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Exploring the impact of silver-based nanomaterial feed additives on green algae through single-cell techniques

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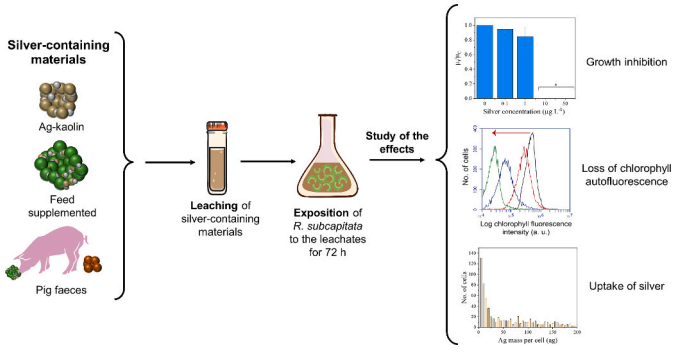
HIGHLIGHTS

- Study of effect of silver-based nanomaterials used as feed additives to algae
- Use of single cell techniques to study the effect on freshwater algae and silver uptake
- Effect of silver-based materials was exposure concentration and time dependent.
- Environmental impact for faeces of pigs fed with silver-based nanomaterial was low.

GRAPHICAL ABSTRACT



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ABSTRACT

Silver in its various forms, including dissolved silver ions (Ag^+) and silver nanoparticles (AgNPs), is a promising alternative to traditional antibiotics, largely used in livestock as feed additives and could contribute to the decrease and avoidance of the development of antibiotic resistance. The present study aims to assess the potential ecotoxicity of a silver-based nanomaterial (Ag-kaolin), the feed supplemented with the nanomaterial and the faeces since the latter are the ones that finally reach the environment. To this end, green alga *Raphidocellis subcapitata* was exposed to the extracts of Ag-kaolin, supplemented feed, and pig faeces for 72 h, along with Ag^+ and AgNPs as controls for comparison purposes. Given the complexity of the studied materials, single-cell techniques were used to follow the changes in the cell numbers and chlorophyll fluorescence by flow cytometry, and the accumulation of silver in the exposed cells by single cell inductively coupled plasma mass spectrometry (SC-ICP-MS). Changes in cell morphology were observed by cell imaging multimode reader. The results revealed a decrease in chlorophyll fluorescence, even at low concentrations of Ag-kaolin (10 μg L^{-1}) after 48 h of exposure. Additionally, complete growth inhibition was found with this material like the results obtained by exposure to Ag^+. For the supplemented feed, a concentration of 50 μg L^{-1} was necessary to achieve complete growth inhibition. However, the behaviour differed for the leachate of faeces, which released Ag_2S and AgCl

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alongside Ag^+ and AgNPs. At 50 μg L^{-1}, inhibition was minimal, primarily due to the predominance of less toxic Ag_2S in the leachate. The uptake of silver by the cells was confirmed with all the samples through SC-ICP-MS analysis. These findings demonstrate that the use of Ag-kaolin as a feed supplement will lead to a low environmental impact.

1. Introduction

During the 20th century, the use of antibiotics as feed additives in livestock to eliminate bacteria from the intestine was a common practice (Fondevila, 2010). They increase the absorption of nutrients and, thereby improve animal efficiency. Nevertheless, the antibiotic can pass the intestinal barrier and accumulate in the animal tissues, which would result in their subsequent consumption by humans and the possible development of antibacterial resistance. To circumvent this issue, the European Union, prohibited antibiotic use as feed additives for animals in 2003 (European Commission, 2003). Consequently, the necessity to explore alternative substances with antibacterial properties emerged. AgNPs have emerged as an alternative to antibiotics owing to their antibacterial properties (Zhang et al., 2018). The research on the incorporation of AgNPs into pig diets has shown promising results, demonstrating their efficacy in inhibiting bacterial reproduction and growth, thus demonstrating a positive impact (Fondevila, 2010; Fondevila et al., 2009). For example, a silver nanomaterial, known as *Argenta*, consisting of 60–100 nm AgNPs adsorbed on sepiolite, a phyllosilicate used as a common animal feed additive, with a concentration of 20–40 mg Ag kg^{-1}, has been shown to improve pig growth and intake by eliminating different bacteria in the ileum (Fondevila et al., 2009). The rise in such nanoparticle (NP) usage prompted research into their environmental risks. Previous studies employed life cycle assessment to evaluate NPs impact from their raw materials to their end-of-life, which includes the synthesis, distribution, use and disposal (Nizam et al., 2021; Temizel-Sekeryan and Hicks, 2020). This last step is important since it ensures the possible risks to the environment are taken into consideration. To evaluate these potential risks, it is essential to investigate both, the quantity, and the forms in which the nanomaterial is released in the environment on one hand and the potential effects to biota on the other. Among the different biota, microalgae have been employed to study the environmental impact of NPs owing to their sensitivity and rapid responsiveness (Lau et al., 2022; Nguyen et al., 2020). Numerous studies have explored the toxicity of silver and AgNPs in green microalgae, such as *Raphidocellis. subcapitata* (Chen et al., 2013; Falanga et al., 2020; Kleiven et al., 2019; Lee et al., 2022; Lekamge et al., 2020; Ribeiro et al., 2014, 2015), *Chlamydomonas reinhardtii* (Bijula et al., 2022; Chen et al., 2013; Guilleux et al., 2022; Liu et al., 2019; Xu et al., 2022; Zhao et al., 2021), or *Chlorella vulgaris* (Khoshnamvand et al., 2020; Mariano et al., 2020; Romero et al., 2020; Shen et al., 2023; Zhang et al., 2020). The results have indicated that the growth of *R. subcapitata* decreased with increasing concentration of both Ag^+ and AgNPs (Lekamge et al., 2020), with Ag^+ exhibiting greater toxicity than AgNPs, even at small sizes, such as 3–8 nm (Ribeiro et al., 2014). Additionally, a relationship between the toxicity of silver and the concentration of exposure has been established (Lekamge et al., 2020; Liu et al., 2020; Ribeiro et al., 2014). Specifically, it was found that 50 μg L^{-1} of Ag^+ completely inhibited algae growth, whereas a concentration of 50 μg L^{-1} of AgNPs resulted in 75 % growth inhibition. Furthermore, exposure of *Poterioochromonas malhamensis* to Ag^+ and AgNPs affected several metabolic pathways, such as nucleotides, amino acids, fatty acids, tricarboxylic acid, antioxidants, photorespiration or photosynthesis in a time-dependent manner (Liu et al., 2020). The size, shape and coating of the AgNPs have also been shown to affect their toxicity (Cao et al., 2021; Kleiven et al., 2019; Lekamge et al., 2020; Nam and An, 2019; Samal et al., 2024). Smaller-size AgNPs were more toxic than larger ones due to their higher surface area-to-volume ratio (Samal et al., 2024). For example, exposure to 5, 20 and 70 nm-sized NPs showed a stronger effect of smaller AgNPs on several metabolite expressions than larger ones in *C. vulgaris*. Nevertheless, the larger particles led to persistent down-regulation of aminoacyl- tRNA biosynthesis. Interestingly, when the cells were no longer exposed to AgNPs, a restoration of the level of the metabolites was observed for 5 and 20 nm-sized AgNPs (Shen et al., 2023). By contrast, 176 nm-sized AgNPs were more toxic than 16 nm AgNPs for *R. subcapitata* (Kleiven et al., 2019). The coating of AgNPs, for example by dissolved organic matter, affected the dissolution and aggregation of NPs and, therefore, their toxicity (Lekamge et al., 2020). Likewise, the presence of sulphide or chloride in the medium reduced the toxicity of the AgNPs (Lekamge et al., 2020; Ribeiro et al., 2014). These anions enhanced the uptake of silver (Fortin and Campbell, 2001; Lee et al., 2004). However, in the presence of thiosulphate, silver was less toxic, due to the formation of thiosulphate complexes inside the algae which remain intact (Lekamge et al., 2020). Other studies showed a decrease in the toxicity of silver in the presence of chloride, due to the formation of AgCl (Nguyen et al., 2020; Ribeiro et al., 2014) and its subsequent precipitation, leading to low bioavailability of silver (Ribeiro et al., 2014). These selected examples demonstrated significant progress in understanding the toxicity of silver-based materials on microalgae. However, the existing understanding has primarily been obtained from studies focussing on dissolved Ag^+ and pristine AgNPs. Yet, silver typically enters the environment as part of complex Ag-containing materials, such as feed additives. Therefore, assessing the impact of silver released from these nanomaterials would be more relevant.

