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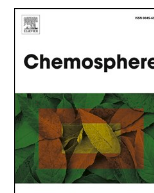

Single cell ICP-MS to evaluate the interaction behaviour for Cd, Ce and U with *Streptomyces coelicolor* spores

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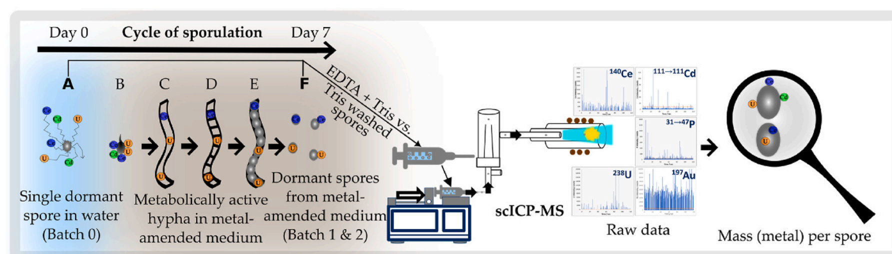
HIGHLIGHTS

- Single cell ICP-MS was used to quantify metal association with spores.
- Distinction between weak adsorption and strong association (metal-spore).
- Ce, U and Cd showed spore-adsorption in aqueous conditions.
- Ce and U were strongly spore-associated after growing in metal-amended solid medium.
- Spore association metabolically controlled for Ce and uncontrolled for U.

GRAPHICAL ABSTRACT



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ABSTRACT

Streptomyces are important soil bacteria used for bioremediation of metal-contaminated soils, however, it is still unknown how metal-selective *Streptomyces* are and which mechanisms are involved during their capture. In this work, we exposed *S. coelicolor* spores to environmentally relevant concentrations (0.1, 1, 10, 100 μM) of Ce, U and Cd in solid medium for one week to investigate the uptake behaviour of hyphae in the newly formed spores. Additionally, metal adsorption onto the spores was explored by incubating inactive, ungerminated spores for one day in aqueous metal solution. The spore-washing treatment was key to distinguishing between strongly spore-associated (e.g. incorporation; Tris-EDTA buffer) and weakly spore-associated metals (Tris buffer alone minus Tris-EDTA). Single cell (sc) ICP-MS was used to quantify metal-associated content in individual spores. Our results revealed element-specific adsorption onto inactive spores showing that out of the total metal exposure, both strongly (Ce: 58%; U: 54%; Cd: 28%) and weakly (Ce: 12%; U: 1%; Cd: 18%) adsorbed metals occur. However, scICP-MS showed that from metal-amended solid medium, only Ce and U were strongly spore-associated (averages 0.040 and 0.062 fg spore^{-1} for 10 μM exposures, respectively) while Cd was below the

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limit of detection (< 0.006 fg spore^{-1}). We propose that hyphae only metabolically interact with Ce in a controlled manner but uncontrolled with U, as 66–73% Ce and only 2–4% U were inherited from adsorbed content. We conclude that *Streptomyces* spore-metal interaction starts with a relevant adsorption step of Ce, U and Cd as presented for aqueous conditions. If spores start to germinate, hyphae are capable of effectively encapsulating Ce and U, but not Cd. This study brings light into the still unknown field of metal interactions with *Streptomyces* and applied understanding for more efficient and metal-specific use of *Streptomyces* in bioremediation of metal-polluted soils.

1. Introduction

Actinomycetes are a large group of gram-positive bacteria that are widely distributed in soils and exhibit a great capacity to survive in metal-contaminated environments (Schütze et al., 2014; Ali et al., 2017). These bacteria are commonly used for many bioremediation studies in various fields, such as in soil or water environments (Nazari et al., 2022; Dobrowolski et al., 2017) and even for wastewater treatment (El Sayed et al., 2015). Bioremediation is a promising alternative, to cost-intensive soil remediation strategies such as soil washing or irrigation, with the advantage of keeping the soil suitable for plant development (Nazari et al., 2022). Moreover, *Streptomyces* are capable of producing extracellular polysaccharides which allow the removal of metals such as Co(II), Cr(VI), Cu(II) and U(VI) either by chelation and/or reduction (Elnahas et al., 2021). Thus, actinomycetes can be considered as potential candidates for bioremediation purposes and promotors for phytoremediation in metal-polluted environments as they increase plant growth and prevent diseases (Seipke et al., 2012). Alongside extracellular biomineralisation, actinomycetes have the ability to remove metals by two main mechanisms; (1) adsorption, by metal association to the cell surface, and (2) bioincorporation, where the metal is taken up into the cells and either stored in cell vacuoles or further bioprocessed into other metallic phases (biomineralisation). Adsorption, on the one hand, is driven by different chemical processes, such as complexation and electrostatic interaction, including both weak and strong bonds influenced by the ligands (ionic and non-ionic) present on the cell wall and the chemistry of the metal ion of interest (Sahmoune, 2018). They are also strongly affected by pH, temperature and metal concentration (Presentato et al., 2020). Bioincorporation, on the other hand, means the intracellular metal uptake by living cells as a consequence of different metabolic pathways (Sharma et al., 2022). It generally starts with the adsorption of metal ions to the bacterial cell wall/membrane through active interactions with various functional groups or through passive channels, by mimicking the behaviour of an essential element for the cell, followed by further transport into the cell. Metal ions can therefore enter bacterial cells through ion channels, active transporters, endocytosis or penetration through the lipid membrane (Tsekhmistrenko et al., 2020). The microbial cell can prevent itself from being metal-intoxicated by releasing metal-binding compounds into the extracellular surrounding, which might reduce adsorption and bioincorporation of metals. The metals are chelated outside the cell and thus blocked from entering it through the unspecific membrane transporters that otherwise would facilitate the influx (Złoch et al., 2016). In a nutshell, bioincorporation suggests a long-term stabilisation of the metals until lysis while adsorption may be a reversible process, depending on how strongly bound the metals are to the spore surface.

Although different adsorption experiments have been conducted to investigate the bacterial retention capacity of metals such as Cd(II) onto dried *Bacillus badius AK* (Vishan et al., 2019), relatively little work has been done to understand the association of metals by actinomycetes investigating systematically both intracellular (bioincorporation) and extracellular (adsorption) distribution in these important soil bacteria. *Streptomyces* is the most studied genus of actinomycetes and are highly abundant in soils and sediments where they contribute to the degradation of organic matter. They are important industrial bacteria since they are the origin of two-thirds of the clinically useful secondary metabolites, including but not limited to antibiotics, antitumoral, immunosuppressant and antifungal compounds (Yagüe et al., 2012). Moreover, *Streptomyces* are multinucleated bacteria that form hyphae, mycelia and spores, and they are considered prokaryotic models of multicellularity. After spore germination, multinucleated branching hyphae grow into the culture media forming the so-called substrate mycelium; when the nutrients begin to become scarce, substrate mycelium differentiates into the aerial mycelium that starts to express hydrophobic covers and to grow into the air. Aerial hyphae trigger a complex cell division that ends in the formation of unicellular spores that are highly resistant to environmental stresses (Piette et al., 2005).

Until recently, the majority of studies on *Streptomyces* reported bulk population behaviour to the exposed metal in the growing medium (liquid or solid), focusing on secondary metabolite production (e.g. antibiotics) or studying their growing behaviour (Abbas and Edwards, 1990; Locatelli et al., 2016). However, none of these have simultaneously investigated (i) exposure to different metals, showing variable interactions and/or extracellular (nano)particle formation, (ii) individual spore behaviour, providing information on the population dynamics, and (iii) the fate or transfer of metals along the metabolic evolution of the *Streptomyces* cycle. A recent study has developed the analytical procedure for the determination of cytosolic copper in hyphae and spores of *S. coelicolor*. This study used inductively coupled plasma - mass spectrometry (ICP-MS) as bulk from a spore suspension and single cell (sc) sample introduction systems to monitor Cu concentrations in individual spores (González-Quiñónez et al., 2019). A follow-up study focussed on intra and extracellular metal (nano)particle formation associated with *S. coelicolor* spores and reported concentrations of both ionic and nanoparticulate cytosolic copper (Cu) (García Cancela et al., 2022). These works were the first scICP-MS applications for *Streptomyces* and provided valuable data about Cu variability in spores and its role in spore germination and secondary metabolite activation.

There is very limited information about the fate of metals in *Streptomyces* and their potential in metal bioremediation. Here, we used a state-of-the-art analytical tool to address different mechanisms of metal interactions (adsorption vs. bioincorporation) of *S. coelicolor* individually exposed to the metals of concern: Ce, Cd and U. Cd and U are well-known environmental pollutants originating from mining and nuclear activities (Lin et al., 2012; Ma et al., 2020). Ce has received much attention over the last few years due to the industrial use of Ce-based compounds, in particular, CeO_2 nanoparticles (NPs) (Dahle and Arai, 2015). These metals can be metabolised and incorporated during the *Streptomyces* developmental cycle, we focused our investigations on metabolically inactive, dormant spores, which are the only unicellular stage very well suited for scICP-MS. We collected spores after seven days from metal-amended (we refer to these spores as metal-spores) and non-amended medium (we refer to these spores as non-metal-spores). Non-metal-spores were incubated in metal aqueous solution for 24 h, and both (metal-spores and non-metal-spores) were washed using either a complex-building (EDTA) or without the complex building agent before scICP-MS to distinguish between metal adsorption and bioincorporation processes. Optimisation of the scICP-MS measurement procedures to analyse Ce, Cd and U as well as data normalisation by using phosphorus (P) as an intrinsic cell constituent to discriminate between metal (nano)particles and cells were implemented. Understanding if metals are adsorbed onto the cell surface or if they weakly or

strongly associated (e.g. incorporation) with *Streptomyces* is crucial to evaluate metal-mobilisation in the environment and is thus important in the evaluation of *S. coelicolor* as an adequate organism for bioremediation applications.

