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Effects of long-term exposure to colloidal gold nanorods on freshwater microalgae

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Abstract: Gold nanorods have shown to pose adverse effects to biota. Whether these effects may be potentiated through prolonged exposure has been rarely studied. Therefore, this work aimed at evaluating the effects of long-term exposure to sublethal levels of cetyltrimethylammonium bromide (CTAB) coated gold nanorods (Au-NR) on two freshwater microalgae: *Chlorella vulgaris* and *Raphidocelis subcapitata*. These algae were exposed to several concentrations of Au-NR for 72h and, afterwards, to the corresponding $EC_{5,72h}$, for growth, during 16 days. The sensitivity of the two algae to Au-NR was assessed at days 0, 4, 8, 12 and 16 (D0, D4, D8, D12 and D16, respectively) after a 72-h exposure to several concentrations of Au-NR. At the end of the assays, effects on yield and population growth rate were evaluated. *Raphidocelis subcapitata* was slightly more sensitive to Au-NR than *C. vulgaris*: $EC_{50,72h,D0}$ for yield were 48.1 (35.3-60.9) and 70.5 (52.4-88.6) µg/L Au-NR, respectively while for population growth rate were above the highest tested concentrations (53 and 90 µg/L, respectively). For *R. subcapitata* the long-term exposure to Au-NR increased its sensitivity to this type of nanostructures. For *C. vulgaris*, a decrease on the effects caused by Au-NR occurred over time, with no significant effects being observed for yield or population growth rate at D12 and D16. The capping agent CTAB caused reductions in yield above 30% (D0) for both algae at the concentration matching the one at the highest Au-NR tested concentration. When exposed to CTAB, the highest inhibition values were 69% (D4) and 21.3%

(D8) for *R. subcapitata*, and 64% (D12) and 21% (D16) to *C. vulgaris*, for yield and population growth rate, respectively. These results suggested long-term exposures should be included in ecological risk assessments since short-term standard toxicity may either under- or overestimate the risk posed by Au-NR.

Keywords: Long-term exposure, *Chlorella vulgaris*, *Raphidocelis subcapitata*, gold nanorods, CTAB

1. Introduction

Nanomaterials (NMs) exist in the environment, being originated either from natural or artificial sources (Rosenkranz, 2010). In the last decades the unique characteristics of NMs started to be exploited by the industry and its production and applications experienced a fast and exponential growth. Although NMs might bring many important benefits to the society (e.g. in areas of diagnosis of diseases), their unique characteristics have also been investigated for potential adverse effects to biota (Farkas et al., 2010; Larguinho et al., 2014; Rosenkranz, 2010; Van Hoecke et al., 2008). Among the wide diversity of NMs being produced, those made of gold (Au-NMs) have been increasingly produced mainly due to the fact that their optical properties may be easily tuned by changing their size, shape or surface chemistry (Barkalina et al., 2014). Such characteristics make Au-NMs attractive for different areas, namely for bioapplications that include the use of Au-NMs in clinical diagnosis and therapeutic procedures (e.g. markers for tumor cells) (Pereira et al., 2014; Salgueiro et al., 2013). However, some works have reported that these NMs may cause adverse effects to biota. The severity of such observed toxic effects is not only influenced by the Au-NMs concentration but also by other characteristics intrinsic to the NMs (e.g. its geometric configuration, size, surface charge, chemical composition including the presence of capping/stabilizing agents) (Nasser et al., 2016; Wan et al., 2015; Yah, 2013; Zhang et al., 2012). Bozich et al. (2014) studied the effects of different gold nanospheres (Au-NS) and nanorods (Au-NR) holding different stabilizing agents: i) trisodium citrate (Cit) with Au-NS, ii) poly(allylamine) hydrochloride (PAH) with Au-NS, iii) mercaptopropionic acid with Au-NS and iv) trimethylammonium bromide (CTAB) with Au-NR, in *Daphnia magna*. After exposure to lethal and sublethal concentrations (below 50 µg/mL) of the two Au-NMs, these authors reported that the positively charged particles (with PAH and CTAB) were more toxic and exhibited a lower aggregation rate than the negatively charged ones. Bozich et al. (2014) suggested that these results were due to the higher affinity of positively charged Au-NMs to the negatively charged cellular membranes. Browning et al. (2009, 2013)

exposed embryos of zebrafish to Au-NS with different sizes (primary size: 11.6±0.9 nm and 86.2±10.8 nm) and observed that the smallest Au-NS caused the highest mortality and deformities rates in the zebrafish embryos.

Although toxic effects of Au-NMs to biota have been reported, most of these studies focus on the effects in cell lines and small mammals aiming to assess their biocompatibility (Conde et al., 2012; Moretti et al., 2013; Rayavarapu et al., 2010). Studies on Au-NMs effects in aquatic biota are scarce and most of them use standard approaches that, though being important for first stages of risk assessment frameworks, lack some ecological relevance and neglect potential adverse effects that may appear or disappear across long-term exposure. Recently, some works began to study the long-term effects of Au-NMs in biota, trying to increase the ecological relevance of the generated ecotoxicity data, but they are focused in a single model organism, the nematode *Caenorhabditis elegans*. Kim et al. (2013) studied the transgenerational effects of *Caenorhabditis elegans* exposure to gold nanospheres (Au-NS). For this, authors fed a parental generation (F0) of *C. elegans* with Au-NS contaminated food (with $5x10^{10}$, $25x10^{10}$ and $50x10^{10}$ particles of Au-NS/mL) and observed the effects of such exposure in survival and reproduction throughout four generations (F1 to F4). Regardless the amount of Au-NS, no significant changes were reported for the lethal sensitivity to Au-NS across generations. However, individuals from F2 reported more effects in reproduction and abnormalities than those of F0; being able to recover in F3 and F4. More recently, Moon et al. (2017) investigated the multigenerational effects of colloidal Au-NS (size ranging from 8.5 to 12 nm) on *C. elegans* after continuous or intermittent supply with food contaminated with Au-NS. Intermittent exposure to Au-NS (exposure of F0 and F3, allowing recovery at F1 and F2) caused a decrease in F3 reproduction, comparatively to the control, of 63%, 53%, and 40%, at concentrations of 5×10^{10}, 25×10^{10}, and 50×10^{10} particles Au-NS/mL, respectively. Under continuous exposure (all generations were exposed to Au-NS contaminated food), an increase in the total abnormalities rate was observed for F1 (13.3%), F2 (15%), F3 (17.5%) and F4 (23.3%) generations of *C. elegans*

exposed at 50×10^{10} particles/mL. This study showed that the effects of multigenerational assay may vary according to different exposure patterns and levels.

Although data on the toxicity of Au-NMs to freshwater organisms was reported, the focus has been on monogenerational and/or short-term exposures by using standard approaches. Information on the effects of long-term exposure to Au-NMs on freshwater organisms is still scarce. In this context, the present work aimed at assessing the long-term effects of sublethal levels of CTAB coated Au-NR in two species of producers, the microalgae *Chlorella vulgaris* and *Raphidocelis subcapitata*. Standard growth inhibition assays with this species recommend a 72h exposure period. However, in the field microalgae may be exposed for much larger periods to sub-lethal concentrations of nanoparticles, which may only cause effects after a long-term. Because these microalgae are primary producers, they represent the group of organisms that constitute the basis of food webs, providing oxygen and energy for higher trophic levels (Yang et al., 2008). Thus, adverse effects that surface coated nanorods may pose to these species may have indirect consequences at higher levels of the food chain (Pakrashi et al., 2014).