In such a context, this study aims to investigate the ecotoxicity of a complex Ag-based nanomaterial, Ag-kaolin, as a feed additive for pigs. Considering the potential silver release into the environment via animal waste products, it is crucial to assess their toxicity. By using a combination of single cell techniques, the specific objective is to investigate the impacts of silver released from AgNPs-kaolin, both from the feed and faeces, on the growth, morphology, and chlorophyll fluorescence of the green alga *R. subcapitata*. This particular alga was chosen because the Organisation for Economic Co-operation and Development (OECD) recommends it as a suitable strain for conducting ecotoxicity tests (OECD, 2011). Additionally, single cell inductively coupled plasma mass spectrometry (SC-ICP-MS) was utilized to examine the uptake of silver by algal cells exposed to these complex materials.

2. Materials and methods

2.1. Ag-based materials

The nanomaterial based on silver used for the feeding of pigs was aluminosilicate, kaolin, on which AgNPs were adsorbed on their surface. Kaolin is usually used as a feed additive for pigs because of its benefits in growth promotion and ability to mitigate the effects of diarrhoea caused by *Escherichia coli* strains (Nadziakiewicz et al., 2022; Trckova et al., 2009). This nanomaterial was provided by Laboratorios ENOSAN (Zaragoza, Spain) and characterized in a previous work (Rodriguez-Garraus et al., 2022). In brief, the predominant size of the AgNPs was between 25 and 35 nm. The total concentration of silver in Ag-kaolin of 8320 ± 350 mg kg^{-1} was determined following the acid digestion with HNO_3 and analysed by flame atomic absorption spectrometry (FAAS) (Perkin Elmer FAAS, Model AAnalyst 200, Toronto, Canada). Ag-kaolin exhibits potent antibacterial activity against a wide spectrum of bacteria as demonstrated previously by (Pérez-Etayo et al., 2021). The material was not genotoxic, as it did not induce chromosome aberrations, gene

mutations or DNA damage in mouse lymphoma cells (Rodriguez-Garraus et al., 2022). Ultrapure water extract of this nanomaterial contained 30 % of the total silver present mostly as Ag^+. AgNPs represented <0.1 % of the total silver as was determined by SP-ICP-MS (Ben-Jeddou et al., 2024b).

The above described AgNPs-based material was added to pig feed in a proportion of 0.2 % (*w*/w). The total concentration of silver in feed was 12.6 ± 1.1 mg kg^{-1}, a value obtained by acid digestion with 7 mL of HNO_3 and 3 mL of HCl, using a microwave and ICP-MS determination (Perkin Elmer NexION 2000 ICP mass spectrometer, Toronto, Canada). The water leachate of the feed contained between 4.6 and 6.6 % of the total silver present in the feed, mostly as Ag^+. Only around 0.02 % of the total silver released was as particles-containing silver. The equivalent size of these particles was around 30 nm.