2. Materials and methods

2.1. Materials and solutions

All solutions were prepared using 18.2 MΩ cm ultrapure water (PURELAB Flex 3, ELGA VEOLIA, Wycombe, UK). External calibrations and bacterial incubations were made with Ce as $Ce(NO_3)_3$ in HNO_3 (2–3%) (Specpure®, Thermo Fisher Scientific, Kandel, Germany), Cd as $Cd(NO_3)_2$ in HNO_3 (0.5 mol L^{-1}) (Certipur®, Merck, Darmstadt, Germany) and U as $UO_2(NO_3)_2$) in 2% HNO_3 (Absolute Standards Inc, Hamden, USA) ICP standards, all 1000 mg L^{-1}.

2.2. Inocculation and incubation of Streptomyces coelicolor

Soya Flour Mannitol (SFM) (Kieser et al., 2000) was used as the growing medium to obtain spores of *S. coelicolor* M145. SFM solid medium was used to represent nutrient-rich topsoils in the environment simulating optimal growing conditions. Ce, Cd and U standards were filtered (< 0.2 μm, PES sterile filters), added in separate batches to the SFM medium supplemented with agar (20 g L^{-1}), and homogenised in order to achieve final concentrations of 0, 0.1, 1, 10 and 100 μM of each metal. Additionally, for Ce and U a concentration of 100 μM without any spores was prepared as a control to investigate the possible formation of Ce/U containing abiotic (nano)particles. These concentrations are representative of environmental systems reported for European top soils (e.g., ranging between 50 and 6000 μM for total Ce, < 1–350 μM for total Cd and 2–600 μM for total U (Salminen et al., 2005)) or ground and soil waters of the same former uranium mine site (Ronneburg, Germany) showing levels < 0.1 and 10 μM (03/2020−05/2022) (e.g. for U (Grawunder et al., 2018)). From each SFM-spiked medium, 25 mL was poured into petri dishes which were inoculated with *S. coelicolor* spores to $3 \bullet 10^7$ spores per plate. Cultures were incubated for one week to complete the development cycle of this bacteria at 30 °C and pH 4.5–7.5 (supplementary material, Table S1). These conditions represent a wide range of expected soil pH values, for sub and topsoils in Europe, ranging from pH 2.9 to 7.9 (Salminen et al., 2005) or more specifically in former uranium mine areas (pH 4.4–5.2, Ronneburg, Germany) (Phieler et al., 2015).

2.3. Harvesting S. coelicolor spores and washing treatments prior to single cell ICP-MS

S. coelicolor spores were harvested by adding 4 mL ultrapure water to the solid plates and were detached using cotton swabs. The spore suspension was transferred into PP centrifuge tubes (Fisher Scientific, Schwerte, Germany), diluted to 7.5 mL with ultrapure water and cotton-filtered to remove the leftover hyphae but also free metals and very weakly bound spore-metals were potentially removed by water and cotton-filtering. The suspension was centrifuged (10 min, 8500 rpm, 20 °C) and the supernatant was disposed of, in order to remove traces of the growing medium. The spores were then re-suspended in 5 mL of washing buffer. In the first batch of experiments (named Batch 1 from now on along the manuscript), two washing buffers were tested on replicate SFM-spiked conditions. The first washing buffer was composed of 10 mM tris(hydroxymethyl)-aminomethanhydrochlorid (Tris-HCl; purity ≥ 99.8 %, Fisher Scientific) and 1 mM ethylenediaminetetraacetic acid (EDTA; ACS reagent, 99.4–100.6 %, Merck), resulting in a pH 7.5 solution, also known as TE buffer. The second buffer solution contained the same Tris-HCl composition but without any EDTA. This centrifugation and washing procedure was repeated two more times. In order to test and ensure the effectiveness of the washing step, the supernatants were individually collected for bulk ICP-MS quantification. The washed spores were re-suspended in 5.5 mL Tris buffered saline (TBS) solution (BioUltra, pH 7.6, Merck). An aliquot of 1.5 mL was transferred to a PP microcentrifuge tube (Labbox Labware, S.L., Barcelona, Spain), and centrifuged once more (10 min, 8500 rpm, 20 °C), disposing of the supernatant and finally resuspending the spores in 1.5 mL ultrapure water for scICP-MS analysis. The remaining spores in the TBS solution were stored at 4 °C. Fig. 1 summarises the procedures followed for the metal-spores (Batch 1 & 2). Batch 2 refers to a second, more exhaustive repetition of the experimental setup, only dedicated to EDTA + Tris washed spores.

2.4. Complementary investigation of metal adsorption onto spores

Adsorption processes onto non-metal-spores (grown in metal-non-amended medium) were also investigated, complementary to the main adsorption and association experiment as described earlier. *S. coelicolor* spores were harvested as described in section 2.3 without exposure to metals. The detailed procedure is schematically shown as a flow chart in the supplementary material (Fig. S1). Spore suspensions were then exposed to 10 μM Ce, Cd and U, respectively, in ultrapure water and left for 24 h in a dark cabinet (room temperature) to exclude any potential light influence on metal adsorption such as increasing temperature. This approach is referred as Batch 0 in the rest of the manuscript. Aliquots were washed with EDTA + Tris vs. Tris buffer and further processed as described in section 2.3. Alongside the supernatant of each washing buffer, the first supernatant of the spore suspension was also collected and investigated using bulk ICP-MS. The final spore suspensions in ultrapure water were measured using scICP-MS.

2.5. Viability analysis of spores

An aliquot of the spores in the TBS solution was stained with a LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, L-13152). The spore viability was investigated using a Nikon eclipse Ci-L fluorescence microscope, equipped with a CoolLED's pE-300 white lamp and a Nikon DS-Fi3 camera using a FITC-A-Basic-000 filter (Semrock, Rochester, USA) (450–490 nm excitation and 505–560 nm emission). LIVE/DEAD BacLight Bacterial Viability Kit consists of two DNA-binding colourants, SYTO9 and propidium iodide (PI), prepared at 6 and 30 μM, respectively. Green fluorescing SYTO9 enters cells with intact membranes, whereas red fluorescing PI only diffuses through cells with damaged membranes, displacing SYTO9. This was done immediately after the washing procedure (on the day of spore harvest, in the freshly resuspended TBS solution) and repeated after 28–37 days from the stored TBS batch to confirm viability even after one-month storage. Additionally, spore sizes were measured from fluorescence microscopy images.

2.6. Cell number concentration

The final spore suspension for scICP-MS in ultrapure water was used for cell number quantification. Cell concentrations were determined via three different methods: photometry, flow cytometry and serial dilution method. For the photometry, spore concentrations were determined spectrophotometrically at 600 nm based on the Beer-Lambert relationship with a UV mini-1240 spectrophotometer (Shimadzu, Kyoto, Japan), using a *S. coelicolor* standard spore concentration vs. absorbance curve as reference. For the flow cytometry, samples were stained using 2.4 μL fluorescence marker SYTO 9 (50 μM) to 200 μL spore suspension and measured using a CytoFLEX S Flow Cytometer (Beckman Coulter, Brea, USA). Unstained samples were measured as controls. Raw data were interpreted using the CytExpert 2.3 software. For the serial dilution method, serial 1:10 dilutions of the suspensions with spores were prepared and 100 μL of each dilution were spread on plates with Glucose, Yeast and Malt (GYM) medium supplemented with agar (20 g L^{-1}). Plates were incubated at 30 °C until single colonies were appreciable for

