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The effect of organic carbon form and concentration on fungal selenite reduction

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ABSTRACT

Fungi are essential to the transformation of carbon (C) and are well-known for degrading recalcitrant plant material. They are also increasingly recognized for their bioremediation potential in contaminated environments. Some fungi transform selenium (Se), an essential nutrient and toxin of growing concern, from an aqueous bioavailable phase (Se(+IV, VI)) to solid or volatile phases (Se(0, -II)). We examined the effect of C on growth and Se transformation for several Ascomycota fungi, including *Alternaria alternata* SRC1lrK2f, *Paraphaeosphaeria sporulosa* AP3s5-JAC2a and *Pyrenochaeta* sp. DS3sAY3a, grown in 0–60 mmol C/L glucose or acetate with 0.1 mM Se(IV) over 23 days. Higher C concentrations coincided with more Se removal, but some species preferred certain C sources. *A. alternata* generally reduced the most Se regardless of C source, but *Pyrenochaeta* sp. reduced more with glucose. *A. alternata* predominantly solidified Se, whereas *P. sporulosa* mainly volatilized Se. Glucose and acetate concentrations in cultures with and without Se trended downward throughout the experiment, suggesting that carbon consumption continues despite Se presence. As biomass did not substantially increase throughout the experiment for any organism with Se(IV) but C consumption continued, it is likely that the fungi experienced a toxicity effect with Se(IV) as observed with increased pigment production, and predominantly consumed C for cell maintenance and repair purposes. This study helps discern the effect of C on fungal Se transformations, providing greater insight into the coupling between C and Se biogeochemical cycling as well as improving Se bioremediation strategies.

1. Introduction

Fungi hold an essential role in the global biogeochemical cycling of many elements, including carbon (C), nitrogen (N), phosphorus (P), manganese (Mn), sulfur (S), and selenium (Se). They play a particularly important and well-known role in the carbon cycle as they are primary decomposers and recyclers of plant material such as cellulose, hemicellulose, pectin, and lignin in the environment (Pérez et al., 2002; Liu et al., 2013; Sun et al., 2017; Zhang et al., 2019; Schilling et al., 2020). The ability of fungi to degrade carbon compounds is performed through the release of extracellular enzymes and metabolites (i.e., the secretome; Tjalsma et al., 2000; Agrawal et al., 2010; Zeiner et al., 2016; Zeiner et al., 2017). Fungi secrete cellulases, hemicellulases, pectinases, and lignin-degrading enzymes into their surroundings in order to break down these recalcitrant C molecules to simpler, more labile compounds such as glucose for use in cellular activity (Tuomela et al., 2000; Ruiz-Dueñas and Martínez, 2009; Schilling et al., 2012; Bi et al., 2016; Daly et al., 2020). In the environment, fungi can extend through areas containing different C substrates and use them, which impacts both gene expression and enzyme production by the fungal cells within that local area (Daly et al., 2020). Analysis of the fungal secretome of *Alternaria alternata* SRC1lrK2f, *Paraphaeosphaeria sporulosa* AP3s5-JAC2a and *Pyrenochaeta* sp. DS3sAY3a, revealed different C utilization strategies for the same C substrate over time and for each species (Zeiner et al., 2017). *A. alternata* and *P. sporulosa* released a series of enzymes depending on the amount of C remaining, whereas *Pyrenochaeta* sp. employed peptidases to rapidly break down C sources (Zeiner et al., 2017). Once in the cell, carbon can be metabolized through glycolysis, glycerol metabolism,

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fermentation, and the tricarboxylic acid (TCA) cycle, among others (de Assis et al., 2015). From these processes, metabolic pathways for the generation of the fungal cell wall and intracellular storage compounds allow for protection against external stresses and adaptation to surrounding environments (Annick Ries et al., 2018).

Glucose ($C_6H_{12}O_6$) and acetate (CH_3COOH) are two common fungal C substrates used in central metabolic pathways and are often provided in fungal growth experiments (e.g., Dijkema et al., 1985; Al-Bader et al., 2010; Hou et al., 2017; Lok et al., 2021). The metabolic flexibility associated with the ability to grow using a range of C substrates allows fungi to adjust their metabolism and nutrient uptake to suit their surroundings (MacCabe et al., 2003; de Assis et al., 2015). Glucose, in particular, is a primary C and energy source essential for cell metabolism, maintenance, and signalling (Forment et al., 2014; Zhang et al., 2015; Dos Reis et al., 2017). Glucose transport in the Ascomycete, *Aspergillus nidulans,* is mediated in part by monosaccharide transporter *mstA* and *mstC* genes that encode sugar permeases (dos Reis et al., 2013; Forment et al., 2014). It is a preferred energetically favorable carbon source for most organisms (Annick Ries et al., 2018; Lok et al., 2021), and can be used in both anaerobic (fermentation) and aerobic (respiration) metabolic pathways to obtain energy (Chambergo et al., 2002). In aerobic respiration, glucose is the substrate in glycolysis, a central metabolic pathway for most organisms (Jørgensen et al., 2007; Dörsam et al., 2017).

Acetate has also been shown to be an effective C source (e.g., Laughlin et al., 2009). Acetate can enter the fungal cell through acetate transporters, such as the membrane protein Ady2 in *Saccharomyces cerevisiae* or AcpA (acetate permease) in *A. nidulans* (Paiva et al., 2004; Robellet et al., 2008; Sá-Pessoa et al., 2015). In the cell, acetate is used by the enzyme acetate kinase, which is used for the synthesis of acetyl-CoA (Ingram-Smith et al., 2006). It can also be completely oxidized as an intermediate within the TCA cycle (Holligan and Jennings, 1973). In some cases, acetate is preferred over glucose as a C source, such as in the presence of Mn (Nüske et al., 2002). Additionally, acetate has also been shown to inhibit glucose uptake by *A. nidulans* when co-present (Romano and Kornberg 1969). Carbon catabolite repression (CCR) allows the selection of the most energetically favorable carbon substrate by suppressing gene expression for other C sources (Annick Ries et al., 2018). In the presence of acetate, fungi can inhibit the utilization of less energy rich (poorer) C substrates such as ethanol as a carbon source through CCR (Simpson-Lavy and Kupiec, 2019). Through these diverse metabolic and enzymatic processes that promote C sequestration and degradation, fungi play an integral role in the global C cycle.

While a large focus of fungal ability focuses on their role in the carbon or nitrogen cycle (Gadd, 2017), a growing body of research shows that fungi have an important role in many other elemental cycles such as manganese (Sutherland et al., 2018; Santelli et al., 2011), copper (Cuevas et al., 2015), silver (Guilger-Casagrande and Lima, 2019), nickel (Magyarosy et al., 2002), gold (Bohu et al., 2019), and tellurium (Gharieb et al., 1999), among others. Fungi also play an essential role in the global selenium (Se) cycle, as a number of fungal species have been shown to facilitate Se reduction, methylation, or oxidation reactions (Barkes and Fleming, 1974; Gharieb et al., 1995; Schilling et al., 2011; Ruocco et al., 2014; Rosenfeld et al., 2018; Liang et al., 2019). Selenium is both a micronutrient required for most life and an element of environmental concern due to its toxicity in high concentrations. In the environment, the mobility and toxicity of Se is closely tied to its redox chemistry (Ralston et al., 2008; Sharma et al., 2015). Selenate and selenite (Se(VI and IV)) are the water-soluble forms most common in oxic environments, whereas elemental Se(0) and organic or volatile Se (-II) are less bioavailable and less toxic to organisms (Lenz and Lens, 2009; Nancharaiah and Lens, 2015b; Chan et al., 2018).

A set of filamentous Ascomycete fungi were shown to effectively transform Se under atmospheric conditions (Rosenfeld et al., 2017), and mediated simultaneous Mn oxidation and Se reduction (Rosenfeld et al., 2020). In previous studies of fungal Se transformations by Rosenfeld et al. (2017, 2020), the fungal isolates were cultured in a HEPES-buffered growth media with acetate (3 mM) and yeast extract (15 g/L) as the C sources. While this growth media provided sufficient carbon and nutrients for substantial Se removal throughout the 30–40 day experiments, the role of carbon in Se reduction could not be discerned due to the undefined composition of yeast extract. Similarly, two *Alternaria* fungi were cultured in Se-contaminated wastewater supplemented with either carbohydrate- or glycerin-based proprietary carbon sources (Sabuda et al., 2020). While the fungi were more successful in Se (IV and VI) reduction and removal from solution with carbohydrates than glycerin, the C source composition was undefined. To date, the impact of defined carbon sources on fungal transformations of Se has not been identified, which limits our ability to understand and predict the impact of C availability on Se biogeochemical cycling.

