed to the relatively small dataset.

RESULTS AND DISCUSSION

For all the experiments carried out, the total Pt concentration in the exposure media remained relatively stable over time. The variation in Pt exposure concentrations measured over time was always below 25% (sampling times = 0, 24, 48, 72 and 96 h). This variation dropped to less than 12% for platinum exposure concentrations of 25 µg Pt L^{-1} and above. At t_0, the measured SRHA concentrations as determined by fluorescence were close to the expected nominal values (Table 2).

Over the 96-h experiments, the pH was stable with variations less than the electrode precision (± 0.05 pH units at 25 °C). The efficiency of the partial ultrafiltration method was deemed satisfactory (Table 2) with good recovery of Pt (in the retentate and ultrafiltrate). Since the molecular cutoff of the ultrafiltration membrane is 3 kDa and SRHA is known to have an average molecular weight in this vicinity (for example, published values between 3.3 and 5.7 kDa have been reported (Beckett *et al.* 1987, Her *et al.* 2002, Guéguen and Cuss 2011)), a small fraction of the organic carbon could pass through the membrane and reach the ultrafiltrate. The measured SRHA concentrations in the ultrafiltrate samples were around 2 mg C L^{-1} for both SRHA concentrations tested (10 and 20 mg C L^{-1}; n = 3), which was deemed acceptable to distinguish

between Pt that is bound or not bound to HA, since the affinity of Pt for SRHA proved to be very low.

Platinum speciation

Figure 1 shows ultrafiltrable platinum concentrations in MHSM-1 medium determined experimentally by PUF. In the absence of SRHA (blue circles), 66 ± 1% of the total measured Pt was determined to be ultrafiltrable using a linear regression (R^2=0.995), suggesting the presence of particulate platinum. As speciation was not experimentally determined for the exposure experiments conducted without SRHA, ultrafiltrable Pt concentrations were calculated based on the slope of this regression . In the presence of SRHA (10 and 20 mg C L^{-1}), ultrafiltrable Pt concentrations were experimentally determined in the media of all experiments, at both the beginning (right before inoculation) and the end (96 h) of the exposure, after algae were removed by filtration. Complexation was very low at 10 mg C L^{-1} SRHA (all triangles), with a proportion of ultrafiltrable platinum of similar to that in the absence of SRHA (64 ± 1%; R^2=0.98), and not significantly different (p-value = 0.11). Note that it is difficult to distinguish between these two regressions in Figure 1 as they are very close to each other. However, the proportion of ultrafiltrable Pt decreased with an increase in SRHA concentration to 20 mg C L^{-1} (all squares), suggesting notable complexation. The proportion of ultrafiltrable platinum was 48 ± 1% (R^2=0.98) and significantly different from both the conditions without SRHA (p-value = 5×10^{-5}) and at 10 mg C L^{-1} (p-value = 3×10^{-5}). Little is known in the literature about Pt complexation by HA, as there is a knowledge gap, especially regarding thermodynamic constants for Pt. Nevertheless, these results show a relatively low affinity of Pt for SRHA under our conditions.The fact that ultrafiltrable Pt concentration can be expressed as a linear function of the total Pt concentration over the entire

range tested suggests that only one type of binding site is involved in Pt complexation and that no saturation of binding sites was reached.

Also, Figure 1 show that the physical speciation of Pt was stable over the 96-hour exposures. In order to check if there were significant changes in speciation during the exposure period, additional regressions (not shown in Figure 1) were performed on the t = 0 and t = 96 h datasets separately for each of the SRHA concentrations (10 and 20 mg C L^{-1}). Linear regressions indicate that 64 ± 1% of Pt was present in the ultrafiltrate for both 0 (yellow triangles; R^2=0.97) and 96 h (black triangles; R^2=0.98) exposure times at 10 mg C L^{-1} of SRHA whereas these percentages were 48 ± 1% for t=0 (red squares; R^2=0.97) and 49 ± 1% for t=96 h (black squares; R^2=0.99) at 20 mg C L^{-1} of SRHA for the entire range of total Pt concentrations tested. There was no significant difference between the regression slopes obtained for t=0 and t=96 h at 10 mg C L^{-1} of SRHA (p-value= 0.91) nor 20 mg C L^{-1} of SRHA (p-value= 0.78). There were, however, statistically significant differences in measured ultrafiltrable Pt concentrations between 0 and 96 h for certain conditions with total platinum concentrations ≤ 50 µg L^{-1}. In these cases, the ultrafiltrable platinum fraction was lower at 96 h, which suggests that equilibrium may not have been entirely achieved prior to the beginning of the experiment and that Pt complexation slightly increased after the 72-hour period of pre-equilibration, especially at the lowest total Pt concentrations tested. This increased complexation after 96 h of exposure could also be due to more organic macromolecules of algal provenance in the medium as exudates or cell debris.