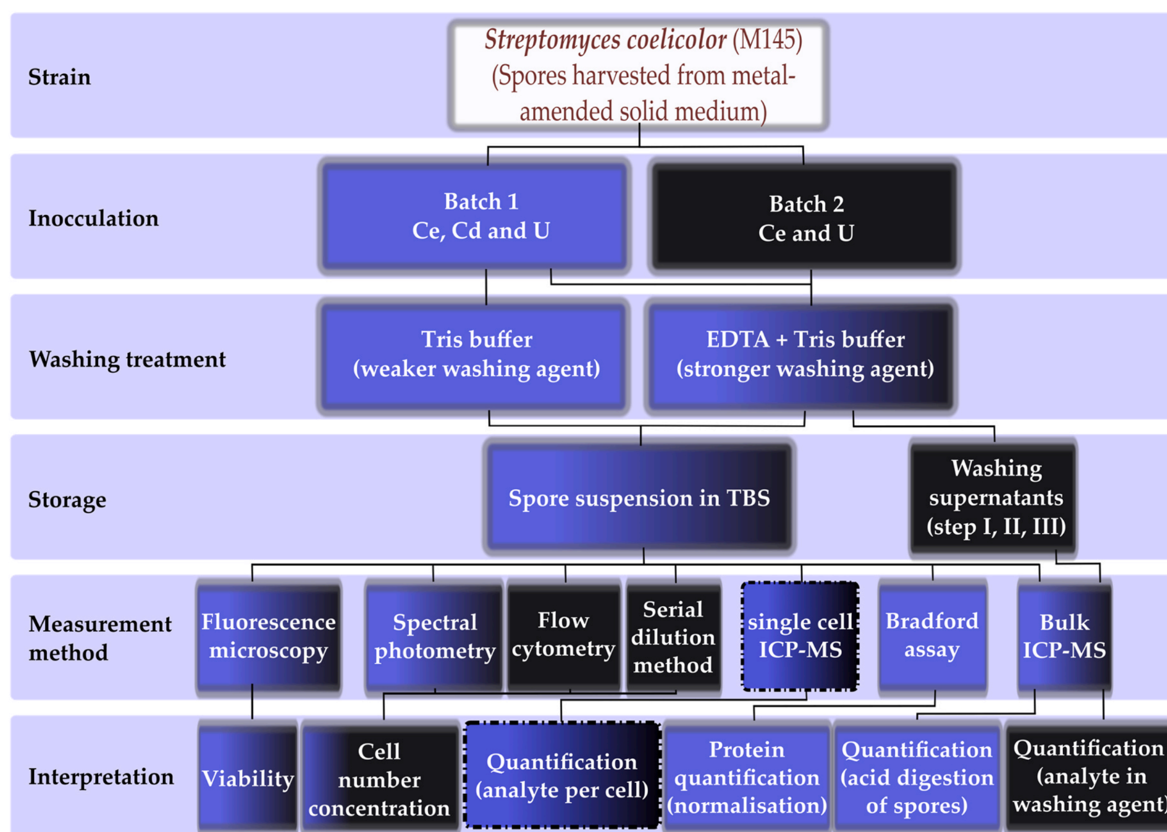

Fig. 1. Schematic flow chart of the method from microbiological batches of the metal-spores obtained from metal-amended solid SFM growing medium, sample preparations, washing treatments to the (quantification) methods and the data interpretation. Batch 1 is marked in blue and Batch 2 in black, representing the main experiments of this study. Boxes including both colours imply common treatments for both batches. The main method of this study (scICP-MS) and its interpretation are highlighted using dashed (–) lines. TBS stands for Tris-buffered saline. The washing steps I–III were three consecutive washing steps. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

counting.

2.7. Single cell ICP-MS analysis

Single cell analysis was performed using the Thermo iCAP-TQ (Thermo Fisher Scientific, Bremen, Germany) equipped with a total consumption spray chamber and a microconcentric nebulizer (High Sensitivity Single-Cell Sample Introduction System for ICP-MS, Glass Expansion, Port Melbourne, Australia). Fresh spore suspensions (10^7–10^9 spores mL^{-1}) were diluted with ultrapure water to final concentrations of 10^4 spores mL^{-1}. For the sample introduction, a Fusion 100-X syringe pump (Chemyx, Stafford, USA) equipped with a 1 mL syringe (Hamilton, Bonaduz, Switzerland) was used to provide a constant flow rate of 10 μL min^{-1}. All parameters are listed in Table S2 and the ionic limits of detection (LOD) and quantification (LOQ) using 3, respectively, 10 • SD + mean (blank) criteria in Table S3.

All measurements were performed using a total acquisition time of 2 min and a dwell time of 5 ms in time-resolved analysis mode. Due to the sequential nature of the measurements in quadrupole-based ICP-MS systems, it is not possible to measure two elements in the same run. Thus, sequential experiments were carried out, analysing the isotopes of $^{140}Ce^+$ and $^{238}U^+$ in No gas mode, and $^{111}Cd^+$ (on-mass) and ^{31}P (with mass-shift to $^{31}P^{16}O^+$) using O_2 as reaction gas in the second quadrupole in triple quadrupole mode to avoid the interference of $^{14}N^{16}O^1H^+$ on $m/z = 31$. P was chosen as a constitutive element to track the spores. Transport efficiency from the syringe to the plasma was determined using citrate-stabilised Au nanoparticles (NPs) with a nominal diameter of 30 nm (LGCQC5050, LGC, London, UK) at a final concentration of 2 • 10^4 NPs mL^{-1}, targeting ^{197}Au in No gas mode via particle number method described elsewhere (Montaño et al., 2016). A daily ionic calibration was prepared for the quantification. For Ce and U, the calibration was prepared in 1 mM EDTA (pH 8) to stabilise the ions in a range from 0 to 10 μg L^{-1} and for Cd in ultrapure water between 0 and 25 μg L^{-1}.

2.8. Data treatment for scICP-MS

Data treatment was carried out following the basic principle described in other publications (García Cancela et al., 2022; Pace et al., 2012; Corte Rodríguez et al., 2017; Corte-Rodríguez et al., 2020). Briefly, an iterative method was carried out based on averaging the whole dataset and extracting all data points higher than the mean (μ) + k • standard deviation (SD). Factor k was individually set for each sample and is based on the background level. The final value was used as a threshold to identify cell events. Finally, outliers that were higher than μ + 3 SD from all cell events were removed. The calculations for the mass of analyte per cell were performed according to a previous study (García Cancela et al., 2022).

2.9. Acid digestion and bulk ICP-MS analysis

For the bulk analysis of the total metal content onto/into spores, an acid digestion of the metal-spores was applied by mixing 100 μL of the TBS spore suspension with 500 μL of sub-boiling purified HNO_3 (65%, Acros Organics, Thermo Fisher Scientific), heated at 70 °C in semi-closed 1.5 mL PP microcentrifuge tubes with a small hole to compensate pressure. After 1 h, 500 μL 30% H_2O_2 was added and left for another 3 h at 70 °C. After cooling down, the samples were diluted to 2% HNO_3

and measured with bulk ICP-MS. The Thermo iCAP-TQ, equipped with a cyclonic spray chamber and concentric MicroMist nebulizer was used, combined with the autosampler ASX-560 (Teledyne, CETAC Technologies, Omaha, USA) for sample introduction. The same measurement mode for the individual isotopes was used for quantification as described in section 2.7. The ICP-MS was run in spectrum mode analysing three replicates using a dwell time of 0.1 s. All parameters are listed in Table S4 and the ionic limits of detection (LOD) and quantification (LOQ) are in Table S5.

2.10. Protein analysis via bradford assay

For the normalisation of the data achieved via acid digestion, the total protein content of the metal-spore suspensions was analysed via Bradford assay (Biorad, Feldkirchen, Germany) (Bradford, 1976). Spore protein was released breaking spores with 1 M NaOH (Merck). For this, a 1:1 v/v mixture of the TBS spore suspensions and NaOH was boiled for 10 min at ca. 100 °C and centrifuged (10 min, 15400 rpm, 20 °C) to remove cell debris. 90 μL of the supernatant was diluted and mixed with 10 μL ultrapure water and 900 μL Bradford reagent (1:5). The Bradford reagent consists of a mixture between 100 mg Coomassie Brilliant Blue G-250 dissolved in 47 mL Methanol (100%) and 100 mL phosphoric acid (85%) to 200 mL of ultrapure water. After 5 min reaction time, the absorbance of the solutions was measured at 595 nm (Shimadzu). A bovine serum albumin standard (Sigma-Aldrich, Saint Louis, MO, USA) was used for the calibration.

2.11. Species and stability analysis of dissolved ions and precipitates using PHREEQC

The free software PHREEQC (Parkhurst and Appelo, 1999) was used to simulate the most probable species to which the spores were exposed. The two tested conditions correspond to the metal-amended SFM growing medium used in Batch 1 & 2 (for the composition of the solid media see Table S6) and to the metal-spiked aqueous solutions from Batch 0 (as presented in Table S7). Briefly, the PHREEQC database "llnl.dat" was used to identify the presence of potential dissolved species and/or formation of solid phases (i.e., precipitation via the saturation index) of U, Cd and Ce. All simulations were performed for pH values between 4 and 8 and focused on the highest concentration conditions (100 μM) in absence of organic components such as mannitol, due to the lack of information in the current thermodynamic databases.

3. Results and discussion

3.1. Viability of the metal-spores obtained from metal-amended SFM medium

A viability analysis was conducted to ensure that spore membranes were intact. This was done directly after spore harvesting (Fig. 2a) and after ca. one-month storage in TBS (Fig. 2b) to verify the long-term viability. The majority of the spores was intact even after one month of storage in TBS at 4 °C. An example of this is shown for the metal-spores obtained using the highest exposure concentration of U (100 μM) (Fig. 2a and b). Almost no damaged spores (PI red staining) were visible and a high number of intact viable spores (SYTO 9 green staining) were detected. Spores are resistant cell structures that remain inactive until germination. Germination cannot start in deionised water or TBS solution, and consequently, the number of spores remained stable after 33 days of storage. This was tested and confirmed for Ce and U via photometric cell number concentration measurement, the spore numbers stayed in the same order of magnitude (Table S8). The viability results of the metal-spores for Ce and Cd amended medium (both 100 μM) (Figs. S2 and S3) were similar to those for the U spores. Spores sizes were measured for Batch 1 (Blank, Ce and U each 100 μM) (Fig. S4). The median spore diameter varied between 0.9 and 1.4 μm independently from the metals added to the growing medium, thus spore size only plays a little or no role in evaluating potentially adsorbed metal content.

