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Research article

Could extracts from spent mushroom materials transform reclaimed water quality? – A pilot study on pathogen suppression, antimicrobial chemical removal, and plant growth enhancement

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1. Introduction

Improving current food production systems is essential to address their various adverse environmental impacts, e.g., they contribute an estimated 19–29% of global greenhouse gas emissions (Mrówczyńska-Kamińska et al., 2021), withdraw 70% of global fresh water (Falkenmark and Rockstrom, 2004) and cause 78% of global ocean and freshwater eutrophication (Mancosu et al., 2015). Water is a vital resource in food production but may be limited due to increased population size, urbanization, climate change and depletion of natural water resources. Hence, it is important to utilize sustainable water management and alternative water sources targeting water scarcity and depletion of natural resources to ensure future food production.

Circular food production systems have been suggested to mitigate these impacts. Industry, society and agriculture produce large amounts of valuable materials and waste that can be utilized and reused in food production. One such resource is wastewater, which can be recycled in food production as an alternative source of water and nutrients. Reclaimed water obtained after purification of wastewater is currently applied in crop irrigation (Expósito et al., 2024), and its use is expected to increase following legislation related to the 2021 EU strategy on adaptation to climate change and the 2020 Circular Economy Action Plan (Expósito et al., 2024). However, it is often difficult to select wastewater purification technologies that ensure the desired water quality owing to the presence of different types of contaminants related to biohazards (Anastasi et al., 2012) and organic micropollutants (Kanan et al., 2022; Richardson and Manafsi, 2024), including antimicrobial chemicals, which can cause health problems. For example, several antimicrobial chemicals, parent compounds and their transformation products, have been detected in effluent water and surface water, increasing the risk of generating antimicrobial resistance (Löffler et al., 2023; Ugolini and Lai, 2024; Khan et al., 2024).

Moreover, the technologies currently used to remove contaminants often necessitate high costs and energy consumption (Corral-Bobadilla et al., 2019). In contrast, bioremediation of wastewater using microbes, such as bacteria, fungi and microalgae, has been proposed as a potentially lower cost alternative to degrade chemical pollutants (Corral-Bobadilla et al., 2019; Cunha–Chiamolera et al., 2024). Application of fungi in myco-remediation is well-documented using different species of non-edible and edible mushrooms, including *Agaricus* and *Pleurotus* species. The potential for using spent material as a waste product from the cultivation of white mushrooms (*Agaricus bisporus*) and oyster mushrooms (*Pleurotus ostreatus*) (in the form of spent mushroom compost (SMC) or spent oyster mushroom substrate (SOS)) in bioremediation (Corral-Bobadilla et al., 2019; Parenti et al., 2013), plant growth promotion and control of plant diseases has also been reported (Khalil et al., 2024). These roles relate to its microbial content and enzyme activities, such as extracellular enzymes that inhibit the spread of plant pathogens (Khalil et al., 2024) and laccase enzyme that promotes the biodegradation of organic micropollutants (Sánchez-San Martín et al., 2024). Our previous study (Khalil et al., 2024) demonstrated the presence of microbial communities and strains with potential bio-degradative activities and antagonistic effects on plant pathogens. Hence, optimization of the biological degradation of organic micropollutants and microbial biohazards using SMC and SOS could provide a novel, low-cost and natural method for the treatment of reclaimed water. The implementation of SMC and SOS to achieve the inhibition potential of biohazards and biodegradation of organic micropollutants has been shown with solid fractions of fungal mycelia (Chang et al., 2018; Hultberg et al., 2020). Water extracts have also been

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demonstrated to have bio-stimulant effects on plant defense mechanisms (Ishihara et al., 2019) and inhibitory effects on plant pathogens (Ebrahimi et al., 2018). In addition, water extraction from *Pleurotus* species has been shown to enhance the production of laccase enzyme, which plays an active role in biodegradation (Parenti et al., 2013). The present study pioneers the use of liquid-phase extracts (water extracts) from SMC and SOS to enhance reclaimed water quality for agricultural irrigation. Unlike prior research that primarily focused on solid-phase applications, we studied the concentration-dependent microbial and enzymatic activity of water extracts to optimize water purification, pathogen suppression and pollutant degradation. Limited knowledge is available about the suppressive potential of reclaimed water with SMC or SOS to eliminate microbial biohazards, the spread of plant pathogens and the presence of antimicrobial chemicals.

To fully understand the role that water extracts of SMC and SOS may play in the treatment of reclaimed water as an additional step in the purification process, and further application in irrigation purposes, it is vital to gain a deeper understanding of the effect of water extract on the degradation of organic pollutants. Moreover, it is important to assess the potential of the treated water to inhibit biohazards, such as the spread of human or plant pathogens, and to enhance plant growth and quality.

This study explored whether the use of water extracts from SMC and SOS affected the potential of reclaimed water to reduce the spread of human and plant pathogens commonly found in cultivation systems and to degrade antimicrobial chemicals. It also examined the potential of reclaimed water treated with SMC and SOS water extracts to enhance seed germination and plant growth. We hypothesized that (i) extra purification of reclaimed water using SMC or SOS water extracts would add value and improve the reclaimed water quality for crop irrigation; (ii) treatment of reclaimed water with SMC and SOS water extracts would increase the production of enzymes that could degrade organic pollutants and inhibit human and plant pathogens; and (iii) reclaimed water treated with water extracts of SMC and SOS would enhance seed germination and plant growth due to the enzymes produced.

Thus, we analyzed different mixtures of water extracts from spent mushroom materials combined with reclaimed water as a potential water purification strategy to remove biohazards, reduce the spread of plant pathogens, degrade antibiotics and improve plant growth and assessed the microbial content and enzyme activities.

2. Materials and methods

2.1. Water extracts of SMC and SOS

SMC and SOS were sourced from a commercial mushroom producer establishment in southern Sweden; the content of the material has been described previously (Khalil et al., 2024). Water extraction of these materials was conducted following the procedure described by Kwak et al. (2015). Briefly, SMC or SOS were mixed with water in 1:4 portions (100 g compost (wet weight) to 400 ml autoclaved water) and left on a shaker for 7 days at room temperature. The mixtures were then filtered using autoclaved microfiber cloth and centrifuged for 10 min to remove the coarse components of the compost material. The extracts were thereafter stored at 4 °C until analysis.

2.2. Treatments and experimental set-up

Reclaimed water was sourced from a commercial purification station in south Sweden (VA-SYD Lund, Sweden). The water was classified as technical water and certified for irrigation in the field. Water extracts from SMC and SOS together with either reclaimed water or Milli-Q (MQ) water (control) were used in the treatments. The treatments comprised (a) 100% reclaimed or MQ water, (b) 20% SMC or SOS mixed with 80% of either reclaimed water or MQ water, and (c) 80% SMC or mixed with 20% of either reclaimed water or MQ water. Each treatment was performed with three replicates (each 400 mL) and evaluated regarding the degradation of antimicrobial chemicals, growth inhibition of human and plant pathogens, and impact on seed germination and plant growth.