Bioaccumulation

According to the BLM, intracellular accumulation depends on the binding of the metal cation to receptor sites (biotic ligands) and this binding is highly dependent on the free metal ion concentration. The addition of a ligand such as HA in an exposure medium results in metal

complexation, and thus decreases the free metal ion concentration. As a result, the bioaccumulation of platinum was expected to decrease in the presence of SRHA. Based on this premise, internalized platinum was plotted as a function of the ultrafiltrable platinum exposure concentrations. We assumed that all Pt was present as $Pt^{(II)}$ as suggested by thermodynamics. Based on this assumption, the proportion of free Pt^{2+} ion concentration with respect to the ultrafiltrable Pt concentration should remain constant in both the presence and absence of SRHA. When expressed as a function of ultrafiltrable Pt, similar bioaccumulation should be observed with and without the presence of SRHA, if the BLM applies.

The platinum accumulated by the algal cells is presented as a function of the ultrafiltrable Pt exposure concentrations for *C. reinhardtii* and *C. fusca* in Figure 2. For both algae, the bioaccumulation data were relatively coherent with toxicity observations (see *Toxicity* section below). With increasing SRHA concentrations, there was an increase in intracellular metal, which led to an increased toxicity for *C. reinhardtii*. A large 2-order of magnitude difference was observed between uptake data at 0 and 20 mg C L^{-1} SRHA whereas the uptake data at 10 mg C L^{-1} overlapped with those for the other two conditions: with the 0 mg C L^{-1} data at low and with the 20 mg C L^{-1} data at high Pt exposure concentrations. The slopes of the linear regressions were significantly different from each other for all SRHA conditions For *C. fusca*, the difference in Pt bioaccumulation is more subtle. There seems to be a slight increase of platinum bioaccumulation with SRHA compared to no SRHA, but the internalized concentrations were very similar. Linear regressions were performed using R software, followed by t-tests on the slope. Results showed that the slopes were significantly different between the experiment without SRHA and those with 10 or 20 mg C L^{-1} SRHA (p-values 0.039 and 0.034 respectively), whereas there was no significant difference between the two SRHA conditions (p-values 0.47). Note that a similar uptake of Pt for

the exposures with 10 and 20 mg C L^{-1} of SRHA is coherent with the similar EC_x values obtained for these two conditions (see below).

Intracellular platinum concentrations seem to reach a maximum that is different for both algal species: 2×10^{-7} µg Pt $cell^{-1}$ for *C. reinhardtii* and 2×10^{-8} µg Pt $cell^{-1}$ for *C. fusca*, i.e., an order of magnitude higher for *C. reinhardtii*. This result suggests higher net metal uptake rates for *C. reinhardtii* than for *C. fusca*, these two species being of similar size and shape.

There are a few other examples of increased bioaccumulation of Pt in the presence of humic substances: the results of *Sures and Zimmermann (2007)* on zebra mussels, as well as those of Diehl and Gagnon (2007) on the aquatic plant *Elodea canadensis*. However, in this latter reference, the authors also determined that Pt bioaccumulation was decreased in the presence of HA in a terrestrial plant. Both these examples show bioaccumulation results. In the present study on unicellular algae, toxicity was also determined.

Toxicity

Toxicity was determined based on final algal yield (cell numbers after 96 h) relative to the control, using the calculated (0 mg C L^{-1}; calculation based on measured total Pt concentrations) and measured (10 and 20 mg C L^{-1}) ultrafiltrable Pt concentrations to determine the 96 h-EC_x values. For both algae, data were relatively coherent with the bioaccumulation observations. Figure 3 shows the dose-response curves and Table 3 the effective concentration values (EC_{50}, EC_{20} and EC_{10}) for the two green algae studied under different SRHA concentration exposure conditions. These EC_x values are expressed as µg L^{-1} of ultrafiltrable Pt or total measured Pt.

