Survival and growth of saprotrophic and mycorrhizal fungi in recalcitrant amine, amide and ammonium containing media

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Short title: Fungal growth in recalcitrant amine, amide and ammonium containing media

Abstract

The elimination of hazardous compounds in chemical wastes can be a complex and technically demanding task. In the search for environmental-friendly technologies, fungal mediated remediation and removal procedures are of concern. In this study, we investigated whether there are fungal species that can survive and grow on solely amine-containing compounds. One compound containing a primary amine group; 2-diethylaminoethanol, one compound with a primary amide group; 2,6-dichlorobenzamide (BAM), and a third compound containing a quaternary ammonium group; N_3-trimethyl(2-oxiranyl)methanaminium chloride, were selected. The choice of these compounds was motivated by their excessive use in large scale manufacturing of protein separation media (2-diethylaminoethanol and the quaternary amine). 2,6-dichlorobenzamide, the degradation product of the herbicide 2,6-dichlorobenzonitrile (dichlobenil), was chosen since it is an extremely recalcitrant compound. Utilising part of the large fungal diversity in Northern European forests, a screening study using 48 fungal isolates from 42 fungal species, including saprotrophic and mycorrhizal fungi, was performed to test for growth responses to the chosen compounds. The ericoid mycorrhizal fungus *Rhizoscyphus ericae* showed the best overall growth on 2-diethylaminoethanol and BAM in the 1-20 gL^{-1} concentration range. A 3500% and 450% increase in biomass, respectively, was observed. For N_3-trimethyl(2-oxiranyl)methanaminium chloride, the peak growth occurred at 1 gL^{-1}. In a second experiment, including three of the most promising species (*Laccaria laccata*, *Hygrophorus camarophyllus* and *Rhizoscyphus ericae*) from the screening experiment, a simulated process water containing 1.9% (w/v) 2-diethylaminoethanol and 0.8% (w/v) N_3-trimethyl(2-oxiranyl)methanaminium chloride was used. *Laccaria laccata* showed the best biomass growth increase (380%) relative to a growth control, while the growth increase for *Rhizoscyphus ericae* and *H. camarophyllus* were 292% and 136% respectively, showing that

also mycorrhizal fungal species can use amine- and amide-containing substrates as nutrients. These results show the potential of certain fungal species to be used in alternative green wastewater treatment procedures.

Introduction

The treatment and destruction of hazardous chemical wastes, such as process water from chemical industries, is a high-cost business connected with environmental risks and considerable energy consumption. This is especially true when desiccation followed by high temperature combustion is used. In the search for more environmental-friendly technologies, fungal mediated remediation and removal procedures are of interest. Bioremediation and biodegradation using for example fungi, bacteria, algae, or plants have developed alongside the commonly used physiochemical technologies [1] and today play an important role in both natural and engineered systems [2]. Fungi are heterotrophic eukaryotes, dependent on organic carbon (C) compounds bio-synthesized by other living organisms. To be able to utilize those, fungi employ powerful enzyme systems to depolymerize and catabolize a plethora of organic compounds. In this capacity they also become of interest from the perspective of possibly catabolizing hazardous chemical compound and transforming them to biomass.

Fungi are of fundamental importance to all ecosystems, with roles as and symbionts, and in elemental cycling, and as such inhabit an immense array of different habitats. They can be split up into large ecological groups - such as saprotrophic and symbiotic fungi - depending on their functions, and evolution of primary lifestyles has occurred repeatedly via loss or reduction of genes for groups of enzymes [3, 4]. Saprotrophic fungi belonging to the phylum *Basidiomycota* primarily facilitate organic matter decomposition, utilizing C and nutrients

from leaf litter and wood for growth [5]. Wood decomposing fungi can be further divided into groups; fungi with the ability to selectively or simultaneously degrade persistent lignin using highly specialized class II peroxidases (white rot fungi), and fungi not able to degrade lignin which instead selectively degrade cellulose (brown rot fungi; use of Fenton chemistry) [3]. Saprotrophic fungi also secrete a range of other enzymes, including cellulose, pectin and hemicellulose degrading enzymes [3]. Mycorrhizal fungi, on the other hand, live in symbiosis with vascular plants, receiving photo-assimilated C from their host plants in return for mineral nutrients and water taken up from the surrounding soil [6]. Fungi belonging either to *Basidiomycota* or *Ascomycota* form symbiosis with boreal trees, and are called ectomycorrhizal (ECM) fungi. Depolymerization of organic matter was earlier assumed to be carried out only by free-living saprotrophic fungi. Although the involvement of ECM fungi in decomposition of soil organic matter remains controversial, recent findings support the view that ECM fungi also have the capacity to oxidize organic matter [4, 7, 8], through enzyme systems similar to those of white rot fungi including peroxidases [9, 10] and brown-rot fungi Fenton chemistry [11].

In addition to their ability to decompose organic matter, fungi were recently high-lighted for their large potential to be exploited further for industrial use; in strategies against human and plant diseases, for production of food and beverages, for enhancing crops and forestry, and for improving waste disposal [12]. This was attributed to their wide-spread distribution and adaptation to all kinds of environments, their competitive ability, and that they can in many cases be cultured with relative ease. Fungi are well known to tolerate and metabolize both recalcitrant and toxic compounds, and are used for bioremediation [13, 14]. Due to their diverse metabolic capacity fungi are therefore good candidates for managing chemical waste.

Fungi with peroxidases are often used in whole cell fungal treatments (*in vivo*) of wastewaters, not at least in those waters that contain pharmaceuticals [15, 16]. The catabolism of the pharmaceutics is in these cases performed by the extracellular enzymes which are secreted from the fungal mycelia [17, 18]. There are also examples of *in vitro* experiments in which solely enzymes and not living fungal cells have been used [19, 20]. It has been demonstrated that fungi that produce these extracellular enzymes can use nitrogen (N) containing aromatic compounds as sole N sources [21], or as both C and N source [22]. However, when it comes to the removal of non-aromatic compounds, the use of these fungi is relevant only when combined with redox-mediators that enhance the oxidation capacity of the enzymes [20] or with reactive oxygen species like the hydroxyl radical [23].

Amines, amides and quaternary ammonium compounds are relatively commonly occurring substances, containing both N and C, which make them interesting from a nutrient point of view. On the other hand, the removal of these substances as contaminants in ground and wastewaters is important since many of them are both toxic and carcinogenic. Amines are used in the syntheses of azo-dyes, polyurethane, pesticides and many other products. The degradation of amines is facilitated both by Advanced Oxidation Procedures (AOPs) and non-AOPs such as biodegradation [24]. AOPs are based on the generation of hydroxyl radicals which can be facilitated chemically (Fenton´s reagent), photo chemically ($UV/TiO_2/H_2O_2$, O_3,/UV) or sonolytically (ultrasound). Although argued that these techniques have an advantage of removing even the non-biodegradable contaminants, there are some drawbacks; reaction products can be even more toxic than the precursors [25], and the presence of organic or inorganic constituents leads to higher oxidant requirements in order to maintain the treatment efficiency [26]. Using biodegradation, the end product (for example via fungal degradation) could be compostable biomass. The majority of amine containing compounds

that so far has been successfully biodegraded using fungi with peroxidases are aromatic amines including azo-dyes [27], tannic and humic acid [28], and pharmaceuticals [29]. Aromatic amines can also be adsorbed to sorbents like activated C or modified chitosan [30]. The possibility to biodegrade non-aromatic amines, amides and quaternary ammonium compounds is less investigated. However, the possibility to remove N-containing recalcitrant compounds in wastewaters using fungi is challenging, and the knowledge is scarce on how to predict the removal efficiency of harmful substances using fungi.

The overall aim of the present study was to test the feasibility of growing fungi for the purpose of metabolizing recalcitrant compounds, utilising part of the fungal species' diversity in Northern European forests and evaluating their growth and survival on relevant N-containing recalcitrant compounds. We wanted to screen a larger number of fungal species with varying taxonomy and ecology, and as a first step we performed a screening study using 48 isolates from 42 species, including both saprotrophic and mycorrhizal fungi. Their ability to survive and grow in high concentration solutions of 2-diethylaminoethanol, N_3-trimethyl(2-oxiranyl)methanaminium chloride and 2,6-dichlorobenzamide (BAM) were evaluated. The chemicals are of interest since they are toxic and difficult to handle in wastewater treatments plants. 2-diethylaminoethanol and N_3-trimethyl(2-oxiranyl)methanaminium chloride are used as ligands in the large-scale manufacturing of weak and strong anion-exchangers in the protein separation field. BAM is a persistent, water soluble degradation product of the pesticide 2,6-dichlorobenzonitrile (dichlobenil), contaminating ground waters [31]. After the screening study a sub-set of species that showed the best growth was chosen for a simulated process water experiment containing both 2-diethylaminoethanol and N_3-trimethyl(2-oxiranyl)methanaminium chloride.

Materials and methods

Fungal isolates and experimental systems

Two experiments were set up; firstly a screening experiment (S1 Fig) to test survival and growth of a wide range of fungal species in the presence of of 2-diethylaminoethanol, N_3-trimethyl(2-oxiranyl)methanaminium chloride and BAM, and secondly a simulated process water experiment (S2 Fig) including 2-diethylaminoethanol and N_3-trimethyl(2-oxiranyl)methanaminium chloride and the top three fungal species able to grow on the investigated N-containing compounds from the screening experiment. A total of 48 fungal isolates and 42 fungal species (S1 Table) were included in the screening experiment. Within-species variation was tested for four mycorrhizal species (*Cenococcum geophilum*, *Laccaria laccata*, *Piceirhiza bicolorata* and *Suillus variegatus*) and two saprotrophic species (*Armillaria mellea* and *Hypholoma fasciculare*). The selection of candidate fungal species for the second experiment was later on based on their overall growth on two of the tested compunds (2-diethylaminoethanol and N_3-trimethyl(2-oxiranyl)methanaminium chloride). Species names, authorities and taxonomical classifications are taken from the Dyntaxa database [32], and information about species ecology from Hallingbäck and Aronsson [33]. The investigated species included both saprotrophic (white rot fungi, brown rot fungi and litter decomposing fungi) and mycorrhizal fungi (ECM and ERM fungi). Fungal isolates were obtained directly from sporocarps collected from forests around Uppsala in 2005 and from fungal culture collections at the Department of Forest Mycology and Plant Pathology, SLU, Uppsala, Sweden (Petra Fransson and Rimvydas Vasaitis). Isolating new isolates from sporocarps were done by removing small pieces of fungal tissue from the sterile inside of the sporocarp and placing them on half-strength modified Melin–Norkrans (MMN) medium [34] in 9 cm Petri dishes, until growth was apparent, and fungi were sub-cultured to new plates.

All fungal isolates were maintained on MMN medium in darkness at 25 °C and had grown on new plates for one month before starting the experiments. For the screening experiment fungal isolates were grown in Petri dishes in 50 mL basal Norkrans medium [35] with a C:N ratio of 15 and pH adjusted to 4.5. One piece of agar containing mycelia was cut out with a corer (∅ 10 mm) from the actively growing mycelial edge of the fungal culture and placed in the liquid medium (one replicate per species and treatment, with three chemicals and three concentrations, giving a total of 432 plates). Growth controls including basal Norkrans medium only were also prepared (n=2). In order to increase survival some of the fungi with slow growth rates, mostly ECM species and the ERM fungus *Rhizoscyphus ericae*, were cut out and put on new agar plates for approximately one week so that growth resumed before the agar pieces were transferred to liquid medium. Petri dishes with liquid isolates were incubated in darkness at 25 °C for one week before chemical exposure.

For the simulated process water experiment three selected fungal species (*Hygrophorus camarophyllus, Rhizoscyphus ericae* and *Laccaria laccata* AT2001038) were grown in autoclaved 1000 mL Erlenmeyer flasks containing 200 mL basal Norkrans medium with a C:N ratio of 15 and pH adjusted to 4.5. Ten pieces of agar with actively growing mycelia were initially transferred to each flask (n=5, giving a total of 15 flasks) with a sterile tool. The flasks were sealed with aluminium foil and kept in dark in closed cardboard boxes at room temperature. After one week's growth in basal Norkrans medium 800 mL of the simulated process water was added to each flask (n=3). Controls (n=2) containing only 200 mL Basal Norkrans medium were included for each of the three fungal species. The chemicals used in the growth media were supplied from Sigma-Aldrich (Switzerland).