2. Materials and methods

2.1. Test substances

Gold nanorods (Au-NR), capped with the surfactant cetyltrimethylammonium bromide (CTAB) (A12-10-780), were purchased from Nanopartz™ (Salt Lake City, UT, USA) as colloids in deionized water with less than 100 mM of CTAB. The concentration of the Au-NR in the dispersion was 35 μg/ml and their primary sizes were 10 nm for width and 38 nm for length (information specified by Nanopartz™, 2006). The surfactant CTAB 95% was supplied by Sigma-Aldrich® (Darmstadt, Germany) as a powder.

2.2. Gold nanorods characterization

The physical-chemical characterization of the stock Au-NR dispersion, of the highest tested concentration for each alga (90 μg/L for *C. vulgaris* and 53 μg/L for *R. subcapitata*), and of the concentrations causing 5% of growth inhibition in each alga (15.54 μg/L for *C. vulgaris* and 21.04 μg /L for *R. subcapitata*) was carried out. The highest value of $EC_{5,72h}$ (21.04) is within the limits of confidence of the lowest value of $EC_{5,72h}$ (CL95: 5.95-25.12) so the physical-chemical characterization was done for the concentration of 22 μg/L. The characterization of the tested Au-NR dispersions was performed at time 0h (just after the preparation of the suspension in Woods hole Marine Biology Laboratory medium-MBL) and after a period of 72h (corresponding to the duration of the toxicity assays). During this period the dispersions were maintained under the same conditions as those used for the ecotoxicity assays. Optical absorption measurements in the UV/VIS/NIR regions were carried out in a spectrophotometer Jasco V-560 to confirm that the spectral features of the Au-NR stock solution comply with those initially provided by the supplier. The concentration of Au in the stock and the tested Au-NR dispersions, previously digested in aqua regia, were analyzed by using Inductively Coupled Plasma Mass Spectrometry in a ICP-MS Thermo X Series to evaluate the concentrations of total gold. The digestion with aqua regia was performed by adding 0.2 mL of HNO3 (65%) and 0.6 mL of HCl (37%) to 10 mL of each Au-NR dispersion (the stock dispersion, the highest tested concentration for each alga - 90 μg/L for *C. vulgaris* and 53 μg/L for *R. subcapitata*, and the concentrations causing 5% of growth inhibition in each alga was prepared). The digestion was performed at room temperature for more than 48h. The surface charge of the Au-NR was given by the zeta potential that was determined through electrophoretic light scattering (ELS) in a Zetasizer Nano ZS with Zetasizer Software (Malvern ZetaSizer, 2013) with a disposable folded capillary cell. The hydrodynamic diameter was measured by dynamic light scattering (DLS) in a Zetasizer Nano ZS with Zetasizer Software. Particle size and morphology were characterized by using Transmission Electronic Microscopy (TEM; TEM

microscope JEOL 200CX operated at 300 kV). For this, 2 mL of the stock dispersion were centrifuged at 10 630 rpm during 30 minutes to decrease the excess of CTAB on the samples and promote a better visualization on the particles. Then, the supernatant was discarded and 0.5 mL of Milli-Q water was added to re-suspend the particles. One drop of centrifuged solution was placed on a carbon coated copper grid and left to dry for 24h. For the Au-NR dispersions that were used to perform the toxicity assays, no previous centrifugation was needed, and a drop of each dispersion was directly deployed in the copper grids.

2.3. Test species

The long-term effects of Au-NR on two species of green microalgae, *Raphidocelis subcapitata* and *Chlorella vulgaris*, were studied. Cultures of *R. subcapitata* and *C. vulgaris* were maintained at 20˚C with continuous light (fluorescent light tubes: OsramL36W/10) and aeration. Four-day inoculates, i.e. in the log growth phase of the algae cultures (EC, 2007; OECD, 2011), were used to run long-term exposures and growth inhibition assays. Woods Hole MBL culture medium was used and prepared according to Stein (1973). The medium, and all the material used to prepare the cultures and assays, were previously sterilized in an autoclave at 121°C and 1 Bar, for 30 minutes.

2.4. 72h-growth inhibition assays with microalgae

Firstly, the effects of Au-NR on yield and population growth rate of microalgae were assessed by exposing *R. subcapitata* and *C. vulgaris* to this nanorod according to the procedures described in OECD Guideline 201 (OECD, 2004). Preliminary assays were carried out to select the concentration range to which algae would be exposed. *Raphidocelis subcapitata* was exposed to seven concentrations of Au-NR ranging from 8 to 53 µg/L, by using a dilution factor of 1.3x, plus a control (consisting of MBL medium). For *C. vulgaris* eight concentrations of Au-NR were tested ranging from 11 to 90 µg/L, by using a dilution factor of 1.3x, plus a control of MBL. To evaluate the effects of the Au-NR capping agent CTAB, both algae were also exposed to the concentration of

CTAB present in the highest tested concentration of Au-NR (55 mg/L for *R. subcapitata* and 94 mg/L for *C. vulgaris*). All assays were conducted at 23±1°C, under continuous light of 4000 lux. Assays were performed in 24-well plates and eight replicates were made for each concentration and control. To minimize evaporation, the outer wells of the plate were filled with 1 mL of autoclaved distilled water and only the inner wells were filled with test solutions. The test wells were filled with 900µl of MBL solution solely (control), MBL with an Au-NR concentration or with MBL with CTAB. To all test wells were added 100µl of algae inoculum, 4-5 days old, at a concentration of 10^5 cell/mL, to start the assay with a cell density of 10^4 cell/mL. To avoid the settling of algae and subsequent shadow effects on their growth during the assay, all plates were re-suspended for a few minutes every day on an orbital shaker. After 72h of exposure the assay ended and absorbance (ABS) was measured for each replicate in a spectrophotometer (Jenway, 6505 UV/Vis) at 440 nm, and converted into cell density per volume according to the following equations (Venâncio et al., 2017; Loureiro et al., 2018):

cell/mL=−17107.5+Abs440 × 7925350 (*R. subcapitata*; r^2=0.98; p≤0.05)

cell/mL=−155820+Abs440×13144324 (*C. vulgaris*; r^2=0.91; p≤0.05)

Yield (biomass produced during the test) was computed according to the following equation:

$$Y = (NF - NI)$$

Where, NF is the biomass of algae at the end of the assay (cell/mL) and NI is the biomass of algae at the start of the assay (cell/mL). The percentage of yield inhibition was calculated according to:

$$I_Y(\%) = \left(\frac{Yc - Yt}{Yc}\right) x100$$

Where Yc is mean value for yield in the control group and Yt is value for yield for the treatment replicate.

The population growth rate (r) was also calculated, according to the following equation:

$$r = \frac{ln\,NF - ln\,NI}{t}$$

where NF is the biomass of algae at the end of assay (cell/mL), NI is the biomass of algae at the start of the assay (cell/mL) and t is the time of exposure (days). The percentage of growth inhibition was calculated according to:

$$I_r(\%) = \left(\frac{\mu C - \mu t}{\mu C}\right) x100$$

where μC is the mean growth rate of algae in control and μt is the growth rate of algae in each replicate.