For *C. reinhardtii*, the EC_x values based on ultrafiltrable Pt decreased in the following order: EC_x (No HA) > EC_x (10 mg C L^{-1}) > EC_x (20 mg C L^{-1}). The EC_{50} and the corresponding 95%

confidence level intervals ($CL_{95\%}$) intervals are respectively 89 [66-120], 36 [32-41] and 0.31 [0.07-1.41] µg L^{-1} for 0, 10 and 20 mg C L^{-1} of SRHA. This shows that the toxicity is increasing with SRHA concentrations, suggesting that the BLM cannot be applied for platinum under these conditions. For *C. fusca*, the toxicity is also greater in presence of SRHA: EC_x (No HA) ≥ EC_x (10 mg C L^{-1}) ~ EC_x (20 mg C L^{-1}). The EC_{50} values are respectively 93 [78-110], 37 [26-52] and 35 [17-70] µg L^{-1} for 0, 10 and 20 mg C L^{-1} of SRHA. For 10 mg C L^{-1} and 20 mg C L^{-1}, the ECx values are relatively close and the $CL_{95\%}$ intervals largely overlap.

The EC_x values expressed as total platinum concentration are also lower in the presence of SRHA for both algal species, which demonstrates that toxicity is greater than predicted even on the basis of total Pt concentrations. For *C. reinhardtii*, the EC_x values decreased following the same sequence as for ultrafiltrable Pt: EC_x (No HA) ≥ EC_x (10 mg C L^{-1}) > EC_x (20 mg C L^{-1}). However, for *C. fusca*, the 95% confidence level intervals largely overlap, except for the EC_{50} values at 0 mg C L^{-1} and 10 mg C L^{-1} of SRHA , indicating no decrease in Pt toxicity despite the addition of SRHA leading to Pt complexation.

Internal dose – response

The relative cell yield after 96 h for both species is plotted in Figure 4 as a function of the platinum accumulated by the algal cells. As EC_x values based on internal dose are close, with 95% confidence level intervals overlapping (Table 4), we conclude that these two algae species have a similar sensitivity to platinum.

The internal dose-response curves could be modelled as sigmoidal functions over the entire range of SRHA concentrations tested for both algal species, suggesting that the toxicity determined under all the exposure conditions is a consequence of the metal accumulation in the cells. It can be concluded that the increased toxicity observed in the presence of SRHA is a consequence of an

enhancement of Pt internalization. Despite the increase in Pt complexation by HA (decrease of ultrafiltrable Pt concentration), there is an increase in both bioaccumulation and toxicity, even on the basis of the total Pt exposure concentration. Not only is this observation in contradiction with the BLM, it also suggests that the observed changes in bioaccumulation and toxicity do not depend on changes in Pt complexation, as we originally postulated. Note that at 10 mg C L^{-1} of SRHA where there was no significant complexation of Pt by SRHA, yet there was a significant increase in toxicity compared to 0 mg C L^{-1} of SRHA for both algal species. This suggest that Pt enhanced accumulation and toxicity are likely due to other factors, such as changes in cell physiology. For example, the interaction of HA with cell membranes could facilitate Pt internalization. Different hypotheses are formulated in the following section.

Permeability change due to HA adsorption on cell membrane. Different responses were observed between the two algae. Internalization of platinum in *C. reinhardtii* increased with SRHA concentrations whereas it did not for *C. fusca* above 10 mg C L^{-1} of SRHA. It is known that HA can adsorb to cell walls and accumulate around the algae (Campbell *et al.* 1997). It is possible that there is a saturation of this adsorption at or below 10 mg C L^{-1} for *C. fusca* and above 20 mg C L^{-1} for *C. reinhardtii*. As a consequence, the quantity of HA surrounding *C. fusca* cells would be the same for concentrations of 10 and 20 mg C L^{-1} of SRHA in solution. These different saturation levels might well reflect the cell wall composition of the two algal species. For *C. fusca* the cell wall is largely composed of carbohydrates (≈ 80%), most of it being mannose and glucose in a 2.7:1 ratio (Loos and Meindl 1982, Takeda 1991) whereas for *C. reinhardtii* it is devoid of carbohydrate polymers and composed mainly of glycoproteins, the majority of which are enriched in the amino acid trans-4-hydroxyproline (Roberts 1974, Adair and Snell 1990). We speculate that the glycoproteins could allow more H-bonding or hydrophobic interactions with HA and possibly

have more binding sites; this could explain why the saturation level of HA adsorption occurs at higher concentrations for *C. reinhardtii* than *C. fusca*.

There are several ways in which HA adsorption could lead to a greater uptake of Pt. Humic substances are amphiphilic compounds and are able to change membrane permeability (Vigneault *et al.* 2000). For instance, Boullemant *et al.* (2011) reported an increased algal uptake of lipophilic Cd complexes in the presence of SRHA. This surfactant-like interaction with the cell membrane might also alter the fluidity of the membrane and affect the metal transporters embedded in the membrane. The mechanisms involved have not been identified, but the increase in Pt uptake caused by adsorbed HA would be coherent with toxicity and accumulation trends observed for both algal species (Figures 2 and 3).

