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Implications of kinetically-hindered metals in ecotoxicological studies: Effect of platinum spike aging on its toxicity to *Dunaliella salina*

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ABSTRACT

Platinum (Pt) is considered an emerging environmental micro-contaminant due to its increasing use in anthropogenic activities during the past decades. However, there are still important gaps in the understanding of its biogeochemical behavior in the aquatic environment – e.g. its speciation, reactivity and fate – mainly as a result of the analytical challenge of the determination of its typical ultra-trace environmental concentrations. Also, Pt is a kinetically-hindered metal displaying slow reaction kinetics, which has important implications regarding ecotoxicological studies. That is, investigation of its toxicity under laboratory-controlled conditions may therefore require ensuring that equilibrium speciation conditions are reached before starting the experiments. In order to shed further light on this issue, in this study we have monitored the speciation changes during aging of the Pt(IV) spikes in controlled media (seawater) using an UV-Vis spectrophotometry. Platinum toxicity to the green microalgae *Dunaliella salina* was then compared, using standardized tests, with fresh and aged Pt(IV) spikes at the mg L^{-1} concentration range. Following 96-hour exposure, ecotoxicological assays consisting in spectrometric measurements of chlorophyll-a concentrations and Effective Concentrations (EC) of Pt resulting in the inhibition of 10% and 50% of algae growth rate were calculated (EC_{10} and EC_{50}, respectively). Daily monitoring of Pt speciation reflected the transition from $PtCl_6^{2-}$ (spike) to hydrolyzed species, probably in the form $[PtCl_{3-n}(OH)_{3+n}]^{2-}$, n = 0–3. Exposure experiments showed that after a short period of aging (10 days), Pt(IV) toxicity increased one order of magnitude compared to freshly spiked media. These results confirm the relevance of considering spike aging to ensure that speciation equilibrium conditions are attained in order to produce environmental realistic eco-toxicological data.

1. Introduction

Platinum (Pt) belongs to the Platinum Group Elements (PGE), which are used in a wide variety of emerging anthropogenic applications. Accordingly, they are considered as Technology Critical Elements (TCE) and, currently, mining activities to address the anthropogenic demand of Pt (and the other PGE) are disturbing the natural Pt cycling and account for more than 70% of the total flux of this metal at the Earth's surface (Mitra and Sen, 2017). In particular, the use of Pt in catalytic converters for the automobile industry (introduced in the US in 1975 and in 1986 in Europe) has led to its environmental contamination both at a local (e.g. Almécija et al., 2015; Neira et al., 2015) and a global scale (Rauch et al., 2005; Soyol-Erdene et al., 2011). To assess the potential adverse effects of elevated environmental Pt concentrations on living organisms, several studies have investigated its bioavailability and bioaccumulation as well as reported specific ecotoxicological endpoints. Such studies were mainly conducted in freshwater media using species such as annelids, isopods, fishes, or bivalves (e.g. Rauch and Morrison, 1999; Brand et al., 2019). Apart from biological uptake rates, different ecotoxicological endpoints including molecular effects (e.g. DNA damage or the induction of heat shock proteins; Osterauer et al., 2011; Singer et al., 2005) or tissue alteration (e.g. cytoplasm vacuolization; Osterauer et al., 2010) revealed the toxicity of Pt.

Regarding marine organisms, previous works have reported studies on Pt exposure to polychaetes, gastropods, and bivalves (Abdou et al., 2018, 2020b; French and Turner, 2008; Mulholland and Turner, 2011), as well as marine micro- and macro-algae (Cosden et al., 2003; Easton et al., 2011; Shams et al., 2014; Turner et al., 2007). Microalgae

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represent indeed excellent model organisms to study exposure processes. In particular, the marine microalga *Dunaliella salina* (Chlorophyceae) is widely distributed in seawater and has been extensively used for ecotoxicological studies to evaluate the toxicity of a variety of harmful chemicals including trace metals and presents high tolerance towards elevated concentrations of these metals (Belghith et al., 2016; Folgar et al., 2009; Nikookar et al., 2005).