2.5. Long-term exposure of microalgae to gold nanorods

The two species of microalgae were exposed to the respective Au-NR concentration causing 20% of reduction in growth rate after 72h of exposure ($EC_{20,72h}$, computed for F0). The $EC_{20,72h}$ was selected because it is considered to correspond to the lowest observed effect concentration. However, following several attempts, it was observed that after being exposed for a period of 5 days to the respective $EC_{20,72h}$, the two microalgae were not capable to attain the log growth phase, which impaired the continuity of the experiment. Therefore, subsequent exposure was performed at the concentration causing 10% of growth inhibition ($EC_{10,72h}$) after 72h of exposure, corresponding to the no observable effect concentration. As for the $EC_{20,72h}$, after 5 days of exposure, mainly visible in the *R. subcapitata*, but affecting both algae, they were not capable to attain the log growth phase. Accordingly, the two algae species were exposed for 16 days, to the respective $EC_{5,72h}$ of Au-NR for population growth rate: 21 (95% confidence limit-CL: 10-32) μg/L and 16 (95% CL: 6.0-25) μg/L Au-NR, for *R. subcapitata* and *C. vulgaris*, respectively. In order to replace the nutrients and avoid medium and culture deterioration, these cultures were renewed at days 4, 8 and

12, following the procedure used by Venâncio et al. (2017). For this, an inoculum of the growing culture, always at a concentration of 10^5 cells/mL, was introduced in a freshly prepared suspension of Au-NR in MBL medium at the corresponding $EC_{5,72h}$. Exposure conditions were maintained during the 16 days, at 20±1ºC with continuous cool-white fluorescent light (fluorescent light tubes: OsramL36W/10; 100 μE/m2/s). During the assays, all plates were re-suspended for a few minutes every day on an orbital shaker to avoid algae settlement and subsequent shadow effects on their growth. To assess the sensitivity of the algae over the 16 days of exposure, growth inhibition assays were performed with inocula sampled at days 4, 8, 12 and 16 (D4, D8, D12 and D16) and compared with the sensitivity of the initial inoculum (not exposed to Au-NR, D0, data obtained in the assays of section 2.4). The procedures used to perform the 72h-growth inhibition assays were the same as those used for assays of section 2.4 and followed the OECD Guideline 201 (OECD, 2004). All the four inocula (D4 to D16) of *R. subcapitata* and *C. vulgaris* were exposed to the same concentrations of Au-NR and of CTAB as those tested with D0 (in section 2.4). All assays were conducted in 24-wells microplates at 23±1°C under continuous light of 4000lx, using the same experimental design as that described in section 2.4. The effects of CTAB were also evaluated in similar conditions.

2.6. Data analysis

For the tests with *R. subcapitata* and *C. vulgaris*, the effective concentration inducing 50, 20, 10 and 5% ($EC_{50,72h}$, $EC_{20,72h}$, $EC_{10,72h}$ and $EC_{5,72h}$ respectively) of yield and population growth inhibition rate was calculated with STATISTICA 8.0 software™ (Zar, 1999) by fitting the data to a logistic model. After confirmed the ANOVA assumptions (Kolmogorov–Smirnov test for normality of data, and Bartlett's test for homoscedasticity of variance), a two-way ANOVA analysis of variance were performed to determine if there were significant differences among treatments, inocula (D0 to D16) and interaction between factors. The two-way ANOVA was followed by the multicomparison Holm-Sidak test. All analyses were done using the SigmaPlot 11.0 software™.

The ImageJ software was used to calculate the mean size value of the gold nanorods for each concentration.

3. Results

3.1. Characterization of gold nanorods dispersions

The optical absorption spectrum of the Au-NR stock colloid exhibited two absorption bands centered at 510 nm and 780 nm, corresponding respectively to the transverse and longitudinal modes of localized surface plasmon resonances (LSPR) (Fig. 1). The average values (± standard deviation) of zeta potential, conductivity, hydrodynamic diameter, measured at pH = 7, in the stock dispersion were as follows: +71.6 mV (±10.9), 0.174 mS/cm (±0.0017), 29.88 (±0.70) nm, respectively (Table 1). As expected, the high positive surface charge is consistent with the presence of a CTAB bilayer, which is coating the nanorods, thus conferring stability to the colloid (Nikoobakht and El-sayed, 2001).

The Au content measured for the tested colloids (13, 35 and 29 μg/L) was systematically lower than the corresponding values of the nominal concentration (22, 53 and 90 μg/L) (Table 1). Also, the concentration of total Au measured in the dispersions decreased with time, with the exception of the nominal concentration 90 μg/L, for which an increase was observed (0h: 29μg/L and 72h: 34μg/L; Table 1). The conductivity values ranged from 0.461 to 0.501 mS/cm (Table 1). As for the zeta potential, the values measured at 0h and 72h, on the tested concentrations, were lower than the zeta potential of the stock dispersion (both comparing with the value provided by the supplier or the valued measured in this study by ELS; Table 1) and were less than 30mV. Although a negative surface charge was initially observed for Au-NR dispersed in MBL (time 0h), it became positive after a period of 72h, at the nominal concentrations of 53 and 90 μg/L (Table 1). The hydrodynamic size of the $EC_{5,72h}$ and of the two highest tested concentrations of Au-NR was much higher (≥631.9 nm) than the primary size reported by the supplier (length=38 nm) and increased with concentration, achieving the highest value at 90 μg/L (Table 1). Also, the hydrodynamic size

of Au-NR increased with exposure time, giving a value at least 2-fold higher at 72h comparatively to 0h (Table 1). The size of Au-NR measured by TEM (Table 1; Figure 2) suggests values lower than those measured by DLS, though, in general, were also higher than the length reported by the supplier. These size values also increased with time, specially at the $EC_{5,72h}$ where it was 2-fold higher at 72h comparatively to 0h. The polydispersity index was high (≥0.573) for the tested Au-NR concentrations. For this reason, the presented hydrodynamic values are only indicative of the observed broad dispersion in size (Table 1). This parameter also registered an increase with time at concentrations 22 and 53 µg/L. The morphology of the nanorods in the MBL medium did not change significantly from 0h to 72h period time (Fig. 3).

3.2. 72h-growth inhibition assays with microalgae (D0)

The Au-NR caused a significant reduction in yield and population growth rate of *R. subcapitata* at concentrations above 11 and 32 µg/L, respectively (Fig. 4A, 5A; $p<0.001$). The values of Au-NR concentrations causing 20% and 10% of effect in this alga were: 19 (95% CL: 8-29) µg/L and 11 (95% CL: 2-20) µg/L for yield and 64 (95% CL: 51-77) µg/L and 36 (95% CL: 26-46) µg/L for population growth rate (Table S1). The $EC_{50,72h}$, could only be computed for yield (48 µg/L with an 95% CL: 35-61 µg/L), since for population growth rate the highest observed effect was below 50% (Fig. 5A).

Exposure to CTAB solely (55 mg/L) significantly reduced yield (35±19 %) and population growth rate (8.5±5.7%) ($p<0.001$) of *R. subcapitata*, but, the percentages of effect were lower than those observed at the highest tested Au-NR concentration (with CTAB at a concentration matching the ones tested as CTAB solely): 55±17 % for yield and 16±6.9 % for population growth rate.

For *C. vulgaris*, a significant reduction in yield and population growth rate was also observed at concentrations similar to those reported for *R. subcapitata*: above 14 and 24 µg/L, respectively (Fig. 4B, 5B; $p<0.001$). The concentrations of Au-NR causing 50%, 20% and 10% reduction in

yield of *C. vulgaris* were: 71 (95% CL: 52-89) µg/L, 13 (95% CL: 6-20) µg/L and 5 (95% CL: 1-9) µg/L, respectively. For population growth rate only the $EC_{10,72h}$ could be computed since the observed effects never exceeded 13%: 52 (95% CL: 35-68) µg/L.

Chlorella vulgaris exhibited a slightly higher tolerance to CTAB than *R. subcapitata* since, though similar percentages of effect were observed (35±12% for yield and 8±4% for population growth rate) those occurred at a higher concentration of this chemical (94 mg/L). As well, the percentages of effects caused by CTAB solely were lower than those provoked by the highest tested Au-NR concentrations (50±1.5 % for yield and 12.0±0.53 % for population growth rate).