In this study, we assessed the effect of two environmentally and metabolically relevant carbon sources, glucose and acetate, on fungal growth, Se tolerance, and Se reduction rates. In a series of culture-based experiments, we separately grew three Se-reducing Ascomycota, *Alternaria alternata* SRC1lrK2f, *Paraphaeosphaeria sporulosa* AP3s5-JAC2a, and *Pyrenochaeta* sp. DS3sAY3a in the presence of 0.1 mM Se(IV) and several carbon concentrations (0, 0.6, 6 and 60 mmol C/L) which span a range of environmental conditions. These experiments were conducted over the course of ~23 days in agar-solidified media to assess radial growth and in liquid media to quantify carbon consumption, Se removal from solution, Se uptake into the solid phase, and the amount of Se lost to volatilization. The results from this study aid in understanding the role that different carbon substrates play in fungal growth and selenite reduction, thus providing greater insight into the coupled C and Se biogeochemical cycling and fate of these elements in the environment. As the genetic mechanism(s) for fungal Se(IV) reduction are currently unresolved, these results provide insight for discerning the mechanism (s) behind these important Se transformations. Further, when designing a treatment strategy for Se-impacted environments, understanding which specific C sources are best for rapid fungal Se transformation to solid or volatile phases will be critical.

2. Materials and methods

2.1. Experiment setup and sampling

2.1.1. Agar-solidified culture experiments

Experiments on agar-solidified growth media were conducted to compare the impact of selenite and both C source and concentration on radial fungal growth rates. Agar (1.5%) plates contained 30 mL of a yeast extract based media containing 0.15 g/L yeast extract, 20 mL HEPES buffer (filter sterile, pH 7, 1.0 M), 1 ml/L trace element stock (10 mg/L $CuSO_4{\cdot}5H_2O$, 44 mg/L $ZnSO_4{\cdot}7H_2O$, 20 mg/L $CoCl_2{\cdot}6H_2O$, 13 mg/L $Na_2MoO_4{\cdot}2H_2O$) and with either acetate (as sodium acetate) or glucose (as dextrose) at 0, 0.6, 6, or 60 mmol C/L. Media was supplemented with or without the addition of 0.1 mM selenite (Se(IV)) and plates were individually inoculated with one of six Se-reducing fungal isolates, *Pyrenochaeta* sp. strain DS3sAY3a, *Acremonium strictum* strain DS1bioAY4a, *Plectosphaerella cucumerina* strain DS2psM2A2, *Stagonospora* sp. strain SRC1lsM3a, *Alternaria alternata* strain SRC1lrK2f, or *Paraphaeosphaeria sporulosa* strain AP3s5-JAC2a (Santelli et al., 2010, 2014). *P. sporulosa* has also been referred to as *Paraconiothyrium sporulosum*, but previous work placed this organism in the genus *Paraphaeosphaeria* (Verkley et al., 2014). These fungal isolates are described in several previous studies for their ability to transform metals and metalloids (Santelli et al., 2010; Rosenfeld et al., 2017; Sabuda et al., 2020). Cultures were inoculated by swabbing a stock fungal isolate plate with a sterile toothpick and stabbing the center of each new agar plate. Two abiotic plates were also produced for each of the four concentrations to ensure that the process was sterile. All growth experiments were conducted in duplicate.

Plates were incubated in the dark at room temperature and five radial growth measurements were collected approximately every five days for each culture. Radial growth was measured from where the toothpick was inserted into the solid media to the point at which the fungi had grown the farthest radially. Growth rates were determined by measuring the radial extent of the fungal hyphae over time. At the final measurement dates, pictures of each culture were taken in order to record color and morphology of the fungi. From the results of these agar plates, three representative fungal isolates were selected for more comprehensive liquid culture experiments based on differences observed in their growth rates and pink color (indicative of elemental Se (0) formation).

2.1.2. *Liquid culture experiments*

Culture experiments with three Se-reducing organisms, *A. alternata* SRC1lrK2f, *P. sporulosa* AP3s5-JAC2a and *Pyrenochaeta* sp. DS3sAY3a, were conducted similar to the solid culture experiments but in liquid (without agar) media so fungal biomass, carbon consumption, and selenite reduction rates could be quantified. Each species was grown in 200 mL of AY media containing acetate (primary C source) and a small amount of yeast extract (primary N and P and micronutrient source that also may serve as a C source) (adapted from Miyata et al., 2004 as described in Rosenfeld et al., 2017) in a sterile 500 mL Erlenmeyer flask for 14 days to allow growth of biomass. After 14 days, the biomass inoculum for each species was centrifuged at 4500 rpm for 7 min and washed with sterile ultrapure (18 mΩ × cm) water. Using aseptic techniques, each inoculum stock was then homogenized with 150 mL ultrapure water in a blender. Blended biomass (200 μL) was then added to 250 mL Erlenmeyer flasks containing 100 mL modified AY media without acetate and incubated in the dark at room temperature for 18 days. While at this stage the media contained yeast extract, it was necessary to permit growth of enough biomass in reliable and repeatable weights for the experiment to proceed, and allow for rapid Se(IV) reduction and Se quantification.

After 18 days of growth, flasks of *A. alternata* and *P. sporulosa* were amended with 0.1 mM sodium selenite and either acetate or glucose at 0, 0.6, 6, or 60 mmol C/L, which indicated the start of the experiments. *Pyrenochaeta* sp. was only cultured at the higher 6 and 60 mmol C/L based on results from the previous two fungal experiments. Note that while concentrations of carbon remained the same for glucose and acetate amendments, as glucose has six C atoms per molecule and acetate has two, the starting concentrations were different (i.e. 0.6 mmol C/L equalled 0.1 mM glucose and 0.3 mM acetate). Triplicate flasks were established for each analysis at every sampling point. Biomass-free controls, cultures without carbon, and cultures without selenite amendments were established as a comparison to Se- and C-amended cultures. A set of flasks was sampled immediately before amendment addition to determine the starting biomass weight. Flasks for each condition were sampled immediately after Se or C addition (within ~5 min), and at 12-h post-amendment addition. As each experiment was offset due to limitations on lab space and supplies, sampling days thereafter are shifted slightly. For *A. alternata* additional samples were collected 1, 2, 4, 8, 10, 23, 29, and 34 days post-acetate amendment and 1, 2, 5, 7, 15, 21, 25, and 30 days post-glucose addition. *P. sporulosa* was sampled at days 1, 2, 3, 4, 9, 14, and 22 days after acetate amendment addition and 1, 2, 4, 7, 11, 17 and 23 days after glucose amendment. Glucose- and acetate-amended *Pyrenochaeta* sp. were all sampled at 1, 2, 4, 7 14, 22, and 30 days after amendment addition. The latest sampling point in common between the cultures was ~23 days, which was used to signify the end of the experiments. At each liquid culture sampling point, the flasks of fungal biomass and media were filtered onto pre-weighed 47 mm 0.22 μm mixed cellulose ester (MCE) filters, air dried overnight, weighed, and preserved as described below for geochemical analyses.

2.2. *Analytical methods*

2.2.1. *Quantitative aqueous chemistry*

Aqueous samples were collected at each sampling point from the liquid culture experiments to assess the amount of Se and either acetate or glucose remaining in solution over time. The 100 mL of filtered (0.22 μm) liquid media from each flask was stored in two sterile 50 mL falcon tubes at −20 °C until selenate, selenite, and acetate concentrations were quantified via ion chromatography (IC; Metrohm, USA). Selenate and selenite anions were measured as described previously by Rosenfeld et al. (2017, 2020), but modified with 3.0 mM sodium carbonate eluent to more effectively separate the peaks. Acetate was measured with an organic acids column 250/7.8 (6.1005.200) using a sulfuric-acid only eluent following manufacturer instructions. As the techniques for detecting the compounds required different columns, one falcon tube of media was used for selenate and selenite quantification, while the other was used for acetate. The limit of detection for Se species and acetate was 0.001 mM. Glucose was analyzed using a D-Glucose - HK assay kit following manufacturer instructions (Megazyme, Co. Wicklow, Ireland), with a detection limit of 0.663 mg/L (3.7 μM) glucose. Samples were analyzed using a UV–vis spectrometer (Agilent Technologies, Cary 60) at 340 nm wavelength.

2.2.2. *Fungal growth and solid-associated Se*

The amount of fungal biomass and Se associated with the solid phase was determined as described previously by Rosenfeld et al. (2017) and Sabuda et al. (2020). To determine the fungal weight at each sampling point, the preliminary weight of each filter was subtracted from the combined filter and air-dried fungal biomass weight at experiment end. Fungal biomass was air dried due to concerns about potentially altering the Se products, either by changing the biomineral form from an amorphous to crystalline form or volatilizing some of the biomass-associated Se. Fungal biomass and filters were then chemically digested in 1 mL hydrogen peroxide and 6 mL concentrated trace metal grade nitric acid in individual polyfluoroalkyl (PFA) bottles overnight. Sample digests were diluted 1:1 with ultrapure (18 mΩ × cm) water and analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES; Thermo-Fisher Scientific) in the Analytical Geochemistry Lab at the University of Minnesota. The limit of detection for this instrument was 0.01 mg/L.

2.2.3. *Selenium volatilization calculations*

A mass balance was calculated to determine roughly how much Se had potentially been volatilized by the fungi from each set of flasks. To calculate mass balances, Se concentrations (mM) were converted to molar quantities (μmol) for easier comparison between Se forms. Se remaining in the aqueous phase at each sampling point was added to the μmoles of biomass-associated Se quantified at each time point. This quantity was subtracted from the amount of Se initially added (0.1 mM; 10 μmol) to determine the amount of Se "missing" or potentially volatilized by the fungi. Each Se fraction was then converted to a percent. This conversion allows the comparison of Se associated with different phases (i.e., solution, solid, and gas) regardless of the volume of each phase.