The formation of ternary complexes at the algal surface, as has been suggested for Pb with the alga *Chlorella kesslerii* (Lamelas *et al.* 2005), does not seem to explain our results, as uptake is not proportional to the SRHA-Pt complex concentration. Moreover, the toxicity response is a function of the intracellular Pt, which suggests that the increased uptake is not due to stronger (non EDTA desorbable) metal adsorption as was suggested for Pb by Lamelas *et al.* (2005).

Platinum is internalized via Cu transporters and Cu complexation by HA favors Pt uptake. Huang *et al.* (2014) showed that gene Ctr2, responsible of regulating Cu^{+} uptake, played a role in the sensitivity of mammalian cells to cisplatin. Another hypothesis would thus be that algal cells accumulate platinum through membrane-bound copper transporters. Internalization of platinum would be favored in the presence of HA, since Cu bioavailability would be lower. Indeed, based on WHAM VII calculations, free Cu^{2+} concentrations in the synthetic growing media were greatly reduced due to complexation with SRHA. Compared to the control media without SRHA, the concentration of free Cu^{2+} decreased by factors of 19 ± 5 and 61 ± 20 respectively for all our

experimental exposure media containing 10 or 20 mg C L^{-1} of SRHA. Copper being an essential metal, a depletion of this element could lead to an increased number of Cu transporters through regulating feedback mechanisms (Blaby-Haas and Merchant 2012, Kochoni *et al.* 2022). This effort of the cell to compensate for the decrease in Cu bioavailability could lead to an increase in platinum uptake (Lavoie *et al.* 2012).

The BLM is not applicable to this situation. The BLM relies on several premises that may not be respected under the studied conditions and thus, the model would be inapplicable in this situation. For example, if the BLM is to be applicable, the rate-limiting step in metal uptake must be the passage across the cell membrane. If the slow step in metal uptake were the diffusion of the metal from the bulk solution across the unstirred layer towards the algal surface, dissolved metal species other than the free metal ion could contribute to metal uptake. This phenomenon has been observed for other soft metals like silver (Fortin and Campbell 2000). In order to verify this hypothesis, the maximum diffusive Pt flux across the boundary layer needs to be calculated and compared to the maximum intracellular Pt uptake flux observed. The maximum diffusive flux was calculated as described by Fortin and Campbell (2000). To do so, we assumed that the Pt diffusion coefficient is half that of Ag (there are no published values for the Pt(II) diffusion coefficient in aqueous solution but Li and Gregory (1974) showed that the coefficient for a monovalent ion is approximately twice that of a divalent ion). The uptake flux was estimated by multiplying our 96-h exposure data by the cellular growth rate (Lavoie *et al.* 2014), estimated to be 1 division per day. The resulting calculated maximum diffusive flux of Pt was 2000 × higher than the observed Pt fluxes, suggesting that a Pt uptake limited by diffusion through the boundary layer is implausible.

Conclusions and perspectives for the BLM

According to the BLM, the complexation of a metal by an organic ligand (such as SRHA) should induce a protective effect that would result in reduced toxicity. However, results obtained in the present work show that the presence of SRHA can significantly and importantly increase platinum uptake and toxicity as determined in two unicellular green algae, *C. reinhardtii* and *C. fusca*. The most dramatic modification was observed with *C. reinhardtii*: EC_{50} values based on ultrafiltrable platinum decreased from to 89 ($CL_{95\%}$ = [66; 120]) µg L^{-1} in the absence of SRHA to 0.31 ($CL_{95\%}$ = [0.07; 1.41]) µg L^{-1} in the presence of 20 mg C L^{-1} of SRHA. Moreover, the toxicity also increased when expressed as the total platinum concentration. These observations raise important questions about the impact of NOM on platinum toxicity under realistic environmental conditions (where NOM is ubiquitous) for primary producers such as green algae. The present work underlines the importance of considering NOM when investigating the applicability of the BLM. Future work should focus on the mechanisms responsible for this Pt internalization enhancement in the presence of HA.

These results show an increase in toxicity of two orders of magnitude in the presence of environmentally realistic HA concentrations. In terms of risk assessment, the study of Pt toxicity based only on laboratory experiments performed in the absence of natural organic matter may be inadequate and could lead to an important underestimation of the ecological impact of the presence of Pt.