Given that the bioavailability and toxicity of a given element greatly depends on its speciation, ecotoxicological studies should be carried out ensuring that chemical equilibrium reflecting ambient speciation is reached at the experimental conditions used (e.g. pH, salinity). An ionic metal spiked to a natural water sample may take times ranging from hours to months to equilibrate, especially for those metals with slow reaction and ligand exchange kinetics. This is the case of Pt which is considered as a kinetically-hindered trace metal (Cobelo-García et al., 2013; Cosden et al., 2003; Cosden and Byrne, 2003). The effect of metal spike aging on the extent of biological responses has already been observed (e.g. Oorts et al., 2006). Yet, the use of 'freshly' spiked test media is common in the literature and may lead to non-equilibrium conditions during static exposure experiments (Sures and Zimmermann, 2007). Generally, in these studies Pt(IV) is spiked in the form of soluble salts (e.g. $PtCl_6^{2-}$); such Pt(IV) speciation in the spike differs significantly from that in natural waters (Catrouillet et al., 2021). Therefore, it appears necessary to study the time required for the spiked metal to attain equilibrium conditions before any toxicity or bioaccumulation study is started.

The present study examines, therefore, the effects of Pt spike aging on its speciation and toxicity to the marine microalgae *D. salina*. Platinum(IV) was used in this study since, although Pt can be present in aquatic systems at ambient conditions in two different oxidation states – Pt(II) and Pt(IV) –, recent thermodynamic calculations suggest that Pt (IV) is the dominant oxidation state in seawater (Catrouillet et al., 2021). The specific objectives of this study are (i) to monitor the change in dissolved Pt(IV) speciation spiked to seawater model solutions over time (> 3 months), and (ii) to evaluate the implications of Pt spike aging on its toxicity to *D. salina*. Results will be valuable in terms of the implications of non-equilibrium speciation conditions on the derived biological endpoints in eco-toxicological studies.

2. Materials and methods

2.1. Spiking and incubation procedures

Filtered (20 μm) and autoclaved seawater from the Vigo Ria (NW Iberian Peninsula; 42°13′45′N; 08°46′01′W; Salinity~32 and a DOC concentration of approx. 85 μM) was used to monitor the change of the spiked Pt(IV) speciation. Platinum was spiked to a concentration of 10 mg L^{-1} (51 μM) through dilution of a Pt atomic absorption standard solution (987 mg L^{-1} in 5.4% HCl, Sigma Aldrich). The solution was kept in a HDPE bottle. The pH of the test solution was adjusted to 8.2 and buffered using TAPS (Sigma Ultra) to avoid variations during the testing period (Turner et al., 2007). Platinum oxidation state was confirmed by UV-spectrometry showing a peak centered at 262 nm ($PtCl_6^{2-}$) but not at 230 nm ($PtCl_4^{2-}$; Gammons, 1996).

The microalgae culture medium (L1) used to perform the toxicity tests was prepared in autoclaved seawater according to reference protocols (Guillard and Morton, 2004; medium composition in the Supporting Information). A second culture medium (LM1), with the same composition as L1 but without EDTA, was also prepared in order to check whether this complexing ligand could have an impact on Pt toxicity. Platinum stock solutions of 400 mg L^{-1} were prepared in both media (L1, LM1) either 10 days before performing the toxicity test (aged media) or the same day of testing (fresh media). Then, serial dilutions were performed in freshly prepared medium to have the following test range of Pt concentrations: 0 (control), 0.5, 1, 5, 10, 20, 50, 100, 200 and 400 mg Pt L^{-1}. The use of such high exposure concentrations compared to natural background levels are relevant in terms of toxicity identification (Sørensen et al., 2016).

2.2. Ecotoxicological tests

Ecotoxicological tests with the green microalga *Dunaliella salina* were performed according to the OCSPP 850.4500 (USEPA, 2012) guideline. The microalgae were obtained from the microalgal collection of the IIM-CSIC (Vigo, Spain). Algae were pre-incubated under controlled conditions (20 ± 2 °C and continuous cool white fluorescent light at 4000 lux) in sterile Erlenmeyer flasks containing L1 nutrient medium until logarithmically growing stock cultures (4-day old) were reached. Toxicity tests were performed in 96-well flat plates with a final volume of 250 μL with the studied medium and initial algae inoculum containing 10^4 cells mL^{-1}. A negative control with L1 without spiked Pt and a positive control L1 spiked with Hg (1, 5, 10 and 20 μM of Hg) were included.