2.3. Microbial enumeration

Microbial content naturally occurred in the SMC and SOS and in their water extracts were enumerated using selective media as described by Khalil et al. (2024). Based on viable count methods and dilution series using 10^0-10^6 dilutions, 1 mL of each of the treatments was diluted in 9 ml of 0.85% NaCl. Thereafter, 200 μL aliquots were spread, in triplicate, on the following media: (i) 0.1% tryptic soya agar (TSA, DIFCO 0369-17-6) complemented with cycloheximide (100 μg mL^{-1}) to enumerate the general bacterial flora using 10^3-10^6 dilutions; (ii) 0.5% malt extract agar (MA, DIFCO 0186-17-7) to enumerate the general fungal flora using 10°-10^3 dilutions; and (iii) King Agar B (KB) with cycloheximide (100 μg mL^{-1}) to enumerate *Pseudomonas fluorescens* using 10^3-10^6 dilutions. MA plates were incubated at room temperature for seven days, whereas TSA and KB plates were incubated for 24 h at 25 °C. After incubation, plates containing 30–300 colonies were counted and the log colony forming unit per ml of each media was calculated. Colonies of *Pseudomonas fluorescens* were counted under UV-light (Spectroline Model CM-10A; Spectronics, Westbury, NY).

2.4. Production of enzymes

The functional characters of the water extract isolates were assessed with respect to enzymatic activities. The extracts were screened for their potential to produce the extracellular enzymes chitinase, protease, phosphatase and cellulase on functional media (Khalil et al., 2024). For protease activity, M9 plates amended with skimmed milk (20 mL L^{-1}) were used and incubated for 24–72 h at 20 °C. For cellulase production, M9 plates amended with carboxymethyl cellulase were used, and after incubation, they were flooded with Congo Red solution (0.2% w/v) for 30 min and washed with 1 M NaCl solution. For phosphatase activity, plates containing tryptose phosphate agar supplemented with Methyl Green (0.05 mg mL^{-1}) were used and were incubated for 5 days at 20 °C.

Production of laccase was assessed as described by Hultberg et al. (2020) to determine whether the water extracts altered the degradation of organic micropollutants and growth of the bacterial strain *E. coli O157:H7gfp+*. For each treatment, 450 μL of the mixture was added to 500 μL of 10 mM DMP in 100 mM acetate buffer. The samples were examined using a spectrophotometer to measure laccase content.

2.5. Analysis of antimicrobial chemicals

Based on European relevance for surface water monitoring (EU Watch list, European Green Deal) (European Commission 2022/0344, 2022/1307, 2018/840), six antimicrobial chemicals were selected for study, i.e., azithromycin (CAS 83905-01-5), ciprofloxacin (CAS 85721-33-1), fluconazole (CAS 86386-73-4), ofloxacin (CAS 82419-36-1), sulfamethoxazole (CAS 723-46-6) and trimethoprim (CAS 738-70-5). Native chemicals were spiked into the samples (10 μg L^{-1}) in triplicate for all treatment types (Section 2.2), representing 1% of the organic solvent content in each sample. After 72 h at 20 °C, a 1 mL aliquot of each sample was centrifuged (8500 rpm, 10 min, 4 °C), the supernatant (160 μL) was transferred to a vial and then spiked with internal standards (IS; 20 μL of 500 ng mL^{-1}) and methanol (20 μL). Antimicrobial chemicals were quantified together with ten calibration standards (0–200 ng mL^{-1}; IS 50 ng mL^{-1}) by direct injection using ultra-high performance liquid chromatography coupled to tandem mass spectrometry (ExionLC™ SCIEX® 3500 Triple Quad™), following a previously validated analytical method (Ugolini and Lai, 2024). The instrumental quantification limit (IQL) and within-run precision (relative standard deviation, RSD%) for each antimicrobial compound were determined as follows: azithromycin (IQL 0.05 ng mL^{-1}; RSD 17%), ciprofloxacin (0.6 ng mL^{-1}; 15%), fluconazole (0.1 ng mL^{-1}; 13%),

ofloxacin (0.5 ng mL^{-1}; 12%), sulfamethoxazole (0.1 ng mL^{-1}; 8%) and trimethoprim (0.1 ng mL^{-1}; 0.3%). MQ water blank samples showed no contamination by the target compounds.

2.6. *In vitro* growth inhibition of *Escherichia coli O157:H7gfp+*

The *Escherichia coli O157:H7gfp+* nonpathogenic strain (Alam et al., 2014; Hartmann et al., 2017; Darlison et al., 2019) was used as a model strain to study the suppressive effects of water extract toward human pathogens. The strain was obtained from the Swedish Institute for Communicable Disease Control, Solna, Sweden (registry no E81186) and was characterized by expression of the *eae* gene but lacked virulence factors verotoxin-1 and -2 (Alam et al., 2014; Hartmann et al., 2017; Darlison et al., 2019). It was labeled with plasmids coding for ampicillin resistance and green fluorescent protein (gfp), which is emitted in the presence of arabinose when exposed to UV light (Hartmann et al., 2017). The strain was induced to fluoresce when grown on Luria–Bertani (LB; L3022- 1 kg; Sigma-Aldrich, St. Louis, MO) agar supplemented with 100 μg ml^{-1} ampicillin and 0.1% L-arabinose.

In the current study, the *E. coli O157:H7gfp+* strain was cultured from a cryo culture (stored at - 80 °C) and propagated overnight on LB agar at 37 °C. A single colony of *E. coli O157:H7gfp+* was picked and propagated in a flask containing 6 mL of LB broth medium amended with arabinose and ampicillin (10 μg ml^{-1}), which was placed on a rotary shaker (Minispin rotary shaker; VWR International AB, Stockholm, Sweden), adjusted at 100 rpm and left to incubate at 37 °C overnight. Afterwards, the *E. coli O157:H7gfp+* suspension was centrifuged at 5000 rpm for 10 min, the supernatant was discarded, and the pellet was washed twice with 0.85% sodium chloride (NaCl). Finally, the pellet was resuspended in 3 ml 0.085% NaCl. The optical density of the bacterial solution was set to an optical density (OD_{620}) of 0.4 by adding 0.085% NaCl, corresponding to 8.5 Log CFU ml^{-1}. The bacterial solution was kept overnight at 4 °C until analysis.

For the trial, 50 mL Falcon tubes were filled with 40 mL aliquots of individual treatment mixtures (reclaimed or Milli-Q water with or without water extract) and inoculated with 200 μL of the *E. coli O157:H7gfp+* suspension. Each treatment was conducted using three replicates. The tubes were then placed on a rotary shaker at 100 rpm for 72 h at 20 °C. Afterwards, growth of the *E. coli O157:H7gfp+* strain was evaluated by preparing a dilution series using 0.85% NaCl and plating 20 μL of each dilution on LB agar medium. Plates containing 30–300 colonies were counted. Green fluorescent colonies of the introduced strain were counted under UV-light (Spectroline Model CM-10A; Spectronics, Westbury, NY) as described by Darlison et al. (2019).