3.3. Long-term exposure of microalgae to gold nanorods

The effects of Au-NR on R. subcapitata yield and growth rate varied across exposure time (Figs. 4 and 5). After exposure for four and twelve days (D4 and D12) to the $EC_{5,72h}$, the sensitivity of *R. subcapitata* to Au-NR increased, as shown by the significant effects in yield, comparatively to the control, observed at concentrations equal or higher than 11 µg/L (Fig. 4 and 5; $p \leq 0.001$). Though, at D8, significant decreases in yield were only observed at concentrations above 24 µg/L (Fig. 4 and 5; $p \leq 0.001$), higher percentages of decrease (63 to 83 %), relatively to the respective control, were registered than at D0 (25 to 55 %) and D4 (54 to 57 %). As well, higher reduction in yield were observed in D16 (50 to 76 %) comparatively to D0 (25 to 55 %), at concentrations above 14 µg/L. The concentrations of Au-NR causing 10%, 20% and 50% of effect in yield were above 3.1, 6.9 and 19.9 µg/L, respectively (Table S1). Exposure to 55 mg/L of CTAB, caused a significant decrease in yield at D4, D8 and D16 ($p \leq 0.003$): 69, 58, 49 %, respectively. While not statistically significant ($p=0.256$), a decrease of 16% in yield was observed at D12.

Although a different pattern of responses was observed over exposure time, the results of population growth rate also suggest an increase of sensitivity of *R. subcapitata* to Au-NR after long-term exposure. At D4, D12 and D16 a significant population growth rate inhibition was observed at concentrations lower (<31 µg/L) than those for D0 (≥40 µg/L) (Fig. 5; $p \leq 0.001$).

Although a tendency for a decrease in population growth rate was observed for D12, no significant differences were observed in comparison to the respective control while increasing the concentration of Au-NR (Fig. 5; $p>0.150$). The concentration causing 10, 20 and 50% of population growth inhibition was above 11.4, 53 and 53 μg/L, respectively (Table S1). Exposure to 55 mg/L of CTAB, caused a significant decrease in population growth rate at all sampled inocula ($p<0.001$) except for D12 ($p=0.279$): 20, 21, 3.5 and 16 %, for D4 to D16 respectively.

Contrasting to *R. subcapitata*, for *C. vulgaris* the sensitivity to Au-NR tended to decrease, after long-term exposure. After exposure for just to the $EC_{5,72h}$, no significant differences were observed in yield for all concentrations comparatively to the respective control (Fig. 4 and 5; $p=0.054$). A similar result was observed for D12 and D16. However, at D8, a significant decrease was observed in concentrations 40, 53 and 90 μg/L (Fig. 4 and 5; $p=0.020$). The concentrations of Au-NR causing 10%, 20% and 50% of effect in yield were above 23, 49 and 90 μg/L (Table S1). Exposure to 94 mg/L of CTAB, induced a decrease in yield for all inocula ($p\leq0.022$): 25, 44, 64 and 48% (for D4 to D16, respectively).

Significant differences in population growth rate, between Au-NR concentrations and control, were only observed for D4 and D8 at concentrations above 40 μg/L (Fig. 5; $p<0.05$). No significant reduction in population growth rate was registered at D12 and D16 (Fig. 5; $p>0.228$). The concentrations of Au-NR causing 10%, 20% and 50% of effect in population growth rate were above 90 μg/L (Table S1). Exposure to 94 mg/L of CTAB induced a significant decrease in population growth rate for all inocula ($p\leq0.042$): 6, 10, 21 and 12%.

4. Discussion

The content of total Au in the several tested samples was lower than the respective values expected from the nominal concentrations, with recovery rates < 65%. Low recovery rates for Au of nanoparticles have been reported by other published works. Using ICP measurements, Allabashi et

al. (2009) have also reported Au contents below the nominal values in suspensions of commercial gold nanoparticles with primary sizes of 5 and 20 nm, with recoveries ranging from 74 to 75.6%. The same authors, reported recovery rates of 62.6% for samples of gold nanoparticles (with primary sizes > 20 nm) that were previously digested with aqua regia. Furthermore, Barreto et al. (2019a,b) reported measured concentrations of total gold lower (92%) than the nominal concentrations, by using gold nanoparticles with primary sizes of 40 nm in seawater. These authors hypothesized that such low recovery rates could be due to the occurrence of aggregation of the nanoparticles in the test media.

For the concentrations of Au-NR tested in MBL medium, the values of hydrodynamic diameter (DLS) increased relatively to the stock suspension and throughout exposure period. This has also been reported by other works; Lopes et al. (2012) measured the hydrodynamic diameter of gold nanorods in two different mediums: Milli-Q water (low ionic strength) and ASTM (high ionic strength) and observed an increase in size from 52 to 308 nm, respectively. The increase in the hydrodynamic diameter registered in the present work, does not necessarily mean that the Au-NR are aggregating (Liu et al., 2012), and could be a result of: i) the DLS apparatus using a fixed DLS angle, which when applied to the rods (non-spherical shape) may not correspond to the real size; (ii) MBL medium having vitamins, which may interact with the surface of Au-NR (Thioune et al., 2013). The values of zeta potential remained in an unstable range. MBL medium has a high ionic strength, in these conditions the electrical double layer at the NR surface may become thinner, with a decrease in the zeta potential, thus, lowering the repulsion between the nanoparticles in suspension.

Raphidocelis subcapitata showed a slightly higher sensitivity to the Au-NR coated with CTAB than *C. vulgaris*. In addition to such higher sensitivity to Au-NR, overall *R. subcapitata* became more sensitive to the Au-NRs after long-term exposure, while *C. vulgaris* was able to acquire an increased tolerance to this NR. The different responses of the two algae may be related

to the different surface area:volume ratios. Based on the size of the two microalgae (given by Nygaard et al., 1986; Geiger, 2014; OECD, 2011) and in the formulas to compute biovolumes and surface areas (Sun and Liu, 2003) the ratio surface area:volume computed for *R. subcapitata* ranges approximately from 5.83 to 199, being higher than those computed for *C. vulgaris*, between 1.5 and 6. This is inline with data reported in the scientific literature, which states that cells with large surface area to volume ratios tend to be more sensitive than those with smaller ratios (Levy et al., 2007). Furthermore, *R. subcapitata*, by exhibiting a higher surface area than *C. vulgaris*, could exhibit more binding sites and receptor–ligand interactions at the cell membrane level promoting a higher biding of Au-NR and a higher internalization of Au-NR (Schwab et al., 2011; Ma and Lin, 2013). Wang et al. (2013) reported aggregates of CTAB coated Au-NR attached to cell membranes and also its internalization by A549 cells. In addition, the consistent higher sensitivity of *R. subcapitata* relatively to *C. vulgaris* to Au-NR could be associated with the fact that *C. vulgaris* tend to form aggregates more easily than *R. subcapitata* (Environmental Protection Series, 2007; Fisher et al., 2016). These aggregates may reduce the exposure of inner cells of the algae-aggregate to the Au-NR, thus, reducing toxicity. The highest sensitivity shown by *R. subcapitata* to Au-NR is inline with the results obtained by Galindo (2014). These authors studied the effects on growth rate of Au-NR capped with CTAB (diameter=10 nm; length=35 nm) for *R. subcapitata* and *C. vulgaris* after 72h of exposure, reporting values of $EC_{20,72h}$ of 59.7 μg/L for *R. subcapitata* and of 95.2 μg/L for *C. vulgaris*, showing similar results to the ones obtained in the present study ($EC_{20,72h}$ of 39 and 79 μg/L, respectively). Additionally, other works have reported a higher tolerance of *C. vulgaris* to chemicals comparatively to *R. subcapitata*. Sohn et al. (2015) evaluated the effects of carbon nanotubes in the growth rate of *R. subcapitata* and *C. vulgaris*. The $EC_{50,72h}$ was higher for *C. vulgaris* ($EC_{50,72h}$ = 24.06 mg/L) than *R. subcapitata* ($EC_{50,72h}$ = 18.32 mg/L), which was thus more sensitive to carbon nanotubes. However, the author did not exploit these differences. Venâncio et al. (2017) studied the effects of NaCl and seawater in the growth rate of these same microalgae,

reporting $EC_{25,72h}$ of 4.63 and 10.3 mS/cm for *R. subcapitata* and of 6.94 and 15.4 mS/cm for *C. vulgaris*. The highest sensitivity of *R. subcapitata* was justified by the authors due to differences in their morphologies and physiological aspects. They also argue that *C. vulgaris* can form colonies as a result of the attachment of daughter cells during cell division and *R. subcapitata* are usually solitary, which may be disadvantageous, since each cell has direct contact with the salt solution.