Plates were incubated during the test in a laboratory growth chamber on a continuous shaker at 60 rpm, constant temperature (20 ± 2 °C) and 12-hour light/12-hour dark photoperiod (4000 lux). The microalgae concentrations were estimated by determining the chlorophyll *a* concentration through spectrophotometric measurements (absorbance wavelength 440 nm, emission wavelength 680 nm) during different incubation periods (0 h, 24 h, 48 h, 72 h and 96 h) using a spectrophotometer (BioTek Synergy H4 Hybrid Microplate Reader) and performing data treatment (Gen5 Data Analysis Software). At the end of the exposure period (96 h), the effective concentration of Pt resulting in the inhibition of 10% and 50% of algae growth rate compared to the positive controls (EC_{10} and EC_{50} respectively; standard ISO tests; e.g. Christensen et al., 2009) and the corresponding 95% confidence intervals (CI) were obtained by fitting a log-logistic model (Doelman and Haanstra, 1989) using the method of Marquardt (SAS software, version 9.1). Total dissolved Pt concentrations obtained by ICP-MS measurements were used in the calculations. Fresh and aged media were tested at the same time using the same algae inoculum and each experiment was repeated twice. The ecotoxicological end-points presented in this study represent the average of both replicates.

2.3. Speciation monitoring and Pt quantification

The UV-Vis spectra for Pt(IV) speciation monitoring were recorded at different time intervals in the wavelength range of 200–500 nm using a spectrophotometer (V-650 UV-Vis Spectrophotometer; JASCO) and a quartz cuvette with light path of 10 mm (SUPRASIL Hellma Analytics, type 100-QS). Non-spiked aliquots were used for background corrections. The pH was checked before each UV-Vis measurement with a precision of 0.1 pH units using a pH-meter (LAQUAtwin B-712; Horiba).

The actual Pt concentrations in the test solutions were determined, after appropriate dilution, by means of ICP-MS (7900 Agilent). Platinum concentrations at the end of the test (96 h) did not show significant variations from the start (0 h).

3. Results and discussion

3.1. Monitoring of Pt speciation evolution with aging

The UV-Vis spectrum of the freshly spiked seawater (Fig. 1, time 0 day) reflects the sole presence of the hexachloroplatinate ($PtCl_6^{2-}$) Pt species, due to the characteristic peak centered around 262 nm with an extinction coefficient of ~ 25.10^3 cm^{-1} M^{-1} (Cox et al., 1972). After 1 day of equilibration, a second species appears; the presence of one isobestic point at ~249 nm until at least the fourth day of equilibration indicates the presence of two Pt species. This second species is expected to be the result of a replacement of one chloride by an hydroxy group leading to $[PtCl_5(OH)]^{2-}$, confirmed by the spectra given by Znakovskaya et al. (2005). After 4 days of equilibration, the appearance of