2.7. *In vitro* inhibition of plant pathogens

A dual culture test was conducted as described by Khalil et al. (2024) using two plant pathogens, which were maintained as follows: leaf pathogen *Botrytis cinerea* was grown on corn meal agar (CMA; Difco 211, 132) for seven days, whereas root *Pythium aphanidermatum* was cultured on potato dextrose agar (PDA; Difco 218,630) for seven days. All the plates were incubated at 20 °C. *In vitro* trials were performed by mixing 20 ml of the investigated treatments with the medium of the respective pathogen. A piece of mycelium of the respective pathogen was placed on the middle of the plate and grown for two weeks. The radial inhibition percentage was determined using an equation described by Khalil et al. (2024): Radial inhibition (%) = $(RC-RT)/RC\ x\ 100$, where RI is the minimal distance between the center and fungal margin and RC is the distance between the center and fungal margin of the control Three replicates per treatment were used in the experiment.

2.8. Germination trial

Sweet basil (*Ocimum basilicum*) seeds were sown in plastic pots using commercial potting soil (SWHorto-Sweden) and irrigated with the respective treatments (Section 2.2). The pots were left to grow in a climate chamber for 4 weeks at a temperature of 20 °C, relative humidity of 80%, light intensity of 200 μmol m^{-2} s^{-1} and with a daylight period of 16 h.

2.9. Statistical analysis

Statistical analysis was undertaken using Minitab version 18. Microbial enumeration data were log transformed to ensure conformity with the assumptions of homogeneity and normality. The effect of the treatments on the microbial count, *in vitro* inhibition of pathogens and seed germination assay was determined using analysis of variance (ANOVA) and Tukey's multiple comparison test at a significance level of $p<0.05$.

3. Results and discussion

3.1. Microbial enumeration

No microbial growth could be enumerated in the reclaimed water or MQ water without the addition of SMC or SOS water extracts. Enumeration of the naturally occurred microbial content in treatments of reclaimed water with SMC and SOS water extracts on selective media revealed a higher content of general bacterial flora in 20% and 80% SMC compared to the respective SOS extracts (Fig. 1). A higher growth count of naturally occurred *Pseudomonas fluorescens* was also indicated in 20% and 80% SMC and 80% SOS than in the other treatments. The highest content was obtained in the 80% SMC treatment. Conversely, 80% of SOS water extract in reclaimed water exhibited a higher general fungal count than obtained with SMC extracts.

Microbial enumeration in MQ water treated with SMC or SOS water extracts indicated lower counts than in reclaimed water. However, treatments in MQ water had higher content of general bacterial flora and *Pseudomonas fluorescens* than that of general fungal flora. The microbial content in MQ water was also higher in treatments with extracts of SMC compared to SOS (Fig. 1). These findings are in line with our previous study (Khalil et al., 2024), which showed that bacterial flora dominated the solid fraction of SMC, whereas fungal flora dominated the solid fraction of SOS. Although, no isolation or identification of bacterial strains was performed in the current study, our previous study (Khalil et al., 2024) indicated the dominance of bacterial isolates (e.g., *Pseudomonas*) that were able to promote plant growth and had diverse metabolic potential, allowing them to thrive in the nutrient-rich environments of SMC and SOS. These findings suggest that it might be possible to use water extracts of SMC and SOS to improve plant nutrition and protection in horticultural production systems, providing an alternative to synthetic fertilizers and pesticides in cultivation systems.

In addition, the results of the current study reveal the effect on microbial growth of treating reclaimed water with spent mushroom material extracts. The higher microbial content in the reclaimed water compared to MQ water demonstrates the potential of reclaimed water to stimulate microbial growth in the presence of SMC or SOS. This might also strengthen the application of reclaimed water in horticultural production as a strategy to stimulate beneficial microbial groups with potential to enhance plant growth and health.

3.2. Production of enzymes

The effects of the water source and cultivated fungal species on extracellular enzyme activities were also investigated (Table 1). Positive cellulase activity was observed in all the treatments of reclaimed water with both SMC and SOS water extracts (Table 1). In addition to cellulase, water extracts of 20% and 80% of SMC actively produced chitinase, protease and phosphatase. Production of phosphatase was also indicated in 80% water extracts of SOS. In contrast, no enzyme activities were indicated in control treatments of MQ water with or without the water

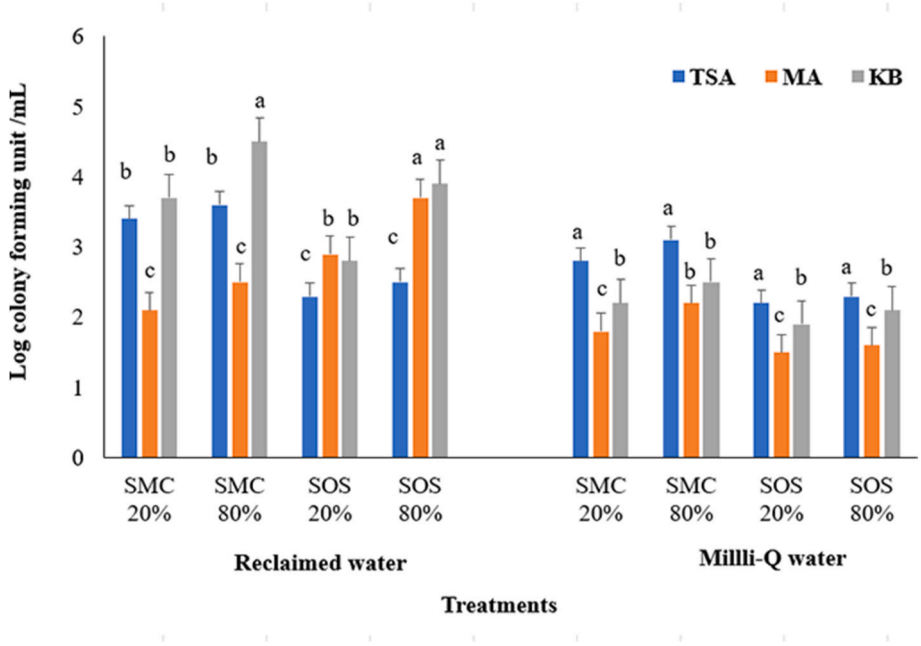

Fig. 1. Microbial content in 20% or 80% water extract of spent mushroom compost (SMC) and spent oyster mushroom substrate (SOS) mixed with 80% or 20% reclaimed water or Milli-Q water (control) determined using the following media: 0.1% tryptic soya agar (TSA) complemented with cycloheximide (100 μg mL^{-1}) for enumeration of general bacterial flora; 0.5% malt extract agar (MA) for enumeration of general fungal flora; and King Agar B (KB) with cycloheximide (100 μg mL^{-1}) for enumeration of *Pseudomonas fluorescens*. Letters above the bars indicate significant differences between the treatments in each water source denoted by Tukey groupings at $p<0.05$. Error bars show SD (n=3).

Table 1
Enzyme production in treatments of reclaimed water and MQ water with different proportions of water extract of spent mushroom compost (SMC) and spent oyster mushroom substrate (SOS). (+) indicates positive and (−) indicates negative enzyme activities.