2.2.4. *Electron microscopy*

At the end of each experiment containing Se (i.e. after 21–23 days growth depending on each fungal species), a small amount of Se-containing biomass was preserved in a fixative solution containing 2.5% glutaraldehyde. After overnight fixation, the samples were prepared as described by Rosenfeld et al. (2017) and Rosenfeld et al. (2020) for transmission electron microscopy (TEM). Briefly, fungal biomass was dehydrated in an ethanol series (75%, 95%, 100%) and placed in epoxy resin. Biomass and resin was cured at both 40 °C and 60 °C overnight before sectioning on a Diatome 45° diamond knife using a Leica UC6 ultramicrotome (Leica Microsystems) and mounted on 100 mesh Cu grids. One replicate from each sample section was kept unstained and the other was post-stained with 2% uranyl acetate before both were imaged at 120 kV with a FEI Tecnai G2 Spirit BioTWIN in the Characterization Facility at the University of Minnesota.

2.3. Statistical analysis

All statistical analyses were performed using the R basic statistics package (R Core Team, 2021), and results were considered significant at p-values <0.05. For aqueous Se, solid-associated Se and biomass weights at experiment end, we compared organic carbon form (acetate, glucose), the three fungal species (*A. alternata, P. sporulosa, Pyrenochaeta* sp.) and four organic carbon concentrations (0, 0.6, 6, and 60 mmol C/L) by two-way and three-way analysis of variance (ANOVA). Tukey's Honest Significant Difference (HSD) post-hoc test was used to examine aqueous Se, solid-associated Se and biomass weights data each for significance of interactions between organic carbon type, fungal species, and carbon concentration at experiment end.

3. Results

3.1. Impact of organic carbon source and concentration on fungal growth

3.1.1. Solid media cultures

When the fungi were grown on agar-solidified media, radial fungal growth in the presence of 0.1 mM Se(IV) was repressed compared to Se-free cultures for all six organisms (Fig. 1; Supplementary Figs. 1–3; Supplementary Table 1). In the presence of Se(IV), increasing the concentration of organic carbon led to more radial fungal growth in *A. alternata*, *P. sporulosa, Pyrenochaeta* sp., and *Stagonospora* sp. (Fig. 1; Supplementary Fig. 2). The exceptions to this C-concentration trend were *A. strictum,* which barely grew throughout the experiment (<5 mm) in the presence of Se(IV), and *P. cucumerina,* which grew the farthest radially and fastest without a carbon amendment. *P. cucumerina* showed a steep increase in radial growth over time and exhibited growth trends in reverse of all other cultures (Supplementary Fig. 2).

For each of the six organisms, growth trends appeared similar between acetate- and glucose-amended cultures. Radial growth generally appeared to increase at different rates over a period of ~2 weeks, then plateaued. In the presence of Se(IV), radial fungal growth is the furthest and faster overall with *A. alternata*, regardless of organic carbon source. *A. alternata* cultures appear light reddish-pink in all Se(IV)-amended cultures, regardless of carbon source or concentration (Supplementary Fig. 4). By experiment end, *A. alternata* in 0.1 mM Se(IV) and acetate grew radially to 50 mm in 60 mmol C/L, but only to ~25 mm for all other acetate concentrations (Fig. 1). With glucose, *A. alternata* grew to ~40 mm with 60 mmol C/L, but to only ~30 mm with all other concentrations. When amended with this highest acetate concentration, *A. alternata* exhibits a blackish-colored pigment. In the equivalent glucose plates, the center of the plate appears black with a ring of pink-red color along the biomass edges where the hyphae are growing through apical extension (Supplementary Fig. 4).

With the exception of *A. alternata* grown under the highest acetate concentration and Se(IV), none of the hyphal growth reached the extent of the Petri dish (50 mm) and most radial growth ceased well before reaching the maximum extent possible. *P. sporulosa*, *Pyrenochaeta* sp., and *Stagonospora* sp. did not grow radially >15 mm when amended with either carbon source and 0.1 mM Se(IV). With both acetate and glucose, *P. sporulosa* and *Pyrenochaeta* sp. grew the most radially with 60 mmol C/L. A few times throughout the experiments, fungal growth in 0.6 mmol C/L exceeded that of 6 mmol C/L (e.g. *Pyrenochaeta* sp. acetate and glucose, *A. alternata* glucose). *P. sporulosa* cultures appear bright pink in the biomass center where plates were inoculated, and slowly fade toward the new growth, regardless of organic carbon source (Supplementary Fig. 5). *Pyrenochaeta* sp. cultures are dark brownish-black overall compared to *A. alternata* and *P. sporulosa* (Supplementary Fig. 6), have a slight orange-pink hue, and have concentric rings of pigment shades and growth.

3.1.2. Liquid media cultures

Fungal biomass in liquid media amended with 0.1 mM Se(IV) held similar trends to their agar-solidified culture counterparts. The three organisms chosen for liquid culture, *A. alternata, P. sporulosa,* and *Pyrenochaeta* sp., grew to the highest biomass in 60 mmol C/L over the course of the experiment, regardless of organic carbon source (Fig. 2; Supplementary Table 2). Without Se(IV), fungal biomass increased or stayed the same throughout time, without fluctuation (Supplementary Fig. 7). With Se(IV) present, fungal biomass appeared to fluctuate over

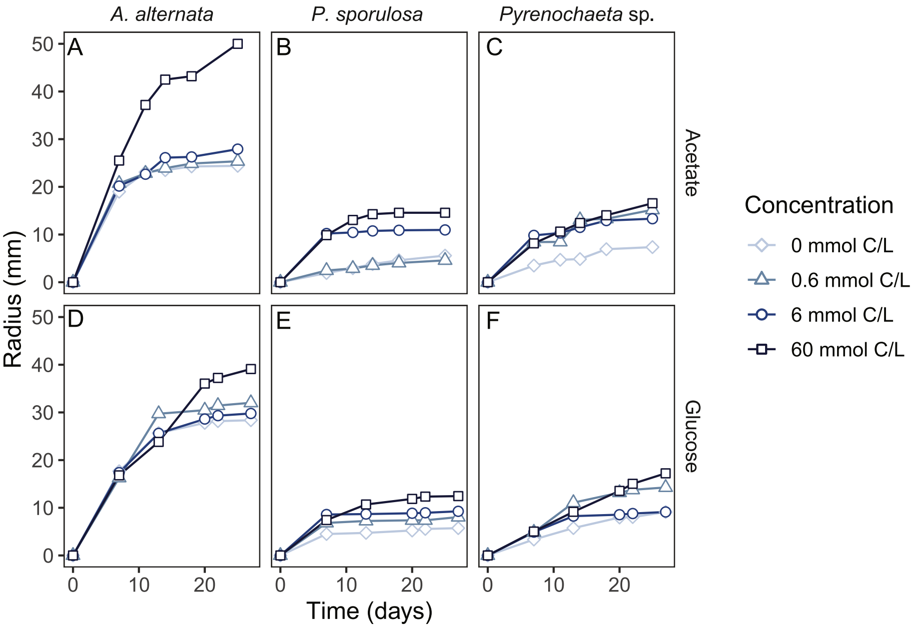

Fig. 1. *A. alternata* (A, D), *P. sporulosa* (B, E), and *Pyrenochaeta* sp. (C, F) growth on solid media containing Se(IV) and either acetate (top panels) or glucose (bottom panels) at 0, 0.6, 6 and 60 mmol C/L.

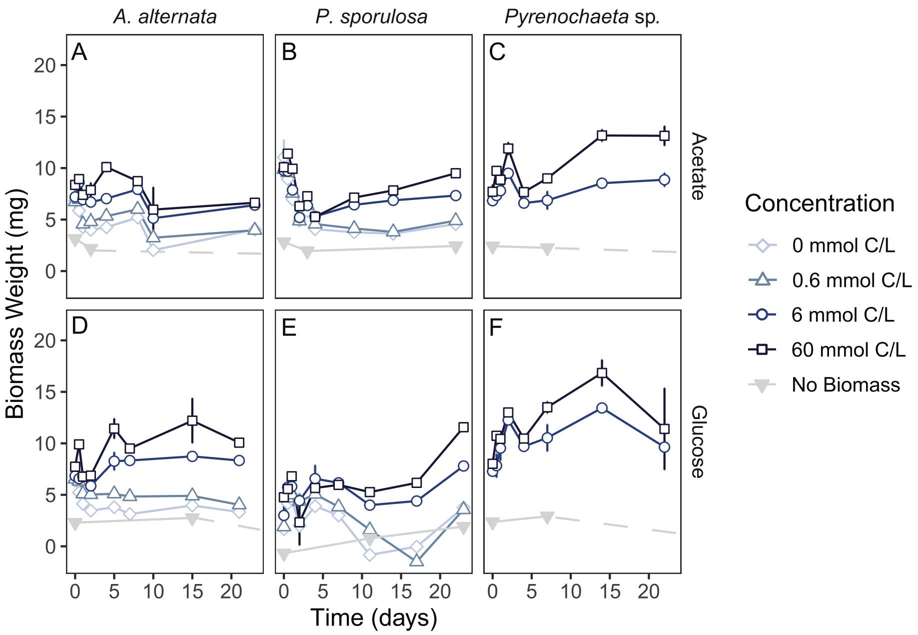

Fig. 2. - *A. alternata* (A, D), *P. sporulosa* (B, E) and *Pyrenochaeta* sp. (C, F) dry biomass weights over time in liquid media containing Se(IV) and either acetate (top panels) or glucose (bottom panels) at various C concentrations, with different shapes representing different C concentrations. Biomass-free controls are also plotted, with dashed lines indicating that the final sampling point was taken just after the ∼23-day experiment end.