198 # Chemical exposure and harvest

199 For the screening experiment standard solutions of 2-diethylamine (Fluka, Switzerland; S1
200 Fig) and N_3-trimethyl(2-oxiranyl)methanaminium chloride (Evonik Industries AG, Germany,
201 trade name; glycidyltrimethylammonium chloride (gly); (S1 Fig) were prepared in autoclaved
202 flasks using autoclaved double distilled water. Both substances were prepared so that in the
203 screening experiment 2 mL added to a Petri dish with 50 mL liquid medium including
204 mycelia, would give the concentrations 1, 10 and 20 g L^{-1}. 2,6-dichlorobenzamide (BAM)
205 (Acros Organics, Belgium; S1 Fig) was not possible to dissolve at a concentration of 2.7 g L^{-1}
206 as previously reported [36]. A saturated solution was prepared by dissolving 250 mg BAM in
207 one litre warm (80° C) double distilled water for 3.5 hours. The undissolved material was
208 removed by vacuum filtration using a 0.45 µm HAWPO4700 cellulose-based Millipore filter.
209 The separate compounds were added to the fungal isolates after a week on liquid medium,
210 during which time the mycelia were adjusted to growing in liquid media and it was assumed
211 that part of the glucose and ammonium sulfate was consumed. After adding the compounds,
212 the fungal isolates were grown for an additional two weeks, giving a total growth period of
213 three weeks.
214
215 The composition and final concentrations of the simulated process water, chosen to reflect
216 conditions at which the amines are present in large-scale manufacturing plants, are found in
217 Table 1. To simulate a harsher environment including substances that are present in process
218 waters NaCl and Na_2SO_4 were added to the 2-diethylaminoethanol and N_3-trimethyl(2-
219 oxiranyl)methanaminium chloride, and the C:N ratio in the mixed water was approximately
220 5.1. After additions to the Erlenmeyer flasks fungi were grown for another three weeks,
221 giving a total growth period of four weeks for the second experiment. The growth controls
222 were harvested already after one week's growth in 200 mL liquid medium. At the end of both

223 experiments the contents of the Petri dishes and Erlenmeyer flasks were vacuum-filtered onto
224 weighed filter papers (Munktell 1003, 9 cm). Filter papers were dried in an oven at 105° C and
225 re-weighed for fungal biomass.
226
227 **Table 1. Composition of simulated process water.**

Composition	% (w/v)[1]	% (w/v)[2]
2-diethylaminoethanol	2.4	1.9
N_3-Trimethyl(2-oxiranyl)methanaminium chloride	1.0	0.8
NaCl	1.9	1.5
Na_2SO_4	0.8	0.6

228
229 [1] Original solution
230 [2] After 4:1 dilution with Basal Norkrans liquid medium pH was finally adjusted to 4.5
231

232 Statistical analysis

233 Growth of each fungal species in the N-containing compounds' treatments was calculated as a
234 percentage of the mean value of the respective growth control (S2 Table). For the screening
235 experiment differences in the average biomass in control treatment between mycorrhizal and
236 saprotrophic fungi, and between types of saprotrophs (white rot, brown rot and generalists)
237 was tested using One-way ANOVA in Minitab 18.1 (Minitab Inc., State College, PA, USA).
238 Ordination analysis was performed using CANOCO version 5.02 (Microcomputer Power,
239 Ithaca, NY, USA). Variation in biomass in controls and all amine treatments (10 response
240 variables) for each fungal isolate (n=48) was visualized using principal components analysis
241 (PCA), without transforming data. We also used the multi-response permutation procedure

(MRPP), a nonparametric procedure in PC-ORD version 5.33 software [37] for testing the hypothesis of no difference between two or more *a priori* assigned groups [38]. This was done to test for the effects of main functional groups (mycorrhizal, saprotroph, saprotroph/parasite and parasite), functional groups (ectomycorrhizal, ericoid mycorrhizal, saprotroph, generalist, white rot, brown rot, litter decomposer and unknown), phylum (*Ascomycota and Basidiomycota*), and order (Agaricales, Atheliales, Boletales, Pezizales, Polyporales, Russulales, and Thelephorales). MRPP provides p-values as well as A-values that measure 'effect sizes,' representing homogeneity within the group compared with that expected randomly. For instance, perfect homogeneity in the group gives A = 1, whereas A values between 0 and 1 indicate that heterogeneity between the groups is greater than that expected by chance. For the simulated process water experiment net growth was calculated by subtracting controls (n=2) from the total four mean weeks growth including three weeks with added SPW (n=3).

Results

Screening experiment – growth controls

In the control treatment fungi produced on average 24.1 ± 1.6 mg biomass when grown for three weeks in a liquid nutrient media, with somewhat higher biomass (but not significantly so) for mycorrhizal fungi (26.0 ± 2.3 mg) compared to saprotrophic fungi (21.8 ± 2.3 mg). Comparing taxonomic groups within the mycorrhizal fungi the five ascomycetes produced on average 28.5 ± 9.7 mg biomass compared to the 21 basidiomycetes which produced 24.4 ± 3.4 mg. All saprotrophic fungi were basidiomycetes. Comparing the different functional groups and rot types within the saprotrophic fungi, the white rot fungi (11 isolates) produced 25.6 ± 3.2 mg biomass, brown rot (5 isolates) 21.3 ± 11.0 mg, and generalists (2 isolates) 5.5 ± 5.5

mg. The largest biomass was produced by the saprotrophic fungus *Ganoderma applanatum* (62.0 mg), followed by the mycorrhizal fungi *Pisolithus arhizus* and two isolates of *Piceirhiza bicolorata* (ca. 50 mg)(S2 Table). Some fungal isolates grew poorly in the control treatment (S2 Table); the mycorhizal fungi *Amanita citrina*, *Laccaria laccata* AT2001038, *Rhizoscyphus ericae*, *Thelephora sp.* and *Tricholoma pessundatum* produced 1.2-6.7 mg biomass (Fig 1), and the saprotrophic fungi *Agaricus arvensis*, *Fistulina hepatica* and *Fomitopsis pinicola* between 1.2-2.8 mg (Fig 2). For the intra-specific comparisons growth in the control treatment were mostly similar between isolates of the same species (Figs 3 and 4, S2 Table), with the exception for *Laccaria laccata* which varied greatly (2.1 mg and 33.5 mg, respectively).

Fig 1. Mycorrhizal fungal growth responses to recalcitrant amine, amide and ammonium containing media.

Biomass responses to treatments were compared to controls for mycorrhizal fungal species in a screening experiment including 2-diethylaminoethanol (2-diet), N_3-Trimethyl(2-oxiranyl)methanaminium chloride (gly) and BAM at three different concentrations. Mycorrhizal species showed positive growth responses to all amine treatments exemplified by (a) *Rhizoscyphus ericae* and (b) *Hygrophorus camarophyllus*, positive growth responses to some amine treatments exemplified by (c) *Lactarius controversus* and (d) *Thelephora sp.*, negative responses to all treatments (e) *Amanita muscaria* and (f) *Cortinarius glaucopus*, (g) represents a commonly occurring species in boreal forests (*Piloderma crocuem*), and (h) *Tricholoma pessundatum* exemplifies a species which produced little biomass in the control and amine treatments.

290 **Fig 2. Saprotrophic fungal growth responses to recalcitrant amine, amide and**
291 **ammonium containing media**.
292 Biomass responses to treatments as compared to controls for saprotrophic fungal species in a
293 screening experiment including 2-diethylaminoethanol (2-diet), N_3-Trimethyl(2-
294 oxiranyl)methanaminium chloride (gly) and BAM at three different concentrations.
295 Saprotrophic species showed positive growth responses to all amine treatments exemplified
296 by (a) *Fomitopsis pinicola* (brown rot) and (b) *Mycena epipterygia* (litter decomposer), two
297 brown rot fungi with positive growth responses to some amine treatments exemplified by (c)
298 *Lycoperdon pyriforme* and (d) *Laetiporus suphureus*, negative responses to all treatments (e)
299 *Ganoderma applanatum* (brown rot) and (f) *Lenzites betula* (white rot), and two parasites (g)
300 *Phanerochaete sordida* and (h) *Rhizina undulata*.
301
302 **Fig 3. Intra-specific mycorrhizal growth responses to recalcitrant amine, amide and**
303 **ammonium containing media similar among isolates.**
304 The intra-specific variation in biomass responses to treatments compared to controls for
305 mycorrhizal fungal species in a screening experiment including 2-diethylaminoethanol (2-
306 diet), N_3-Trimethyl(2-oxiranyl)methanaminium chloride (gly) and BAM at three different
307 concentrations. (a) and (b) *Cenococcum geophilum*, (c) and (d) *Laccaria laccata*, (e) and (f)
308 *Piceirhiza bicolorata*, (g) and (h) *Suillus variegatus*.
309
310 **Fig 4. Intra-specific saprotrophic growth responses to recalcitrant amine, amide and**
311 **ammonium containing media similar among isolates.**
312 Intra-specific variation in biomass responses to treatments compared to controls for
313 saprotrophic fungal species in a screening experiment including 2-diethylaminoethanol (2-

diet), N_3-Trimethyl(2-oxiranyl)methanaminium chloride (gly) and BAM at three different concentrations. (a) and (b) *Armillaria mellea*, (c) and (d) *Hypholoma fasciculare*.

General growth responses to N-containing compounds

When fungi were exposed to individual compounds for two weeks most of the 48 isolates were able to survive in liquid media containing amines (S2 Table), and many species were either restricted compared to controls or inhibited. Average biomass production across all treatments was 15.1 ± 0.7 mg, similar between mycorrhizal and saprotrophic fungi (15.9 ± 0.9 mg and 14.2 ± 1.0 mg, respectively), and ranging from no growth (values around zero; Fig 1h) up to 65 mg (*Schizophyllum commune*, see S2 Table). The biomass values correspond to a growth increase relative the controls up to a 3640% increase (S2 Table, Figs 1-4). For some treatments where the final biomass production was around zero at harvest, the biomass from the first week of growth on basal Norkrans medium decreased when exposed to the selected compounds. In general, there were more negative growth responses to all three compounds than positive (S2 Table). Biomass production was positively affected by all three compounds for the mycorrhizal *Rhizoscyphus ericae* (up to 3600% growth increase; Fig 1a) and *Hygrophorus camarophyllus* (up to 150%; Fig 1b), and for the saprotrophic *Fomitopsis pinicola* (up to 1500% increase; Fig 2a), *Mycena epipterygia* (up to 450% Fig 2b) and *Rhizina undulata* (up to 150% Fig 2h). Negative effects by all three N-containing compounds at all three concentrations compared to controls were found for eight mycorrhizal fungi (*Amanita muscaria*, *Cortinarius glaucopus*, *Hebeloma sp. 1*, *Laccaria bicolor*, *Paxillus involutus*, *Piloderma byssinum*, *Suillus bovinus*, and *Suillus luteus*; S2 Table, Figs 1e and f), and for three saprotrophic fungi (*Ganoderma applanatum*, *Lenzites betulina* and *Hypholma sp.*; S2 Table, Fig 2e, Fig 2f and Fig 4e). The phylogenetic distribution of the 48 fungi plotted against the growth responses to the three highest compound concentrations showed that the ability for

fungal isolates to increase growth in the presence of the substances varies across the range of systematic entities and species (S3 Fig). Hence, no single evolutionary group seem to have a clear advantage in biodegrading these compounds, but rather that is appears to be important to apply an evolutionary broad screen when selecting suitable taxa. The multivariate analysis revealed no patterns in growth response for the different functional groups (Fig 5), neither for the main groups nor for the detailed rot types etc within the saprotrophic fungi. For systematic levels, the MRPP analyses showed significant differences between the two phyla *Basidiomycota* and *Ascomycota* (MRPP analysis; p=0.045, A=0.025), and between fungal orders (MRPP analysis; p=0.0067, A=0.093). The average biomass responses for each species to both controls and all treatments are shown in S4 Fig.

Fig 5. Overall fungal biomass responses to the three tested recalcitrant compounds in the screening experiment.

Principal component analysis (PCA) showing the variation in biomass responses for 48 fungal isolates when grown for three weeks in control treatment with nutrient solution and N-containing compounds' treatments (2-diethylaminoethanol [2-diet], N_3-Trimethyl(2-oxiranyl)methanaminium chloride [gly] and BAM) at three different concentrations. Species differences are visualized by (a) a sample plot with the vector length indicating the relative importance of the amine treatments, and (b) a sample plot with species coded according to functional groups. The first three axes together explained 87.5% of the total variation (84181.4).