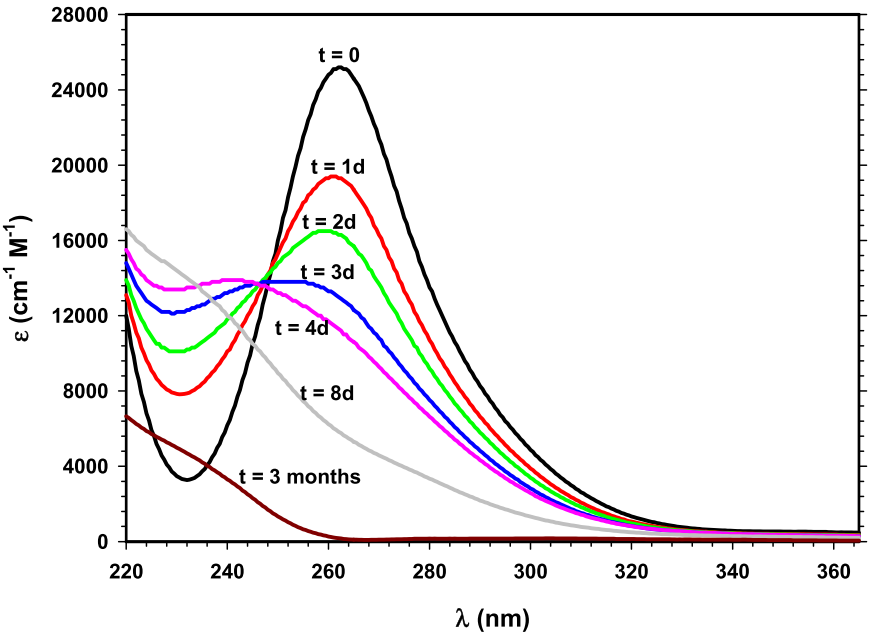


Fig. 1. UV-Vis spectra of the autoclaved seawater spiked with 10 mg L^{-1} Pt as $PtCl_6^{2-}$ recorded at different equilibration times.

further hydrolyzed species is suggested by lower absorption coefficients at these wavelengths (Cox and Peters, 1970).

In a Pt-H_2O-Cl system, Pt speciation will be dominated by the formation of hydroxides, chlorides and mixed-hydroxy-chlorides (Fig. 2), depending on pH and chloride concentration. In seawater, the chemical speciation of inorganic Pt(IV) has been suggested to be dominated by the $PtCl_5(OH)^{2-}$ species (Cobelo-García et al., 2013). However, a recent re-evaluation of the thermodynamic data supports the presence of higher hydrolyzed species in the form of $[PtCl_{3-n}(OH)_{3+n}]^{2-}$, n = 0–3 (Catrouillet et al., 2021; Fig. 3) at typical seawater pH. This is consistent with the appearance of further species after 4 days of speciation monitoring (Fig. 1). Similar long equilibration times are to be expected in freshwater, where Pt(IV) is fully hydrolyzed ($Pt(OH)_5^-$; Catrouillet et al., 2021).

3.2. Effects of Pt spike aging on its toxicity to D. salina

3.2.1. Evolution of Pt speciation in the exposure medium

Total dissolved Pt concentrations in test systems for L1 and LM1 media, without algae addition, were quantified by ICP-MS and, importantly, no significant changes during the 96 h duration of the toxicity tests were quantified. However, there were important differences in the UV-Vis spectrum of the media (Fig. 4). In the aged media, the UV spectra remained fairly constant after the first day, indicating no further speciation changes. Yet, in the freshly spiked medium significant changes were observed during the duration of the toxicity test, indicating non-equilibrium conditions. Similar results were obtained for LM1 (not shown). It is interesting noting the similar UV-Vis spectra and its evolution obtained in the test media (Fig. 4) compared to autoclaved seawater (Fig. 1) despite of the presence of organic substances (e.g. EDTA, vitamins) that could potentially interact and form complexes with Pt(IV). Although the formation of a Pt(IV)-EDTA complex has been reported in the literature (Ayres and Glass, 1972), the authors noted the extremely slow reaction of Pt(IV) with EDTA. Also, given that the UV-Vis spectra of the Pt(IV)-EDTA complex (Ayres and Glass, 1972) is similar to that of the hydrolyzed Pt(IV) species given in Fig. 1, it is not possible to conclude whether an interaction of Pt(IV) with EDTA (or vitamins) in the test media was occurring.

Such result highlights the advantage of performing static exposure experiments i.e. without water and spike renewal during the testing