the first 5–10 days, before showing clearer growth trends of steady or increasing biomass for nearly all higher C experiments, except *Pyrenochaeta* sp. in glucose (i.e., 6 and 60 mmol C/L). In many cases, the biomass decreased over time in most low C experiments (0 and 0.6 mmol C/L). For example, despite showing the greatest radial growth in the solid culture experiments, *A. alternata* showed minimal to no growth relative to the starting weight of ∼7 mg. In 60 and 6 mmol C/L acetate, *A. alternata* weighed ∼7 mg by experiment end, and weighed ∼4 mg with 0.6 and 0 mmol C/L (Fig. 2; Supplementary Table 2). With glucose, *A. alternata* biomass was greater than with acetate at the highest C concentrations, weighing 10 mg with 60 mmol C/L, ∼8 mg with 6 mmol C/L, and ∼4 mg with 0.6 and 0 mmol C/L. When grown on acetate, *A. alternata* biomass exhibited as one coherent biomass and increased in red color with higher acetate concentrations, from a very light pink to a dark red-orange with black pigment around the biomass edges (Supplementary Fig. 8). In glucose, the color change was similar but the black pigment appeared randomly in small clusters throughout the biomass (Supplementary Fig. 8).

The biomass trends for *P. sporulosa* were similar to those of *A. alternata*, with very small differences in their masses at experiment end. When grown in acetate, *P. sporulosa* biomass weighed ∼10 mg with 60 mmol C/L, ∼7 mg with 6 mmol C/L, and ∼5 mg for both 0.6 and 0 mmol C/L after 23 days. With glucose, *P. sporulosa* biomass weighed ∼11 mg, 8 mg, 4 mg, and 4 mg for each respective decreasing C concentration (Fig. 2). *P. sporulosa* biomass appeared as clumpy or flaky and white with spots of pink-red color as organic carbon concentration increased (Supplementary Fig. 9). The pink-red color was only evident in 6 and 60 mmol C/L acetate and glucose flasks. Acetate-amended flasks appeared more pink-red, and glucose-amended flasks appeared more orange-red (Supplementary Fig. 9).

The trends for *Pyrenochaeta* sp. were similar in that there was measurable biomass growth in all experimental conditions, but there was greater overall biomass reached compared to the other species. The biomass levels peaked (∼17 mg) around 15 days for 60 mmol C/L glucose and then decreased substantially (11 mg) at the end of the experiment. This general trend was similar for *Pyrenochaeta* sp. in 6 mmol C/L glucose, but weights only reached ∼13 mg before dropping to ∼10 mg. Compared to a starting weight of ∼7.5 mg, in acetate *Pyrenochaeta* sp. weighed ∼13 mg when grown with 60 mmol C/L and weighed ∼9 mg when exposed to 6 mmol C/L after 23 days. In glucose at 23 days, *Pyrenochaeta* sp. biomass appeared as one coherent mass, with occasional medium-sized clumps of pink-orange biomass. A layer of black pigment was observed at the top of glucose-amended flasks. In acetate, *Pyrenochaeta* sp. showed only a slight pink hue but, similar to glucose cultures, also had a thick layer of brownish-black pigment along the top of the biomass (Supplementary Fig. 10). Comparisons between fungal species, carbon concentration, and carbon form for biomass weights that were statistically significant at experiment end (i.e. $p < 0.05$) are listed in Supplementary Table 3. All cultures were visibly clear of bacterial contamination throughout the experiment, except *Pyrenochaeta* sp. at experiment end, where biomass-free replicate cultures showed some cloudiness indicative of bacterial contamination. These flasks were discarded and not measured in the experiment. Overall, addition of a C source did not substantially increase growth throughout experiments, and there were only slight improvements in growth when higher quantities of C were amended.

3.2. *Organic carbon removal over time*

3.2.1. *Acetate*

When amended with acetate in liquid media, *A. alternata, P. sporulosa,* and *Pyrenochaeta* sp. generally consumed carbon to near-depletion by ∼23 days whether they were grown with or without Se(IV), except for those cultures grown under the highest acetate concentration (60 mmol C/L; Fig. 3). However, patterns of acetate removal for each fungal species with and without Se(IV) varied. For all 0.6 and 6 mmol C/L fungal cultures tested, between 71% and 99% of initial acetate concentrations were removed. With both 0.6 and 6 mmol C/L concentrations for *P. sporulosa*, acetate was depleted to ∼0 μmoles by 11 days in the absence of Se(IV), but at this time there was still considerable acetate (79–91%) remaining in solution when grown with Se(IV) (Fig. 3; Supplementary Table 4). For *A. alternata*, the reverse pattern is apparent in the 6 mmol C/L experiments, where more acetate was removed (54%) within the first 11 days in the presence of Se(IV) compared to cultures

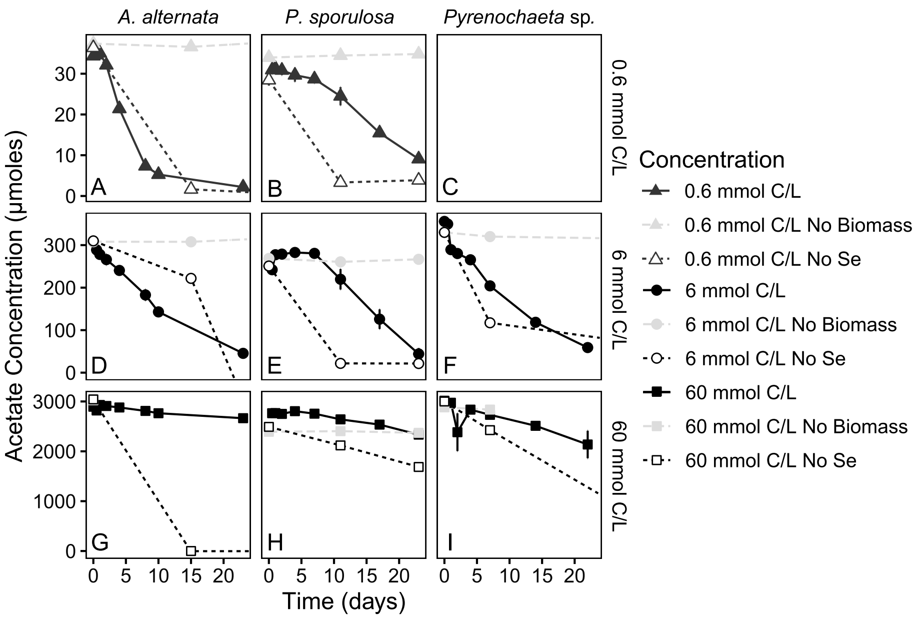

Fig. 3. - Acetate concentrations in aqueous media through time for *A. alternata* (A, D, G), *P. sporulosa* (B, E, H), and *Pyrenochaeta* sp. (C, F, I) grown with and without Se(IV) at 0.6 (top panels), 6 (middle panels), and 60 mmol C/L (bottom panels). Biomass-free controls are plotted as gray symbols, and Se(IV)-free controls are plotted as open green symbols. *Pyrenochaeta* sp. no biomass flasks at day 23 were visibly contaminated with bacteria and the data is not plotted here. Dashed lines extended beyond 23 days indicate control data points that were sampled at day 30, but did not fit within the main data time frame. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

without Se(IV) (28%). *Pyrenochaeta* sp. 6 mmol C/L acetate removal trended similarly through time for both the Se(IV)-supplemented and Se (IV)-free experiments. The acetate concentrations for the biomass-free and acetate-free experiments (Fig. 3; Supplementary Table 4) either remained unchanged through time or remained below detection, respectively, for all experimental conditions.

When grown with 60 mmol C/L, more acetate was removed in the Se (IV)-free experiments than those supplemented with Se(IV) by all three fungi. In the presence of Se(IV), *A. alternata* removed 230 µmoles (8%), *P. sporulosa* removed 430 µmoles (16%), and *Pyrenochaeta* sp. removed 878 µmoles (29%) by experiment end. However, acetate concentrations were never fully depleted by day 23 like in the lower C experiments. The exception to this was the *A. alternata* Se(IV)-free control which removed all acetate (60 mmol C/L) by day 15. Comparisons between fungal species and carbon concentration for acetate that were statistically significant at experiment end (i.e., $p < 0.05$) are listed in Supplementary Table 3.