Growth responses to specific N-containing compounds

There were some general response patterns for growth on the individual compounds (see S2 Table). For 2-diethylaminoethanol 19 out of 26 mycorrhizal isolates and 16 out of 22

saprotrophic isolates showed a negative growth response for all concentrations. For BAM 15 mycorrhizal fungi and 15 saprotrophic fungi were negatively affected by all concentrations, followed by N_3-trimethyl(2-oxiranyl)methanaminium chloride where eight mycorrhizal and three saprotrophic fungi were negatively affected. Among the mycorrhizal fungi, positive growth responses (for one or more concentrations) were most common when grown on N_3-trimethyl(2-oxiranyl)methanaminium chloride; 13 mycorrhizal species (incl. *Amanita muscaria*, *Cenococcum geophilum*, *Hygrophorous amarophyllus*, both isolates of *Laccaria laccata*, *Lactarius controversus*, *Leccinum scabrum*, *Piloderma croceum*, *Pisolithus arhizus*, *Rhizopogon roseolus*, *Rhizoscyphus ericae*, *Suillus variegatus* (1st Sept 04), *Thelephora sp.* and *Tricholoma pessundatum*). Similarly, for saprotrophic fungi positive growth responses were most common when grown on N_3-trimethyl(2-oxiranyl)methanaminium chloride; 19 fungi with the exception of *Ganoderma applanatum* and *Hypholoma sp.* When comparing the intra-specific variation in biomass and growth responses to the N-containing compounds, patterns were very similar for all species except *Laccaria laccata* (Figs 3-4).

Simulated process water experiment

Based on growth data in the experiment, in combination with how easy the isolates were to grow in liquid culture, the mycorrhizal species *Rhizoscyphus ericae*, *Hygrophorus camarophyllus*, and *Laccaria laccata* AT2001038 were chosen for the simulated process water experiment. All three isolates survived and grew on the simulated process water (Fig 6). Net biomass and growth responses corresponded to 179 mg (380%), 292 mg (136%) and 336 mg (292%) for *Laccaria laccata*, *Hygrophorus camarophyllus*, and *Rhizoscyphus ericae*, respectively.

388 **Fig 6**. **Biomass production for three mycorrhizal fungal species grown for three weeks in**
389 **simulated process water.**
390 White bars show controls (one week in Basal Norkrans medium; n=2), grey bars show the
391 simulated process water treatment (one initial week in Basal Norkrans medium followed by
392 three weeks in amine solution (n=3).
393

394 # Discussion

395 In a first experiment, the growth and survival of 48 fungal isolates with varying taxonomy and
396 ecology on three different N-containing compunds (2-diethylaminoethanol, N_3-trimethyl(2-
397 oxiranyl)methanaminium chloride and BAM) at three concentrations were evaluated. The
398 isolates belonged to the two main functional groups saprotrophic and mycorrhizal fungi,
399 which are known for their complex enzyme systems used for depolymerizing organic matter
400 [5, 8] ability to compete for nutrients in soil and woody substrates, as well as being relatively
401 easy to grow in pure culture. Although many isolates were partly restricted or inhibited in
402 growth in the presence of the selected substances, most survived. A subset of three
403 mycorrhizal isolates, which were further tested in a simulated process water experiment,
404 produced large biomass despite exposure to harsh conditions at which the compounds are
405 present in large-scale manufacturing plants.
406

407 ## Do fungal functional groups differ in their responses to individual
408 ## N-containing compounds?

409 Comparing the main functional groups, mycorrhizal and saprotrophic fungal isolates were
410 able to produce similar amounts of biomass when grown in control treatments, and among the

411 saprotrophic fungi wood decomposing species with and without peroxidases (white and
412 brown rot fungi, respectively) tended to grow better than the few species that are generalists.
413 There was large inter-specific variation in growth among the tested isolates, which is in line
414 with earlier studies conducted in pure culture [39, 40]. Low biomass production in some
415 isolates may reflect slow growth rates for some species when grown in pure culture or
416 indicate use of an unsuitable substrate for other isolates. Although many species were partly
417 restricted or inhibited in growth, most survived when the selected compunds were added. This
418 indicates an ability to utilize the compounds as substrates, and a large biomass was assumed
419 to indicate fungal use via either enzymatic biodegradation, biosorption or bioaccumulation. In
420 a previous study including 44 fungal isolates from vineyard soil and grapevine the ability to
421 degrade biogenic amines was noteworthy for many fungi, and independent of the amine
422 incorporated into the culture medium [41]. In the present study, mycorrhizal fungi showed
423 generally more negative responses to all three N-containing compounds compared to the
424 saprotrophic fungi, which probably reflects a higher ability of for example wood decomposers
425 (white rot fungi) to tolerate toxic chemicals and environments within e.g. wood [42]. The
426 non-specific degradation mechanisms using extracellular enzymes, allow lignolytic fungi to
427 degrade a wide range of recalcitrant pollutants [1, 43-45]. Despite this general pattern when
428 comparing responses to all three individual substances, some mycorrhizal isolates also coped
429 well. For example, suilloid species (*Suillus spp.* and *Rhizopogon roseolus*) are well known to
430 produce large amounts of biomass (e.g. [39]) and do so when exposed to amines, and these
431 results were confirmed in the present screening study. Among the mycorrhizal species
432 included in the study, we only had one isolate of the ascomycete forming ericoid mycorrhiza
433 (*Rhizoscyphus ericae*), which was chosen for the simulated process water experiment due to a
434 strongly positive biomass response to the N-containing compounds. This species belongs to
435 an aggregate of species [46] also including *Piceirhiza bicolorata* with yet unclear systematic

affinities, which can form ECM associations. The species aggregate is of special interest in the context of withstanding or metabolising N-containing compounds of the type included in our study, since they possess a wide range of biochemical and physiological attributes enabling the fungus to cope with the harsh and stressed habitats of ericoid plants [47]. It was clear from the screening experiment that all three isolates from this species aggregate produce very large amounts of biomass in the current set-up. Experimental studies have confirmed their saprotrophic capabilities [48] with a wide range of extracellular enzymes, and they are known to utilize ammonium, nitrate, organic substances like amino acids [49] and their amides [50], and proteins [51]. Further, *R. ericae* is able to mobilize organic N also from even more recalcitrant sources such as lignin [52] and chitin [53, 54]. *Laccaria laccata*, one of the ectomycorrhizal species included in the simulated process water experiment, is mainly an early succession ectomycorrhizal fungus ranging widely over the world and known to be easy to grow in liquid culture [55]. The species, which is used and commonly occurring in nursery seedlings [56], has a wide host range, ease of forming ectomycorrhizal synthesis and potential as biological control agent against disease causing fungi such as members of the genus *Fusarium* [57]. Although ectomycorrhizal fungi such as *Laccaria laccata* degrade pollutants and expedite removal of persistent organic pollutants [58, 59], it is unknown whether the species can metabolise amines, amides or quaternary ammonium compounds. However, the closely related species *Laccaria bicolor* was previously shown to be unable to grow on media containing amines as sole N sources [60] and is suggested in nature to use the ammonium produced either by microbial or chemical amine decomposition since it has been shown to have little or no ability to grow on organic N sources [61]. The main conclusion of the growth experiment including either 2-diethylaminoethanol, N_3-trimethyl(2-oxiranyl)methanaminium chloride or BAM was that many fungal isolates survived and grew in the presence of these N-containing compounds.

Simulated process water experiment

In the simulated process water, all three tested species grew well, and the addition of salts did not seem to significantly prevent their growth. The fungi most likely continued to use the Basal Norkrans medium as nutrients in the three weeks period including the simulated process water, however, the excess of test solution in combination with the large biomass indicated that the fungi used the N-containing compounds in the process waters as substrates for growth. *Rhizoscyphus ericae* produced the largest biomass (ca. 340 mg) and *Laccaria laccata* showed the highest growth increase. The observed growth of the fungi on the N-containing substrates was most likely explained by either extracellular or endo-enzymatic degradation mechanisms. In the first case, the products from the biodegraded compounds must penetrate the fungal cell bi-layers, and in the endo-enzymatic mechanism, the native substances are transported through the membranes for further degradation within the cells. Several kinds of filamentous fungi are known to produce amine oxidase activity when using amines as a sole N source for growth [62-64]. Two kinds of amine oxidases were the first to be purified and characterized from fungi [65, 66], later followed by studies revealing other types of amine oxidases (e.g. [67]). The enzymes catalyze the oxidative deamination of terminal amino groups, allowing the fungi to degrade an amine as a source of ammonium for growth. This would explain the ability of many fungal isolates to increase biomass in the presence of amines, since N often is the most growth-limiting nutrient. In the present study, the C:N ratio of the simulated process water was low (5.1) and most of the N was not present in a directly available form, thus the fungi must have the ability to metabolise the selected substrates to promote uptake and biomass production. This, however, needs to be confirmed by for example analyzing residual N-containing compunds in the liquid media or investigating the potential presence of amine oxidases and other relevant enzymes than can catalyze the

investigated compounds. Amine oxidase activity was first observed in strains of *Aspergillus niger, Aspergillus fumigatus, Penicillium chrysogenum* and *Penicillium notatum* [65], which are well-known representatives of the order Eurotiales in *Ascomycota.* In a more recent study evaluating the ability of vineyard soil and grapevine fungi to degrade biogenic amines *Penicillium* spp., *Alternaria* sp., *Phoma* sp., *Ulocladium chartarum* and *Epicoccum nigrum* showed high capacity to *in vitro* amine degradation in a microfermentation system [41]. These are also species within *Ascomycota*, where all (except *Penicillium*) belong to the order Pleosporales. In the present study we did not include any species from these orders, since we focused mainly on fruitbody forming saprotrophic and mycorrhizal fungi belonging to *Basidiomycota*, with a few exceptions found in *Ascomycota*. The species included here represent other ecological groups of fungi compared to the examples from Pleosporales and Eurotiales. Amine oxidase activity was previously detected in one basidiomycotous species, which is also included in the present study, *Armillaria* (saprotroph/parasite), in a large screening study investigating 85 fungal isolates [66], along with a number of species belonging to *Ascomycota*. Beside from these studies, little is known about the distribution of the enzyme systems in fungal strains from different ecosystems, and as far as we are aware, it is unknown whether amino oxidases are present in most saprotrophic or mycorrhizal fungi. In future studies, it would be of interest to design the experiments so that concentration changes in N-containing compounds can be measured, requiring lower substrate amounts.

In summary, the feasibility of growing fungi for metabolizing recalcitrant N containing compounds, including an amine, an amide and a quaternary compound, in wastewater was tested, utilising part of the large fungal species diversity in Northern European forests. The species included in the present study differed from earlier studies of filamentous fungi in the context of e.g. amine oxidation of these substances, since they belong to the functional groups

511 wood and litter decomposers, and mycorrhizal fungi. Although many isolates were partly
512 restricted or inhibited in growth, most survived in the presence of 2-diethylethanolamine, N_3-
513 trimethyl(2-oxiranyl)methanaminium chloride and BAM. The observed growth on these
514 compounds is to our knowledge not previously reported. The most promising fungi of those
515 were tested, when growth data were considered, was the ECM fungus *Laccaria laccata* and
516 the ericoid mycorrhizal fungus *Rhizoscyphus ericae*. In addition to the saprotrophic fungi,
517 especially fungi with peroxidases, which are used in whole cell fungal treatments within
518 industry, mycorrhizal fungi showed potential as alternatives for treatments of wastewater
519 containing the investigated N containing substances. However, this first screening study needs
520 to be followed by more in-depth studies confirming decreased concentrations of these
521 substances.
522