Pig faeces were collected in an in vivo experiment in which pigs were fed with the nanomaterial (Ben-Jeddou et al., 2024a). Two types of faeces, labelled as Ag20 and Ag200, were collected. The total silver concentration in faeces was 155 ± 30 and 1026 ± 178 mg kg^{-1} for Ag20 and Ag200 faeces, respectively. These values were obtained by ICP-MS measurements following the microwave-assisted digestion with 7 mL of HNO_3 and 3 mL of HCl (Ben-Jeddou et al., 2024a). A sequential extraction using tetrasodium pyrophosphate (TSPP) and Na_2S (Hong et al., 2021) was employed to obtain speciation of the different silver species in faeces (Jiménez et al., 2023). This sequential extraction, in addition to the analysis by inductively coupled plasma optical emission spectrometry (ICP-OES), was demonstrated to be a suitable alternative to the use of X-ray absorption near edge spectroscopy (XANES) for speciation analysis (Hong et al., 2021). This method is based on using TSPP and NH_3 to extract the fractions corresponding to Ag^+, AgCl and AgNPs and with Na_2S to extract the fraction of Ag_2S. The total silver present in every fraction was quantified by ICP-MS. It was found that silver in faeces was present as Ag_2S (44.8 ± 17.3 %), dissolved silver (34.2 ± 8.0 %), AgClNPs (23.3 ± 6.1 %) and AgNPs (6.59 ± 0.35 %). Additionally, after the leaching in ultrapure water, the concentrations of the released silver were 3.75 and 20.3 mg kg^{-1} for Ag20 and Ag200, respectively. The presence of Ag_2S species in the leachates of the faeces was also revealed by transmission electron microscopy energy dispersive spectroscopy (TEM-EDS) (Jiménez et al., 2023).

2.2. Chemicals

For the ecotoxicity studies, a standard solution of Ag^+ with a concentration of 1000 mg L^{-1} (Sigma-Aldrich, Sta. Louis, USA) was utilized. A commercial suspension of AgNPs with a size of 50 ± 4 nm, stabilized with citrate (Nanocomposix, San Diego, CA, USA) and with a concentration of 1.07 mg mL^{-1} (1.5×10^{12} particles mL^{-1}) was also used for comparative purposes. For the analysis by SC-ICP-MS, Ag^+ (994 ± 3 mg L^{-1}) standard solution (Sigma-Aldrich, St. Louis, USA) was employed for the construction of the calibration curves. For the determination of the transport efficiency, a microparticle size standard based on polystyrene (PS) monodisperse with a size of 10 ± 0.15 μm and 2 % of solid content (Fluka, Steinheim, Germany) was used. Ultrapure water (Milli-Q Advantage, Molsheim, France) was used for the preparation of all the solutions.

2.3. Bioassays with green alga R. subcapitata

2.3.1. Algal culture

The alga *R. subcapitata* was cultured in Tris-acetate-phosphate (TAP) medium (Table S1) starting with an inoculum of 5×10^4 cells mL^{-1}. Cultivation took place in a specialized incubator (Binder GmbH, Tutiilingen, Germany) with a temperature of 20.1 ± 0.1 °C and a light:dark cycle of 16:8 h. After 72 h of growth, the algae were centrifuged (1300 ×*g* for 10 min) and the resulting pellet containing the cells was collected. The pellet was then resuspended in 5 mL of modified TAP exposure medium (Table S2), and the density of cells was quantified by flow cytometry (FCM, BD Accuri C6 Plus flow cytometer, BD, New Jersey, USA). The initial cell density in the exposure experiment was 3×10^5 cells mL^{-1}.

2.3.2. Algae growth inhibition

The algal growth inhibition tests of the leachate of different Ag-based materials were carried out according to the OECD guidance document (OECD, 2020). The leachates were prepared in ultrapure water using 1 h mixing for Ag-kaolin and feed, and 48 h for the faeces. The solid residue in the leachates was separated by centrifugation (4700 ×*g* for 15 min) with a Multifuge X4 Pro Centrifuge (Thermo Fisher, New Jersey, USA). The appropriate amount of the leachate supernatant was added to the cultures to study their effect on algal growth. The cell numbers and the cell fluorescence were followed by FCM for 72 h. The gating strategy was designed to discriminate between the algal cells and other particles eventually present in the medium of these complex materials (Figs. S1 and S2). The growth inhibition was calculated following Eq. S1 in SI. In parallel, to monitor the changes in the cell morphology, the cultures were analysed by a cell imaging multimode reader (Cytation 5 Image Reader, Biotek, Santa Clara, USA) with the software Gen 5 version 3.11).

2.3.3. Silver uptake by the exposed cells

The uptake of silver by the algae was determined following the 72 h-exposure of the algae to 10 μg L^{-1} of total silver the sample leachates by SC-ICP-MS (Perkin Elmer NexION 2000 (Perkin Elmer, Toronto, Canada) with an Asperon™ linear pass spray chamber (Perkin Elmer, Toronto, Canada) and a flow-focusing nebulizer (Ingeniatrics, Sevilla, Spain)). At the end of 72 h-exposure, the algae were freeze-dried and then, resuspended in 50 mL of ultrapure water and introduced directly to the instrument to study the uptake of silver. Before the analysis, the performance of the SC-ICP-MS instrument was optimized with a solution with a concentration of 1 μg L^{-1} of Be, Ce, Fe, In, Li, Mg, Pb and U, provided by the manufacturer. Besides, the acquisition parameters for silver were also optimized with a standard solution of Ag^+ of a concentration of 1 μg L^{-1} in 1 % of HNO_3, prepared daily. The conditions used for the analysis of the algal suspensions are provided in Table S3. The sample flow rate and the transport efficiency were also obtained. The flow rate was calculated by gravimetry, obtaining a value of 13 μL min^{-1}. Furthermore, the transport efficiency was calculated following the frequency method procedure (Pace et al., 2011). For that, a suspension of 3.5×10^7 particles mL^{-1} of 10 μm of PS particles was used. A calibration curve of Ag^+ in 1 % HNO_3 with concentrations between 0 and 1 μg L^{-1} was also used. The transport efficiency obtained was 10.0.