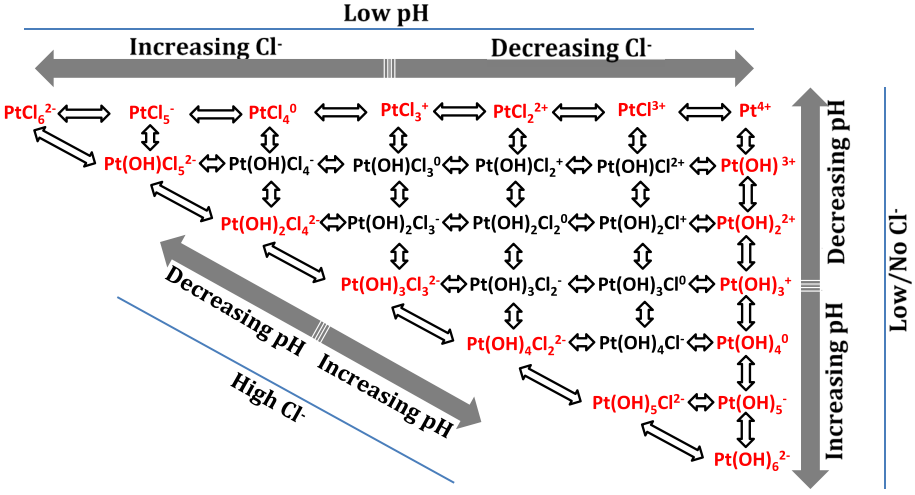


Fig. 2. Different inorganic Pt(IV) complexes possibly existing in a Pt-H_2O-Cl system.

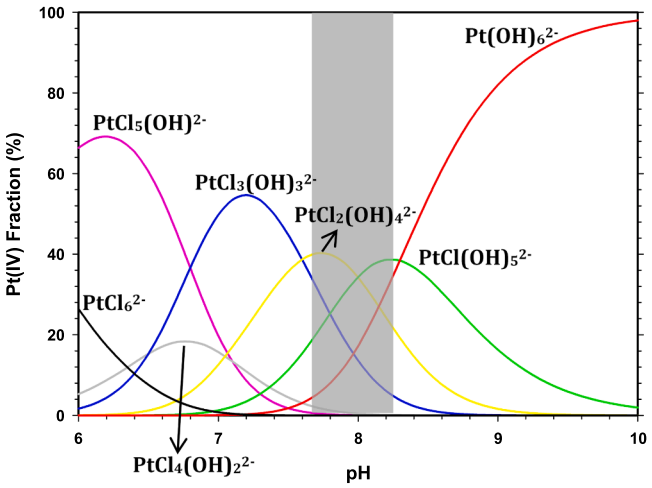


Fig. 3. Inorganic speciation in a Pt-H_2O-Cl system at a chloride concentration of 0.5 M according to Catrouillet et al. 2021. The shaded area corresponds to typical seawater pH.

period rather than static-renewal or flow-through exposure systems. These later would in any case be difficult to apply with algae as test organisms. It also reflects the importance of allowing sufficient equilibration time of the test medium prior the beginning of the experiment, as further discussed below. Static experiments are usually advantageous compared to semi-static tests with respect to cost effectiveness, the volume of contaminated waste water to be discharged and the period to reach steady-state conditions (Zimmermann and Sures, 2018). Same authors observed in a static experiment with mussel shells exposed to $PtCl_4$, that the time needed to reach the steady state of Pt concentration in freshwater was $\geq$ 1 week and was clearly influenced by the water type (Sures and Zimmermann, 2007). They, therefore, recommend equilibrating the test system with metals to allow for more or less constant metal concentrations in the tank water before exposure of the test organism. System equilibration is not only important for metal spike speciation but also for potential abiotic losses that may occur during the experiments (Cobelo-García et al., 2007). Accordingly, trace metal precipitation or adsorption onto tank walls can cause significant reduction of concentration. Turner et al. (2007) observed in exposure experiments conducted in seawater that in control conditions without macroalgae, ~ 20% of Pt (spiked as $PtCl_6^{2-}$) was lost from the aqueous phase over the period of incubation (100 h), potentially due to adsorption on the container surfaces (static experiment). Even in semi-static Pt exposure experiments on oysters involving daily spike and seawater renewal, Abdou et al. (2018) observed a decrease of around 30% in dissolved Pt concentration 20 h after spiking seawater.

3.2.2. *Influence of Pt spike aging on its toxicity*

The toxicity of Pt was investigated with the marine algae *D. salina* in two different batches. A positive control with Hg was also checked in order to determine the viability of the algae and the test. The EC_{50} average values obtained from both batches for Hg was 4.5 μM (3.6–5.6, 95% CI). These values are close to those obtained by Fisher et al. (1984) with the species *Dunaliella tertiolecta* (EC_{50} = 5.01 μM). The logarithmic growth in cell counts in negative controls had an average factor of 134 and the pH was maintained constant (7.9 ± 0.6) during the incubation period, indicating appropriate conditions for the performance of such tests (USEPA, 2012).