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527

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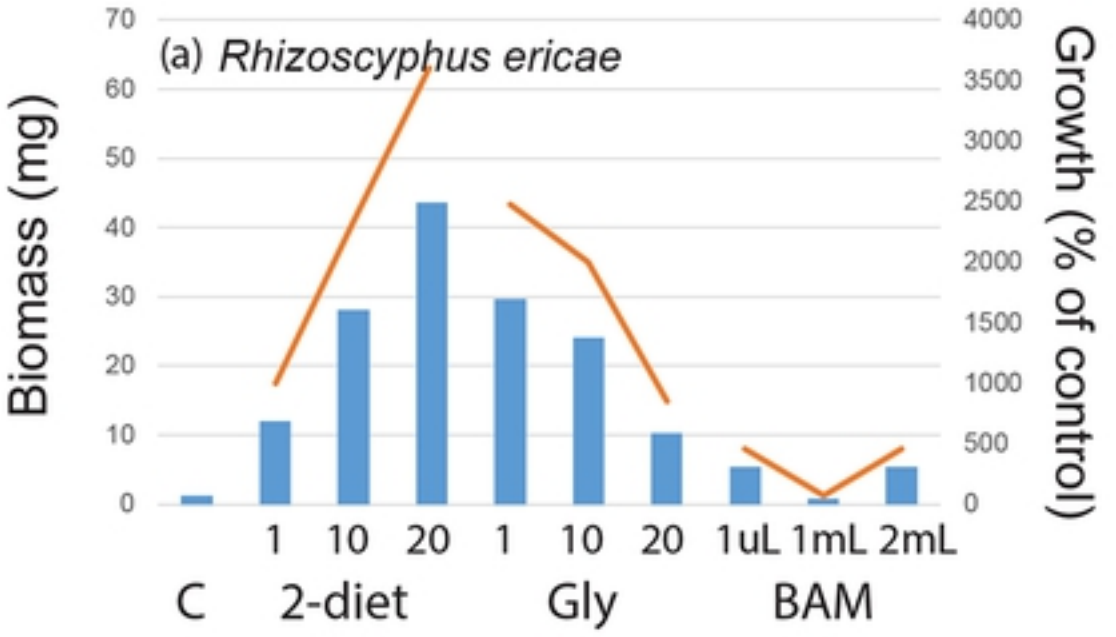
Supporting information

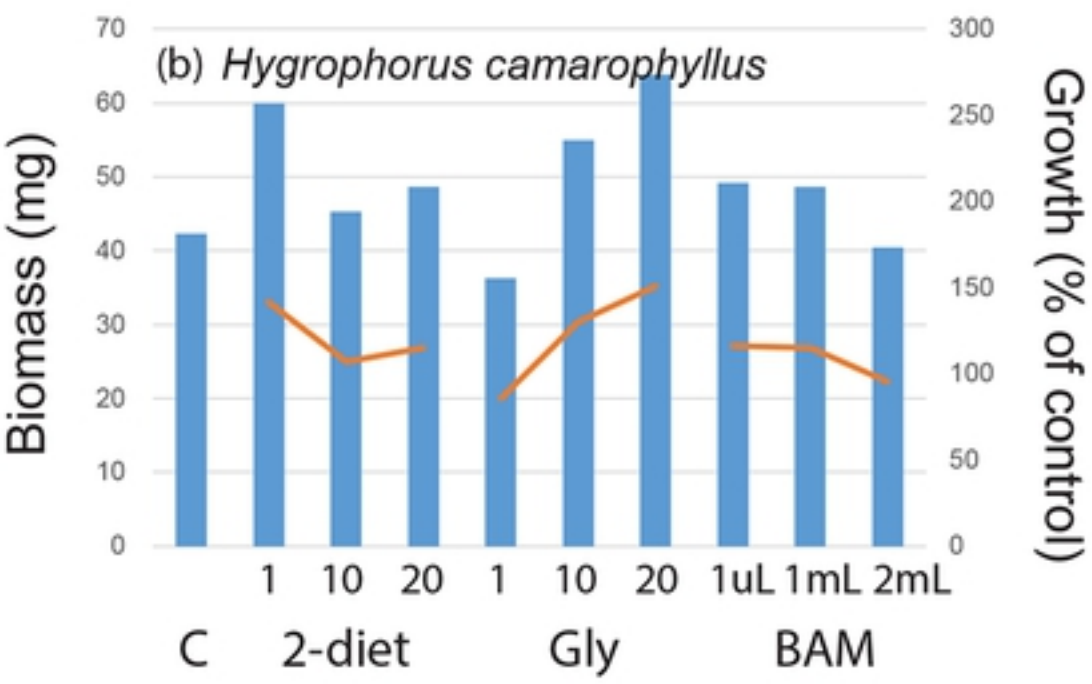
S1 Fig. Experimental scheme for the screening experiment.
A total of 48 fungal isolates where grown in Petri dishes for a total of three weeks in liquid growth media containing individual recalcitrant compounds. Concentrations for the N-containing compounds were 1 g/L, 10 g/L and 20 g/L for 2-diethylaminoethanol and N_3-Trimethyl(2-oxiranyl)methanaminium chloride , and for BAM 1 µL, 1 mL and 2 mL were added from a saturated solution. No substance was added to the growth controls. The recalcitrant N-containing compounds are depicted to the right.

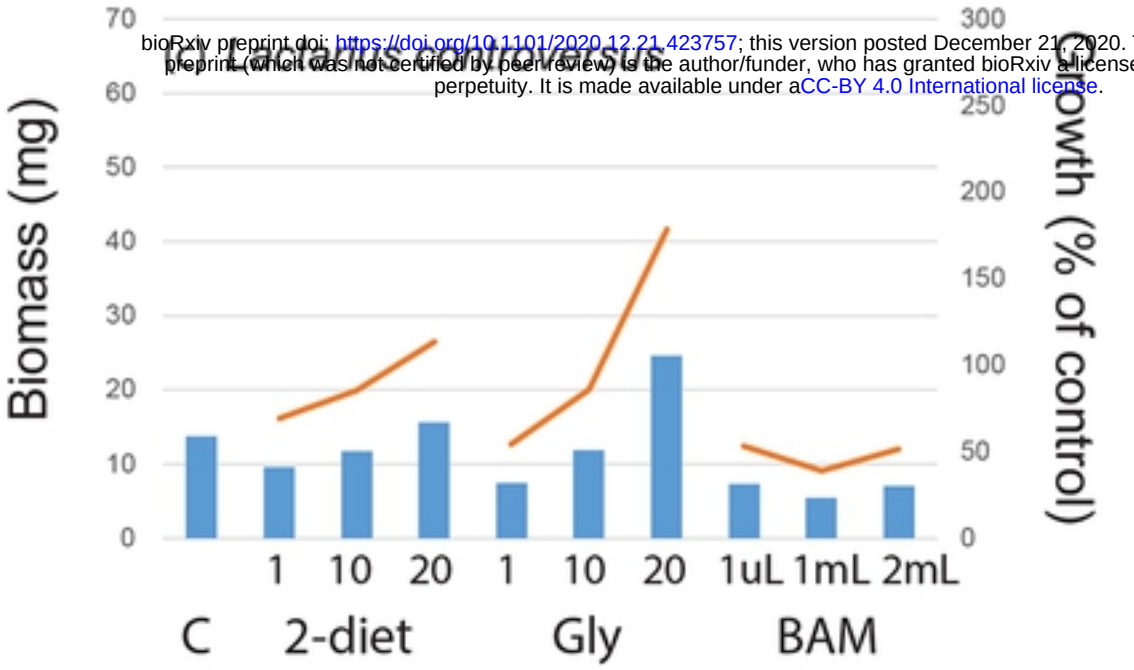
S1 Table. **Mycorrhizal and saprotrophic fungal isolates.**
The fungal isolates were used to screen for survival and growth in liquid media containing recalcitrant amine, amide and ammonium compounds.

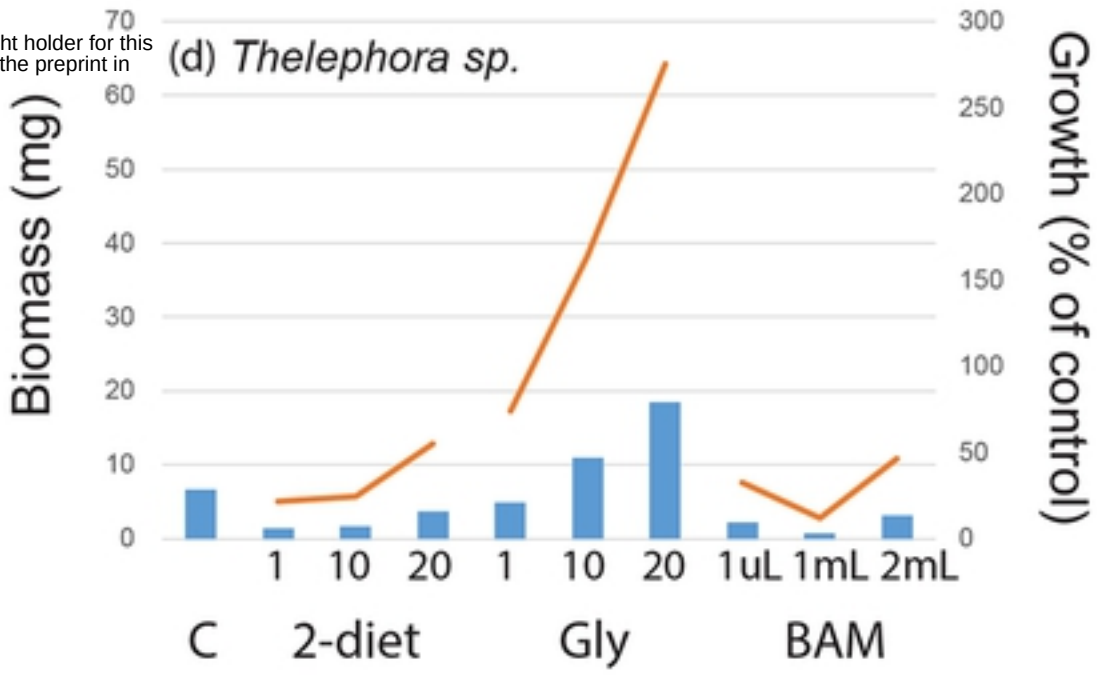
S2 Fig. Experimental scheme for the simulated process water experiment.

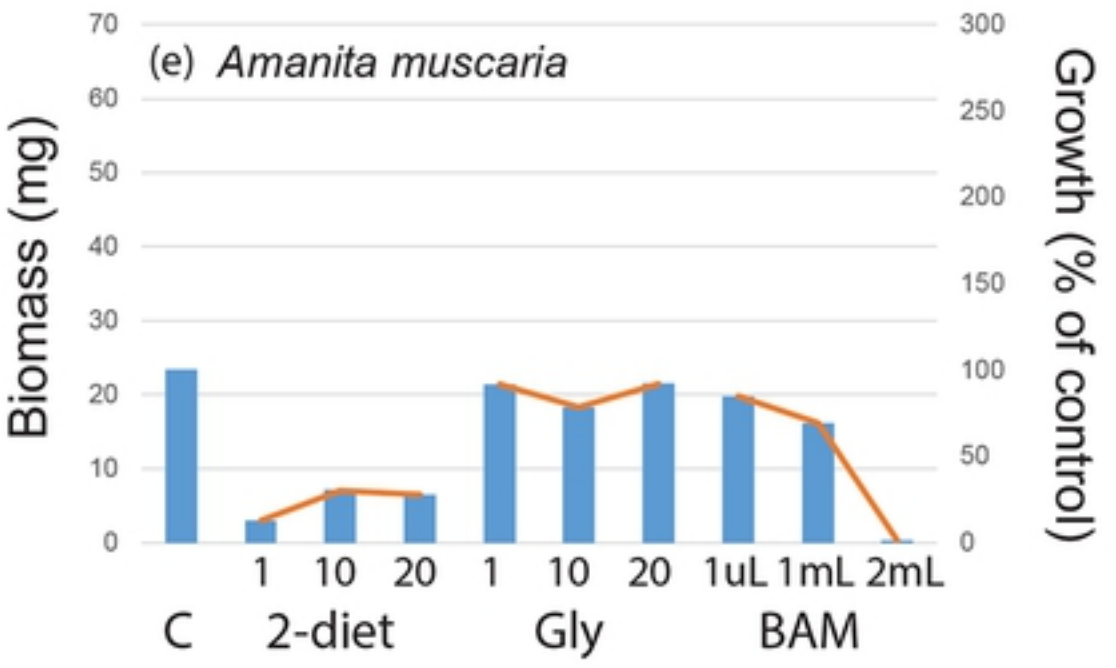
826 Three mycorrhizal fungal species (*Hygrophorus camarophyllus*, *Rhizoscyphus ericae* and
827 *Laccaria laccata*) were grown for a total of four weeks in Erlenmeyer flasks containing a
828 recalcitrant amine/amide mixture. For composition of mixture see Table 1.
829
830 **S2 Table. Biomass production by 48 fungal isolates grown for three weeks in liquid**
831 **culture in the presence of three individual recalcitrant N-containing compounds.**
832
833 **S3 Fig. The fylogenetic distribution of 48 fungal isolates included in the screening**
834 **experiment, plotted against the growth responses.**
835 The growth responses represent the isolates (% of controls) containing the three highest N-
836 containing compounds' concentrations. Concentrations corresponded to 20 g/L for 2-
837 diethylaminoethanol (blue bars) and N_3-Trimethyl(2-oxiranyl)methanaminium chloride
838 (orange bars), and addition of 2 mL saturated BAM solution (red bars). Values over 100%
839 means that fungi grew better with the amines present. The red branches are ECM fungi, the
840 blue saprotrophs and the green ericoid mycorrhiza. The diagram was cut at 500%, missing
841 values and negative values were set to 0%.
842
843 **S4 Fig. Principal component analysis (PCA) showing the overall variation in fungal**
844 **biomass responses to three recalcitrant N-containing compounds.** 48 fungal isolates when
845 grown for three weeks in control treatment with nutrient solution and amine treatments (2-
846 diethylaminoethanol [2-diet], N_3-Trimethyl(2-oxiranyl)methanaminium chloride [gly] and
847 BAM) at three different concentrations. Species differences are visualized by a sample plot
848 with circle size depicting the average biomass response across all treatments. The first three
849 axes together explained 87.5% of the total variation (84181.4).