2.4. Data analysis

All the experiments were carried out in triplicate. To test for significant differences between the treatments, a one-way analysis of variance (ANOVA) was performed. A p-value <0.05 was considered statistically significant. The Tukey Honestly Significant Difference was performed as a post-hoc test. For the representation of SC-ICP-MS data, Origin 9.6.5.169 (OriginLab Corporation, MA, Northampton, USA) software was utilized.

3. Results and discussion

3.1. Effect of the different silver-containing materials on the algal growth

Algae were exposed to the leachate of the different Ag-based materials - Ag-kaolin, feed supplement and pig faeces - and analysed by FCM for 72 h. In Fig. 1 the ratio between the growth rate of the exposed and the unexposed cells (μ_t/μ_c) for the different silver exposure concentrations is represented. Exposure to 10 μg L^{-1} of silver from Ag-kaolin (Fig. 1a), resulted in complete growth inhibition. Since most of the silver released from Ag-kaolin was as Ag^+ (Ben-Jeddou et al., 2024b), the observed effect is consistent with the results obtained for the exposure to

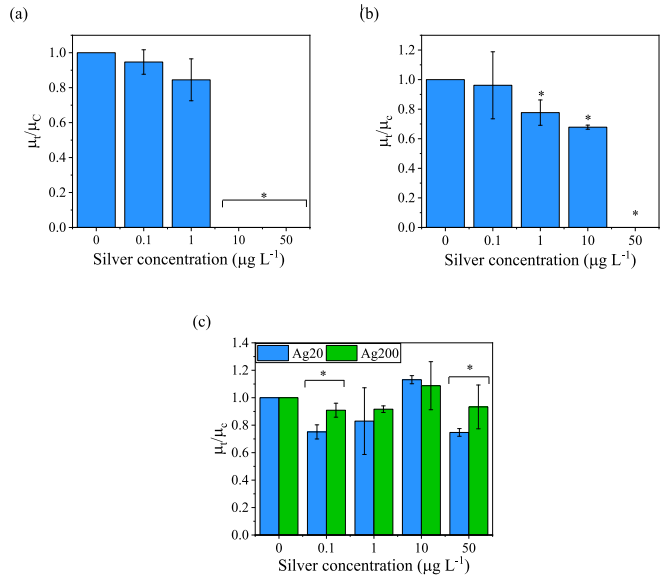

Fig. 1. Ratio between the growth rate of the exposed and the unexposed cells (μ_t/μ_c, where μ_t is the average growth rate of the treated cells and μ_c is the average growth rate of the unexposed cells) for different silver-containing materials. (a) Leachate of Ag-kaolin (b) Leachate of feed (c) Leachate of Ag20 and Ag200 faeces (mean ± standard deviation, $n = 3$). Asterisks indicate significant differences between the different exposure concentrations compared with the unexposed cell obtained by one-way ANOVA followed by a Tukey test ($p < 0.05$, n = 3). Ag20 faeces corresponds to faeces with a silver concentration of 155 ± 30 mg kg^{-1} and Ag200 faeces to faeces with a concentration of 1026 ± 178 mg kg^{-1} of silver.

dissolved Ag^+, where at a concentration of 10 µg L^{-1}, the inhibition was complete. The concentration inducing growth inhibition effect in 50 % of the algal population, EC_{50}, for Ag^+ would be between 1 and 10 µg L^{-1}, corresponding to the hazard ranking level "very toxic", according to the EU-Directive 93/67/EEC classification (CEC, 1993). For comparison, exposure to a suspension of pristine AgNPs at a concentration of 10 µg L^{-1} (Fig. S3b), resulted in minimal growth inhibition of 1.35 ± 0.17 % and for 100 µg L^{-1} a growth inhibition of 13.6 ± 0.8 %.

These findings agree with the reported literature demonstrating that Ag^+ was more toxic than AgNPs (Khoshnamvand et al., 2020; Kleiven et al., 2019; Lekamge et al., 2020; Ribeiro et al., 2014). Indeed, for *R. subcapitata*, a concentration of 50 µg L^{-1} of Ag^+ was needed to cause growth inhibition of almost 100 %, whereas for AgNPs, a minimum concentration of 100 µg L^{-1} was necessary to observe an inhibition of 100 % (Ribeiro et al., 2014). Similar results were found for EC_{50} values. For Ag^+ the EC_{50} obtained for *R. subcapitata* was 7.09 µg L^{-1}, while the EC_{50} was 9.74 µg L^{-1} for 16 nm AgNPs and 24.18 µg L^{-1} for 176 nm AgNPs (Kleiven et al., 2019). For another green alga *C. reinhardtii*, at 300 µg L^{-1} of AgNPs, the growth was inhibited completely, and at 100 µg L^{-1}, the change of the growth (μ_T/μ_C) was <0.5. However, for the brown-yellow alga *Ochromonas danica* the effect on the growth was lower, for 100 µg L^{-1} of AgNPs, μ_T/μ_C the change of the growth is >0.5 (Huang et al., 2019).

For the feed leachate, it was observed that the silver concentration in feed leachate needed to achieve 100 % inhibition was 5 times higher (at 50 µg L^{-1}) than the concentration in Ag-kaolin treatments (Fig. 1c). Although most of the silver released from the feed was in the form of Ag^+, like Ag-kaolin, the toxicity to algae was lower for the feed than for the nanomaterial itself. This result could be attributed to differences in the matrix of the leachate of feed and Ag-kaolin. Indeed, the presence of a higher amount of organic matter in the leachate of feed than in the leachate of Ag-kaolin would result in a decrease in the toxicity of silver probably due to the complexation of Ag^+, stabilization of AgNPs (Hu et al., 2018) and avoiding the aggregation of cells (Khoshnamvand et al., 2020).