3.2.2. Glucose

Similar to acetate-amended growth, all three fungal species depleted glucose to near 0 µmoles over the course of ~23 days for all conditions except those supplemented with 60 mmol C/L. For the 0.6 and 6 mmol C/L experiments, glucose removal trends were more similar between the Se(IV)-enriched and Se(IV)-free conditions compared to the acetate experiments (Fig. 4; Supplementary Table 5). Each organism varied slightly in removal efficiency, but the glucose was generally depleted faster than acetate for all three fungi. For example, when *A. alternata* and *P. sporulosa* were exposed to Se(IV) and 0.6 mmol C/L glucose, both organisms removed almost all glucose (97%) within the first 7 days. In contrast, 7 µmoles (21%) and 29 µmoles (93%) were still remaining when *A. alternata* and *P. sporulosa*, respectively, were grown with acetate (Fig. 4). For the 6 mmol C/L, nearly 100% glucose was removed within 15 days for both *A. alternata,* and *Pyrenochaeta* sp., regardless of Se presence. Glucose depletion proceeded a little more slowly when *P. sporulosa* was supplemented with Se(IV) and 6 mmol C/L, as 12.5

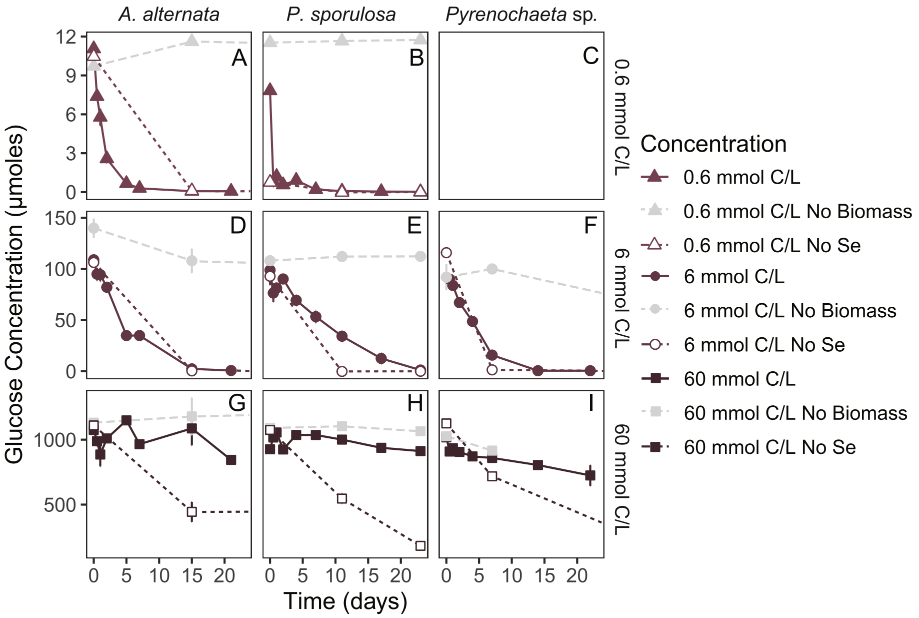

Fig. 4. - Glucose concentrations through time for *A. alternata* (A, D, G), *P. sporulosa* (B, E, H), and *Pyrenochaeta* sp. (C, F, I) grown with and without Se(IV) in liquid media at 0.6 (top panels), 6 (middle panels), and 60 mmol C/L (bottom panels). Biomass-free controls are plotted as gray symbols, and Se(IV)-free controls are plotted as open pink symbols. *Pyrenochaeta* sp. no biomass flasks at day 23 were visibly contaminated with bacteria and the data is not plotted here. Dashed lines extended beyond 23 days indicate control data points that were sampled at day 30, but did not fit within the main data time frame. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

μmoles (13%) glucose remained after ~15 days, and only 1.2 μmoles remained (1%) after 23 days. Glucose concentrations in cultures containing both Se(IV) and glucose but no fungal species remained steady throughout the experiment.

At the highest concentration (60 mmol C/L), glucose removal trends largely paralleled those of acetate, where *A. alternata, P. sporulosa,* and *Pyrenochaeta* sp. all removed glucose considerably faster in the Se(IV)-free conditions than those containing Se(IV) (Fig. 4). In the presence of Se(IV), *A. alternata* glucose concentrations fluctuated compared to the other cultures, though there was still a net loss (230 μmoles; 21% removed) of glucose by experiment end. *A. alternata* without Se(IV) removed over half of the glucose by 15 days (from 1110 to 444 μmoles; 60% removed). *P. sporulosa* without Se(IV) removed almost all (892 μmoles; 83%) glucose from solution but only 15 μmoles in the presence of Se(IV). *Pyrenochaeta* sp. removed 732 μmoles (65%) vs. 158 μmoles (16%) glucose from solution for Se(IV)-free and Se(IV)-supplemented conditions, respectively (Fig. 4). Comparisons between fungal species and carbon concentration for glucose that were statistically significant at experiment end (i.e. $p < 0.05$) are listed in Supplementary Table 3.

3.3. Fungal Se(IV) removal in the presence of different organic carbon sources and concentrations

When grown in liquid culture with 0.1 mM Se(IV) and varying carbon concentrations, fungal Se(IV) removal generally increased with increasing organic C concentrations (Fig. 5; Supplementary Table 6). For example, *P. sporulosa* growing with acetate removed 2% (0.2 μmoles) of added Se(IV) at 0.6 mmol C/L, 19% (2 μmoles) Se(IV) at 6 mmol C/L, and 31% (3 μmoles) Se(IV) at 60 mmol C/L. An exception to this trend is with *Pyrenochaeta* sp. growing in acetate, where 6 mmol C/L cultures removed only slightly more (0.9 μmoles) Se(IV) than the 60 mmol C/L (0 μmoles). Additionally, *A. alternata* supplemented with glucose removed similar amounts (~4.5 μmoles) of Se(IV) by experiment end in the presence of 6 and 60 mmol C/L, but still considerably greater than 2.5 μmoles Se(IV) removed at 0.6 mmol C/L glucose.

Another general trend was that more Se(IV) was removed by fungi growing with glucose than acetate as their C source, especially at higher C concentrations (Fig. 5). As described above, in acetate, *Pyrenochaeta* sp. removed very little selenium (~1 μmole) when exposed to either concentration. With glucose, *Pyrenochaeta* sp. removed 66% (6.1 μmoles) Se(IV) in 60 mmol C/L and 29% (2.8 μmoles) Se(IV) in 6 mmol C/L. Between organisms, *A. alternata* generally removed the most Se(IV) regardless of organic carbon source. In 60 mmol C/L acetate conditions, for example, *A. alternata* removed 32% Se(IV) while *P. sporulosa* and *Pyrenochaeta* sp. removed 31 and 0%, respectively. Comparisons between fungal species, carbon concentration, and carbon form for Se(IV) concentrations that were statistically significant at experiment end (i.e., $p < 0.05$) are listed in Supplementary Table 3.

3.4. Solid (biomass)-associated Se

For all organisms, solid-associated Se (total Se adsorbed to or incorporated into the fungal biomass) generally increased with higher concentrations of C (Supplementary Fig. 11; Supplementary Table 7). When *P. sporulosa* was grown in glucose, for example, solid-associated Se concentrations measured 0.2 μmol at 0.6 mmol C/L, 1.5 μmol at 6 mmol C/L, and 2 μmoles for 60 mmol C/L conditions at experiment end. *A. alternata* grown with glucose at 0.6, 6, and 60 mmol C/L incorporated and/or adsorbed 2, 3.5, and 4 μmoles Se, respectively, into the solid phase, which was the most out of all the experimental conditions (Supplementary Fig. 11). This removal trend was not always consistent for other conditions. *A. alternata* growing in acetate incorporated similar quantities (~2 μmoles) of Se for all three C-supplement concentrations (0.6, 6, and 60 mmol C/L), and only 0.6 μmoles Se was associated with the solid-phase when grown without a C amendment (0 mmol C/L).

Similar to trends of greater aqueous Se(IV) removal by fungi growing with glucose relative to acetate, more solid-associated Se was generally measured in glucose-amended conditions than for acetate. For example, in the presence of acetate, *Pyrenochaeta* sp. incorporated very little Se into the solid phase (<0.5 μmoles; Supplementary Fig. 11). Solid-associated Se levels relatively skyrocketed up to approximately 2 and 4 μmoles respectively for 6 and 60 mmol C/L glucose. Comparisons between fungal species, carbon concentration, and carbon form for solid-associated Se values that were statistically significant at experiment end (i.e., $p < 0.05$) are listed in Supplementary Table 3. Visualization of fungal cells under transmission electron microscopy shows