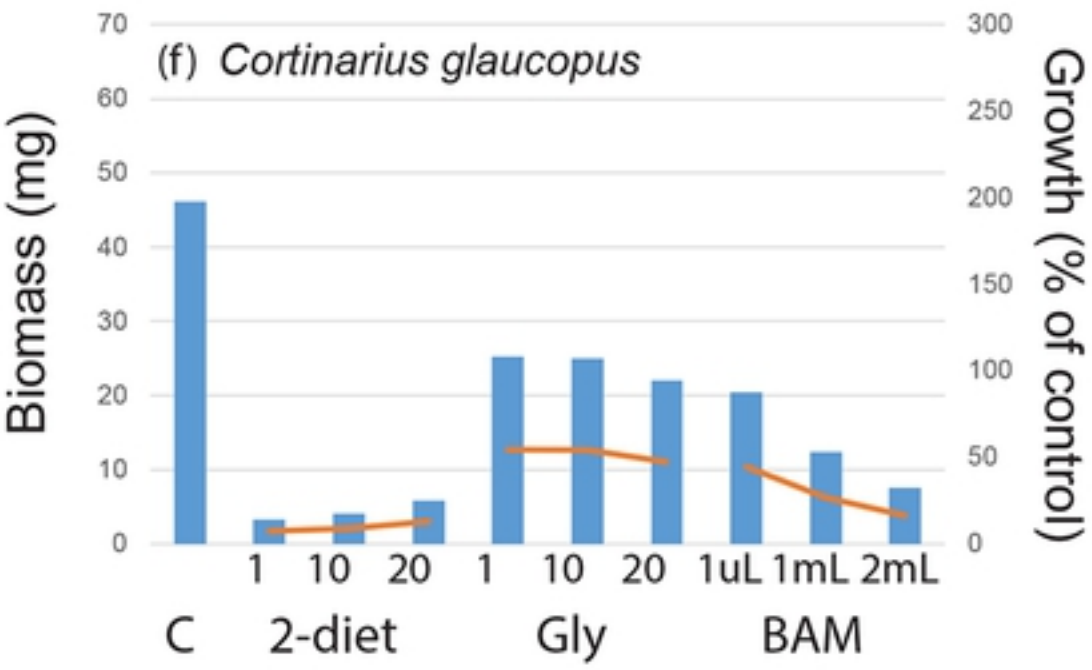


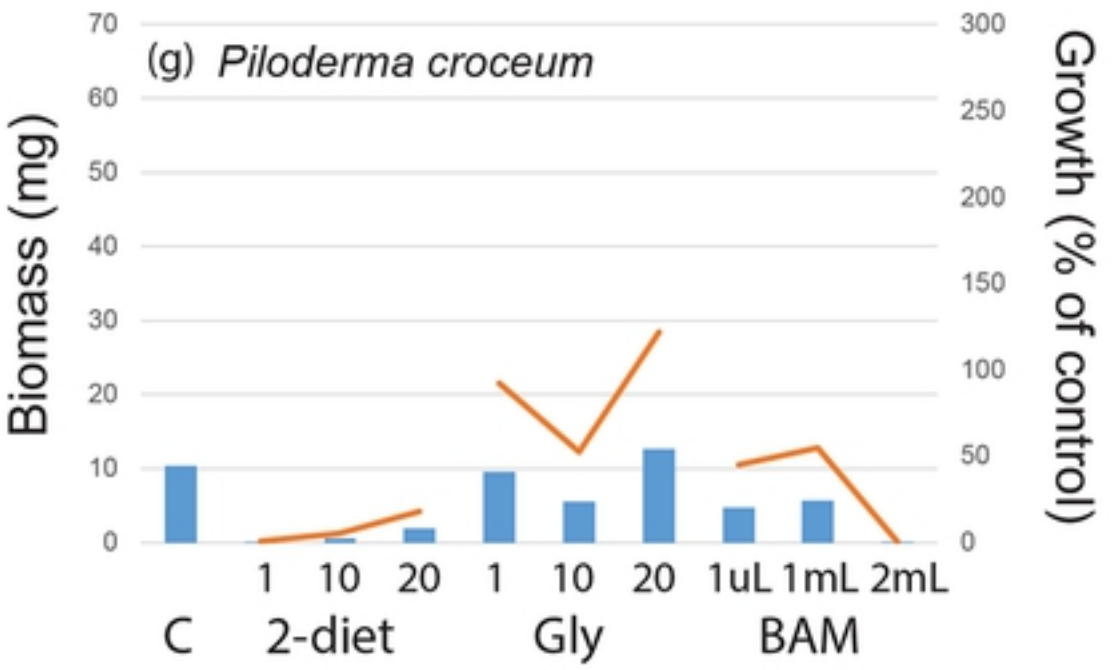


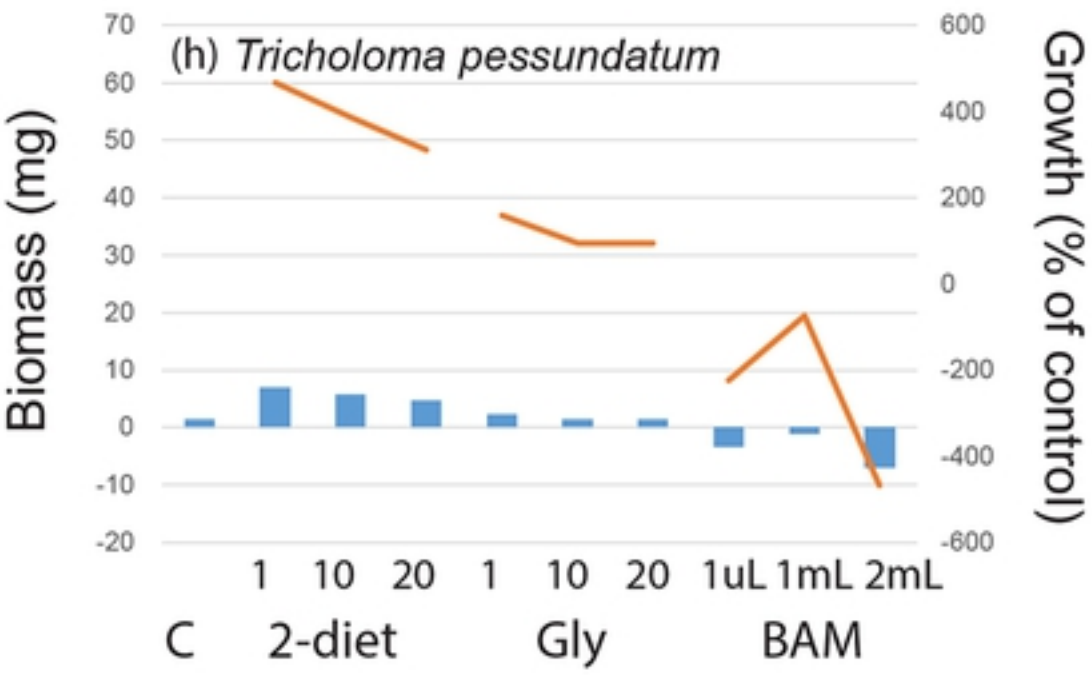












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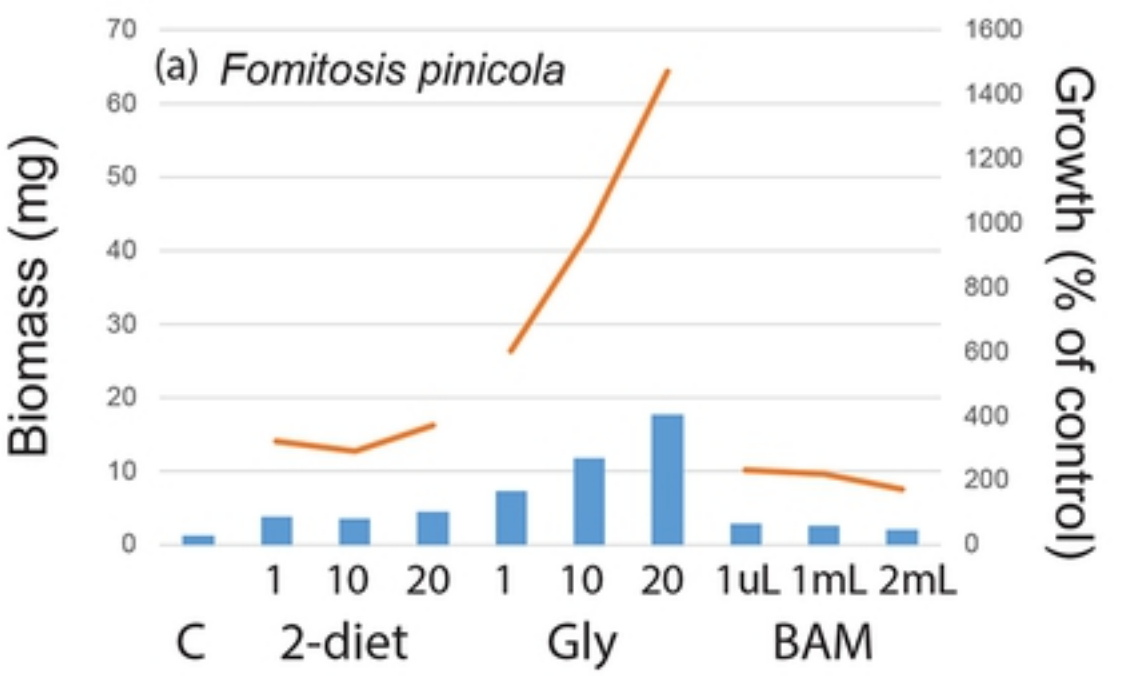