Relatively to the CTAB (capping agent of the Au-NR), the tested concentrations induced significant toxicity to both algae species, but at a lower intensity than the toxicity caused by the highest Au-NR tested concentration (with the matching CTAB concentration). Based on these results, it is hypothesized that the toxicity observed at the highest tested concentration of Au-NR was caused by the presence of the two chemicals (either independently or through interactions, one influencing the toxicity of the other) and not only by CTAB. Other works have already reported the toxic effects of CTAB to biota at much lower concentrations than the ones tested here. For example, Mesquita et al., (2017) reported that a CTAB concentration of 2.92 mg/L induce 100% mortality in embryos of *Danio rerio* after and exposure of 30 min. The effects of CTAB on the survival of *D. magna* have also been studied, Sandbacka et al. (2000) observed 50% mortality in *D. magna* exposed to 0.058 mg/L CTAB, while Bozich et al. (2014) reported mortalities of 93% and 100% at CTAB concentrations of 10 and 50 µg/L, respectively. Furthermore, it was observed that the long-term exposure to the respective $EC_{5,72h}$ for Au-NR (capped with CTAB) increased the sensitivity of the two tested algae to CTAB, as a higher percentage of effect was observed in yield and population growth rate over exposure time. The surfactant CTAB is the most used to synthesize Au nanorods, as it controls the growth of Au into rod-shaped particles and prevents the aggregation of the NR. However, toxicity data obtained in the present work jointly with those already available in the literature reinforce the need to develop surrogates to CTAB, with similar efficiencies, in the production of Au-NR or to promote surface exchange reactions that use such alternative capping agents.

The long-term effects of nanoparticles, namely Au-NR, to biota have not been much studied, and no works have been published with microalgae. In the present work, the long-term exposure to Au-NR caused intensification of the effects observed over time in yield and population growth rate for *R. subcapitata* while a decrease in effects was observed for *C. vulgaris*. This pattern of response was also observed for these two species of microalgae exposed to other stressors. Venâncio et al. (2017) reported a decrease in the sensitivity of *C. vulgaris* to NaCl after a long-term exposure while reporting an increased sensitivity of *R. subcapitata* to this salt, after the same exposure period. However, when the two algae were exposed to seawater, both increased their sensitivity to this water after a long-term exposure (Venâncio et al. 2017).

Studies published with other species report both the maintenance and the increase of sensitivity to nanoparticles. Moon et al. (2017) evaluated the effects of long-term exposure to Au-NR (colloids; size between 8.5 and 12 nm) on *Caenorhabditis elegans*, after continuous or intermittent exposure through Au-NR contaminated food. Both continuous and intermittent exposure to Au-NR caused adverse effects in reproduction. They observed a decrease in reproduction and an increase in abnormalities with time. But, in both types of exposure (continuous or intermittent), *C. elegans* did not acquired and increased tolerance to Au-NP exposure. Ma et al. (2016) studied the effects on plant growth and the oxidative stress after long-term exposure to cerium oxide nanoparticles (spherical CeO2-NP; size=20 nm) over a range of concentrations (0–1000 mg/L) on the terrestrial plant *Brassica rapa*. During the exposure, CeO2-NP caused a significant reduction of seed yield and seed quality, which is critical for continued food security, altered physiological and biochemical responses in subsequent generations of plants, caused greater reductions in plant growth and development and an increase on the ROS production, i.e. increased the sensitivity of the plants to the NP.

Though no studies on the long-term effects of Au-NR exist for microalgae, the effects that such type of exposure on this group of organisms to some chemicals (including metals) has already been

reported, and no clear pattern of increasing sensitivity or tolerance to the chemical can be identified. For example, Muyssen and Janssen (2001) exposed *R. subcapitata* and *C. vulgaris*, to a concentration of 65μg/L of cationic zinc and observed if these algae were capable to acquire an increased tolerance to this metal comparatively to the initial inoculum (not exposed to increased zinc concentrations). They observed that both algae species acquired and increased tolerance to zinc through physiological acclimation. However, Stachowski-Haberkorn et al. (2013) evaluate the capacity of microalgae *Tetraselmis suecica* to develop long-term tolerance to the herbicide diuron. During the first 25 generations this alga exhibited a high sensitivity to this pesticide, by exhibiting a 2 to 2.5-fold increase in the doubling-time of growth when exposed to the pesticide. But, after being exposed for 25 to 32 generations to of 5 μg/L to diuron, *T. suecica* was able to tolerate the pesticide, showing doubling-time of growth similar to that in the control. Considering the above data, it is suggested that responses of biota to chemicals long-term exposure is both chemical and species dependent, which may lead to the need of performing specific assays to be used for ecological risk assessment frameworks.

Conclusion

The research reported here suggests that the long-term exposure of the two microalgae to Au-NR resulted in distinct effects in yield and population growth rate over time. These observed differences were species dependent; while for *R. subcapitata* an increase in effects occurred with prolonged exposure, for *C. vulgaris* a decrease was observed. These results suggest that the standard 72h growth inhibition assay may under- (for *R. subcapitata*) or overestimate (for *C. vulgaris*) the long-term effects that Au-NR may pose to microalgae, highlighting the need to assess long-term effects of NRs when conducting risk assessment approaches. Furthermore, since different responses were observed for the two species, under an environmental risk assessment framework it may be needed

to consider the sensitivity of different species from the same taxonomic and functional level to drive environmental safety levels for these NR.

Acknowledgements

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Figure 1 – UV-VIS-NIR spectrum of the stock dispersion of gold nanorods showing the transverse and longitudinal localized surface plasmon resonance (LSPR) bands at 514 and 780 nm, respectively.

Figure 2 – TEM image of the Au nanorods deposited from the stock colloid onto a carbon coated Cu grid.

Figure 3 – Gold nanorods in MBL medium under the transmission-electron microscope: a and b – EC5,72h (22 µg/L) at 0h and 72h, respectively. c and d - highest tested concentration for *Raphidocelis subcapitata* (53 µg/L) at 0h and 72h, respectively. e and f - highest tested concentration for *Chlorella vulgaris* (90 µg/L) at 0h and 72h, respectively.

Figure 4 – Average of yield of *Raphidocelis subcapitata* (A) and *Chlorella vulgaris* (B) after being exposed for a period of 72h, to several concentrations of gold nanorods. D0 to D4 represent the inoculum obtained from cultures exposed at the respective $EC_{5,72h}$ of Au-NRs (21 µg/L for *R. subcapitata* and 16 µg/L for *C. vulgaris*) for zero (D0), 4 (D4), 8 (D8), 12 (D12) and 16 days (D16). Error bars represent the standard deviation. * means significant differences relatively to the control.