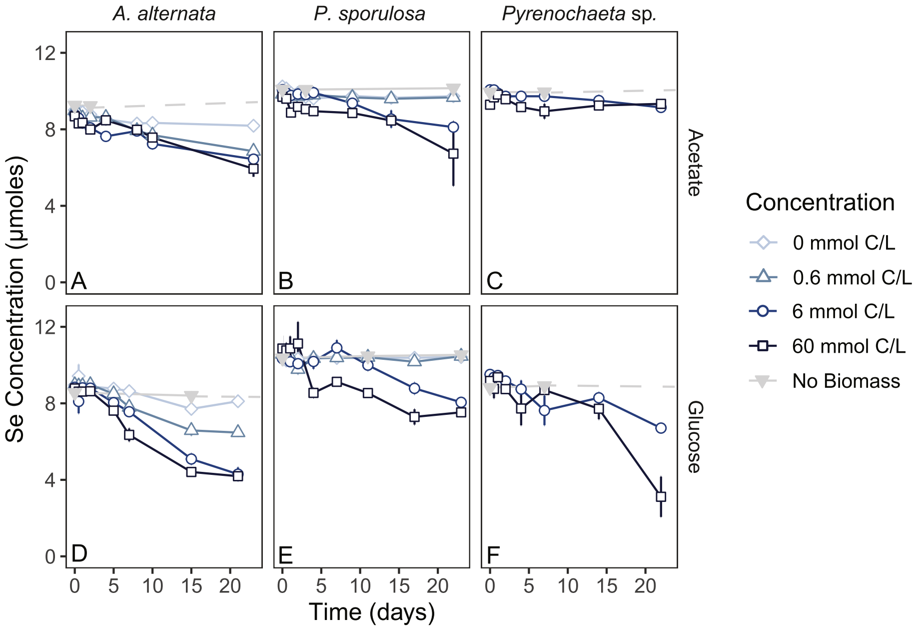

Fig. 5. - Aqueous Se(IV) concentrations for *A. alternata* (A, D), *P. sporulosa* (B, E), and *Pyrenochaeta* sp. (C, F) grown in liquid media containing Se(IV) and either acetate (top panels) or glucose (bottom panels) at 0, 0.6, 6, and 60 mmol C/L. Note that *Pyrenochaeta* sp. was only cultured at 6 and 60 mmol C/L. Dashed lines extended beyond 23 days indicate control data points that were sampled at day 30, but did not fit within the main data time frame.

electron dense (dark) nanoparticles of amorphous elemental Se for all three fungi (Supplementary Fig. 12).

3.5. *Mass balance of Se removal and redistribution*

As time progressed, all fungi transformed at least some Se from an aqueous Se(IV) phase to solid-associated and volatile forms, but average percentages varied for each organism, C form, and C concentration with time (Fig. 6; Supplementary Fig. 13; Supplementary Table 8). Due to the experimental setup where the volatile Se forms (e.g., methylated Se(-II) compounds) are able to escape the loosely capped flasks, values for volatile Se were not able to be directly measured. Instead, they were calculated through mass balance of measurements made from the solid-associated and aqueous phases as performed previously (Rosenfeld et al., 2017).

The amount volatilized remained fairly constant after the first few days. By experiment end, *P. sporulosa* grown in glucose volatilized the most Se for both 6 and 60 mmol C/L conditions. Generally, the amount of Se removed from solution via volatilization was greater in higher C conditions regardless of C source, although more Se volatilization typically occurred for fungi growing in glucose compared to acetate. For example, *P. sporulosa* volatilized an average of 2% with 0.6 mmol C/L acetate, 4% with 6 mmol C/L, and 7% with 60 mmol C/L. With glucose, volatilization percentages by *P. sporulosa* at experiment end measured 2% for 0.6 mmol C/L, but increased to ~23% for 6 and 60 mmol C/L.

Very little Se was removed from solution either through volatilization or partitioning to the biomass when *Pyrenochaeta* sp. was grown in acetate. By experiment end only between 6 and 3% Se was volatilized for 6 and 60 mmol C/L, respectively and ~2% was solid-associated under both conditions by experiment end. When *Pyrenochaeta* sp. was grown in glucose at these concentrations, however, 15% and 22% was volatilized and 17% and 38% Se was solid-associated by the end of the experiment. In the 60 mmol C/L glucose, a rapid decrease in aqueous Se concentration between 15 and 23 days was associated with a spike in both the volatile (increase from 9 to 22%) and solid-associated Se (11–38%) phases (Fig. 6; Supplementary Fig. 13).

While volatile Se levels did not change dramatically over time in most of these experiments, the proportion of solid-associated Se increased substantially as aqueous Se levels decreased. Selenium measurements in *A. alternata* experiments with 60 mmol C/L glucose reflect this trend, where 9% was volatilized by day 5, 11% by days 10 and 15, and 14% by experiment end. On day 1 of these experiments, <1% Se was in the solid phase, but by experiment end, 44% was solid-associated, exceeding aqueous Se at 42%. When exposed to increasing levels of acetate, *A. alternata* partitioned 6–24% Se (0–60 mmol C/L) to the solid-associated phase and volatilized 5–8% by 23 days (Fig. 6; Supplementary Fig. 13), with little change in percent volatilized between days 5 and 23.

4. Discussion

4.1. *Carbon concentration, not substrate, influences fungal growth in the presence of Se(IV)*

Filamentous Ascomycete fungi are capable of using a diverse range of C compounds and concentrations to gain energy, grow, produce spores, and maintain cellular function (Jouhten et al., 2009; de Oliveira Costa and Nahas, 2012; Rojas-Jimenez et al., 2017; Wu et al., 2020; Sánchez, 2020). While one carbon substrate can be preferred for growth or a specific function (e.g., Holligan and Jennings 1973), it was unexpected that the radial growth in Se(IV)-free agar media was not substantially improved by either carbon source or concentration (Supplementary Figs. 1 and 3). In these solid cultures, the fungi were grown with the respective carbon source and concentration (and Se(IV) when relevant) from the experiment start (i.e. initial inoculation), unlike liquid cultures where the fungi were grown for two weeks in modified AY media before any amendment was added. In these solid cultures without Se(IV), most fungi grew to the same extent at 60 mmol C/L as 0 mmol C/L (i.e. without Se).

Detoxification is a cellular function relevant to the presence of metals and metalloids, including selenium. It is well-documented that Se can be toxic to diverse life forms upon exposure to high concentrations (Lemly, 1993; Hamilton, 2004; Lemly, 2018). Previous studies of filamentous Ascomycete fungi have observed Se toxicity effects such as inhibited growth (Rosenfeld et al., 2017). In the current study, cultures on agar-solidified media without Se(IV) grew radially larger and faster than those grown with Se(IV), providing further evidence of its toxicity. Regardless, all fungi were able to grow radially to some extent in the

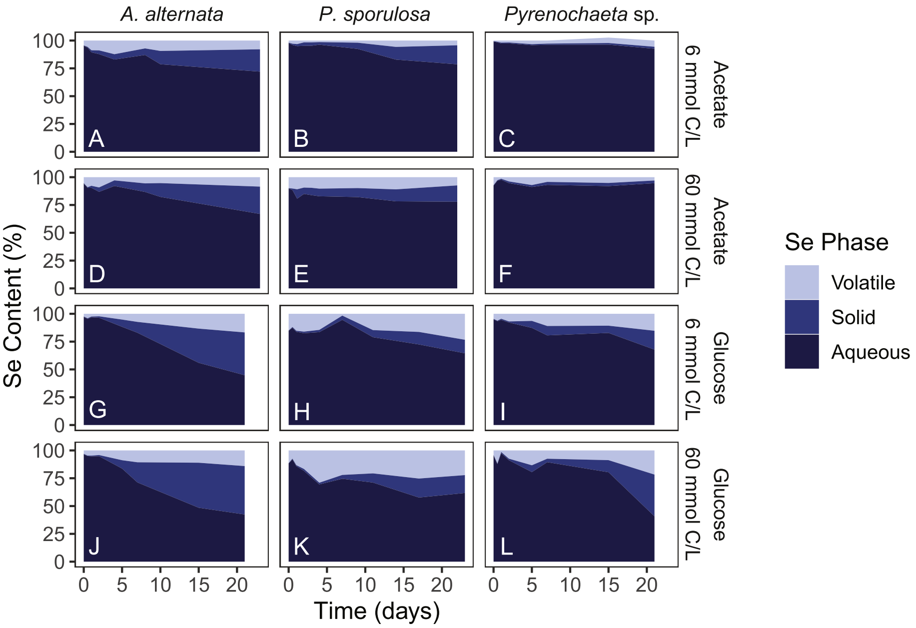

Fig. 6. - *A. alternata* (A, D, G, J), *P. sporulosa* (B, E, H, K), and *Pyrenochaeta* sp. (C, F, I, L) mass balances of Se between the aqueous, solid, and volatilized phases over time for liquid cultures containing Se(IV) and either acetate (top panels) or glucose (bottom panels) at 6 and 60 mmol C/L.

presence of Se(IV) when given either acetate or glucose.

With Se(IV) present, the two C substrates generally had similar impacts on growth trends for each respective organism. The substantial differences in growth for Se(IV)-amended cultures resulted from changes in C concentration where there was generally greater growth with higher levels of C. An exception to this was interestingly with *P. cucumerina*, which exhibited a reversal of growth trends relative to the other five cultures. *P. cucumerina* with 0 mmol C/L showed resilience and increased growth relative to 60 mmol C/L of either C source (Supplementary Fig. 1), and could be a focus of future research. In the presence of Se(IV) and 60 mmol C/L acetate on solid media, *A. alternata* seemed to overcome any deleterious effects of Se(IV) and grow to the plate edge. Combined, these solid cultures showed that carbon concentration, not necessarily substrate, matters more for improved growth in the presence of Se(IV).