In the case of the faeces leachate, it was observed that silver concentration higher than 50 µg L^{-1} was necessary to obtain 100 % growth inhibition. At exposure with 50 µg L^{-1}, the ratios of growth rates between the exposed and the unexposed cells were 0.74 and 0.93 for the Ag20 and Ag200 faeces (Fig. 1c), respectively. The algal growth inhibition was lower than the obtained for Ag-kaolin and the supplemented feed. The lower toxicity could be explained by the different physicochemical speciation of the faeces leachate. Indeed, the presence of Ag_2S and AgClNPs in the leachate of faeces was demonstrated, with the presence of S in the TEM-EDS spectrum and the sequential extraction with TSPP and Na_2S (Jiménez et al., 2023). In the literature, it has been demonstrated that exposure to Ag_2S induced lower toxicity than Ag^+ (Kang and Park, 2021). The formation of this silver species was responsible for the reduction of the uptake of Ag^+ in numerous organisms and the decrease in its toxicity (Jagadeesh et al., 2015; Rajan et al., 2022). The inhibition of different enzyme activity of <20 % of *Mougeotia sp*. cells was obtained during exposure with 100 µg L^{-1} of Ag_2S (Jagadeesh et al., 2015). Moreover, the adsorption of Ag^+ on the different particles released from the faeces could significantly reduce the uptake of the silver by the cells (Jiménez et al., 2023). AgCl was also found as less toxic than Ag^+ since AgClNPs would be less bioavailable for algae (Yue et al., 2017). In addition, both for Ag20 and Ag200, with a concentration of 10 µg L^{-1}, the μ_t/μ_c ratio was higher than 1. This could be due to a hormesis effect. Hormesis is a stress-protective response, which shows stimulatory or inhibitory effects depending on the contaminant exposure concentrations (Agathokleous et al., 2023; Iavicoli et al., 2018). The presence of the organic matter and the sulphur in the faeces (Jiménez et al., 2023), could contribute to the appearance of this hormesis effect (Guo et al., 2016).

3.2. *Effect of the different Ag-based materials on the chlorophyll fluorescence*

The effect of different Ag-based materials on the chlorophyll fluorescence was exposure time (Fig. 2) and concentration (Fig. 3) dependent. For the exposure to 0.1 and 1 µg L^{-1} of Ag^+ (Fig. S4) and silver from Ag-kaolin (Fig. 2), no loss of the intensity of the cellular chlorophyll fluorescence was found. When the concentration increased to 10 µg L^{-1}, the loss of the fluorescence intensity after 48 h of exposure was observed, with a shift of the cell distribution toward low values. For the algal treatment with 50 µg L^{-1} of Ag^+ and Ag-kaolin, there was a loss of chlorophyll fluorescence during the first 24 h of exposure. The chlorophyll fluorescence cytogram shifted toward lower values as the time of exposure increased to 48 h and 72 h. For a given exposure time, as the concentration of silver increased, the loss of intensity of fluorescence was higher (Fig. 3), in agreement with the results obtained for growth inhibition (Fig. 1). This observation is consistent with the literature demonstrating that exposure to Ag^+ decreased algal chlorophyll autofluorescence primarily due to bleaching and the inhibition of its synthesis (Nowicka, 2022; Pillai et al., 2014). In addition, deformation and loss of the C-cell shape and swelling were observed in the first 24 h of exposure to Ag^+ and the leachate of Ag-kaolin for 10 and 50 µg L^{-1} (Fig. 4). For 0.1 and 1 µg L^{-1}, these changes were not appreciable, which indicates that the alteration of the morphology of the cells depends on the exposure concentration (Romero et al., 2020). In the literature, interactions between silver and the thiol groups of glycoproteins of the membrane cell have been demonstrated for *Euglena gracilis* (Li et al., 2015; Yan and Wang, 2021). These interactions caused an irregular morphology and algal sizes increased until they doubled their normal volume, as stress response induced by the exposure to silver was also observed for cells (Li et al., 2015).

For AgNPs, no loss of algal fluorescence was observed in the exposure with 50 µg L^{-1} or lower concentrations (Fig. S4), confirming that nanoparticles are less toxic than Ag^+. Nevertheless, when algae were exposed to a higher concentration of 100 µg L^{-1}, a loss of chlorophyll