Figure 5 – Average of daily growth rate of the four generations of *Raphidocelis subcapitata* (A) and *Chlorella vulgaris* (B) after being exposed for a period of 72h, to several concentrations of gold nanorods. D0 to D4 represent the inoculum obtained from cultures exposed at the respective $EC_{5,72h}$ of Au-NRs (21 µg/L for *R. subcapitata* and 16 µg/L for *C. vulgaris*) for zero (D0), 4 (D4), 8 (D8), 12 (D12) and 16 days (D16). Error bars represent the standard deviation. * means significant differences relatively to the control.

Table 1: Physical-chemical characterization of gold nanorods dispersions. (±\s.d)

	$EC_{5,72h}$ (22 µg/L) (both microalgae)		**53 µg/L (*Raphidocelis subcapitata*)**		**90 µg/L (*Chlorella vulgaris*)**		**Stock solution (35 000 µg/L)**	
	0h	**72h**	**0h**	**72h**	**0h**	**72h**	**Supplier information**	**Measured in this study**
Concentration (µg/L)	13	9.6	35	19	29	34	-	-
Zeta Potential (mV) (pH ≈7)	-6.6(±10.2)	-14.1(±4.29)	-6.7(±9.01)	-12.4(±5.67)	6.5(±7.77)	-11.0(±6.05)	38	71.6(±10.9)
Hydrodynamic diameter (nm)	632(±46)	1834(±152)	673(±98)	1121(±153)	714(±16)	2323(±343)	10	30.81(±0.70)
Polydispersity index (PDI)	0.741	0.922	0.596	0.799	0.573	0.589	-	1.0

Highlights

- Long-term effects of gold nanorods (Au-NR) on two microalgae species were assessed.
- *Raphidocelis subcapitata* showed higher sensitivity to Au-NR than *Chlorella vulgaris*.
- The capping agent CTAB of Au-NR exerted toxicity on both microalgae.
- Effects of long-term exposure to low levels of Au-NR revealed to be species-dependent.
- Sensitivity of *R. subcapitata* to Au-NR increased with long-term exposure, while that of *C. vulgaris* decreased.

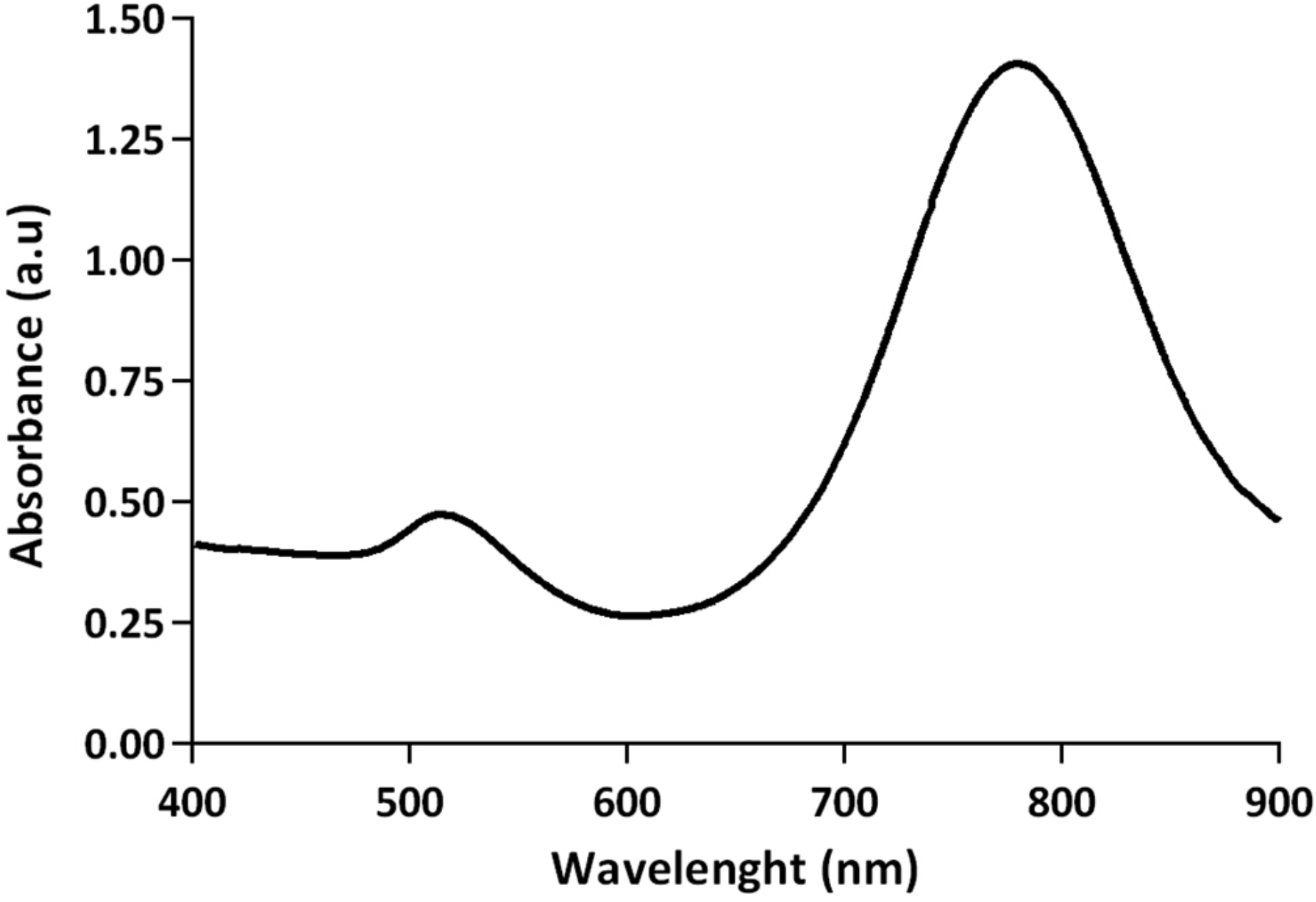
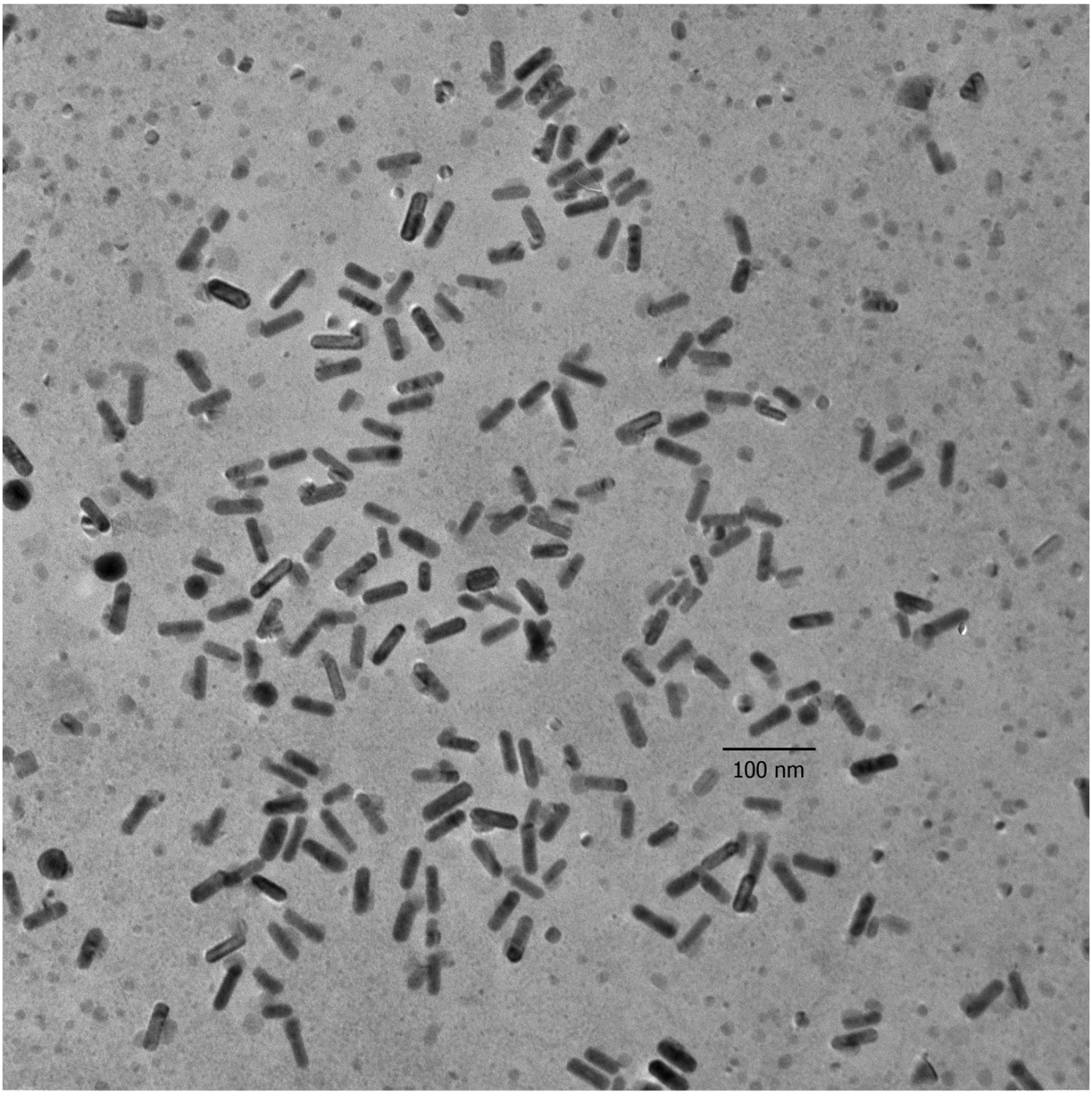