Treatments		Cellulase production	Chitinase production	Protease production	Phosphatase production
Reclaimed water	SMC 20%	+	+	+	+
	SMC 80%	+	+	+	+
	SOS 20%	+	−	−	−
	SOS 80%	+	−	−	+
Milli-Q water	SMC 20%	−	−	−	−
	SMC 80%	−	−	−	−
	SOS 20%	−	−	−	−
	SOS 80%	−	−	−	−

extracts (Table 1). Production of extracellular enzymes is a key mechanism applied by microorganisms in their antagonistic potential against plant pathogens (Ghorbanpour et al., 2018) and in nutrient breakdown (Khalil et al., 2024). Importantly, our study revealed that reclaimed water treated with SMC extracts showed significantly improved enzymatic activity beyond water purification. The production of cellulase, chitinase, protease and phosphatase by SMC suggests additional bio-stimulant properties that could contribute to enhanced seed germination and plant health, enabling its dual role in water purification and sustainable crop production. The diversity of enzyme production by SMC has been indicated in other studies (Wang et al., 2021; Khalil et al., 2024). Treating reclaimed water with 20% or 80% water extracts increased this diversity compared to MQ water (Table 1). This highlights the potential of treating reclaimed water with SMC water extracts to enhance production of valuable enzymes with diverse applications (Chang et al., 2021).

The obtained results indicate the benefits of using reclaimed water mixed with SMC water extract as an irrigation source not only on the microbial content (Fig. 1), but also microbial activities related to enzyme production. Although the effects of reclaimed water on microbial activities in soil have been reported in earlier studies (Leogrande et al., 2022), our study provides evidence of the benefits of using both reclaimed water and SMC extract as an irrigation source.

Next, we examined the production and activity of laccase enzymes that can interact with the bacterial strain *E. coli O157:H7 gfp*+. No laccase activity was found in the water sources with only *E. coli O157:H7 gfp*+ inoculation (Fig. 2). Addition of water extracts of SMC or SOS enhanced laccase activity in both the reclaimed and control water, regardless of whether *E. coli O157:H7 gfp*+ was inoculated. The highest activity occurred in the water sources treated with 80% SOS water extract without the presence of *E. coli O157:H7 gfp*+. On the other hand, the introduced *E. coli O157:H7 gfp*+ strain reduced laccase activity in 80% SOS in both the reclaimed and MQ water, indicating an effect of the inoculated strain rather than the water source. Laccase activity in treatments with 20% or 80% SMC water extract were also high in both the reclaimed and MQ water, regardless of inoculation with *E. coli O157:H7 gfp*+ (Fig. 2). However, inoculation with *E. coli O157:H7 gfp*+ enhanced laccase activity in treatments of reclaimed water with either 20% SMC or 20% SOS water extract, showing that both the water source and inoculation strain affected laccase activities. On the other hand, laccase activity in the treatment with 80% SMC water extract increased due to the extract rather than the inoculation strain. These results demonstrate the impact of water sources, sources of water extract and their concentration on laccase activity. The production and activity of laccase enzymes in both SMC and SOS extracts agree with previous studies showing the ability of *Agaricus* and *Pleurotus* species to produce laccase enzymes (Mohammadi et al., 2022; Hultberg et al., 2020). In general, laccase production by these species has been connected to colonization of the substrate with active mycelia at an early growth

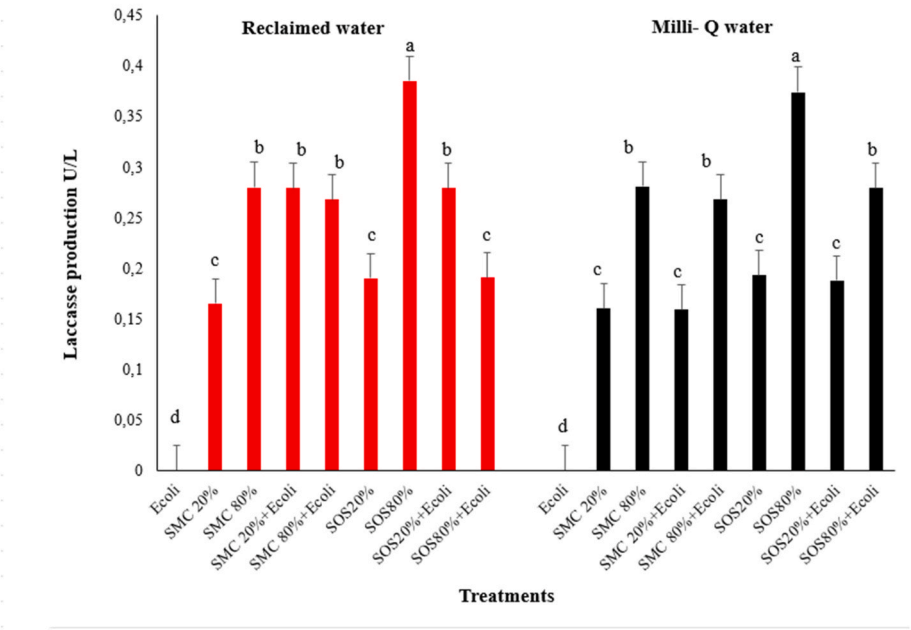

Fig. 2. Laccase activity in U/L in treatments of reclaimed water (red) and MQ water (black) with spent mushroom compost (SMC) and spent oyster mushroom substrate (SOS) water extracts (20% or 80%), controls (without SMC or SOS), and with and without inoculation with nonpathogenic strain *Escherichia coli O157:H7gfp+ (E. coli)*. Letters above the bars indicate significant differences between the treatments in each water source according to one-way ANOVA followed by Tukey's test ($p<0.05$). Error bars show SD (n=3).

phase of *Agaricus* and *Pleurotus* species (Mohammadi et al., 2022; Hultberg et al., 2020). Compared to studies performed on laccase activities from active mycelia of *Pleurotus* species (Hultberg et al., 2020), our findings indicated low laccase activity in water extracts of both SMC and SOS, but with potential to be utilized at concentrations of both 20% and 80%. This suggests that water extracts of spent mushroom material could be used as a strategy for wastewater purification and bioremediation, where laccase activity can be utilized. Nevertheless, the possibility of utilizing laccase activities in water extracts of SMC and SOS might be enabled by conditions in the spent material. SMC is characterized by high organic matter and nutrient availability (Khalil et al., 2024), which may enhance laccase activities, whereas SOS has lower organic matter content but is rich in fungal mycelia related to *Pleurotus* cultivation, which might be a causal factor for laccase activities (Hultberg et al., 2020).