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Data availability statement

All raw data used to prepare figures are publicly available at

https://doi.org/10.5683/SP3/LWHVU9

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Table 1: Examples of BLM exceptions in the presence of natural organic matter for unicellular algae

Metal	**Ag**	**Al**	**Cd**	**Pb**
Exposure concentrations	$[Ag^+]$ up to 160 nmol L^{-1}	$[Al]_{mononuclear\ inorganic}$ = 6 µmol L^{-1}	$[Cd]_{tot}$ = 3.6 10^{-8} to 3.6 10^{-6} mol L^{-1}	[Pb]tot = 10^{-6} M ; $[Pb^{2+}]$ = 3 10^{-8} M
Exposure time	≤60 min	96 h	24 h	≤ 60 min
Exposure medium and pH	MHSM-E; pH = 5.5 and 7	AAP; pH = 5	Filtered lake water with addition of K_2HPO_4 (0.2 mg L^{-1}) and $NaNO_3$ (2 mg L^{-1}) or Fraquil	OECD; pH = 6
Type of organic ligand and concentration	SRHA (0, 5, 10 mg C L^{-1})	SFA (Soil Fulvic Acid) 1.2-11 mg C L^{-1}	NOM from the filtered lake water (18 to 25 mg C L^{-1})	SRHA, SRFA and polysaccharides (2.5 to 100 mg C L^{-1})
Speciation determination method	IET (ion exchange technique)	Cation exchange resin and PCV (automated pyrocatechol violet)	ISE (ion-selective electrode)	ISE (ion-selective electrode) and ultrafiltration (30 kDa)
Species	*Chlamydomonas reinhardtii* and *Pseudokirchneriella subcapitata*	*Chlorella fusca*	*Selenastrum capricornutum*	*Chlorella kesslerii*
Response	Apparent increase in uptake	Increased uptake	Reduction of ^{14}C uptake relative to the control	Increased uptake
References	Chen *et al.* (2013)	Parent *et al.* (1996)	Laegreid *et al.* (1983)	Lamelas *et al.* (2005), Lamelas *et al.* (2009) and Slaveykova *et al.* (2003)

Table 2: Concentrations of humic acid (HA) determined in ultrafiltrates at the beginning and end of exposures using fluorimetry, and average recoveries of platinum (in the retentate and ultrafiltrate) after partial ultrafiltration was performed. Values are means ± standard deviation; n = 3.

Nominal [SRHA] (mg C L^{-1})	10	20
Measured [HA] at t = 0 (mg C L^{-1})	9.8 ± 1.6	18.5 ± 3.1
[HA] detected in ultrafiltrates at t = 0 (mg C L^{-1})	2.0 ± 0.9	2.0 ± 0.8
[HA] detected in ultrafiltrates at t = 96 h (mg C L^{-1})	2.5 ± 1.2	2.5 ± 0.8
Recovery of total Pt (%)	97.5 ± 3.1	99.8 ± 2.5

Table 3: Effective ultrafiltrable and total Pt concentrations (EC_{50}, EC_{20} and EC_{10}) calculated with TRAP (Erickson 2015) based on growth inhibition after a 96-h exposure for *C. fusca* and *C. reinhardtii* for three SRHA concentration conditions in MHSM-1 medium; pH = 6.00 ± 0.05; n = 3.

Ultrafiltrable [Pt] (µg L^{-1})

Effective concentrations and 95% confidence intervals (µg L^{-1})

Alga	*Chlamydomonas reinhardtii*			*Chlorella fusca*		
[SRHA] (mg C L^{-1})	0	10	20	0	10	20
EC_{10}	35 [21-60]	15 [11-20]	0.38 [0.01-0.15]	34 [21-54]	10 [5-21]	6 [1-37]
EC_{20}	50 [37-67]	21 [17-26]	0.08 [0.02-0.32]	49 [35-69]	16 [10-28]	12 [3-41]
EC_{50}	89 [66-120]	36 [32-41]	0.31 [0.07-1.41]	93 [78-110]	37 [26-52]	35 [17-70]
Relative slope S at x(EC50)	3.2	2.7	0.3	3.3	1.5	0.9

Total [Pt] (µg L^{-1})

Effective concentrations and 95% confidence intervals (µg L^{-1})

Alga	*Chlamydomonas reinhardtii*			*Chlorella fusca*		
[SRHA] (mg C L^{-1})	0	10	20	0	10	20
EC_{10}	53 [31-92]	23 [17-32]	0.15 [0.01-4.41]	51 [32-82]	20 [10-39]	15 [3-84]
EC_{20}	75 [55-102]	32 [26-40]	0.66 [0.06-7.61]	74 [33-104]	31 [19-51]	27 [8-95]
EC_{50}	135 [100-183]	56 [50-64]	2.46 [0.48-12.67]	140 [119-167]	67 [47-93]	79 [39-160]
Relative slope S at x(EC50)	3.2	3.2	0.6	2.3	1.8	0.9