4.2. Se(IV) toxicity impacts fungal growth despite carbon substrate consumption

In every Se-containing liquid culture experiment, dry fungal biomass weight fluctuated over the first 10 days before leveling out or minimally increasing (Fig. 2). As the fungal cultures were grown in medium with trace amounts of yeast extract for 14 days before the experiment started in order to obtain enough biomass for the experiment, the cultures may have been deprived and starved of carbon for growth before the experiment began. Yeast extract is complex and concentrations during this initial growth period were not measured, however those trace amounts did support substantial biomass growth (~7.5 mg). This was observed previously in fungal carbon starvation experiments with *Aspergillus* species, where secondary growth was fueled by carbon recycling (Nitsche et al., 2012). The relative abundance of fungal taxa in C-amended soil microcosms has been shown to change based on the availability of different carbon substrates, due to different resource utilization capabilities between taxa (Hanson et al., 2008). The yeast *Mortierella isabellina* was shown to simultaneously use glucose and acetate in the presence of inhibitors such as phenolic compounds related to lignocellulose hydrolysis (Ruan et al., 2015). In that study, substrate consumption and cell growth was delayed, but improved over time. In the current study, the simultaneous spiking of C and Se(IV) may have forced the fungi to divert energy from immediate growth to a strategy to tolerate and/or eliminate the toxic substance. Evidence for this is visible between the Se(IV)-free liquid culture experiments, where growth substantially improved over time relative to the respective culture with Se (IV) (Supplementary Fig. 7).

Glucose and acetate concentrations in fungal cultures grown with and without Se(IV) trended similarly downward throughout the experiment timeline, suggesting that carbon consumption continues on despite the presence and active removal of Se(IV) (Fig. 3; Fig. 4). The exception to this similarity occurred in the highest C concentration (60 mmol C/L) for both glucose and acetate, where all three fungal species grown with Se(IV) removed substantially less C over time than the respective cultures without Se(IV). It is important to note that the quantities of C removal at this highest concentration are greater than the other starting C concentrations (0, 0.6, and 6 mmol C/L). In these highest C concentrations, biomass weights, Se(IV) removal, and solid-associated Se percentages were slightly improved compared to those in the presence of lower C concentrations.

However, as biomass did not substantially increase throughout the experiment for any organism in the presence of Se(IV) but C consumption continued, it is likely that the fungi were predominantly consuming C for cell maintenance, protection, and repair purposes rather than growth. The highest C concentrations may have provided the fungi with enough C to grow slightly more and reduce more Se(IV) while also diverting resources to cell maintenance tasks. The energy required for these various biochemical processes may have impacted their ability to do everything simultaneously, especially when given lower C concentrations. The presence of environmental stressors such as elevated levels of metals like lead, copper, and cadmium have been shown to affect cell membranes and transport processes (Du et al., 2015) and induce DNA damage (Collin-Hansen et al., 2005). For example, in a study of the yeast *Pichia kudriavzevii*, membrane permeability and cell growth were impacted by cadmium, lead and zinc (Mesquita et al., 2015). Lead, nickel, and zinc, among others have also been shown to disrupt intracellular activity and impact hyphal formation (Lanfranco et al., 2002; Słaba et al., 2005; Tuszyńska, 2006). Examination of fungal gene expression through transcriptomics analyses would aid in understanding how fungal carbon use changes between the presence and absence of Se(IV). It would also help to elucidate the role that C plays in the fungal ability to both tolerate and detoxify Se(IV) and in increasing concentrations of solid-associated selenium (such as intracellular Se(0) nanoparticles).

4.3. Fungi transform and redistribute Se over time

Most organisms, such as bacteria, archaea, humans and plants, require Se for cellular processes (Labunskyy et al., 2014). For example, some bacteria are known to transform Se(IV or VI) to solid or volatile forms by respiring Se oxyanions to gain energy (Nancharaiah and Lens, 2015a) or through reduction of Se oxyanions via genes such as selenate reductase (*serABC;* Butler et al., 2012). Ascomycota fungi are not known to require Se, as the selenocysteine (Sec) insertion machinery is missing (Mariotti et al., 2019). Previous work has shown the ability of common filamentous Ascomycete fungi to transform Se from an aqueous bioavailable form (Se (IV or VI)) to less-bioavailable solid or volatile forms (Se(0 or -II)) under atmospheric conditions (Rosenfeld et al., 2017; Sabuda et al., 2020). To date, the mechanisms behind fungal selenium transformations remain unresolved. As these fungi are grown in the presence of oxygen, the most energetically favorable electron acceptor, they are hypothesized to reduce Se oxyanions as a detoxification mechanism (Rosenfeld et al., 2017) or incidentally as a byproduct of their metabolism. The results of this study coincide with this idea, as fungal growth did not improve much throughout the experiment and the fungi had to divert resources to either grow or remove Se(IV).

Despite minimal biomass growth, higher concentrations of carbon generally coincided with increased Se(IV) removal from solution compared to lower concentrations (Fig. 5), and the amount of Se incorporated into the solid and volatilized phases increased over time (Fig. 6). Without an additional organic carbon source beyond the trace amount in yeast extract (i.e. 0 mmol C/L), *P. sporulosa* left 95–99% of the Se(IV) in solution after experiment end, indicating that the fungi were not able to transform Se(IV) well without a sustained C source. *A. alternata* was not much better without C, with 91% aqueous Se(IV) remaining. With a small carbon supplement (i.e. 0.6 mmol C/L) these Se (IV) concentrations did not change for *P. sporulosa*, but did aid *A. alternata* in Se(IV) removal, where Se(IV) remaining in solution dropped to 72% for glucose and 76% for acetate.

The fate and distribution of Se speciation over time changes substantially based on organic carbon form and concentration (Fig. 6). Generally, the data indicate that the presence of elevated C concentrations is conducive to increased transformation of Se to less toxic phases either volatilized and lost from solution or sequestered with the solid biomass. The solid-associated phase contains red-colored nanoparticulate elemental Se(0) as shown previously (Rosenfeld et al., 2020), and which appears as electron-dense black circles in the electron microscopy images presented here (Supplementary Fig. 12). Organo-Se forms such as Se-cystine have also been identified in cultures amended with Se(IV), and some Se(IV) could also be adsorbed to the biomass, though the latter is expected in only minimal amounts based on prior analyses (Rosenfeld et al., 2020).

Previous work studied the same six fungal isolates used here in liquid and solid AY media containing 0.001–1 mM Se(IV or VI). AY media contains 6 mmol C/L acetate, and our 6 mmol C/L agar-solidified radial growth results trend well with their agar-solidified radial growth data as expected (Fig. 1 Rosenfeld et al., 2017, Fig. 1 and Supplementary Figs. 1–3 this study). For the liquid cultures in Rosenfeld et al. (2017), rather than growing the fungi first for 14 days without Se(IV) to accumulate biomass, they added a small amount of biomass to flasks with Se oxyanions already present in the AY media, indicating the start of their experiments (Rosenfeld et al., 2017). The current study allowed liquid culture growth for 14 days in modified AY media (i.e. yeast extract-only) before C and Se(IV) amendment addition. When comparing Se(IV) removal from solution in these two studies, Rosenfeld et al. (2017) saw complete removal after ~10 days for *A. alternata* and by ~20 days for *P. sporulosa*, whereas in the current study removal was incomplete with only a few μmol transformed after ~20 days. With *Pyrenochaeta* sp., Se (IV) levels dropped from 10 μmol to 4 μmol after the experiment ended (40 days; Rosenfeld et al., 2017). In the current study, *Pyrenochaeta* sp. removed <1 μmol with acetate, but with 60 mmol C/L glucose this species would likely outperform the results of Rosenfeld et al. (2017). Combined, this indicates that the fungi perform faster Se(IV) removal from solution when acetate is present at the first onset of fungal growth. Based on the results of the current study, Se(IV) removal may be faster and in higher quantities if glucose is present as a C source from the start.

4.4. Clues about metalloid-induced oxidative stress response

With both acetate and glucose, *A. alternata* converted Se(IV) predominantly to a solid-associated phase that increased with time, though was also able to volatilize a substantial percentage. In cultures amended with higher levels of C, solid-associated and volatile Se for *A. alternata* was elevated in the presence of glucose compared to acetate. Se(0) nanoparticles produced by *A. alternata* appeared to be both intra- and extracellular under TEM. Alternatively, *P. sporulosa* and *Pyrenochaeta* sp. transformed Se(IV) mostly to a volatile form with glucose, though some solid Se was also created. *Pyrenochaeta* sp. exhibited minimal removal into solid or volatile forms in the presence of acetate (less than ~10% removal) but improved when grown with glucose, transforming over 50% of the initial Se(IV) to other forms. This was consistent with physical observations of the cultures, where less pink color was evident in these two organisms than with *A. alternata*. Similarly, while *P. sporulosa* did have a substantial amount of intra- and extracellular Se (0) nanoparticles (especially with glucose) under TEM, *Pyrenochaeta* sp. did not (Supplementary Fig. 12). Based on these results, the fungi appear to remove more Se(IV) from solution and transform it to less toxic phases more efficiently in the presence of glucose, which is a preferred energetically favorable carbon source (Annick Ries et al., 2018; Lok et al., 2021).