Regarding the effect of laccase activity on biohazards related to the growth of human pathogens, the results showed various impacts with respect to biohazards and the use of reclaimed water. Overall, laccase activity in the presence of *E. coli O157:H7 gfp+* was mostly affected by the water source and source of water extract rather than the presence of *E. coli O157:H7 gfp+* (Fig. 2). However, the inoculated strain influenced some treatments. Treatments with 20% SMC or 20% SOS showed enhanced laccase activity in the presence of *E. coli O157:H7 gfp+*, whereas the treatment with 80% SOS in the presence of *E. coli O157:H7 gfp+* showed reduced laccase activity (Fig. 2). Earlier studies have suggested different mechanisms related to laccase activity and *E. coli* interactions. For example, laccase activity can degrade nutrients, reducing their availability to bacteria. Other mechanisms relate to the degradation of bacterial cell membranes by laccase activities (Li et al., 2018). The enhanced laccase activity in the treatment with 20% SMC (Fig. 2) in the presence of *E. coli O157:H7 gfp+* might correlate to growth reduction of the investigated strain (Fig. 5). These findings indicate the potential of SMC water extract to reduce biohazards related to contamination in reclaimed water (Heng and Wen, 2016). However, further investigations into the relationship between laccase activity and growth of *E. coli* strains are crucial to confirm whether SMC water extracts are suitable for water treatment and explore the potential of laccase activities for treating biohazards in reclaimed water to develop sustainable crop irrigation strategies.

Regarding organic micropollutants, 20% of mixtures of SMC or SOS showed much lower laccase activities than 80% mixtures (Fig. 3). The highest activity was indicated in 80% SOS treatments in both control and reclaimed water. This is in line with the well-documented role of *Pleurotus* species in biodegradation processes of organic pollutants (Parenti et al., 2013; Janusz et al., 2023). However, in most previous studies, the production of laccase was investigated in an active fungal mycelium of *Pleurotus* species (Hultberg et al., 2020; Silva et al., 2022). Our study provides new knowledge regarding the potential to detect laccase production in water extracts of SMC and SOS. Our results suggested also that laccase production in the presence of organic micro-pollutants was largely affected by the fungal species rather than the water source, since no significant differences in laccase production were detected between the two water sources (Fig. 3). Nevertheless, the results showed that in the presence of organic micro-pollutants, laccase activities were highest with the highest concentration of water extract (80% SMC or SOS), whereas the highest laccase activities toward biohazards were achieved with lower concentrations of SMC or SOS extract (20%). This might reflect the different content of the spent material and its water extract. The rich nutrient content of SMC likely enhances laccase activity and reduction of microbial biohazards, whereas the dominance of fungi in the SOS water extract of and *Pleurotus* species in SOS (Khalil et al., 2024) enhances biodegradation processes. Further, the difference in biocatalyctic activity stems from the differences in microbial community, as investigated in Khalil et al. (2024), where the fungal community in SMC was richer and more varied, potentially leading to a richer enzymatic production. The same was also true for the bacteria, which then naturally would cause a difference in the substrates' ability to produce enzymes. This demonstrates the benefits of applying water extracts of SMC and SOS in water purification and biodegradation to enable its use in crop irrigation.

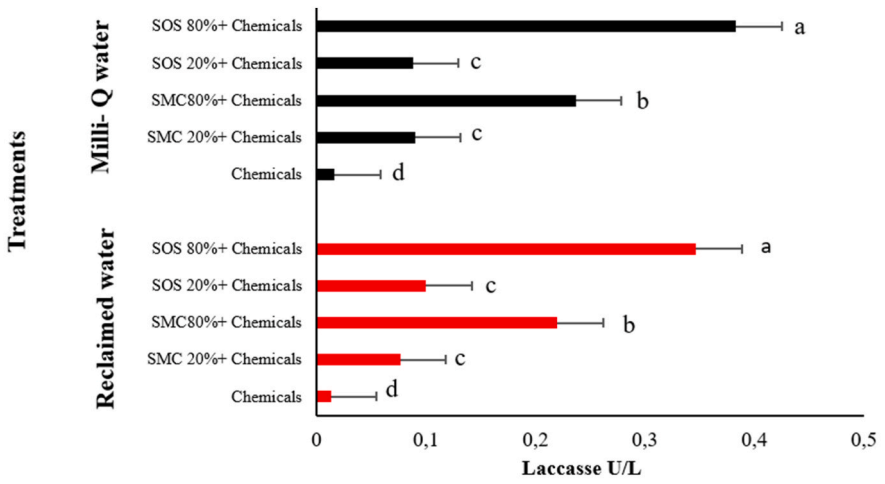

Fig. 3. Laccase activity in U/L in treatments in reclaimed water (red) and MQ water (black) in the presence of spent mushroom compost (SMC) and spent oyster mushroom substrate (SOS) water extract (20% or 80%) and controls (without SMC or SOS) in the presence of antimicrobial chemicals. Letters above the bars indicate significant differences between the treatments in each water source according to one-way ANOVA followed by Tukey's test ($p<0.05$). Error bars show SD (n=3).

3.3. *In vitro degradation of antimicrobial pollutants*

Of the six antimicrobial chemicals tested, two (azithromycin and fluconazole) were not removed (with >80% remaining) in any of the treatments (reclaimed or MQ water), whereas the other four showed variable degradation and removal potential (Fig. 4). Occasional values exceeding 100% of antibiotics remaining in treatments are consistent with expected experimental and analytical variability. Ofloxacin was more degraded in SMC (20% and 80%) than SOS treatments in both MQ water and reclaimed water, with 55–72% *vs.* 83–113% remaining, respectively. Ciprofloxacin was degraded most effectively in the presence of SMC (20% and 80%), leaving less than 60% at the end of the experiment. Sulfamethoxazole was substantially degraded across all treatments, with 80% SOS and 80% SMC yielding the lowest residuals in reclaimed water, where only 12% and 27% remained, respectively. Trimethoprim removal was greatest in reclaimed water with 80% SOS, with around 60% still present. Degradation of various antibiotics using SMC has been shown to involve various enzymatic reactions of laccases in the nutrient-rich environment of spent materials (Chmelová et al., 2024). In the present study, the observed degradation of antibiotics might be related to the presence of water extracts and laccase activities rather than the impact of water sources. Both SMC and SOS treatments showed laccase activities at concentrations of 20% and 80% in both reclaimed and control water (Fig. 3). Laccase activity has been suggested as a possible tool to degrade the antibiotics of ofloxacin and ciprofloxacin (Sánchez-San Martín et al., 2024).