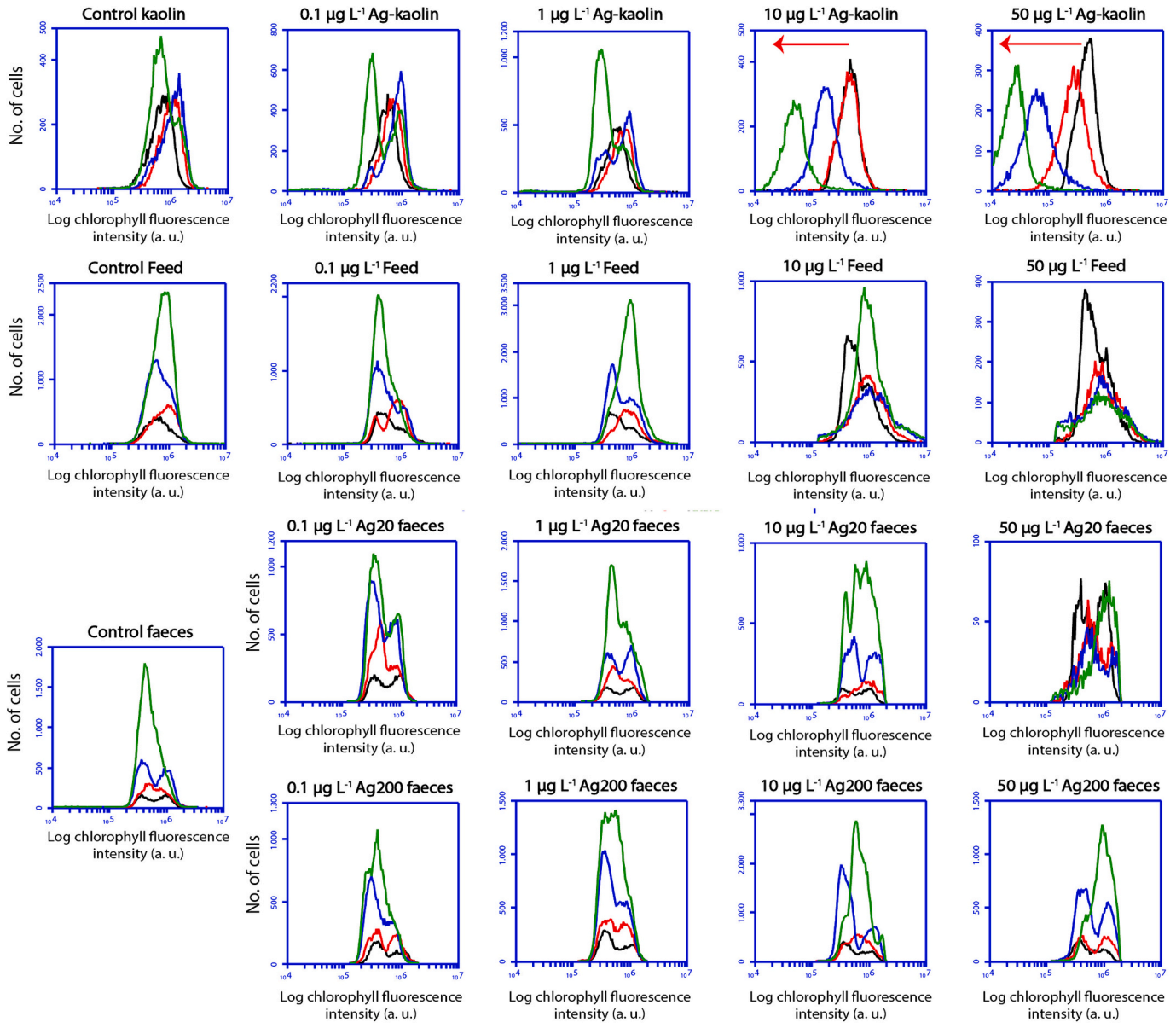

Fig. 2. Cytograms of chlorophyll autofluorescence for *R. subcapitata* during 72 h exposed to the leachates of the samples. Black line: 10 min. Red line: 24 h. Blue line: 48 h. Green line: 72 h. Red colour arrows show the shift in the intensity of the chlorophyll autofluorescence through the exposure time. Ag20 faeces corresponds to faeces with a silver concentration of 155 ± 30 mg kg^{-1} and Ag200 faeces corresponds to faeces with a concentration of 1026 ± 178 mg kg^{-1} of silver.

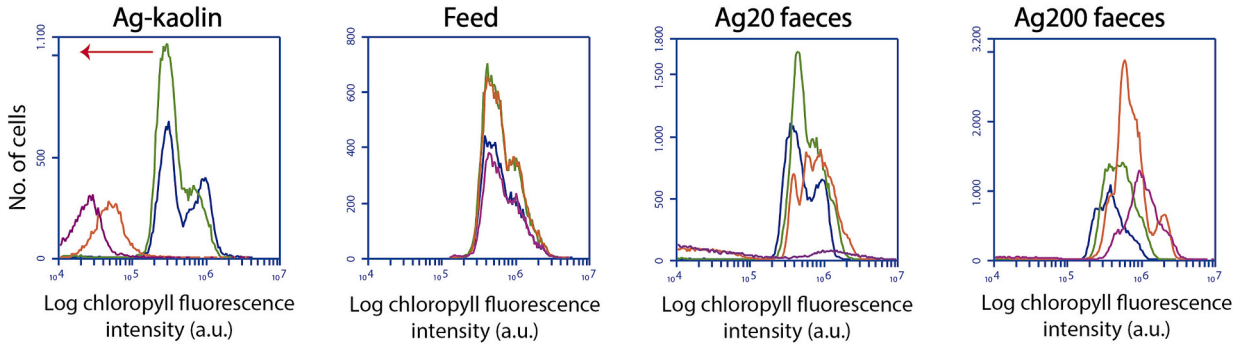

Fig. 3. Cytograms of chlorophyll autofluorescence for *R. subcapitata* after 72 h exposed to the leachates of the samples. Blue line: 0.1 μg L^{-1}. Green line: 1 μg L^{-1} Orange line: 10 μg L^{-1} Purple line: 50 μg L^{-1}. Red colour arrows show the shift in the intensity of the chlorophyll autofluorescence with the silver concentration of exposure. Ag20 faeces corresponds to faeces with a silver concentration of 155 ± 30 mg kg^{-1} and Ag200 faeces corresponds to faeces with a concentration of 1026 ± 178 mg kg^{-1} of silver.