Obtained toxicity data demonstrate that after a short period of aging (10 days) the toxicity increased significantly, with EC_x values one order of magnitude lower in aged media than in the freshly-spiked (Table 1). These marked differences demonstrate that, according to the slow Pt reaction kinetic, chemical equilibrium is not achieved in commonly applied freshly-spiked Pt(IV) tests.

This trend was observed in both the L1 and LM1 media (without EDTA), where the EC_x values obtained were similar (Table 1). The EDTA is a chelating agent able to complex trace metals and therefore acting as a potential competing agent in exposure media. Our results suggest that no significant complexation occurred with this chemical, as already observed in exposure studies involving marine microalgae *Chlorella stigmatophora* (Shams et al., 2014). Accordingly, the kinetics of Pt-algal interactions appeared to be unaffected by the presence of EDTA as complexing agent in the experimental medium. The EDTA concentration used in test media may has also been unsufficient to significantly chelate the relatively elevated Pt spike.

3.3. *Ecotoxicological implications of the Pt(IV) toxicity to algae*

Results from the exposure experiments provided EC values close to 10 mg L^{-1} for the aged Pt spike incubations, which is considered as more relevant from equilibrium speciation conditions. Standard EC test results classify a compound as hazardous if the EC_{50} is $\leq$ 100 mg.L^{-1} in tests involving algae, therefore Pt(IV) can be considered as toxic for organisms. Values obtained for freshly-spiked conditions, considering the 95% CI, would provide a different interpretation, classifying Pt(IV) as a non-hazardous substance. Ecotoxicological studies on Pt exposure effects in the literature were mainly conducted on freshwater organisms and only few studies report Pt toxicity to algae. Using another Pt spike source – Pt nanoparticles (PtNPs) – similar EC_{50} were observed for the green microalgae *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii*. Authors obtained EC_{50} values of ~15 and 200 mg L^{-1}, respectively, after 48 h of incubation (Sørensen et al., 2016). These results classify PtNPs as 'harmful' to *P. subcapitata* algal species. Similar growth inhibition rate were observed for *P. subcapitata* in another study exposing algae for 72 h to PtNPs (EC_{50} ~17 mg L^{-1}), which is yet twelve-fold higher (less toxic) than silver (Ag) NPs (Książyk et al., 2015). In freshwater organisms, Pt toxicity for the crustacean *Daphnia magna* has been evaluated reflecting EC_{50} ranging between ~276 μg L^{-1} and 110 μg L^{-1}, after 24 h and 48 h, respectively (Zimmermann et al., 2017).

Despite the scarcity of reported EC_{50} values for Pt in marine organisms, the comparison of the results obtained in this study using *D. salina* with other more commonly studied metals indicate a similar or higher Pt toxicity. For example, for *D. salina* a EC_{50} value of 218 mg L^{-1} and 48.9 mg L^{-1} were reported for Ag (using $AgNO_3$; Johari et al., 2018) and Cd (using $CdCl_2$; Folgar et al., 2009), respectively. Similar values than for Pt are obtained with *D. salina* exposed to Cu(II) (EC_{50} of ~ 18 mg L^{-1}; Gallo et al., 2020).

Apart from growth inhibition, several Pt-algae exposure studies reported its sorption, uptake kinetics, and adverse physiological endpoints. Accordingly, Pt in algal cells following short-term exposure indicated a quasi-linear accumulation isotherms with a significant decrease of accumulation with increasing density of the marine algae *C. stigmatophora* (Shams et al., 2014). At cellular level, significant oxidative stress following Pt exposure was reported in marine algal species (Sørensen et al., 2016). In freshwater periphyton community, Rauch et al. (2004) observed a linear increase of uptake with increasing exposure concentration suggesting high affinity of dissolved Pt to the periphyton matrix, composed of various diatom species, as well as a linear decrease of the photosynthetic activity. Marine microalgae exposure to Pt NPs also influenced negatively their chlorophyll content (Książyk et al., 2015). Other studies report Pt toxicity to marine macroalgae, especially *Ulva lactuca*. While slow complexation and adsorption reactions of Pt(II) and Pt(IV) were observed, these species proved to mediate the partial oxidation of Pt(II) to Pt(IV) (Cosden et al., 2003). Internalized Pt (after EDTA leaching) was estimated to be 95% of Pt since in seawater, complexation of dominant Pt(IV) species at the organic-rich algal cell surface is expected to be kinetically constrained, while a pH dependence on Pt uptake was observed (Turner et al., 2007); however, the authors indicated that these results could be overestimated