The results obtained indicate the potential of using SMC to produce laccase enzyme and degrade the antibiotics ofloxacin and ciprofloxacin. The combination of laccase production and a rich nutrient environment (Chmelová et al., 2024) might explain the higher degradation potential of SMC compared to SOS toward the investigated antibiotics. Our results show the potential of SMC and SOS to degrade antimicrobial chemicals

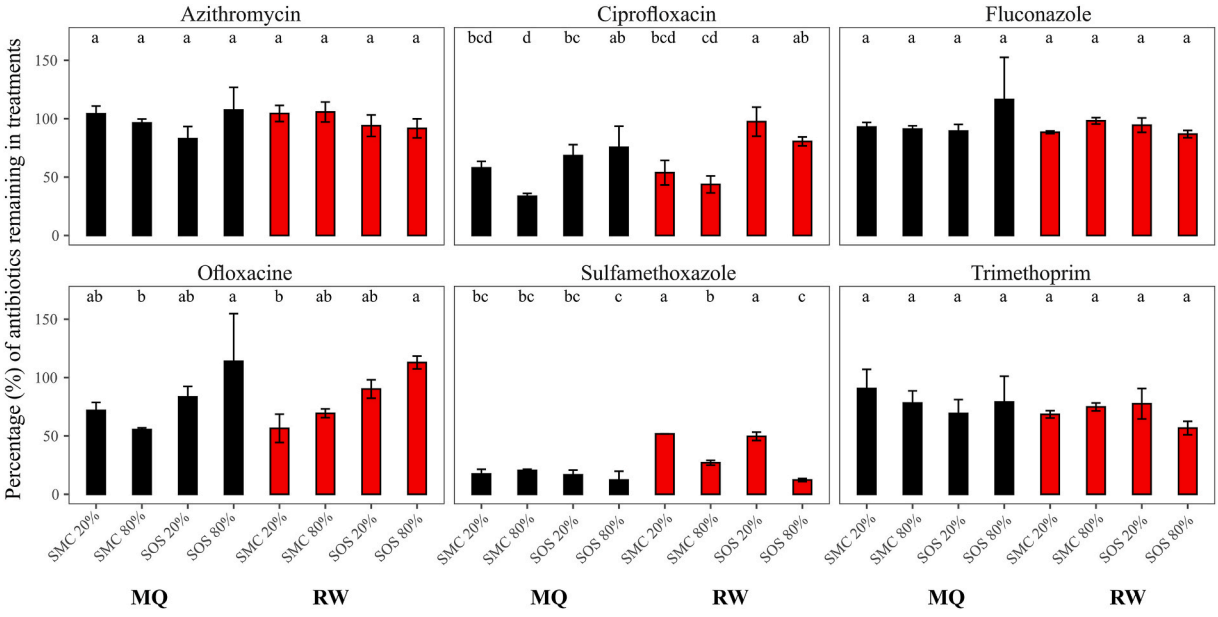

Fig. 4. Percentage (%) of antibiotics remaining after treatment with spent mushroom compost (SMC) and spent oyster mushroom substrate (SOS) water extract in Milli-Q water (MQ, black bars) and reclaimed water (RW, red bars). Letters above each bar indicate significant differences between the treatments according to one-way ANOVA followed by Tukey's test ($p<0.05$). Error bars show SD (n=3). Lower percentages indicate greater antibiotic degradation, i.e., more effective removal.

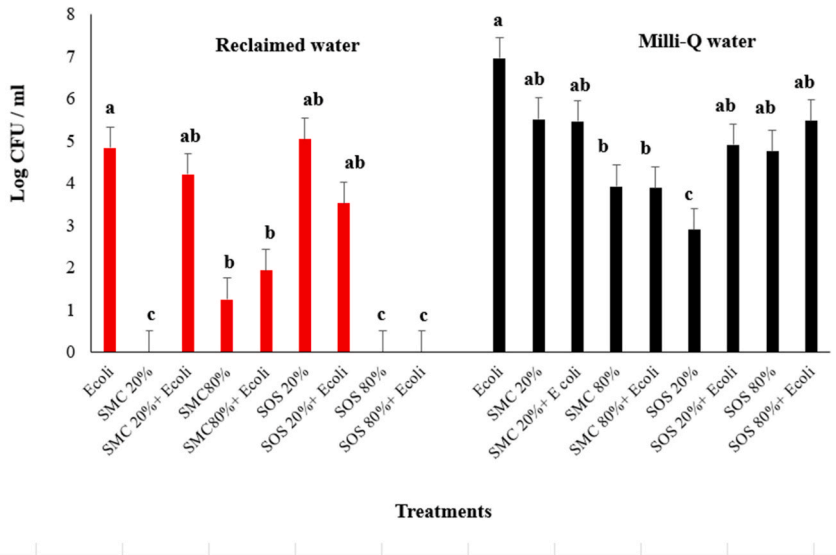

Fig. 5. Growth of nonpathogenic strain *Escherichia coli O157:H7gfp+* (*E. coli*) expressed as colony forming unit (CFU) per mL in treatments of reclaimed water (red) and Milli-Q water (black) in the presence of spent mushroom compost (SMC) and spent oyster mushroom substrate (SOS) water extracts (20% or 80%) and controls (without SMC or SOS) with/without the presence of *E. coli*. Letters above the bars indicate significant differences between the treatments in each water source according to one-way ANOVA followed by Tukey's test ($p<0.05$). Error bars show SD ($n=3$).

in reclaimed water, with SMC being a better candidate in this context. A previous study by van Brenk et al. (2024) also indicated the potential of using enzyme activities in spent material of *Agaricus bisporus* to purify water by removing organic pollutants, while other studies have examined laccase-mediated mycoremediation and wastewater purification (Ge et al., 2023; Mathur et al., 2023).

3.4. Growth inhibition of *E. coli O157:H7 gfp+*

The results showed lower growth of the bacterial strain *E. coli O157:H7 gfp+* in the treatments of reclaimed water than those of control MQ water (Fig. 5). Water extracts of SMC and SOS in treatments of reclaimed water and inoculated with *E. coli O157:H7 gfp+* exhibited the lowest growth. In the control MQ water, the lowest growth was shown by treatments with 20% or 80% SMC water extract and inoculated with *E. coli O157:H7 gfp+*. These results indicate that water extracts of SMC and SOS may have suppressive effects on *E. coli O157:H7 gfp+* growth in reclaimed water.