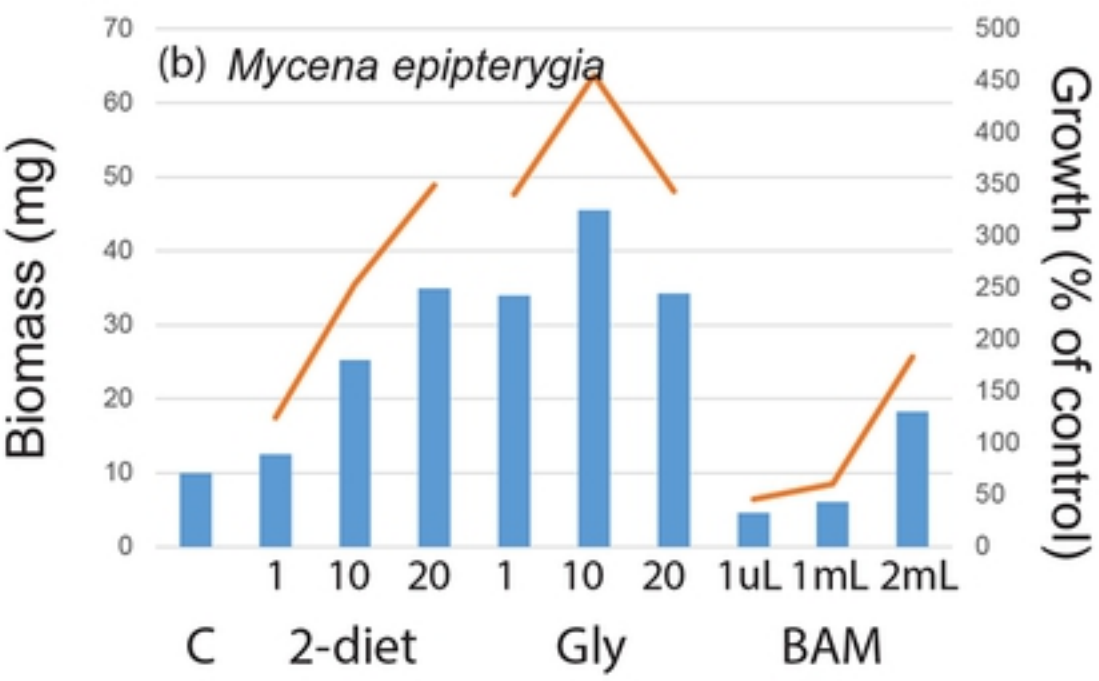

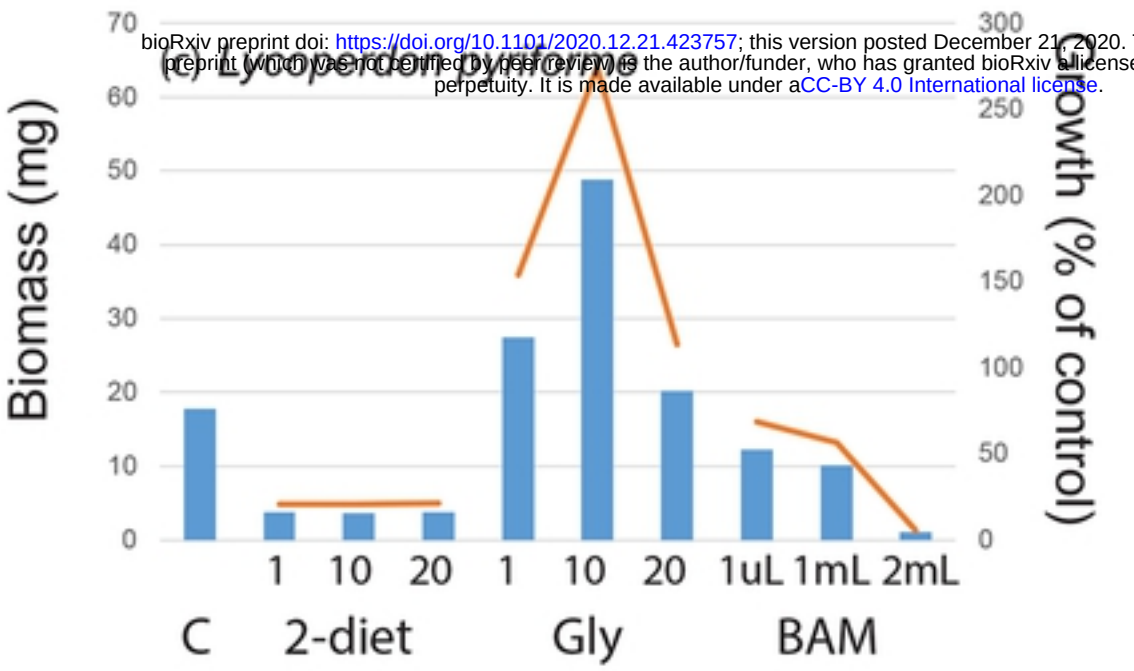

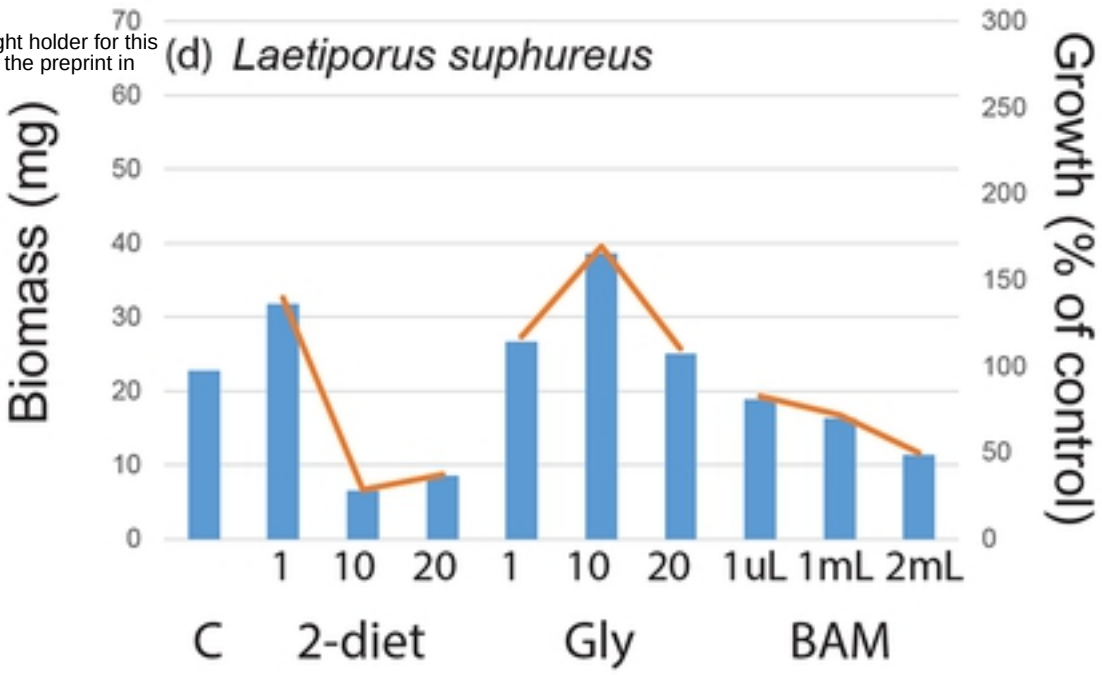

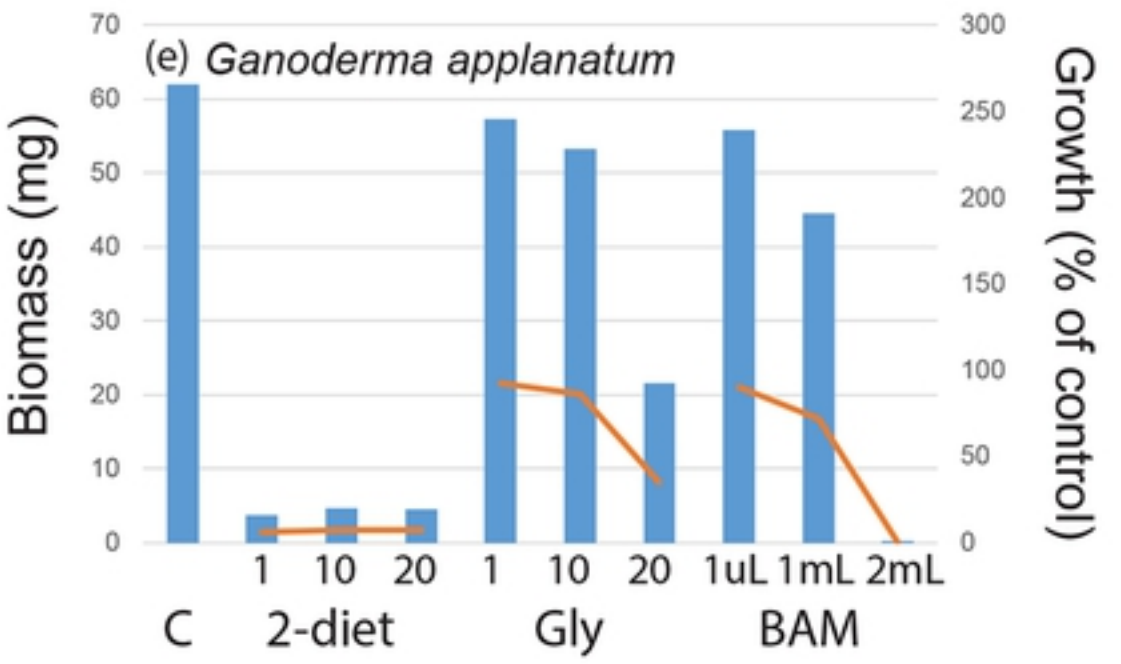

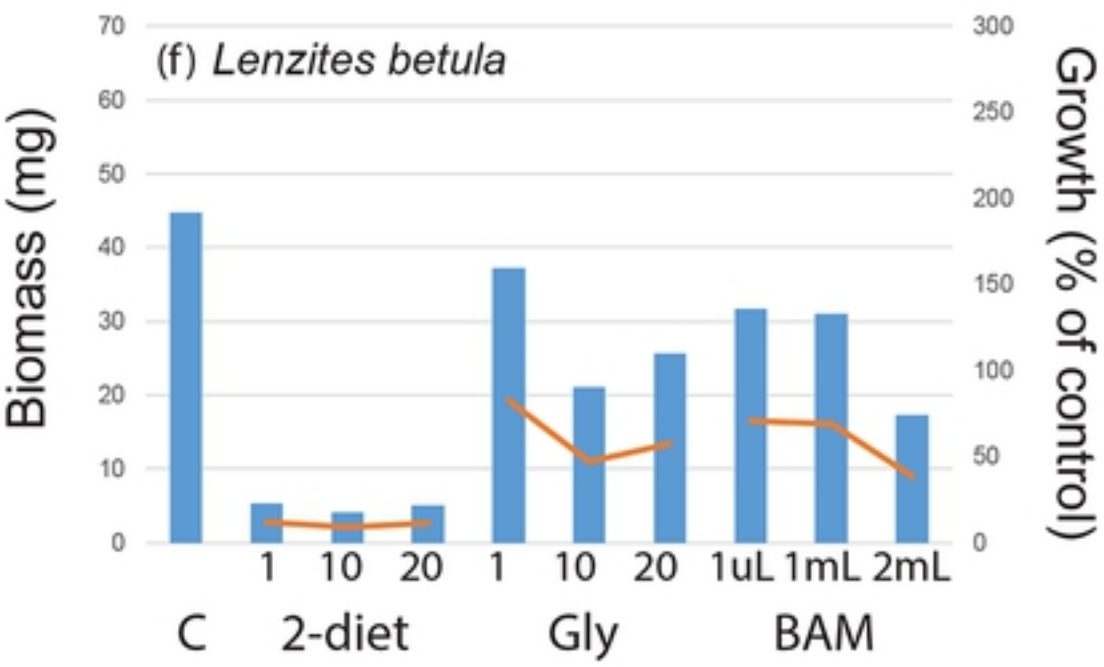