1.50
1.25
1.00
0.75
0.50
0.25
0.00
Absorbance (a.u)
400
500
600
700
800
900
Wavelenght (nm)

Figure 1



AuNR_1.tif
Print Mag: 152000x @ 7.0 in
TEM Mode: Imaging

100 nm
HV=300.0kV
Direct Mag: 20000x
University of Aveiro

Figure 2

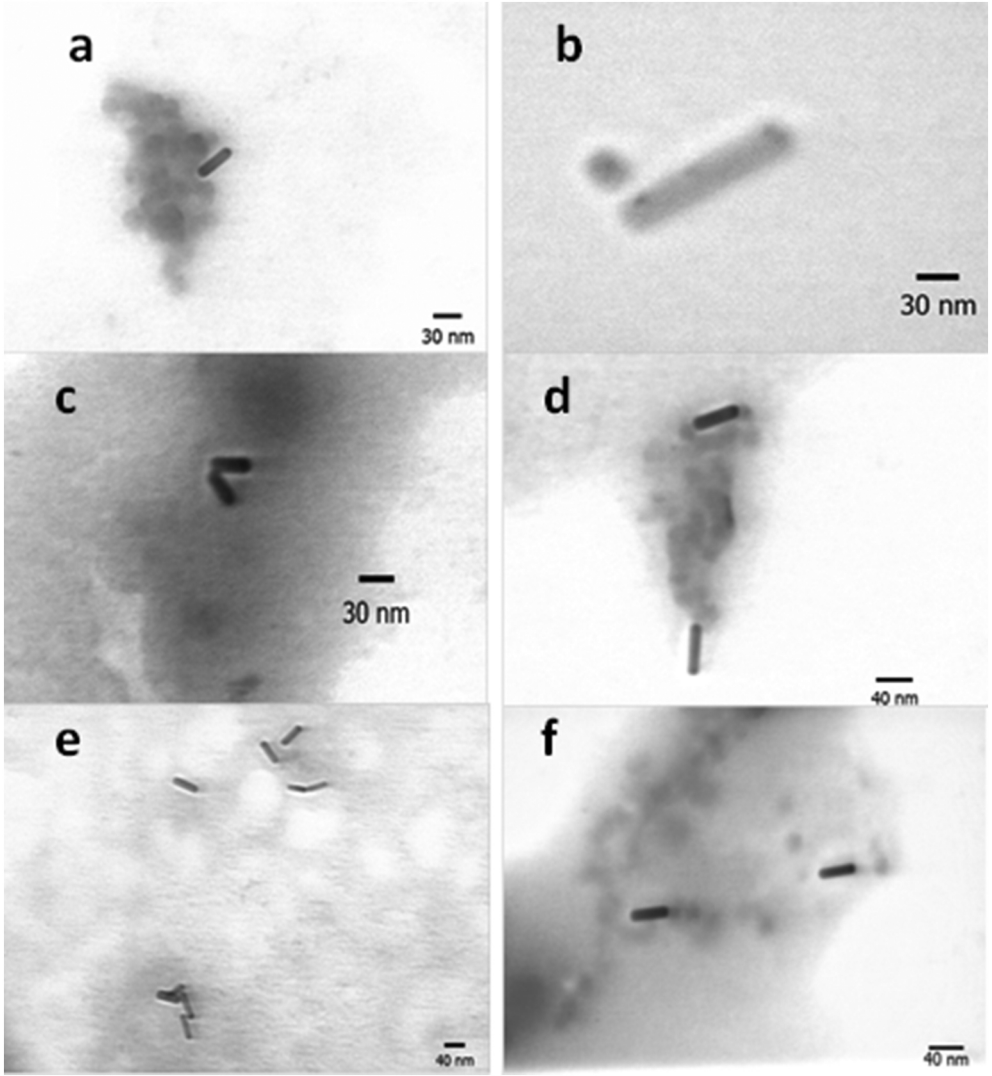
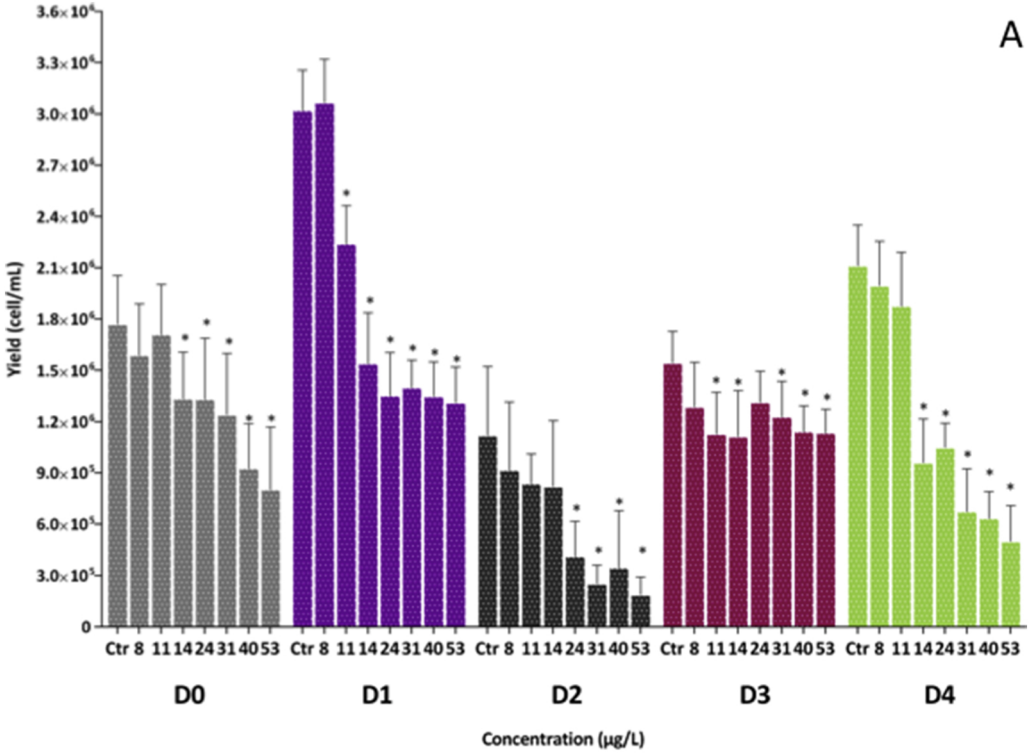