3.2. Single cell ICP-MS: metal association with metal-spores obtained from metal-amended cultures

After confirming the spore viability and estimating the harvested spore number concentrations (supplementary material, section S1, Fig. S5, Table S8), further investigations using scICP-MS were performed. This provides quantitative information about spore-associated metals and spore-to-spore variations, which is mandatory to further understand the uptake of metals into/onto *S. coelicolor*. In this section, we address the metal-spores which were obtained from SFM metal-amended cultures meaning that metals can be either adsorbed onto spores or be metabolically accumulated in sporulating hyphae forming these spores. The washing treatment was key to differentiate between weakly and strongly spore-associated metals. At the end of the exposure time, two sets of washing conditions were applied and compared using two buffers (Tris vs. EDTA + Tris buffer) in a preliminary batch (Batch 1) and repeated in a more exhaustive batch for only EDTA + Tris treatment (Batch 2) (Fig. 1). To simplify the following sections, we do not differentiate between Batch 1 & 2 when not required, but rather between washing conditions used. As EDTA is a complexing agent for many analytes (and specifically for our metals of interest (Ce as Ce(III) (Rajpal

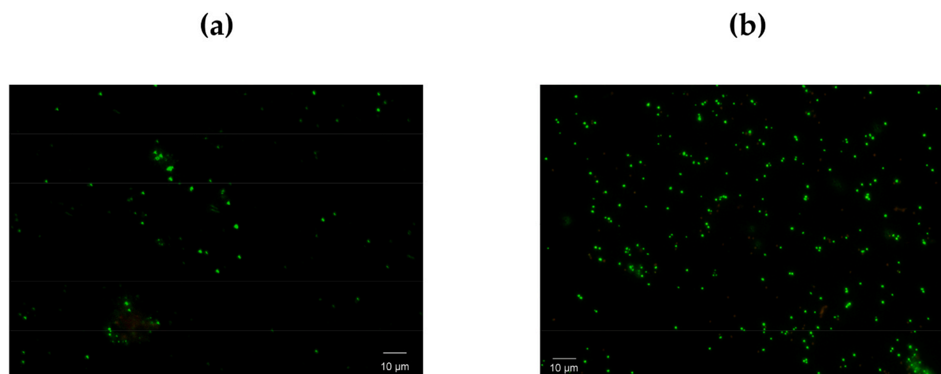

Fig. 2. Viability of *Streptomyces coelicolor* spores (SYTO9 green stained: intact (viable) spores; PI red stained: damaged (dead) spores) for U (100 μM) after **(a)** 0 days (harvest); **(b)** 33 days (note: the picture was taken near the edge of the object plate where spores accumulate to ensure good spore visibility). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

et al., 2022) or Ce(IV) (Sumaoka et al., 2006); Cd as Cd(II) (Engström et al., 1980); and U as uranyl ($U(VI)O_2^{2+}$) (Wang et al., 2022)), it was assumed that the EDTA + Tris washing buffer is a comparably stronger washing agent that would remove adsorbed ions from the spore surfaces. In fact, it was previously reported that Ce (as Ce(III)) forms strong EDTA complexes in an acidic range (pH 4–7), while at pH higher than 7, the complex stability with EDTA significantly decreases (Rajpal et al., 2022). U (as $U(VI)O_2^{2+}$) and Cd (as Cd(II)) both build complexes with EDTA in a wide pH range, between pH 2 and 12 (Wang et al., 2022; Pinto et al., 2014). In contrast, Tris is a comparably soft washing agent which would remove none or only weakly adsorbed ions. Although it was reported in a previous publication that Ce(III) can build complexes with Tris at our experimental conditions (pH 7.5) (Gamov et al., 2020), we expect this effect to be lower compared to the EDTA + TRIS treatment. This is in accordance with another publication, where the authors reported that trivalent actinide or lanthanide ions (in this case Cm(III)) show no evidence for complexation with Tris (pH 8.6) and further concluded that Tris buffer acts only as a pH buffer but does not influence the speciation of trivalent metal ions (Weiser et al., 2011). In the following sections, we refer to strongly spore-associated (EDTA + Tris treatment; incorporation or irreversibly bound) vs. weakly spore-associated (Tris buffer alone minus EDTA + Tris treatment; adsorption or reversibly bound). In the first case, a clear distinction between irreversibly bound (outside the spore) and incorporated (inside the spore) content of metals onto/into the spores is, however, not possible using this experimental setup. The results showing the differences between washing buffers are presented for each element in the following sections.

As an example, mass histograms for the detected cell events are shown for spores after washing with EDTA + Tris in the supplementary material (Ce: Fig. S6, U: Fig. S7). Furthermore, a preliminary screening using PHREEQC gave hint of the expected speciation in the aqueous phase and possible precipitates (i.e., predicting the most probable, thermodynamically stable solid phases) of Ce, Cd and U in the growing medium (Fig. S8).

3.2.1. *Cerium*

Ce is known for its industrial applications, in particular CeO_2 nanoparticles. The PHREEQC simulation suggested that Ce(III) at the experimental pH conditions (pH 4–8, Table S7) could be present as ionic Ce^{3+} or aqueous Ce-phosphates species (Fig. S8a). This means that the metal-spores obtained from metal-amended cultures were most likely exposed to free Ce^{3+} ions and phosphate cation/neutral species, given the phosphate content in the SFM composition (Table S6). The scICP-MS investigations (Fig. 3a) revealed no significant trend of higher masses Ce per spore from the Tris washed spores than EDTA + Tris washed spores, indicating that all Ce was strongly associated with metal-spores. As the data are not normally distributed, a non-parametric statistical test (Mann-Whitney-U-test) was performed for the highest concentration (100 μM) with a p-value = 0.1 to account for biological variabilities. This test confirmed that there is no significant trend of washed spores using Tris being higher than EDTA + Tris. The strong Ce-spore

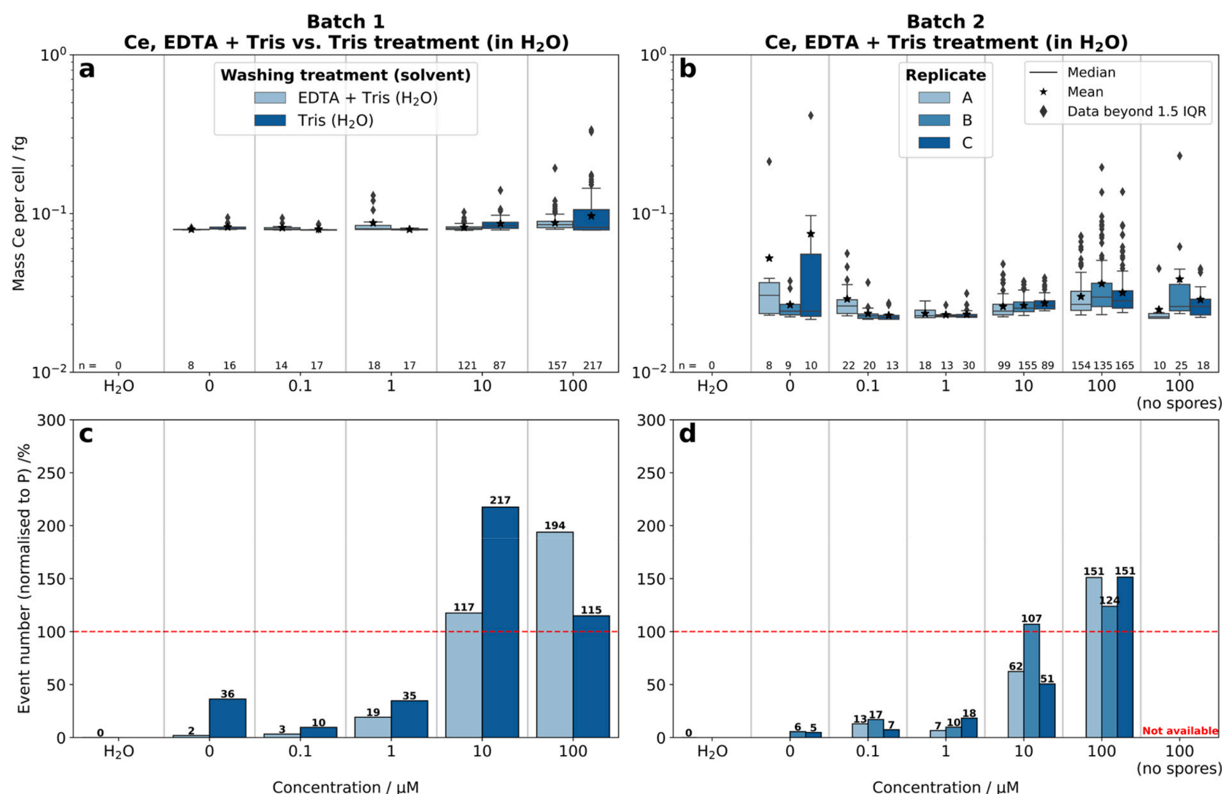

Fig. 3. ScICP-MS investigation for Ce of metal-spores obtained from metal-amended cultures. The boxes include data points between the 1st quartile (25%) and the 3rd quartile (75%) and the whisker range represents the 1.5 interquartile range (IQR). The dots outside the boxes show spores with concentrations above (or below) the whiskers limits. "n" values represent detected event numbers for Ce per run. The red dashed line indicates that all spores (100%) were associated with Ce. **(a)** Ce mass per spore vs. exposure concentration in the medium for metal-spores washed with EDTA + Tris vs. Tris buffer alone; **(b)** Ce mass per spore vs. exposure concentration in the medium for metal-spores washed with only EDTA + Tris buffer and an additional set with metal-amended medium (100 μM) but no spores. The detected events correspond to potential Ce containing particles formed in the SFM medium; **(c)** Ce event number normalised by the P event number (Ce events/P events) vs. exposure concentration in the medium for metal-spores washed with EDTA + Tris vs. Tris buffer alone; **(d)** Ce event number normalised by the P event number (Ce events/P events) vs. exposure concentration in the medium with only EDTA + Tris buffer and an additional set with metal-amended medium (100 μM) but no spores. As no P events could be found for "100 μM Ce (no spores)", no results were achieved for this data series. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