However, irrigation water has previously been identified as a source of *E. coli* (Truchado et al., 2018) as well as reclaimed water (Heng and Wen, 2016). Our findings highlight the possibility of using water extracts as a strategy to suppress the growth of the pathogen in reclaimed water. However, the enhanced growth of the bacterial strain *E. coli O157:H7 gfp+* in water extracts of SMC and SOS without inoculation of *E. coli* is of concern and might suggest a contamination source of *E. coli* bacteria. This conclusion needs to be investigated further to assess whether the extracts themselves are a source of *E. coli* and they support their proliferation or suppression. Future applications should carefully evaluate risks of microbial contamination, especially the potential presence or regrowth of human pathogens like *E. coli*. Field-scale systems could integrate UV treatment or filtration post-SMC/SOS extract application to mitigate these risks in line with EU Class A–D standards (European Commission, 2017). In addition, in the current EU regulation on water reuse (European Commission, 2017), reclaimed water quality is primarily evaluated based on *E. coli* (from $\leq$10 units/100 mL for Class A to $\leq$ 10,000 units 100 mL^{-1} for Class D), together with a few water parameters. Our results indicated lower growth of *E. coli O157:H7 gfp+* in treatments with 20% and 80% SMC water extract (Fig. 5), which could comply with the safety regulations. Nevertheless, further investigations are required to strengthen these results, particularly with respect to cross-contamination due to the use of spent material. Risk assessment studies (Mulaosmanovic et al., 2019) that can facilitate further application of reclaimed water for larger-scale irrigation purposes are thus crucial. However, the main aim of the present study was to investigate if water extracted from SMC and SOS could be applied as an extra purification step prior to its application in crop irrigation. This could not be confirmed in relation to the growth of the bacterial strain *E. coli O157:H7 gfp+* and raises questions regarding food safety aspects and the need to apply other methods (e.g., filtration and UV radiation) to control contamination prior to the implementation of reclaimed water in field applications. A study performed by Kim et al. (2020) highlighted the possibility to use Zero-valent iron sand filtration to reduce the occurrence of *E. coli* and improve water quality in agricultural irrigation without the need to add disinfectant chemicals. This could be one strategy to be applied when reclaim water and water extract of SMC or SOS are scaled up as a source of irrigation water in field applications. Other tools such as temperature treatment, membrane filtration, Ozon and UV radiation as well as photocatalyst treatments also hold promising potential (Banach and van der Fels-Klerx, 2020). However, the radiation and sterilization techniques would have a direct effect on the reduction of the microbial population and suppressive potential of the water extracts towards pathogens and should carefully be optimized to keep good microbial quality. In addition to such chemical and physical tools, elimination of biohazards in field application needs to be integrated with monitoring and decision support tools applied by the growers to identify factors that either contribute or reduce the load of biohazards (Olivera et al., 2017). However, these areas need further investigations to develop an effective strategy towards elimination of biohazards from the spent mushroom compost and reclaimed water. Moreover, the enzyme activities (Table 1) and laccase activities (Fig. 2) indicated in the present study in the treatments with SMC water extract could help to reduce pathogen growth. These results are of interest and need to be developed in further studies to examine the role of laccase in food safety.

3.5. In vitro growth inhibition of plant pathogens

The lowest inhibition of the root pathogen *Pythium aphanidermatum* (PA) was indicated in the reclaimed water and control water, respectively, with inoculation of the root pathogen PA (Table 2). However, the

Table 2
In vitro growth inhibition (%) of root pathogen *Pythium aphanidermatum* (PA) in treatments of reclaimed water or Milli-Q water in the presence of spent mushroom compost (SMC) or spent oyster mushroom substrate (SOS) water extract (20% or 80%) and controls (without SMC or SOS) after inoculation with PA. Letters above the bars indicate significant differences between the treatments according to one-way ANOVA followed by Tukey's test ($p<0.05$).

Treatments		Inhibition in (%)
Reclaimed water	RW + PA	29^{c}
	RW + SMC 20% + PA	75^{a}
	RW + SMC 80% + PA	51^{b}
	RW + SOS 20% + PA	63^{a}
	RW + SOS 80% + PA	39^{c}
Milli-Q water	MQ + PA	19^{c}
	MQ + SMC 20% + PA	68^{a}
	MQ + SMC 80% + PA	48^{b}
	MQ + SOS 20% + PA	70^{a}
	MQ + SOS 80% + PA	40^{b}

inhibition of PA was enhanced after the addition of SMC or SOS water extract to either reclaimed water or control water. Higher inhibition was found in treatments using 20% SMC or SOS than in treatments using 80% SMC or SOS. The results suggested that the type of water extract affected the growth inhibition of PA, whereas the type of water source had little effect. Conversely, the impact of the water extract decreased as the amount of extract increased in the treatment. This might indicate a lower suppression effect with a higher proportion of the spent material. Our results agree with previous studies showing the potential of SMC in growth suppression of the root pathogen *Pythium ultimum* (Ebrahimi et al., 2018). Furthermore, a negative correlation between disease inhibition and the amount of compost was observed, which is in line with another study (Goonani et al., 2011) showing stronger inhibition of the root pathogen *Phytophthora drechsleri* with 15% SMC than with higher amendment ratios.

The inhibition potential of water extracts of SMC was also examined in *in vitro* investigations toward the leaf pathogen *Botrytis cinerea* (BC) (Table 3). Similarly to PA, the inhibitory effect was largely due to the addition of water extract and not due to the type of water source (reclaimed or control). This implies that reclaimed water without water extract has no potential to suppress the plant pathogen. The suppressive potential of compost water extract toward leafy pathogens has been highlighted in previous studies (Welke, 2005). However, the non-suppressive effect of SOS toward BC is of interest, particularly as SOS has shown to enhance the degradation of organic pollutants, and little is known about their antagonistic role. Our previous study (Khalil et al., 2024) indicated the potential of bacterial isolates from SOS to suppress the growth of plant pathogens. However, this potential was reduced when water extract was used in the current study. Treatments with water extracts of SMC also showed a higher potential to produce extracellular enzymes than treatments with SOS (Table 1). This might explain the observed suppressive potential toward the root pathogen PA but not the leaf pathogen BC. The root pathogen PA belongs to the fungus-like class oomycetes, members of which contain cellulose as a key component in their cell-wall (Hardham, 2007). Therefore, antagonistic activity may induce production of cellulase, which can degrade the pathogen cell-wall. On the other hand, the leaf pathogen BC contains chitin as a cell-wall component. However, production of the enzyme chitinase was not indicated by SOS. This might explain the non-efficient effect of SOS toward BC.

Table 3
In vitro growth inhibition (%) of leaf pathogen *Botrytis cinerea* (BC) in treatments of reclaimed water or Milli-Q water in the presence of spent mushroom compost (SMC) or spent oyster mushroom substrate (SOS) water extract (20% or 80%) and controls (without SMC or SOS) after inoculation with BC. Letters above the bars indicate significant differences between the treatments according to one-way ANOVA followed by Tukey's test ($p<0.05$).

Treatments		Inhibition in (%)
Reclaimed water	RW + BC	19^{c}
	RW + SMC 20% + BC	85^{a}
	RW + SMC 80% + BC	71^{b}
	RW + SOS 20% + BC	21^{c}
	RW + SOS 80% + BC	24^{c}
Milli-Q water	MQ + BC	15^{c}
	MQ + SMC 20% + BC	58^{b}
	MQ + SMC 80% + BC	45^{b}
	MQ + SOS 20% + BC	20^{c}
	MQ + SOS 80% + BC	27^{c}

3.6. Germination trial

The seed germination assay revealed higher seed germination in treatments with reclaimed water mixed with 20% or 80% SMC water extract compared with SOS treatments (Fig. 6). The lowest germination was indicated in the control treatments without water extract. Therefore, for the first time, our study demonstrated that use of reclaimed water treated with SMC extract significantly improved seed germination and plant growth. This indicates additional biostimulant properties beyond pathogen suppression and pollutant degradation, suggesting potential applications for enhancing crop resilience in sustainable agricultural systems. Similar results have been reported in other studies. For example, Amarasinghe and Jayaweera (2022) showed a higher germination index of plants treated with SMC. The type of water source also had an influence on our results. Use of reclaimed water with water extract showed the highest germination, in line with earlier studies indicating positive effects on plant growth when using reclaimed water (Fotia et al., 2017). The positive effect when using reclaimed water mixed with SMC might be related to the higher bacterial content in SMC water extracts compared to SOS water extracts (Fig. 1), which could stimulate nutrient availability and uptake by the plant (Khalil et al., 2024).