Figure 3


A
Yield (cell/mL)
Ctr 8 11 14 24 31 40 53
D0
D1
D2
D3
D4
Concentration (µg/L)

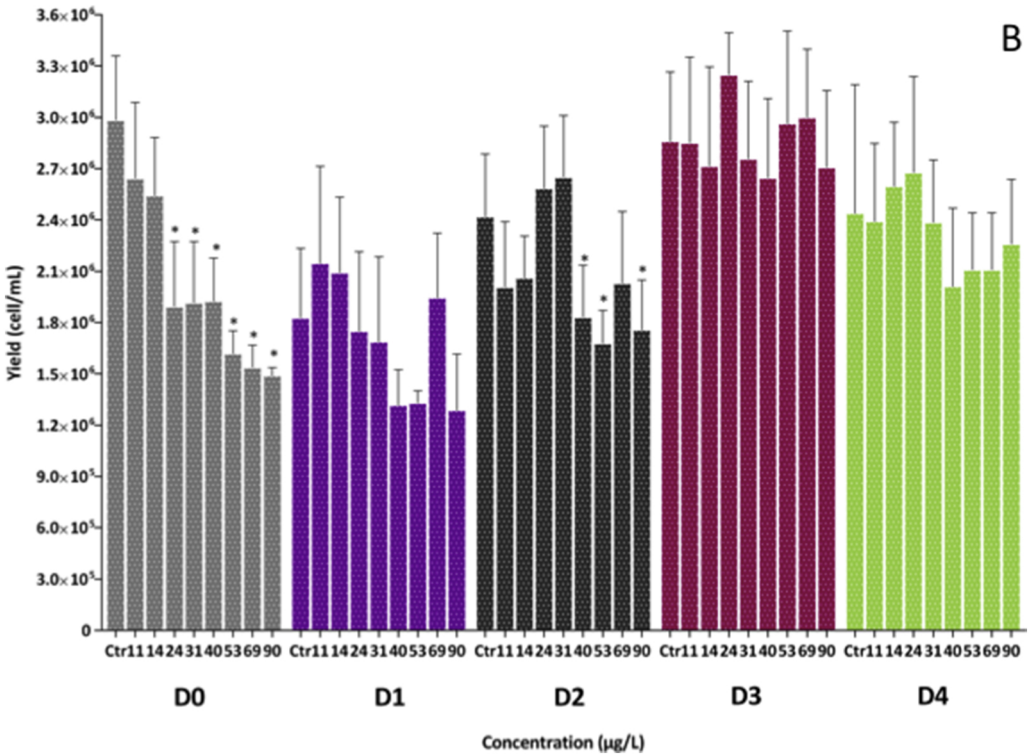

B
Yield (cell/mL)
Ctr 11 14 24 31 40 53 69 90
D0
D1
D2
D3
D4
Concentration (µg/L)

Figure 4

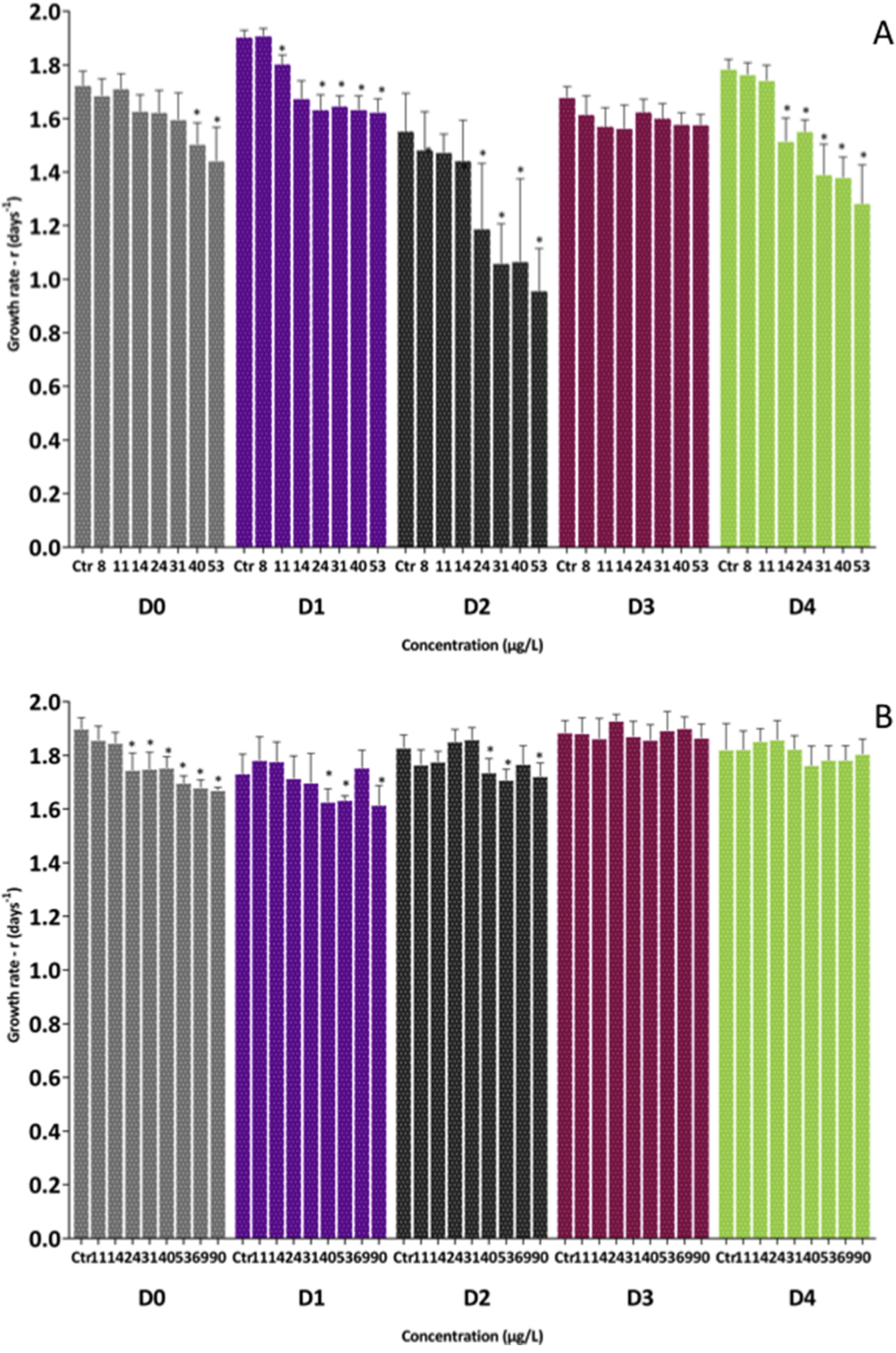

A
B
Growth rate - r (days-1)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Ctr 8 11 14 24 31 40 53
Ctr1114243140536990
D0
D1
D2
D3
D4
Concentration (µg/L)

Figure 5