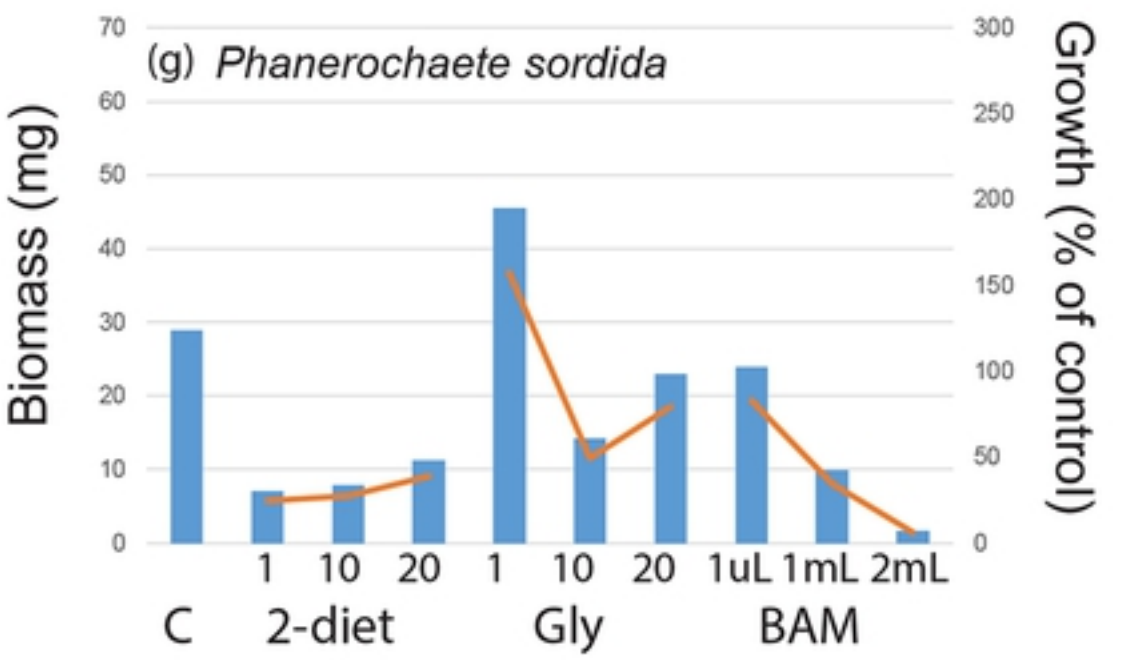

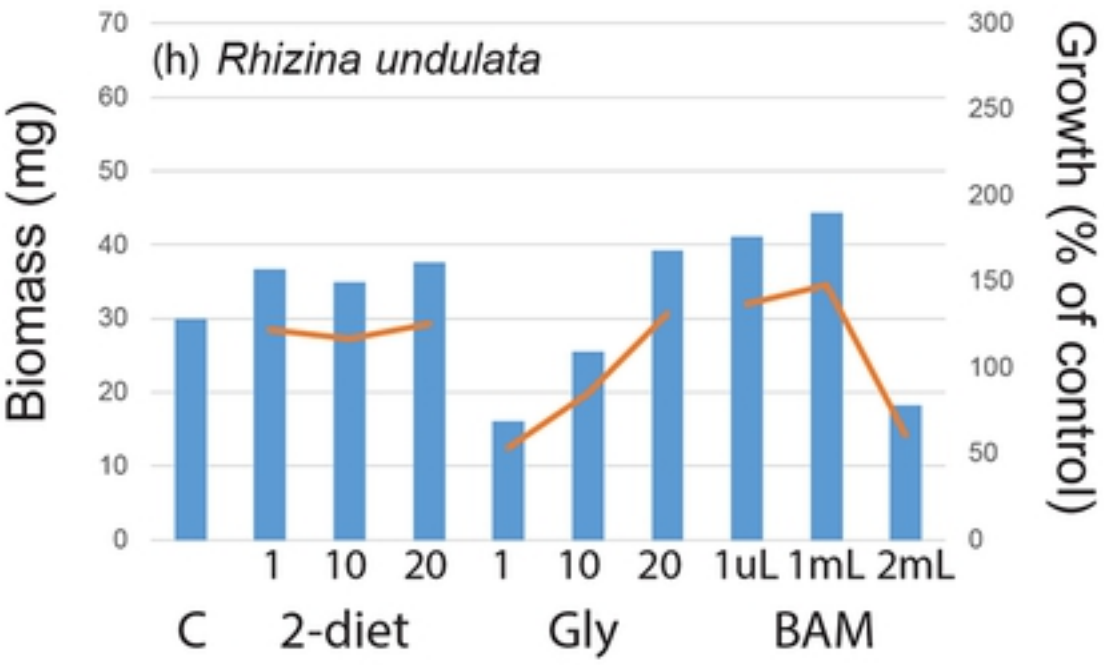

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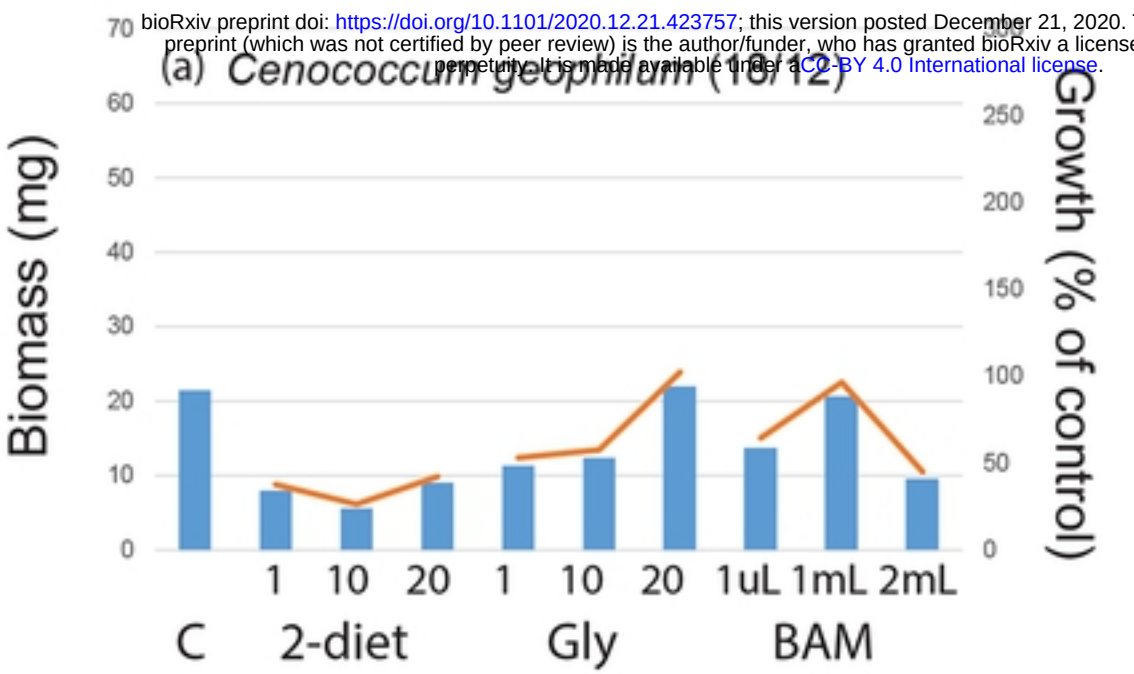