Table 4: Effective internal Pt concentrations (EC50, EC20 and EC10) calculated with TRAP (Erickson 2015) based on growth inhibition after a 96-h exposure for *C. fusca* and *C. reinhardtii* in MHSM-1 medium all SRHA concentration conditions combined; pH = 6.00 ± 0.05; n = 3.

intracellular Pt (µg cell^{-1}) 10^{-9}

Effective concentrations: EC (%)

Alga	*Chlamydomonas reinhardtii*	*Chlorella fusca*
EC_{10}	0.9 [0.6-1.2]	1.4 [0.7-2.9]
EC_{20}	1.7 [0.1-2.1]	2.6 [1.7-4.4]
EC_{50}	5.2 [4.3-6.1]	7.8 [6.1-1.0]
Relative slope S at x(EC50)	0.7	2.2

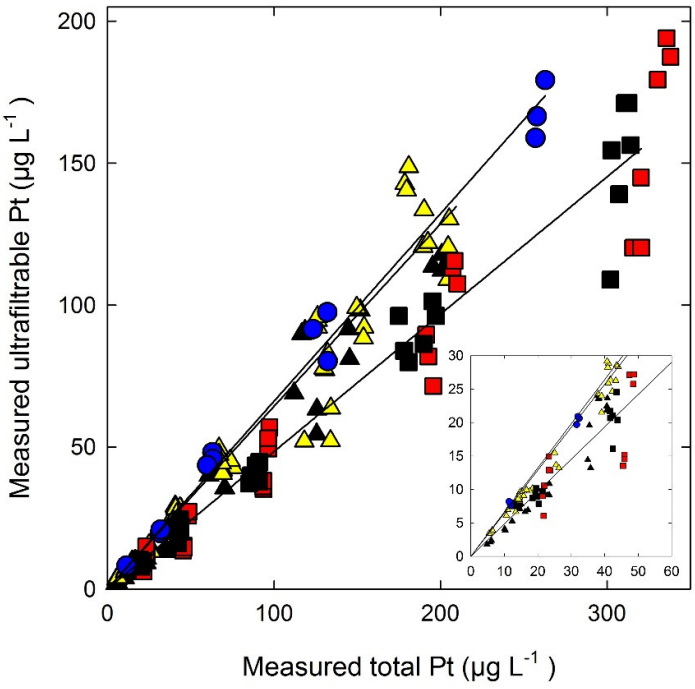

Figure 1: Measured ultrafiltrable platinum concentrations as a function of total measured platinum concentrations in MHSM-1 medium for 0 (blue circles) 10 (triangles) and 20 (squares) mg C L^{-1} SRHA concentrations at the beginning (t = 0; coloured symbols) and end of exposure (t = 96 h; black symbols). Lines are linear regressions through each SRHA concentrations tested.