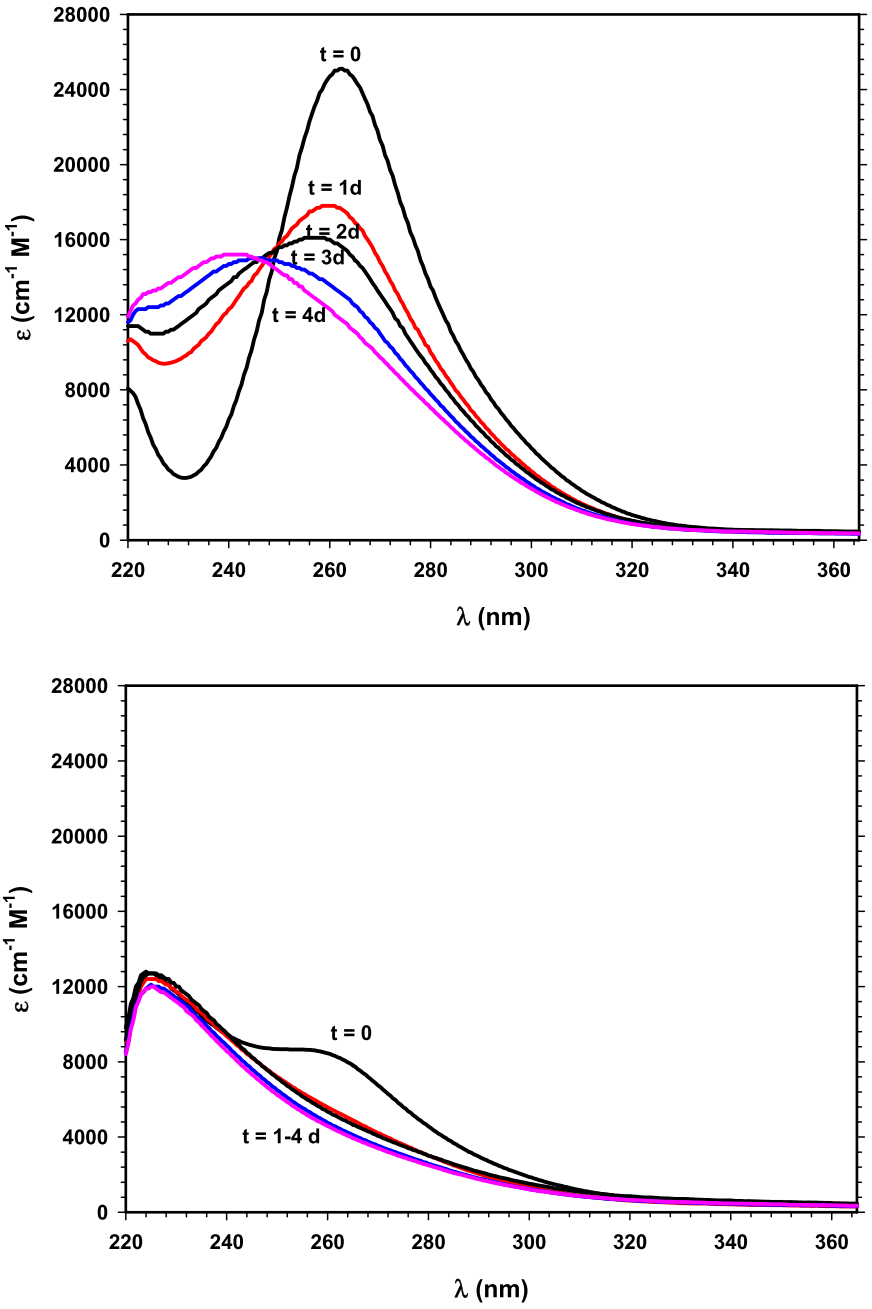


Fig. 4. Monitoring of the Pt speciation evolution by UV spectra for L1 media during the duration of the toxicity test (from 0 to 96 h), using a freshly (upper panel) and aged (lower panel) spiked solution (10 mg L^{-1} Pt spikes). Note that the spectra were recorded in the absence of algae.