Under some liquid culture experimental conditions, the highest carbon concentrations were not always the most effective for Se(IV) transformations. In some cases, fungi exposed to 6 mmol C/L outperformed 60 mmol C/L. For example, *Pyrenochaeta* sp. grown in acetate at 60 mmol C/L removed more Se over the first 10 days (Fig. 5). However, after this time, 6 mmol C/L proved to be the most effective carbon concentration for Se(IV) reduction. This indicates that there may be a limit to the amount of carbon required for fungal growth and Se(IV) removal, and adding carbon above these limits may not substantially improve Se(IV) reduction.

This trend may also signify increased levels of oxidative stress, where the fungi were able to divert more energy toward cellular responses that provide protection and tolerance to metal(loid) toxicity, such as the synthesis of melanin (Gadd, 1980; Gadd, 1984; Seyedmousavi et al., 2011; Eisenman and Casadevall, 2012; Eisenman et al., 2020). To this end, in Se(IV)-containing solid and liquid cultures of *A. alternata* exposed to high C concentrations (60 mmol C/L) and *Pyrenochaeta* sp. exposed to 0.6–60 mmol C/L, the presence of black pigmentation (i.e. melanin) also became apparent (Supplementary Figs. 4, 6, 8, 10). While Se(0) amorphous particles can be black in color, this only happens after heating red amorphous particles to >30 °C (Kessi et al., 1999), which was not done here. As previous electron diffraction analysis has not revealed any structure to the fungal Se(0) nanoparticles, black vitreous Se(0) was not a contributor to the black color observed, indicating that a pigmentation such as melanin is the likely color. Melanin, a pigment stored in the fungal cell wall, has been shown to positively correlate with fungal redox reactions and cell signaling, and negatively correlate with DNA replication and hyphal growth (Siletti et al., 2017). While indicators of oxidative stress were not directly measured, the presence of elevated metal(loid) levels is a known biological stressor (e.g., Pócsi et al., 2004). The melanized cell wall can act as a barrier to metal ions (Gadd and Griffiths, 1980), and melanin can also bind to metals, enhancing cell survival under metal-induced stress (Apte et al., 2013). It is unclear whether the presence of melanin or the melanin synthesis pathways are promoting Se(IV) tolerance or detoxification as it has been shown to do with other metals such as copper (Gadd and Griffiths, 1980), or whether it simply allows for survival without substantial Se(IV) chemical transformations. The latter may be a reason for the subdued Se(IV) reduction performance in some cases by *Pyrenochaeta* sp. Regardless, further research is warranted for understanding the role of melanin in fungal Se(IV) transformations.

While the fungal mechanisms for Se(IV) transformation are not yet resolved, previous work has shown that fungal adaptation to stress from carbon deprivation allowed for increased tolerance to oxidative stress (Pócsi et al., 2004; Petkova et al., 2010; Petti et al., 2011), which could include toxic levels of a metalloid such as Se. To this end, in response to glucose starvation and acetate growth stress, the antioxidant enzymes catalase and glutathione peroxidase were shown to increase in activity in the fungus *Phycomyces blakesleeanus* (Rúa et al., 2014). Glutathione (GSH) has many roles in a cell including cellular maintenance under oxidative stress and detoxification of metals (Pócsi et al., 2004), and earlier work implicated GSH in the reduction of Se(IV) in yeast (Nickerson and Falcone, 1963; Falcone and Nickerson, 1963). It is unclear why there are noticeable differences in Se chemical speciation and location of Se(0) nanoparticles between organisms over time, but this may be due to disparities in their individual tolerance of different Se forms, production of melanin, glutathione, and other protective defense mechanisms, or perhaps variations in Se transformation abilities. Extracellular Se(0) production from Se(IV) reduction is possible via sulfhydryl sites, extracellular polymeric substances, and/or iron siderophores, and others (Yu et al., 2018; Nancharaiah and Lens, 2015), and is a potential mechanism for the extracellular Se(0) particles observed under TEM here. Future work should investigate the mechanisms of fungal Se(IV) transformation and explore these differences within and between fungal species.

4.5. Implications for Se(IV) bioremediation strategies

In the environment, Se is an element of increasing concern, especially in areas of anthropogenic activity such as those related to coal mining, processing, and combustion (Yudovich and Ketris, 2006; Pond et al., 2008; Arnold et al., 2014; Naslund et al., 2020). While abiotic technologies exist to remove Se from the environment, they can be expensive and time consuming. Bioremediation strategies, or treatment strategies involving biology, have been discussed for Se-contaminated environments (e.g., Cantafio et al., 1996; Soda et al., 2011; Tan et al., 2016). Fungi have been used to remediate co-contaminated sites, where both organic and inorganic pollutants persist (Ceci et al., 2018).

In a recent study, supplemented carbohydrates were found to aid in removal of Se(IV) from wastewaters by two *A. alternata* strains, including the same strain (SRC1lrK2f) used here (Sabuda et al., 2020). However, while this carbohydrate product could be helpful in a water treatment strategy, the proprietary nature of the amendment used did not allow further insight into its role in fungal growth or Se(IV) reduction. The specific concentrations of defined carbon sources (i.e. acetate or glucose) used here help to more directly understand the role of carbon in this process. With the results of the current study, it is evident that supplementation of C regardless of source substantially improved the amount of Se(IV) removed by fungi relative to cultures without acetate or glucose (i.e. 0 mmol C/L). Higher C concentrations improved Se(IV) removal relative to lower C concentrations, but there may be a limit to how much C is necessary to add for optimal Se removal. Additionally, fungi grown with glucose generally removed more Se(IV) than with acetate. Looking forward, these findings should be considered when applying fungi in Se(IV) treatment strategies. Finally, the previous study supplemented carbon in only wastewaters that were filtered. Future work should examine the role of supplemented carbon in unfiltered wastewaters, and the possible synergies of a mixed community where bacteria and other organisms are also present.

The results of Sabuda et al. (2020) showed that both *Alternaria* strains produced more biomass when grown in wastewater amended with carbohydrates compared to wastewater alone, whereas biomass in this study with synthetic media remained relatively steady or exhibited slight increases. Based on this, carbon (and possibly nutrients such as nitrogen and phosphorus) supplementation should be added at the beginning of any treatment strategy to enhance fungal growth and removal efficiency, and be occasionally replenished over time to aid in continued removal. Selection of a fungal isolate for water treatment purposes depends in part on the C source provided and the desired end-product of Se, as in this study *A. alternata* transformed Se(IV) to predominantly a solid phase regardless of C substrate, whereas *P. sporulosa* mainly volatilized Se(IV) with glucose and solidified Se(IV) with acetate. Ultimately, these fungi are well adapted to transform and remove Se(IV) from solution in a bioremediation strategy, especially when C supplementation is implemented.

5. Conclusions

This biogeochemical study of carbon and selenium transformations by several Ascomycota fungi revealed that carbon source and concentration have a substantial impact on fungal Se(IV) reduction. It is evident from both solid and liquid cultures that the presence of Se(IV) impacts fungal growth. As biomass did not substantially increase throughout the experiment for any organism in the presence of Se(IV) but C consumption continued, it is likely that the fungi experienced a toxicity effect with Se(IV) as observed with increased pigment production, and predominantly consumed C for cell maintenance and repair purposes rather than growth. Higher carbon concentrations (6 and 60 mmol C/L) regardless of source, were generally coincident with more fungal Se(IV) removal from solution than the lower C concentrations (0.6 and 0 mmol C/L). Glucose and acetate levels in fungal cultures grown with and without Se(IV) trended similarly throughout the experiment timeline, suggesting that carbon consumption continues despite the presence of Se (IV). Regardless of carbon source and concentration, glucose is a more effective carbon amendment than acetate for all three fungal species in terms of Se(IV) removal from solution. With glucose, *A. alternata* was able to incorporate the most into the solid phase, whereas *P. sporulosa* mainly volatilized Se(IV), and *Pyrenochaeta* sp. transformed Se(IV) to half solid and half volatile forms. On a molecular level, this study lends foundational knowledge to understanding the genetic mechanism(s) for fungal Se(IV) reduction. The results also provide new insight and considerations for engineering a more efficient fungal remediation strategy for Se-impacted environments. In terms of biogeochemical cycles, it is evident that the transformations of C and Se by fungi in the environment are tightly linked.

Data availability

All information related to results are included in this manuscript as supplemental data sheets, which are also available on Figshare for additional accessibility at the following DOIs: Supplementary Table 1 -10.6084/m9.figshare.14714340; Supplementary Table 2 - 10.6084/m9.figshare.14714355; Supplementary Table 3 - 10.6084/m9.figshare.14714373; Supplementary Table 4 - 10.6084/m9.figshare.14714358; Supplementary Table 5 - 10.6084/m9.figshare.14714361; Supplementary Table 6 - 10.6084/m9.figshare.14714364; Supplementary Table 7 - 10.6084/m9.figshare.14714367; Supplementary Table 8 - 10.6084/m9.figshare.14714370.

Author contributions

MCS, JM, and CMS created and designed the study. BK, MRW, JM, and MCS performed laboratory experiment set-up and conducted experiments. JM, TX, MRW, and MCS conducted sample analysis. MCS created the figures and tables and wrote the manuscript with input, critical discussion, and edits from JM, MRW, BK, TX, and CMS. All authors have seen and approved the final version of this manuscript.

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Additional information

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apgeochem.2021.105163.

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