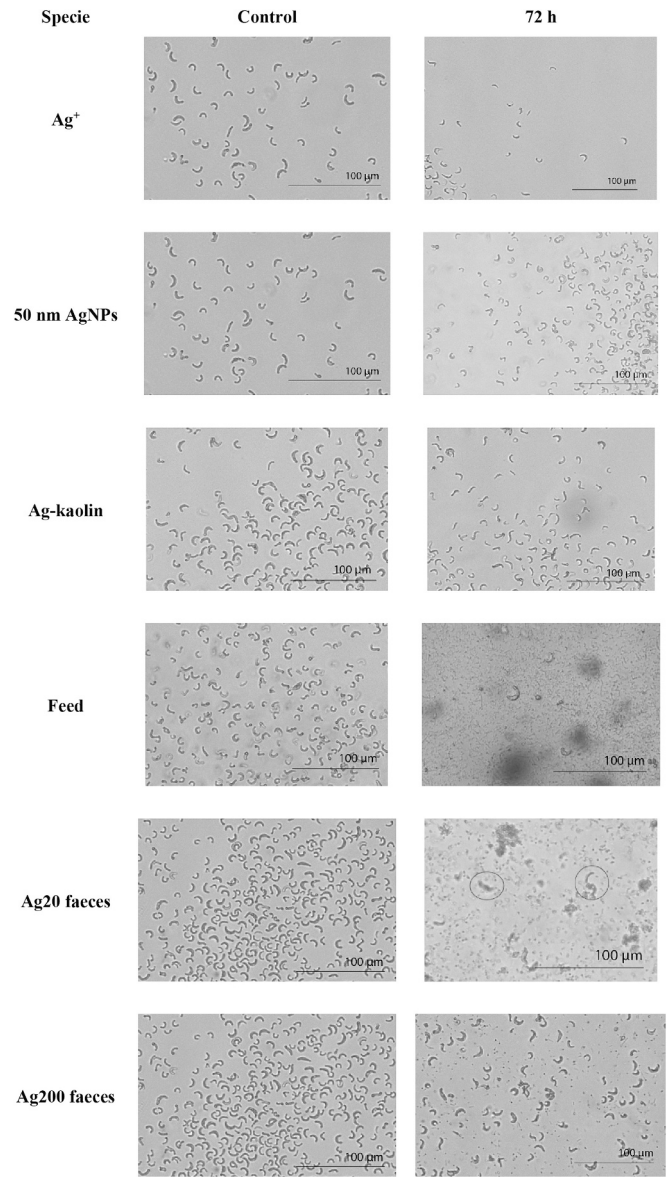

Fig. 4. Brightfield images of *R. subcapitata* exposed to 50 µg L^{-1} of standard Ag^+ and 50 nm AgNPs and the leachates of the silver-based materials and unexposed controls. Ag20 faeces corresponds to faeces with a silver concentration of 155 ± 30 mg kg^{-1} and Ag200 faeces corresponds to faeces with a concentration of 1026 ± 178 mg kg^{-1} of silver. The red circles in Ag20 faeces images indicate the algae.

fluorescence was observed after 48 h. This observation agrees with the literature reporting that the decrease in the fluorescence of *C. reinhardtii*, was more pronounced after 48 h and 72 h of exposure to AgNPs (Zhao et al., 2021). Also, the exposure to 100 µg L^{-1} of AgNPs resulted in the appearance of a pronounced second peak corresponding to the cell population exhibiting lower chlorophyll fluorescence (Fig. S4).

For the exposure to leachate of the feed and both types of faeces, there was no loss of chlorophyll fluorescence in the studied concentration range (Fig. 3) and exposure times (Fig. 2). However, although there is no loss of intensity of fluorescence for feed, the growth inhibition was complete at 50 µg L^{-1} (Fig. 1c). A deformation of the morphology of the cells was also visible in the exposure of the faeces (Fig. 4).

3.3. Silver uptake in algae exposed to the leachate of different Ag-based materials

For the algae exposed to different Ag-based materials (Fig. 5), the most frequent silver mass obtained was between 5 and 15 ag per cell, confirming the cellular uptake. As Ag^+ was the prevailing form in the leachates, it can be concluded that the uptake was due mainly to the Ag^+. In the literature, several pathways for the uptake of silver by green algae have been described. The use of the same transport channel used by Cu^+ was manifested since the transporter could not distinguish between them since they have similar chemical reactivity (Leonardo et al., 2016; Pillai et al., 2014; Ribeiro et al., 2015; Yue et al., 2017). Algal cells are also capable of absorbing silver as neutral complexes, such as $AgCl^0$, diffusing directly through the lipid bilayer (Yue et al., 2017). The formation of different negatively charged silver species, such as silver chloride or silver-thiosulfate complexes and their internalization via anion transporters, would be another uptake pathway of silver (Fortin and Campbell, 2001; Wang et al., 2016).

For Ag-kaolin (Fig. 5b) the most frequent mass of silver per cell was 5 ag, while for Ag^+ (Fig. 5a), the most frequent mass was 10 ag. This means that, although their behaviour was similar, in terms of growth inhibition and loss of chlorophyll autofluorescence, the silver accumulated in algae was much lower for the exposure to a comparable concentration of Ag-kaolin. The kaolin particles in the leachate of Ag-kaolin could adsorb Ag^+, thus decreasing the silver uptake by cells. Nevertheless, for feed (Fig. 5c), the most frequent mass was 15 ag, higher than the most frequent mass observed for exposure to Ag^+ and Ag-kaolin. The presence of different substances in the feed matrix, in addition to Ag-kaolin, may contribute to enhancing the uptake of silver by cells. Although the uptake was higher, the toxicity for feed was lower than the toxicity of Ag^+ or Ag-kaolin, since at 10 µg L^{-1}, the growth inhibition for feed was 32.2 % (Fig. 1b), so the substances of the matrix may lead to a lower effect in growth and chlorophyll fluorescence.

For faeces (Fig. 5d), the most frequent mass was 10 ag, as the one found for Ag^+ standard (Fig. 5a), although a low growth inhibition was observed by FCM (Fig. 1c). This could be explained by the presence of Ag_2S in faeces, which would be less toxic than Ag^+, but the algae are capable to uptake them and remain in this form in the cytosol of the algae (Hiriart-Baer et al., 2006; Lekamge et al., 2020), preventing the diffusion through the cytosol and maintaining the photosynthetic activity unaffected (Leonardo et al., 2016).