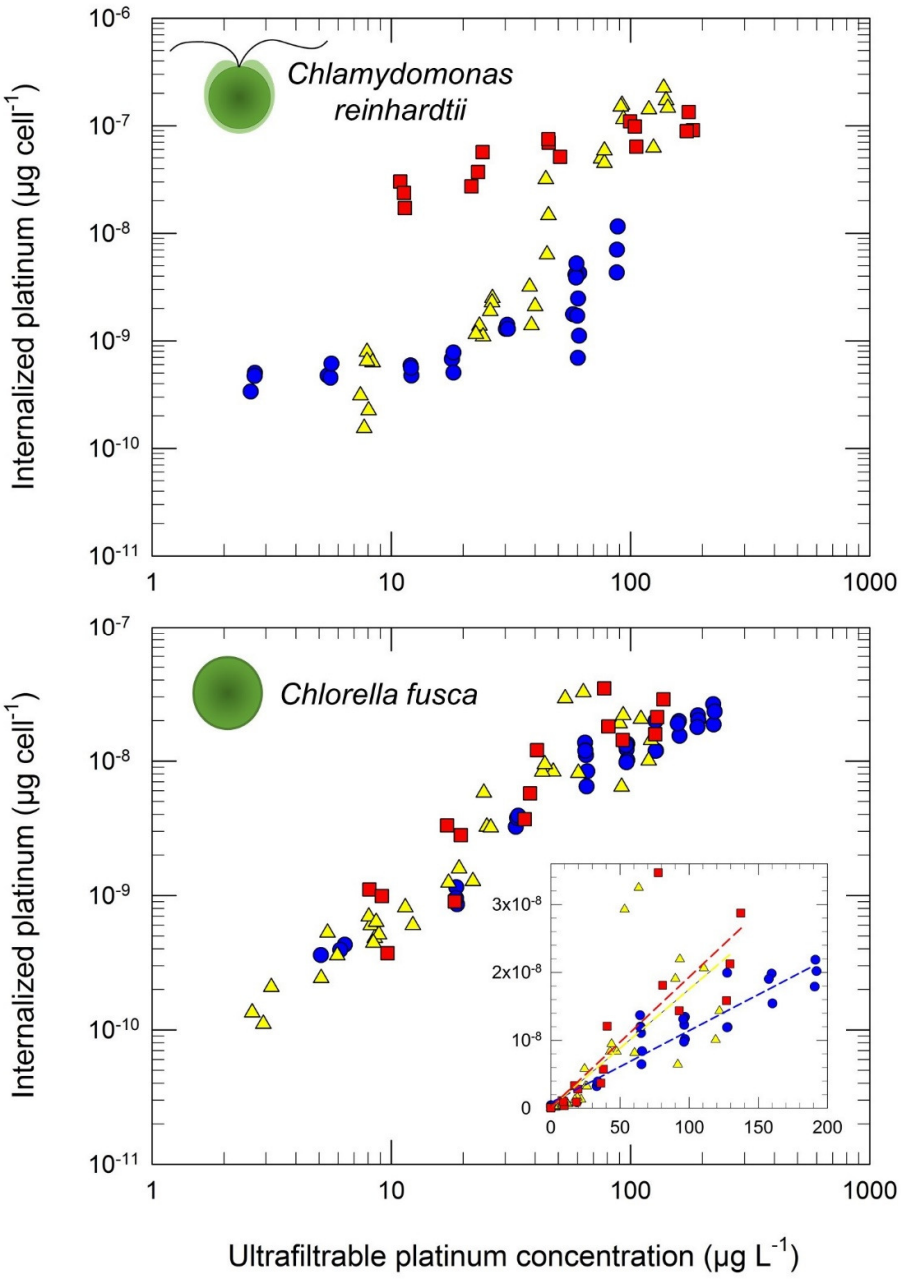

Figure 2: Platinum quantity internalized by the two green algae species: *C. reinhardtii* (top) and *C. fusca* (bottom) after a 96-h exposure. Three different SRHA concentrations were tested: No SRHA (blue circles); 10 mg C L^{-1} (yellow triangles); 20 mg C L^{-1} (red squares); pH = 6.00 ± 0.05; error bars represent standard deviations around the mean (n = 3). Insert shows data at Pt concentrations below 150 µg L^{-1} and regressions on a linear scale.