association is in agreement with the literature where Ce was incorporated into the cytoplasm and not associated with the cell walls of fungus spores (Owens and Miller, 1957) confirming our indication of strong association or even possible incorporation. Given these outcomes, the rest of the experiments were performed with only the EDTA + Tris washing buffer and three replicates to validate repeatability. For metal-spores washed with EDTA + Tris, the content of strongly associated Ce mass per spore was independent of the exposure concentration (0.1–100 μM) in the growing medium indicating a plateau in Ce accumulation per spore for each Batch (mean: 0.097 (plateau at Batch 1) vs. 0.036 (plateau at Batch 2) fg $cell^{-1}$) in our exposure concentration range (Fig. 3a and b). This was the first indication that *Streptomyces* sporulating hyphae are able to actively control the Ce association process resulting after a complete sporulation cycle as spore-associated Ce. Nevertheless, the Ce per spore concentration was systematically lower in the repetition Batch 2 (Fig. 3b) for all replicates A–C compared to the preliminary Batch 1 (Fig. 3a). Given that both, pH and temperature conditions, were kept constant, the observed difference can potentially be caused by biological variability.

As P is a major constituent of spores, $^{31}P^{16}O^{+}$ detection was used to track individual spores. The number of observed P events within a given time suggested the number of spores, whereas tracing the events of $^{140}Ce^{+}$ indicated the number of spores containing this element (Fig. 3c and d). Results beyond the 100% (red dashed line) imply that there were more Ce events than spores. These are the cases for exposure concentrations ≥ 10 μM implying all spores were associated with Ce (Fig. 3c and d). In addition, one experiment with 100 μM Ce but no spores was conducted to investigate the potential formation of Ce-containing particles in the growing medium. Results showed some spurious Ce events with similar concentrations as those found with the presence of spores, in accordance with the PHREEQC calculations pointing towards potential CeO_2 or $CePO_4 \bullet 10\ H_2O$ precipitation at 10 and 100 μM (Fig. 3b, d, Fig. S8d). Nevertheless, these events account for < 12% of the mean number of events for 100 μM with spores, which can also be included within the analytical variability. This suggests that there was a potentially enhanced formation of extracellular Ce-containing (nano)particles due to the activity of *S. coelicolor* and not due to the saturation conditions in the medium.

Overall, our findings suggest that *S. coelicolor* can effectively interact with Ce, more efficiently and in a controlled manner when the environment contains phosphate-rich Ce species. This suggests that Ce might be a part of a metabolic pathway for *S. coelicolor*. In any case, highly Ce-concentrated systems can result in extracellular Ce (nano)particle formation after interaction with *S. coelicolor*.

3.2.2. Uranium

U is known for its ecotoxic and radioactive implications. The PHREEQC simulations suggested that U(VI) is dominating as aqueous uranyl-phosphate species (Fig. S8b) in the growing medium (pH 4–8; Table S7). Similar to Ce, no significant trend of Tris > EDTA + Tris was detected in the single cell experiments between washing treatments, indicating that all U is strongly associated with the spores (Fig. 4a). This was statistically confirmed by applying the Mann-Whitney-U-test for the 100 μM concentration, using a cut-off p-value = 0.1 to account for

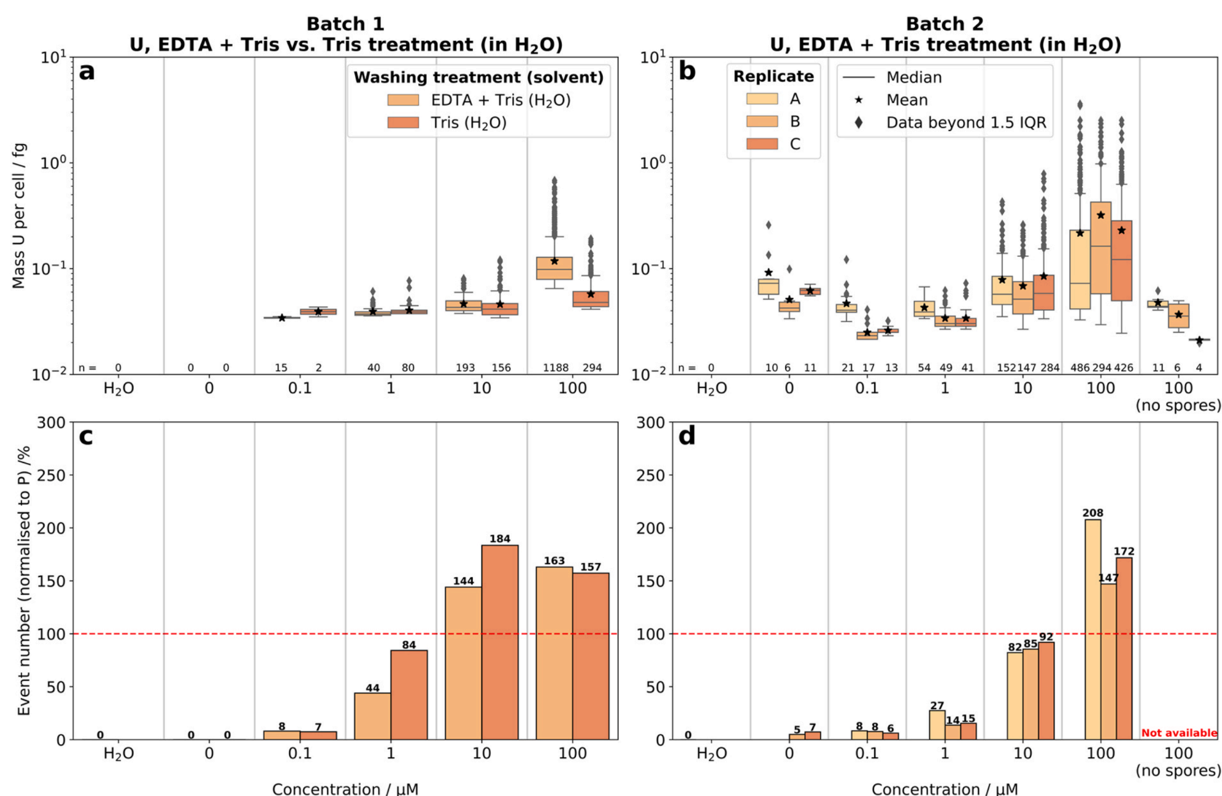

Fig. 4. ScICP-MS investigation for U of metal-spores obtained from metal-amended cultures. The boxes include data points between the 1st quartile (25%) and the 3rd quartile (75%) and the whisker range represents the 1.5 interquartile range (IQR). The dots outside the boxes show spores with concentrations above (or below) the whiskers limits. "n" values represent detected event numbers for U per run. The red dashed line indicates that all spores (100%) were associated with U. **(a)** U mass per spore vs. exposure concentration in the medium for metal-spores washed with EDTA + Tris vs. Tris buffer alone; **(b)** U mass per spore vs. exposure concentration in the medium for metal-spores washed with only EDTA + Tris buffer and an additional set with metal-amended medium (100 μM) but no spores; **(c)** U event number normalised by the P event number (U events/P events) vs. exposure concentration in the medium for metal-spores washed with EDTA + Tris vs. Tris buffer alone; **(d)** U event number normalised by the P event number (U events/P events) vs. exposure concentration in the medium with only EDTA + Tris buffer and an additional set with metal-amended medium (100 μM) but no spores. As no P events could be found for "100 μM Ce (no spores)", no results were achieved for this data series. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

biological variabilities. The strong association of U-phosphate species was not controlled by the spores, as a dependency was observed between the spore-associated U and the exposure concentrations during both washing conditions (Fig. 4a and b). This was especially clear when comparing the median from the highest exposure concentration (100 μM) to those from the lowest concentration (0.1 μM) washed using the EDTA-containing buffer. Contrastingly to Ce, this was our first indication showing that metabolically active sporulating hyphae are not able to restrict the U association process resulting after a complete sporulation cycle as spore-associated U. Thus, the U spore-association process was rather uncontrolled, only restricted by the U concentration added to the growing medium. Similar findings were also reported in a study analysing U bulk intracellular uptake as UO_2^{2+} in *Streptomyces* sp. (Golab et al., 1991), where UO_2^{2+} uptake increased 10-fold from 0.1 to 1 mM spiked solutions. Similarly to Ce, at concentration levels ≥ 10 μM all spores at those concentrations were over the 100% line and therefore U-associated (Fig. 4c and d).