Moreover, SC-ICP-MS does not distinguish between the adsorbed and intracellular silver, so the presence of silver adsorbed in the walls also needs to be considered. Indeed, silver was adsorbed to the surface of another green alga *C. reinhardtii* through the coordination bonds with amine and carboxyl groups of the proteins present in the cell surface (Xu et al., 2022); electrostatic interactions with negatively charged functional groups of the exopolysaccharides and proteins (Xu et al., 2022; Zhang et al., 2020). Silver adsorbed on the surface of the cell could also cause cell membrane damage and lead to the death of the cells (Jagadeesh et al., 2015; Xu et al., 2024). Indeed, in our study, changes in the morphology of the cells were visualized (Fig. 4). Besides, 50 nm AgNPs did not internalize by other green algae *Chlorella vulgaris* and *Dunaliella tertiolecta*, they could be adsorbed in the cell membrane, leading to the formation of aggregates of cells, which would cause growth inhibition (Oukarroum et al., 2012). This inhibition would be caused by the interruption of cell division and the creation of shading effects, which would lead to the inhibition of photosynthesis (Ribeiro et al., 2014, 2015). The interactions with proteins and extracellular enzymes, such as alkaline phosphatase, β-glucosidase or L-leucine, would also lead to inhibitory effects (Yue et al., 2017).

4. Conclusions

The toxicity of different Ag-based materials - Ag-kaolin, supplemented feed and faeces from pigs fed with the supplemented feed - was

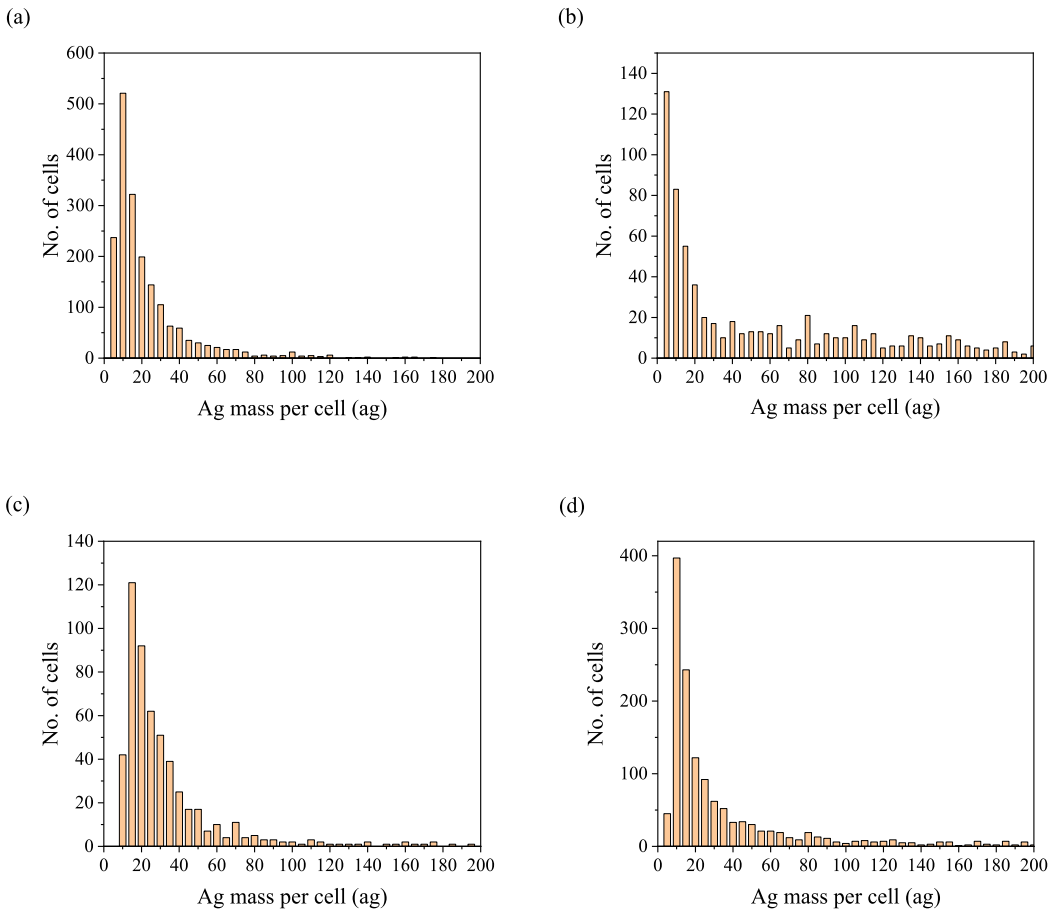

Fig. 5. Mass distributions of Ag obtained by SC-ICP-MS in *R. subcapitata* exposed to 10 μg L^{-1} of (a) a standard solution of Ag^{+} (b) leachate of Ag-kaolin (c) leachate of Ag-containing feed (d) leachate of Ag20 faeces.

studied in green alga *R. subcapitata,* exposed to leachates of these materials by utilizing single cell techniques (FCM and SC-ICP-MS). The silver in the leachate from these materials inhibited the growth and decreased the chlorophyll autofluorescence of microalgae. The impact of silver released from the leachates of the faeces was less pronounced compared to the effect of the feed itself, which in turn was also less significant than the effect of Ag-kaolin.

At a concentration of 10 μg L^{-1} of Ag^{+}, the leachate from Ag-kaolin caused total growth inhibition and loss of fluorescence. At lower concentration of 10 μg L^{-1}, cell morphology changes were noted, with cells losing their typical "C" shape. For the faeces leachate, a growth inhibition occurred at a concentration of 50 μg L^{-1}, though it was not complete. At a concentration of 10 μg L^{-1}, a potential hormesis effect was evidenced. Research findings indicate that despite the pronounced toxicity of Ag-kaolin, the effect of pig faeces is comparatively lower than the effect of Ag^{+} alone. The presence of low solubility species like Ag_2S and the complex matrix of the materials contribute to the reduced toxicity of faeces compared to pristine materials. This study highlights that the potential impact of faeces leachate on green algae would likely be low if Ag-kaolin were used in pig feed. However, further investigations are necessary to guarantee minimal environmental impact.

CRediT authorship contribution statement

Mariam Bakir: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. **María S. Jiménez:** Conceptualization, Supervision, Validation, Writing – review & editing. **Francisco Laborda:** Conceptualization, Supervision, Validation. **Vera I. Slaveykova:** Conceptualization, Supervision, Validation, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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