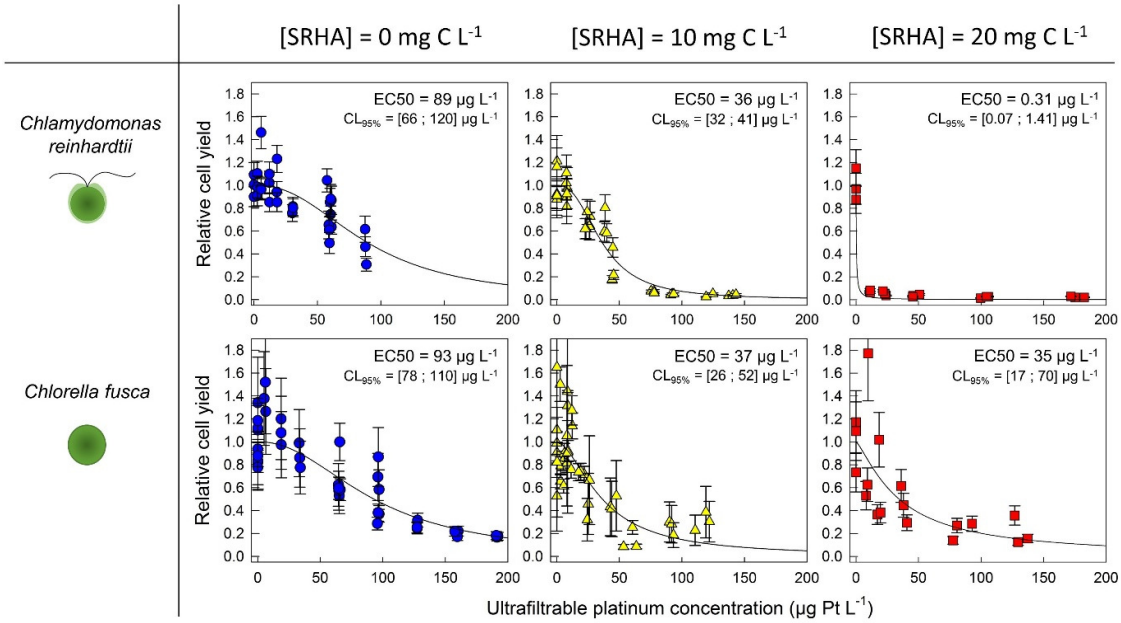

Figure 3: Cell yield after 96 h relative to control as a function of ultrafiltrable platinum for *C. reinhardtii* (upper panels) and *C. fusca* (lower panels) for three different SRHA treatments: 0 (left; blue circles); 10 (middle; yellow triangles); and 20 mg C L^{-1} (right; red squares). Associated EC50 values as well as corresponding 95% confidence intervals are provided in top right corner of every figure; pH = 6.00 ± 0.05; error bars represent standard deviations around the mean (n = 3).

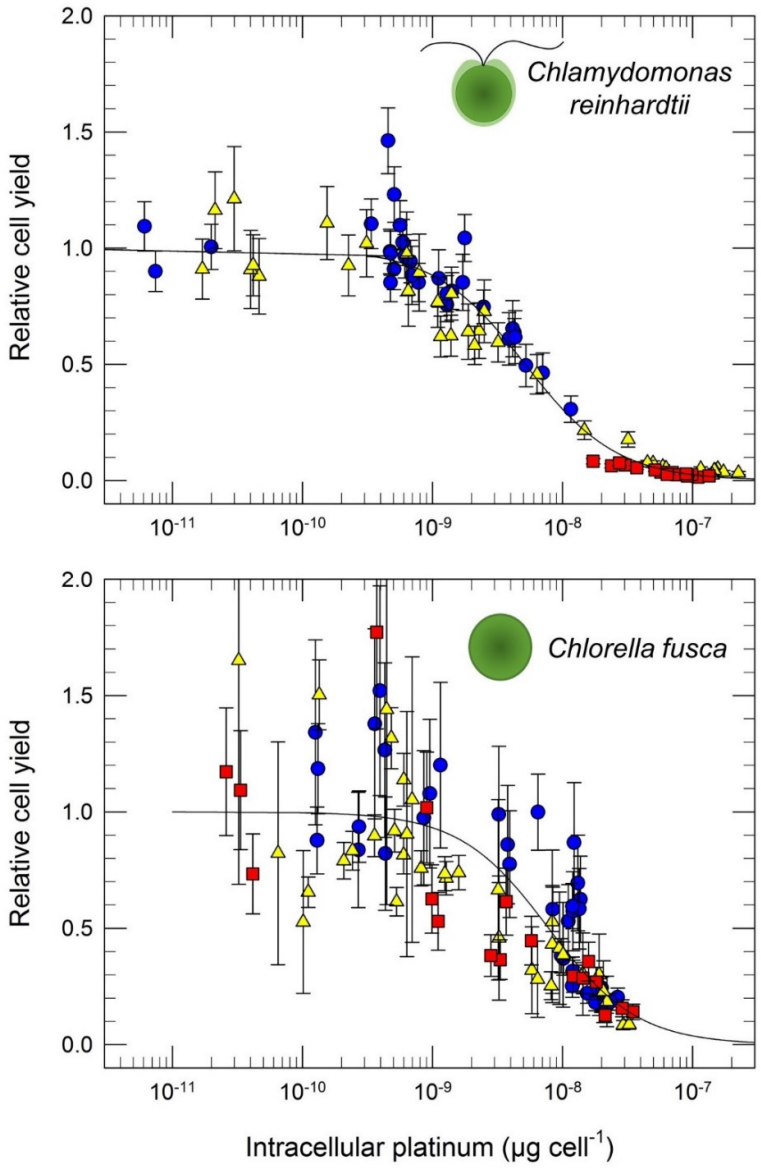

Figure 4: Cell yield after 96 h relative to control as a function of platinum internalized dose by the two green algae species: *C. reinhardtii* (top) and *C. fusca* (bottom) at three different SRHA concentrations: 0 mg C L^{-1} (blue circles), 10 mg C L^{-1} (yellow triangles) and 20 mg C L^{-1} (red squares); associated internal EC50 values with their corresponding 95% confidence intervals are 5.2 [4.3-6.1] × 10^{-9} µg $cell^{-1}$ for *C. reinhardtii* and 7.8 [6.1-10.1] × 10^{-9} µg $cell^{-1}$ for *C. fusca* ; pH = 6.00 ± 0.05; error bars represent standard deviations around the mean (n = 3).