Regarding spurious U precipitation, the PHREEQC calculation suggested that there could be uranyl phosphates, Ca/Mg-based and/or silicon-based precipitated species for an exposure concentration of 100 μM in the growing medium (Fig. S8e). However, only very few events were found in the absence of spores (Fig. 4b), more clearly represented for the case of U than that of Ce. Nevertheless, the P normalised events (Fig. 4c and d) also point towards the presence of biologically formed extracellular U (nano)particles for the highest exposure concentrations. All in all, despite the common presence of (nano)particles, it seems that U spore association was uncontrolled contrastingly to the Ce-phosphate-regulated association. This suggests that *S. coelicolor* can remediate more efficiently U-contaminated sites compared to Ce-contaminated areas, potentially related to alternative metabolic pathways for U and Ce within the spores as suggested in (Golab et al., 1991) for UO_2^{2+} in *Streptomyces* sp. biomass. However, in another publication, the influence of metabolic inhibitors (dinitrophenol and sodium azide) on actinomycetes was investigated and no negative effect after treating the cells was observed, concluding the U uptake is a non-metabolic process (Horikoshi et al., 1981). The authors further suggested that the U association mainly depends on the physicochemical adsorption at the cell surface (confirmed via Freundlich-isotherm) and is highest at pH 6 and low carbonate concentration due to the formation of complexes which cannot be taken up by actinomycetes. While for Ce, the medium pH was quite constant (4.5–5.5) for all exposure concentrations, for U, pH varied greater (4.5–7.5). As metal solubility and therefore bioavailability depends on pH this should be taken into account if comparing the U spore-association at the different concentrations (Table S1). However, as the highest pH (7.5) was found for the 10 μM exposure concentration but the U spore-association still increases compared to the lower exposure concentrations, this effect is thought to be low. It can be further concluded, same as for Ce, that the strong U-spore association leads to an immobilisation of metals and therefore reduces metal groundwater leaching which further explains the ability of *Streptomyces* to stabilise metals in soils, important for remediation.

3.2.3. Cadmium

The PHREEQC simulation suggested that the potentially ecotoxic Cd (II) species dominates mainly as Cd^{2+} and $CdCl^{+}$ (Fig. S8c) in the hypothetical aqueous phase of the SFM conditions, assuming similar pH values as for Ce and U. In such conditions, there are also no expected Cd precipitates at 10 μM nor 100 μM (i.e., low saturation indices (1–3) were only found at ≥ pH 7 for $Cd_3(PO_4)_2$, Fig. S8f). The scICP-MS measurement showed no events for Cd under any washing conditions (data not shown) above the background equivalent mass (mass limit of detection 0.006 fg cell^{-1}) calculated as in a previous publication (Corte Rodríguez et al., 2017). Therefore, we concluded that Cd neither adsorbs nor is weakly or strongly associated with *S. coelicolor* metal-spores obtained from metal-amended cultures. However, previous reports investigating *Streptomyces zinciresistens* hyphae (not spores), have found Cd accumulation mainly on the cell wall followed by intracellular accumulation performed under comparable pH conditions (pH 7.5) to our work (Lin et al., 2012). Assuming that both strains behave similarly, this could mean that the Cd can be associated with the metabolically active, sporulating hyphae, but not with the spores. Further work will be necessary to estimate if Cd is accumulated into *S. coelicolor* hyphae as is the case for *S. zinciresistens*. Another hypothesis is that the Cd^{2+} concentration in the medium is comparably lower than those of other divalent ions such as Ca^{2+}. Even the highest Cd concentration (100 μM) is a factor of 420 lower than the Ca concentration (42,000 μM) (Table S6). Assuming that both ions would compete for the association with *S. coelicolor* under our experimental conditions, much more Ca would be spore-associated and almost no Cd. This could consequently mean that depending on the ions present in the metal-contaminated soil site also other ions could interfere the bioremediation efficiency in the same way as demonstrated for Cd vs. Ca. To summarise, *S. coelicolor* metal-spores were not associated with Cd. Therefore, we conclude *S. coelicolor* cannot be used for the bioremediation of Cd-contaminated soil environments. Thus, Cd was not further investigated in the repetition, Batch 2.

3.3. Bulk analysis: acid digestion of metal-spores obtained from metal-amended cultures

We further investigated if Ce, U and Cd are strongly associated (incorporated and/or irreversibly bound) to the metal-spores by looking at the results from the acid digestions of the spore suspensions analysed via bulk ICP-MS (Fig. 5a–c). As spore suspensions had different spore number concentrations (Table S8 & Fig. S5), metal concentrations were normalised to the protein concentration using the Bradford assay.

For all analytes, there was no systematic trend of a higher concentration obtained after the Tris treatment between both washing treatments (EDTA + Tris vs. Tris). This strengthens the hypothesis that Ce and U are only strongly associated with the metal-spores (Fig. 5a and b) as previously seen in the scICP-MS investigations. Moreover, there is a linear concentration increase of all elements up to 10 μM. After that, a plateau was reached, which was especially clear for Ce and less clear for Cd. Although there were no Cd events in the scICP-MS measurement, Cd was found via acid digestion (Fig. 5c). This can be explained by comparing the dilution factors (DF) used for scICP-MS (DF: 3500–100,000) vs. acid digestion (DF: 15). Even for the highest Cd exposure concentration (100 μM, EDTA + Tris treatment) a raw concentration of only 0.45 μM was measured for the acid digestion of 100 μL spore suspension via bulk ICP-MS. The same spore suspension was diluted (DF: 10,000) for the scICP-MS. This would result in a concentration of around 0.05 μM which cannot be distinguished from the ionic background. This suggests that the Cd measured via acid digestion is ionic/dissolved and not associated with the metal-spores, despite the washing treatment.

The overall Ce association with the metal-spores obtained from metal-amended solid SFM medium accounted for 0.1–1.1% of the original spike (mass (Ce) from the bulk investigation divided by mass (Ce) of the original spike to the medium). These values are much lower than found in literature, where three out of four tested *Streptomyces* strains (*S. albus*, *S. viridans*, *S. flavovirens* and *S. viridoflavus*) retained 71–79% of Ce after 16 days of exposure at pH 6–7 in liquid medium (nutrient broth + 1 mM citrate) (Johnson and Kyker, 1961). Contrastingly, *S. viridoflavus* only took up 21% of the Ce. A similar trend was found regarding U association with the spores (< 0.1–2.4% of the original spike), which was also much lower than reported in literature for *Streptomyces* sp. hyphae (~60% uptake from U spiked solution) (Golab et al., 1991). For Cd, those values were even lower (< 0.1–0.2% of the original spike). Compared to literature, the removal efficiency by *S. zinciresistens* from Cd spiked solution ranged from 5 to 77% and was very sensitive to competing ions. There are two possible explanations for the lower retentions in our work. Firstly, we only accounted for the spores, not for the (leftover) hyphae. However, high quantities of metals

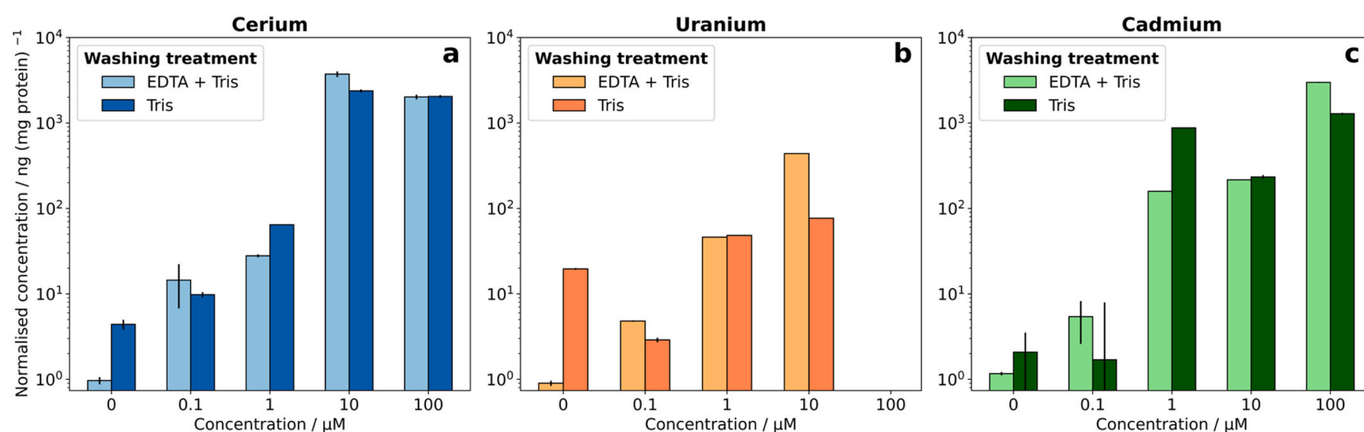

Fig. 5. Acid digestion of 100 µL spore suspension (Batch 1, metal-spores from metal-amended solid SFM medium) normalised per mg protein/100 µL spore suspension achieved using Bradford assay shown for **(a)** Cerium; **(b)** Uranium (for the 100 µM concentration, the spore number concentration was too low for protein to be quantified and therefore the acid digestion could not be normalised); **(c)** Cadmium. The bars are mean values and the error bars show the expanded uncertainties (k_{95} = expansion factor = 2) of three replicates.

potentially accumulated there as well. Secondly, in our work, a solid growing medium (SFM) was used, which is especially suitable to maintain the spores. Other studies in literature used a liquid medium, which results in a faster diffusion speed and higher bioavailability. It confirmed that Ce and U are both strongly associated to the metal-spores obtained from metal-amended cultures that even the comparably stronger EDTA + Tris washing buffer was not able to remove it. Further details on the EDTA + Tris supernatant investigations can be found in Fig. S9.