A key finding of this study was the functional divergence between SMC and SOS extracts, i.e., SMC enhanced bacterial-driven pathogen suppression and enzyme activity, whereas SOS promoted fungal-mediated degradation of antimicrobial chemicals via laccase production. This suggests that a tailored approach may be needed to optimize microbial interactions for improved reclaimed water purification and disease control. Water extracts of SMC and SOS serve as valuable sources for biocatalysis and can provide cultivation systems with an alternative to address challenges associated with synthetic fertilizer and pesticide use as well as a method for the biodegradation of organic micro-pollutants. However, each extract offers distinct benefits owing to their specific properties and variations in microbial composition and content of organic matter after mushroom or oyster cultivation. SMC is typically preferred for its wider range of beneficial microbial activities, whereas SOS is often chosen for its potential in bioremediation. This was confirmed in our studies, since SOS water extract showed the highest laccase activities in the biodegradation of organic micro-pollutants, whereas SMC water extract offered a high microbial content and potential for the suppression of plant pathogens. Upscaling the use of water extracts as a purification step of reclaimed water, and thereafter application for irrigation purposes, is feasible and would benefit horticultural practices by providing organic nutrients and microbial tools with potential to suppress pathogens and promote plant growth and health. However, careful planning, monitoring and risk management related to the occurrence of biohazards are crucial. Although the use of water extracts of SMC and SOS is a resource efficient method in water purification, it may not be as economical for biodegradation processes as using active mycelia with high laccase activity.

From a practical perspective, preparing SMC and SOS extracts is straightforward. The extraction and filtration process can be completed within a few hours using standard laboratory or on-farm equipment. Once prepared, the extracts remain active for a few weeks when stored at 4 °C, consistent with previous studies showing that aqueous

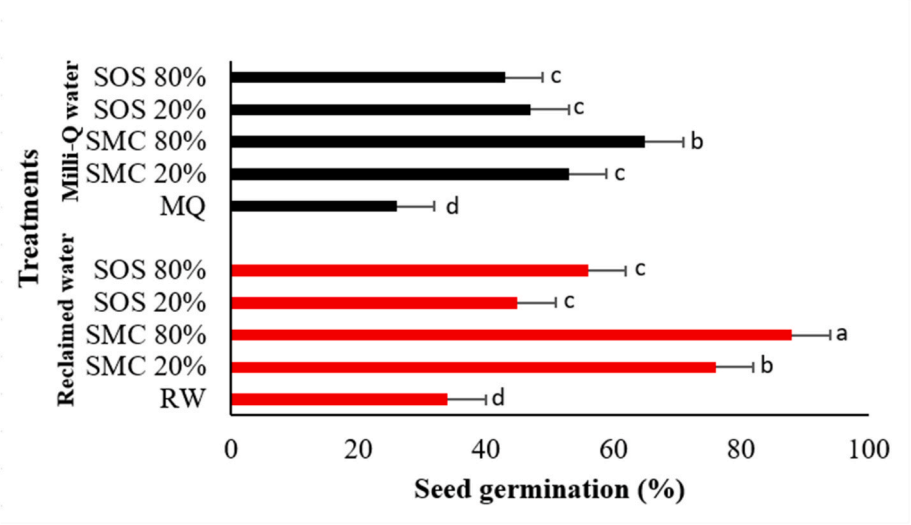

Fig. 6. Seed germination (%) in treatments of reclaimed water (RW, red) and Milli-Q water (MQ, black) in the presence of spent mushroom compost (SMC) and spent oyster mushroom substrate (SOS) water extract (20% or 80%) and controls (without SMC or SOS). Letters above the bars indicate significant differences between the treatments in each water source according to one-way ANOVA followed by Tukey's test ($p<0.05$). Error bars show SD ($n=3$).

dispersions derived from spent mushroom substrate were stable under agitation for at least one week under varying temperature conditions (Beckers et al., 2019), and fungal water-soluble extracts maintained inhibitory activity for a few weeks (Coda et al., 2008). Since SMC and SOS originate from spent mushroom substrate, a nutrient-rich, widely available, low-cost agricultural by-product and the overall production cost is expected to potentially remain low. This cost efficiency potentially makes the approach competitive with, or even cheaper than, conventional water purification methods currently used for reclaimed water treatment. While these operational features indicate promising scalability, further studies are needed to optimize large-scale extraction protocols, evaluate shelf-life under variable environmental conditions, and perform comprehensive cost–benefit analyses across diverse water treatment settings.

The distinct performance characteristics of SMC and SOS can be attributed to their compositional differences. SMC, derived from *Agaricus* cultivation, contains more bacterial taxa capable of extracellular enzyme production (cellulase, chitinase, etc.), leading to strong biocontrol effects. In contrast, SOS, enriched with *Pleurotus* mycelia, contributes more strongly to laccase-mediated oxidative degradation of antibiotics. These functional divergences support the need for tailored use depending on the target contaminants.

4. Conclusions

This study demonstrates the effect of using water extracts sourced from spent mushroom compost (SMC) or spent oyster substrate (SOS) on the suppressive potential of reclaimed water toward biohazards related to human and plant pathogens as well as its removal efficiency of organic pollutants. Our findings show that when reclaimed water is mixed with water extract derived from SMC or SOS, it can suppress the growth of the model human pathogen *E. coli O157:H7 gfp+*, plant pathogens related to root and leaf diseases and eliminate some high priority antimicrobial chemicals. The results suggest that dual-purpose applications of SMC and SOS extracts may support sustainable agriculture by enhancing water irrigation safety while simultaneously supporting plant health and promoting circular bioeconomy practices. Our findings emphasize that tailored use of SMC and SOS extracts can optimize microbial-driven pathogen suppression and pollutant degradation, paving the way for innovative solutions for reclaimed water purification. Additionally, our findings advocate an eco-friendlier approach to provide alternative water sources for irrigation purposes in horticulture and deal with risks related to biohazards and microbial and chemical contamination. Water extracts of SMC and SOS were found to enhance the production of enzymes that increased the antagonistic potential toward pathogens and degradation potential toward organic pollutants. The results obtained also show that reclaimed water treated with SMC extract could be used to enhance seed germination and plant growth. However, issues related to biohazards and risks from pathogens contained in the spent material need to be examined. Nonetheless, our work provides a robust baseline for developing new water sources for plant irrigation purposes and tools to deal with risks and contamination sources. Further studies are required to verify these findings in large-scale systems and assess the impact of other abiotic and biotic factors.

CRediT authorship contribution statement

Samar Khalil: Writing – original draft, Validation, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Valentina Ugolini:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Julia Forsbacka:** Writing – review & editing, Methodology, Investigation. **Maria Karlsson:** Writing – review & editing, Methodology. **Ramesh R. Vetukuri:** Writing – review & editing, Validation, Methodology, Investigation, Conceptualization. **Foon Yin Lai:** Writing – review & editing, Methodology, Investigation, Conceptualization.

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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Data availability

Data will be made available on request.

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