Table 1
Mean ECx values (algae growth inhibition) calculated using the total dissolved Pt concentrations (mg L^{-1}; obtained by means of ICP-MS measurements) spiked in two different media (fresh and aged). The 95% confidence intervals of the EC values are given in the brackets.

		Fresh	Aged (10 days)
L1 (mg L^{-1})	EC_{10}	81.2 [55.5–119]	7.8 [6.7–9.0]
	EC_{50}	99.7 [43.9–227]	9.1 [7.4–11.1]
LM1 (mg L^{-1})	EC_{10}	83.2 [61.2–113.0]	6.1 [5.2–7.2]
	EC_{50}	134.4 [124–145]	12.5 [11.6–13.3]

due to the slow kinetics of Pt(IV) complexation and dissociation as indicated in this study. When comparing Pt(II) and Pt(IV) removal rate in seawater by six macroalgal species, greater and faster removal was observed for *U. lactuca*, while slow sorption kinetics of Pt were again observed (Pinto et al., 2021).

Relatively elevated spiked Pt concentrations were applied in our exposure experiments and this is associated to regulatory requirements when evaluating the toxicity of a compound applying the EC_{50} test with algal species (Sørensen et al., 2016). In fact, the limited environmental information regarding Pt toxicity on marine algae is of concern since Pt also proved to be as potentially toxic to algae as other metals of concern that have been monitored for decades in coastal environments. Accordingly, microalgae should be considered as decaying and settling particles and therefore represent an important vehicle for the vertical transport of contaminants in the marine environment (González-Dávila, 1995; Twining and Baines, 2013). Field studies on the interactions between marine phytoplankton and Pt have been conducted, revealing that in coastal systems, primary producers' activity partially controls Pt

cycling and partitioning during daytime while during the night, zooplankton grazing may release Pt into the dissolved phase (Abdou et al., 2020a). Besides Pt scavenging following algal cell decay, trophic transfer is therefore another important environmental aspect to consider, despite no biomagnification was observed between phyto- and zooplankton (Abdou et al., 2020a). Platinum levels were also quantified in marine macroalgae confirming its bioavailability and bioaccumulation in natural environments (Hodge et al., 1986; Yang, 1989). Such environmental observations highlight the importance of identifying the potential Pt toxic effects in different marine organisms and especially in algae as primary producers. To date, data are too scarce to allow a complete risk assessment of the effects of this emerging contaminant and, to this aim, relevant data on Pt speciation and reactivity in seawater is needed in order to allow addressing the environmental regulations. As reported in Sørensen et al. (2016), while the use of high Pt exposure concentrations in experiments is questionable, it is of high regulatory relevance for toxicity identification, ranking as well as classification, and labeling within the current regulatory framework.

4. Conclusions

Our results revealed the influence of Pt spike aging in exposure studies, as Pt speciation equilibrium may need from days to months to be attained. Platinum toxicity to marine algae was dependent on its speciation and it increased one order of magnitude in aged Pt spiked seawater. Special care should then be taken therefore to ensure that spiked species equilibration is reached before test organism exposure experiments are conducted; although this can be challenging for such kinetically-constraint element as Pt, this is needed in order to ensure that the eco-toxicological endpoints obtained are produced by environmentally-relevant Pt species. Pt(IV) toxicity obtained towards *D. salina*, ~10 mg L^{-1} as EC_{50}, is several orders of magnitude higher than typical dissolved concentrations in estuarine and coastal systems which are generally at the sub ng L^{-1} range (e.g. Cobelo-García et al., 2013); however, this represents a similar or higher toxicity than those reported for other metals which are known to be toxic for phytoplankton at elevated concentrations (e.g. Cd, Cu, Ag).

CRediT authorship contribution statement

A.R.-F. and A.C.-G. designed the study. A.R.-F. performed the eco-toxicological tests and initially drafted the manuscript. A.R.-F., A.C.-G. and M.A. have jointly interpreted the data, revised and completed the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2021.112924.

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