3.4. Adsorption of Ce, Cd and U onto non-metal-spores obtained from metal-non-amended cultures but incubated in aqueous metal solution

Additional investigations were conducted as described in section 2.4 to assess the adsorption of metals onto the non-metal-spores which were obtained from SFM non-amended cultures and left in aqueous metal-spiked ultrapure water (Fig. S1, Batch 0). Dormant spores have no or very limited metabolism (Bobek et al., 2017). Consequently, *S. coelicolor* spores can passively adsorb metals but not actively bioaccumulate them, without going through germination and the formation of metabolically active, sporulating hyphae. As pH conditions were kept comparable (pH 5.5–6.0) to the previous metal-spore experiment, the comparison between metal-spores and non-metal-spores was possible. This allowed us to distinguish between metal adsorption and weak or strong metal association with spores. Previous publications in adsorption kinetics with *Streptomyces* sp. *K33* and *HL-12* revealed that the adsorption equilibrium was reached after 30–60 min incubation (Yuan et al., 2009). To ensure that the adsorption equilibrium was reached, the non-metal-spores were left for 24 h in ultrapure water spiked with the sought metals at 10 µM; spores were then washed with EDTA + Tris vs. Tris and the total Ce, U and Cd quantified in the aqueous phases (the first supernatant (equilibrium supernatant) and the three supernatants from each washing step after centrifugation) via bulk ICP-MS (Fig. 6). The four aforementioned fractions were subtracted from the total content of metals added to the spore suspensions to estimate the “remaining spore associated” fraction.

In the case of Ce, for those conditions (ultrapure water spiked with 10 µM Ce and exposed to atmospheric CO_2), the PHREEQC species simulations suggested that the spores would be exposed to ionic Ce^{3+} or Ce(III) carbonate species (Fig. S10a). We found that 25% of the Ce added was in the aqueous phase of the (first) equilibrium supernatant after centrifugation. The uncertainties for all percentages in this section were below 0.5%. The EDTA + Tris washing buffer removed ca. 12% more Ce (sum washing step I–III: 17%) from the spores than the Tris buffer (sum washing step I–III: 5%) (Fig. 6a). This 5% can be considered as weakly adsorbed Ce while the remaining 12% are considered as strongly adsorbed with the spores. The remaining 58% (EDTA + Tris) and 70% (Tris) spore-associated Ce is in accordance with the literature, they

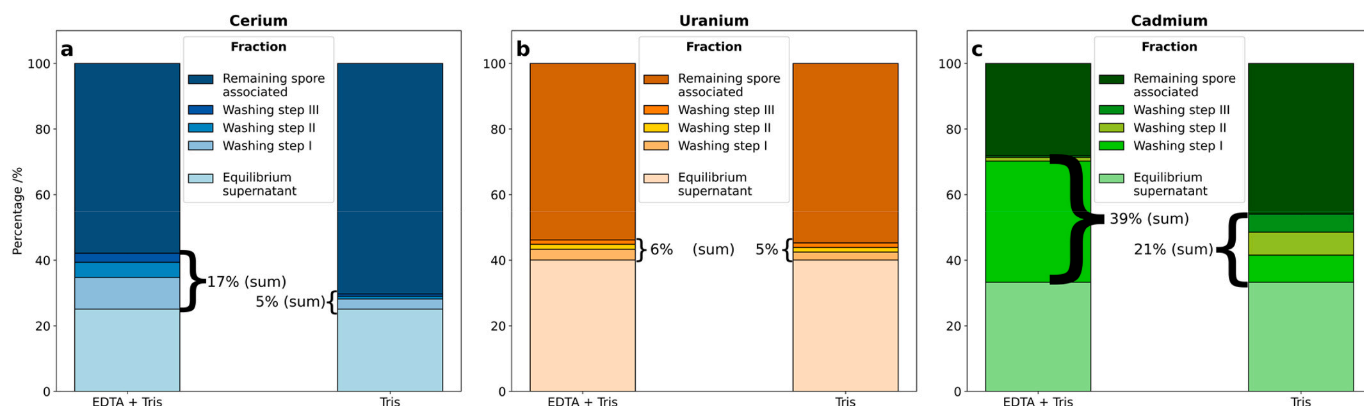

Fig. 6. Mass balance of the metal adsorption onto the non-metal-spores obtained from metal-non-amended cultures but incubated in metal aqueous solution (10 µM). “Equilibrium supernatant” represents the first supernatant which is re-dissolved after centrifugation. “Washing steps I–III” represent the three consecutive washing supernatants which contain the re-dissolved metals after centrifugation. “Remaining spore associated” is the difference between the metals added to the spore suspension (10 µM) minus the equilibrium supernatant and the three washing supernatants. The uncertainty is < 0.5% for all percentages. **(a)** Cerium; **(b)** Uranium; **(c)** Cadmium.

reported retention capacities from 71 to 79% for three out of four *Streptomyces* strains (hyphae) using a liquid medium (Johnson and Kyker, 1961).

Contrastingly, the PHREEQC simulation for U showed that spores would be exposed to ionic UO_2^{2+}, hydroxide or carbonate U(VI) species (Fig. S10b). 40% U remained in solution after the first centrifugation. After washing with EDTA + Tris (sum washing step I–III: 6%) vs. Tris (sum washing step I–III: 5%), we found that both solutions provided comparable results (Fig. 6b). This can either mean that U does not have a high affinity for complexation with EDTA under those conditions, or that the majority of U is so strongly associated with the spores that the washing steps are not able to remove it 54% (EDTA + Tris) and 55% (Tris) remaining spore-associated content suits well to an adsorption study, the authors reported that 60% of the UO_2^{2+} (exposed to 1 mM U) were removed from the liquid medium by *Streptomyces* sp. (hyphae) (Golab et al., 1991). Another crucial parameter to take into account is the pH. For this experiment, pH was between 5.5 and 6.0 (20–25 °C). Previous studies showed for UO_2^{2+} that this is the optimal pH range for an ideal adsorption process on *Streptomyces* hyphae (not spores), thus our study confirms the strong association of U with *Streptomyces* spores (Golab et al., 1991).

In the case of Cd, the PHREEQC simulation indicated that spores would be mainly exposed to ionic Cd^{2+} (Fig. S10c). As expected from the previous results, 33% of the Cd remained in solution after the first centrifugation (Fig. 6c). Using EDTA + Tris treatment, a high percentage (37%) of the Cd was removed in the first washing step (sum washing step I–III: 39%) showing a high affinity to be complexed by EDTA under our experimental conditions. Compared to the Tris treatment (sum washing step I–III: 21%), 18% more Cd was removed with the EDTA + Tris treatment. Thus, 28% (EDTA + Tris) respectively 46% (Tris) remained as spore-associated which is in accordance with previous literature, 26–55% (calculated from their data, Cd uptake from cells divided by the total Cd spike) of the Cd was taken up by the mycelia using liquid medium (Dimkpa et al., 2009).

Overall, 70±0.5% Ce, 55±0.5% U and 46±0.5% Cd out of the total spike are captured by the non-metal-spores: 12±0.5% Ce, 1±0.5% U and 18±0.5% U were weakly absorbed (i.e., difference Tris minus EDTA + Tris treatment); and 58±0.5% Ce, 54±0.5% U and 28±0.5% Cd were strongly absorbed (i.e., spore remaining after EDTA + Tris treatment). 30±0.5% Ce, 45±0.5% U and 54±0.5% Cd were not spore-associated and remained in the aqueous phases (equilibrium and washing supernatants).

The remaining spore-associated fraction was further investigated using scICP-MS. A high number of events was observed for Ce and U (n = 133–206) after both washing treatments, confirming metal adsorption onto non-metal-spores (Fig. 7a and b). The mean and median values after the Tris washing treatment showed higher adsorbed masses of Ce (rel. percentages 58% respectively 43% higher) and of U (19% respectively 47% higher) per spore than after the EDTA + Tris treatment, indicating that some metals were reversibly bound to the spores and could be removed by EDTA + Tris. In contrast, only very few events were found for Cd (n = 17–25) suggesting (almost) no adsorption onto non-metal-spores (Fig. 7c). This was confirmed by looking at the Cd event numbers, normalised by P (Fig. 7f). Only 12–13% of the spores were

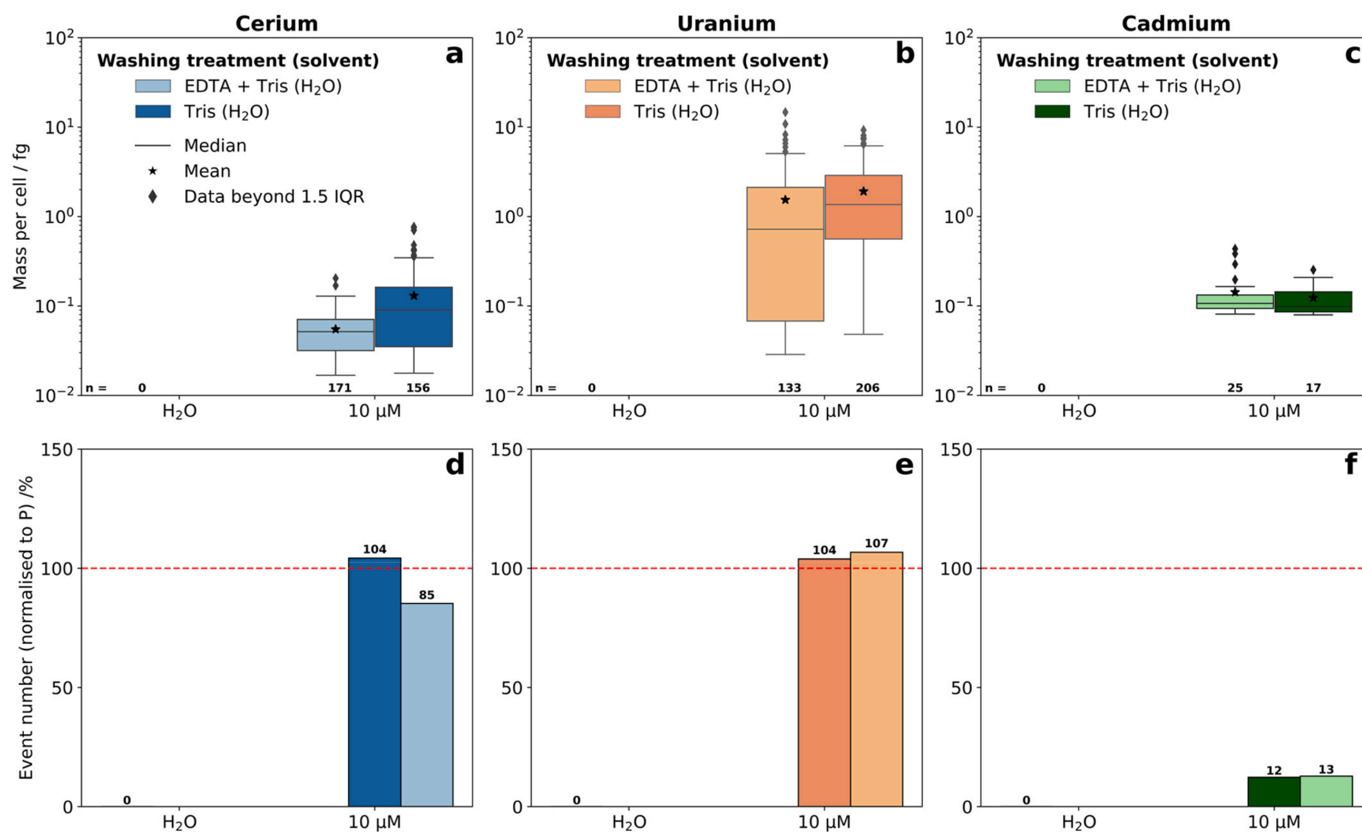

Fig. 7. ScICP-MS investigation of non-metal-spores obtained from metal-non-amended cultures but incubated in metal aqueous solution (10 μM). The boxes include data points between the 1st quartile (25%) and the 3rd quartile (75%) and the whisker range represents the 1.5 interquartile range (IQR). The dots outside the boxes show spores with concentrations above (or below) the whiskers limits. "n" represents the event number for Ce, Cd or U detected per run. The red dashed line means that all spores (100%) were associated with U. **(a)** Adsorbed Ce mass per spore vs. exposure concentration in suspension; **(b)** Adsorbed U mass per spore vs. exposure concentration in suspension; **(c)** Adsorbed Cd mass per spore vs. exposure concentration in suspension; **(d)** Ce event number normalised by the P event number (Ce events/P events) vs. exposure concentration in suspension; **(e)** U event number normalised by the P event number (U events/P events) vs. exposure concentration in suspension; **(f)** Cd event number normalised by the P event number (Cd events/P events) vs. exposure concentration in suspension. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

associated with Cd. Contrary to Cd, all spores were associated with Ce and U (ca. 100%) (Fig. 7d and e). The event numbers found for Ce and U normalised by P for non-metal-spores showed comparable percentages to metal-spores (obtained from the metal-amended SFM solid medium) exposed to 10 μM concentration, indicating that, in both cases, all spores were associated with Ce and U, respectively. In the case of Cd, almost no events were detected (around 20) showing the lack of adsorption in this case.

Comparing the adsorbed masses onto non-metal-spores (Fig. 7) with the strongly associated masses with metal-spores (Figs. 3 and 4), we found for Ce that only slightly lower masses were strongly associated with metal-spores compared to the adsorbed masses onto non-metal-spores (66–73% from the adsorbed mass). Contrastingly for U, results revealed that only 2–4% of the adsorbed masses onto non-metal-spores were strongly associated with metal-spores. Differences can be related to the variability of aqueous speciation. For Ce, metal-spores incubated in SFM medium, exposed to phosphate would generate phosphate species while non-metal-spores were only exposed to ultrapure water + Ce spike, forming mainly ionic Ce^{3+} species according to the pH conditions between 4 and 6. This confirms our initial indication (section 3.2.1) that *S. coelicolor* can control the spore-association processes for Ce^{3+} or Ce-phosphate species from the medium and the exposed species are bioavailable for the spores. In contrast, the much higher adsorbed U content for non-metal-spores (aqueous setting) than for metal-spores (metal-amended setting) suggests that spores can adsorb high quantities of U which does not necessarily mean they are bioavailable. Only small percentages were associated with metal-spores also confirming our initial indication (section 3.2.2) that *S. coelicolor* cannot control U-association processes. Additionally, if Ce or U phosphates are built in the SFM medium, both are poorly water-soluble. This could also lead, in both cases, to a reduced bioavailability for metal-spores.

4. Conclusions

Our study shows the great potential of a relatively novel method, single cell ICP-MS, to investigate metal-associated content in individual biological cells including cell-to-cell variations. This work presents the feasibility of pre-washing experiments to investigate association processes with biological cells using complex building washing agents such as EDTA.

Despite the expected dormant phase of *Streptomyces coelicolor*, we found that fresh spores obtained from metal-non-amended cultures but incubated in metal aqueous solution are capable of mainly strong adsorption (i.e., 58% Ce, 54% U and 28% Cd of total spike) but also weak adsorption (12% Ce, 1% U and 18% Cd) of free ions, hydroxide and carbonate species from target metals representing aqueous systems with low ionic strength. However, evaluating spores from metal-amended cultures, all Ce and U were incorporated in or strongly associated with *S. coelicolor* spores after growing in a metal-spiked phosphate-rich solid medium. As the speciation of the metals is different under aqueous conditions than in the solid soya flour mannitol (SFM) growing medium, we point out that speciation of the metals is key for spore-association processes and bioavailability. While only slightly less Ce (66–73% from the adsorbed mass onto non-metal-spores) was found spore-associated, much less spore-associated U (2–4% from the adsorbed masses onto non-metal-spores) was found when comparing spores obtained from the metal-amended solid SFM medium (weak or strong spore association) with ungerminated spores obtained from not amended medium but incubated in metal spiked aqueous medium (adsorption). This could mean that during the life cycle of *S. coelicolor*, the hyphae are capable of controlling Ce but not U association processes. Once the next generation of spores is formed in the solid growing medium, they inherit the adsorbed Ce content but only minor parts of the adsorbed U. With increasing metal exposure concentrations in the growing medium, a comparable mass of Ce but more U per spore was spore-associated. In both cases, the number of spore-associated metals increased with exposure concentration.

To summarise, the strong spore-associated content of Ce and U implies that *S. coelicolor* effectively traps and therefore immobilises them (not the case for Cd), making it a viable organism for remediation without aiming to remove but to stabilise Ce and U. This is crucial information if using *Streptomyces* for bioremediation purposes to prevent ecotoxicological critical processes such as further leaching of metals into the groundwater or remediation in soils. We present an adsorption process of Ce and U onto metal-non-amended spores if exposed to metal aqueous solution, followed by biological active uptake and processing during incubation in the growing medium, resulting in the presence of biological-associated metals in the newly formed spores, effectively encapsulating Ce and U retention.

Author contributions

Conceptualisation, J.B., A.M., T.S. and M.M.-B.; methodology, S.H.; validation, S.H. and M.C.-R.; investigation, S.H., P.G.C. and S.A.F.; resources, J.B., A.M. and M.M.-B.; writing—original draft preparation, S. H.; writing—review and editing, T.S., A.M., D.M., T.G.D., M.C.-R., P.G. C., S.A.F. and M.M.-B.; visualisation, S.H.; supervision, T.S. and M.M.-B. All authors have read and agreed to the published version of the manuscript.

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Declaration of competing interest

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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