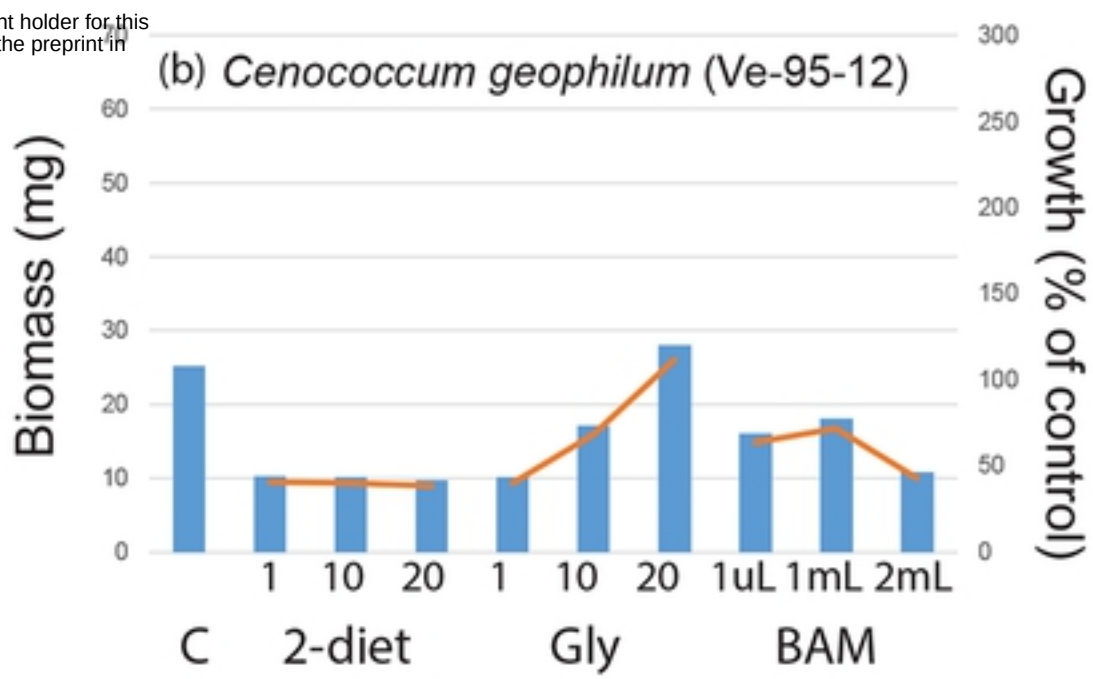

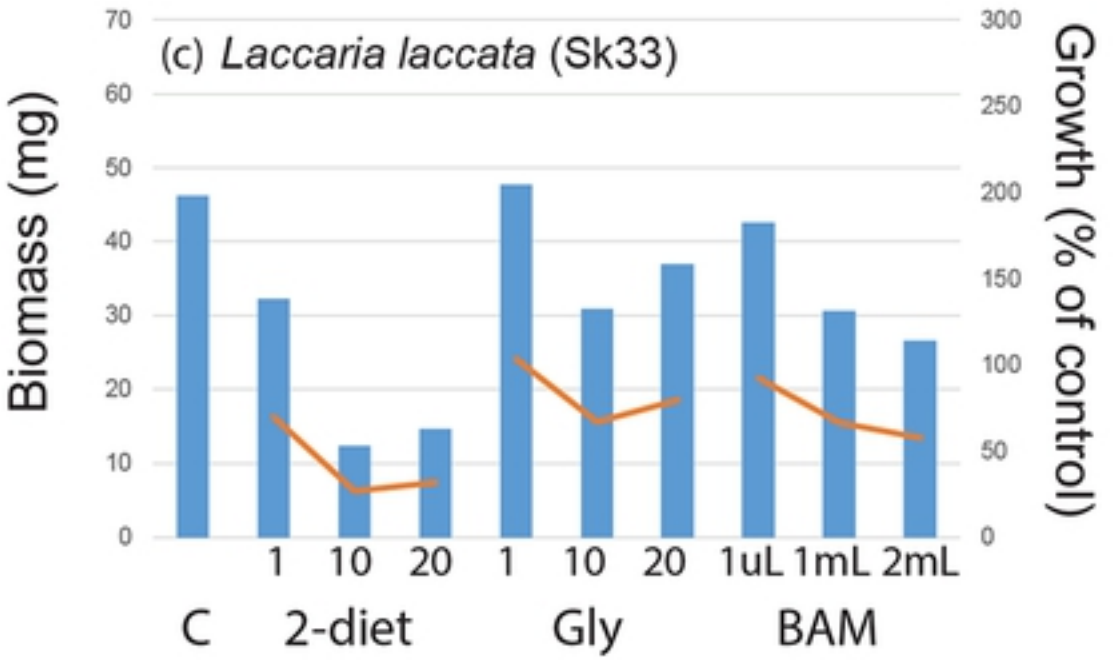

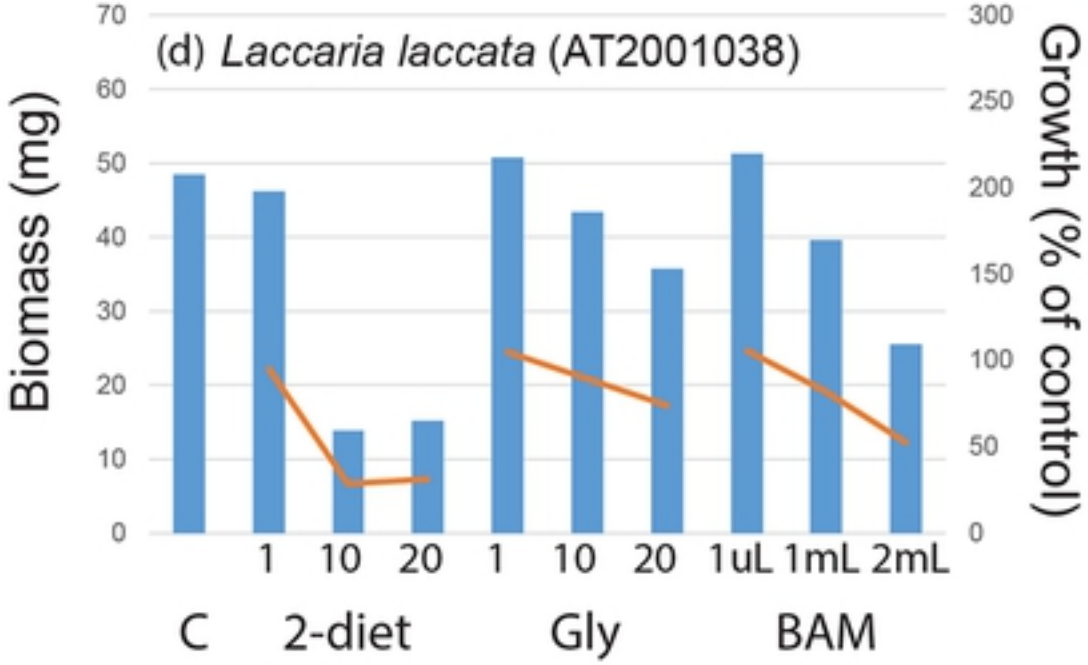

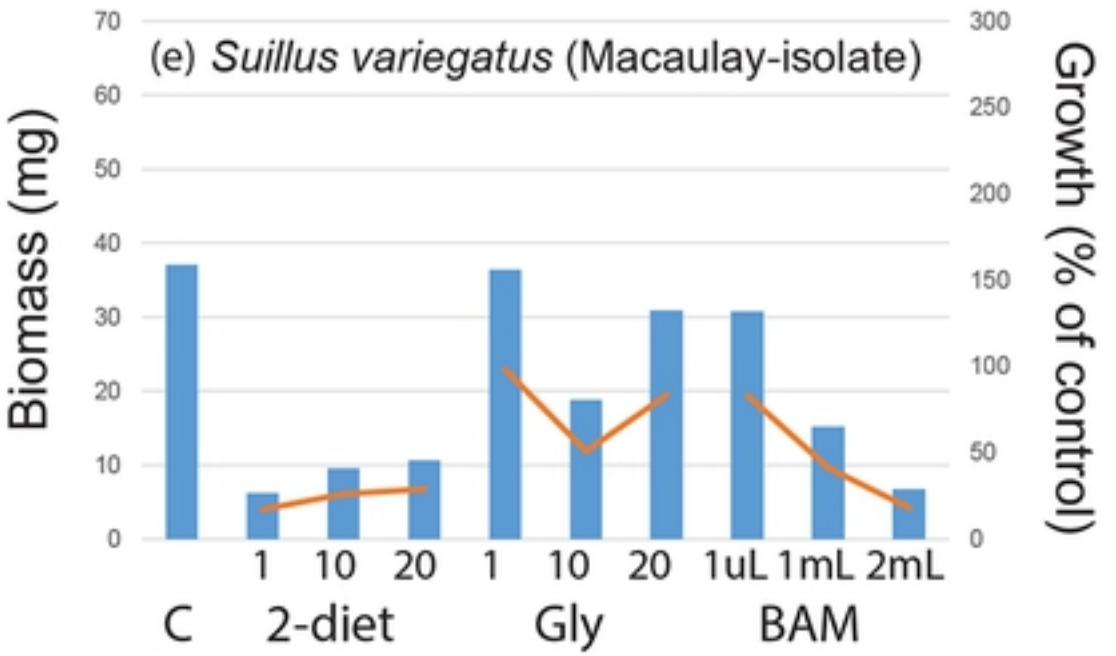

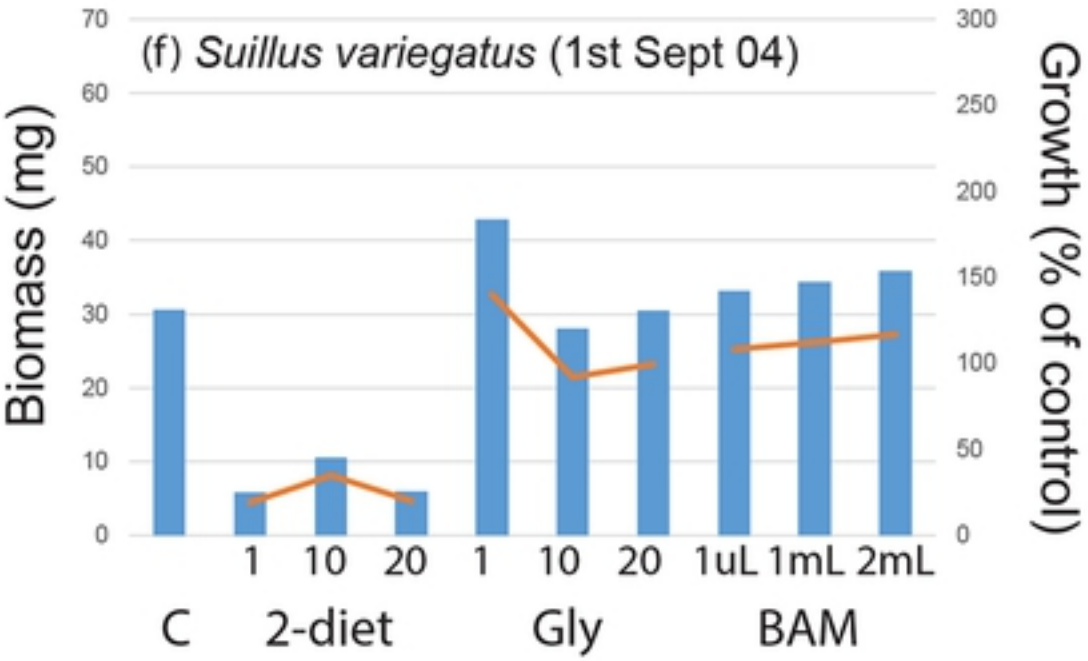

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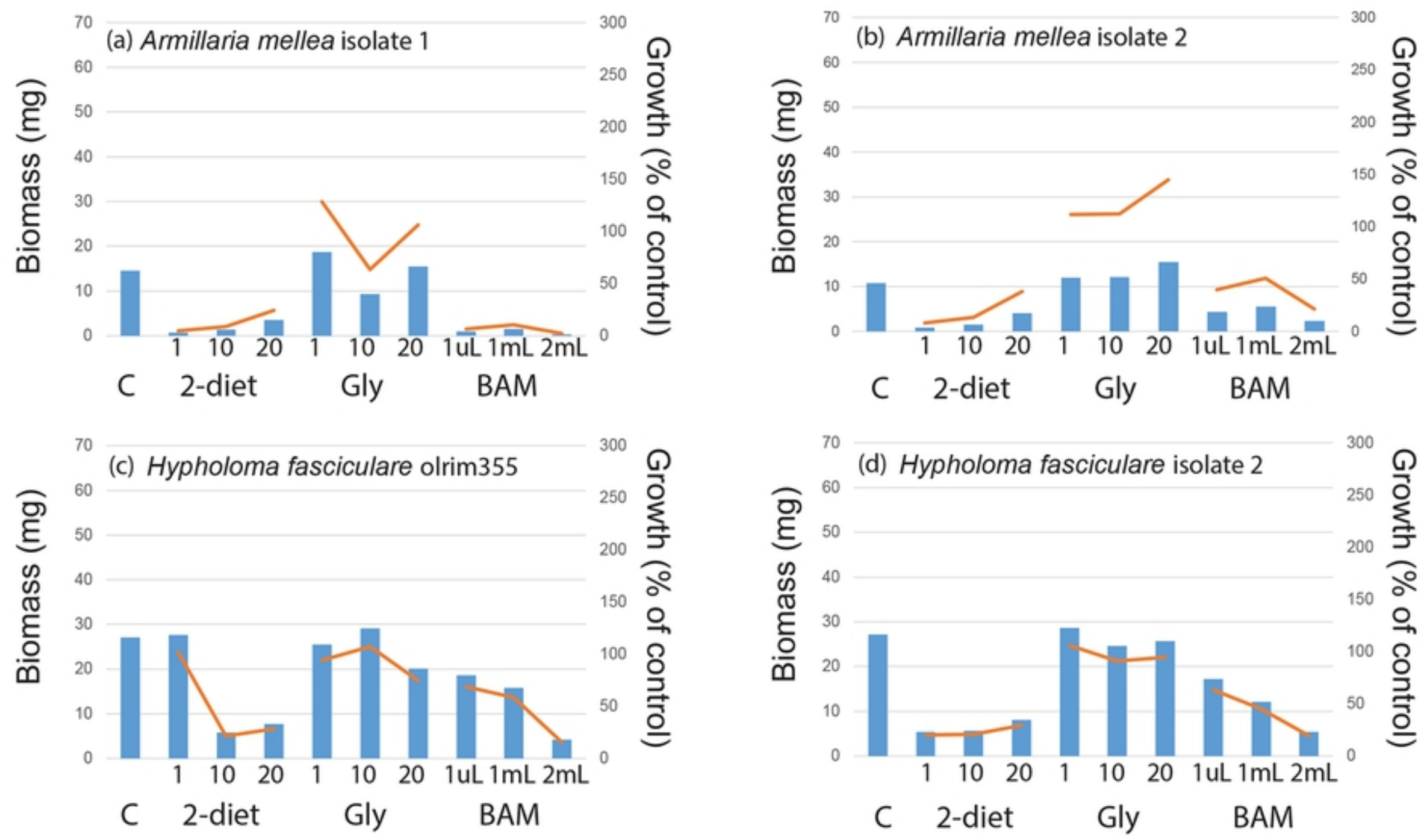

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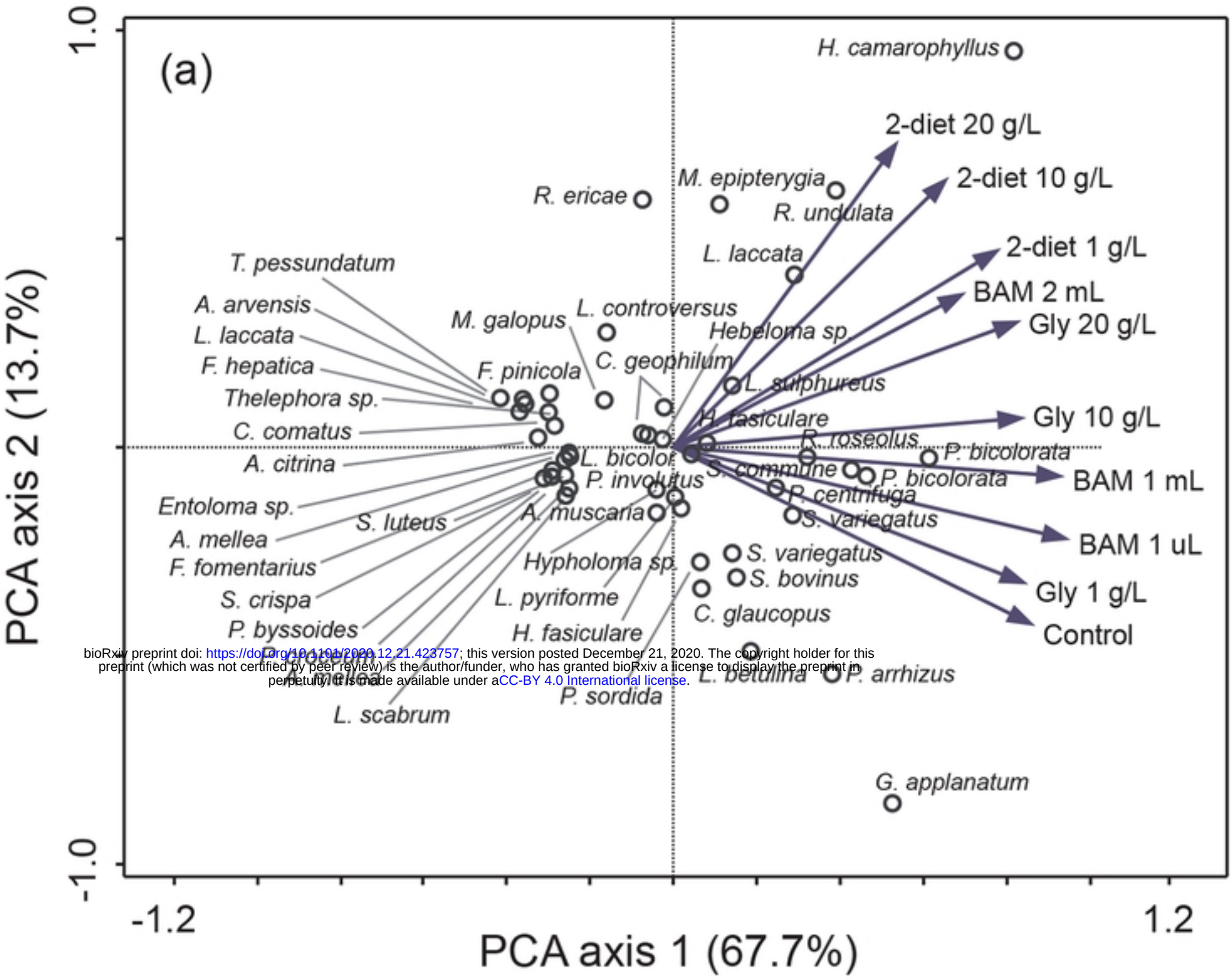

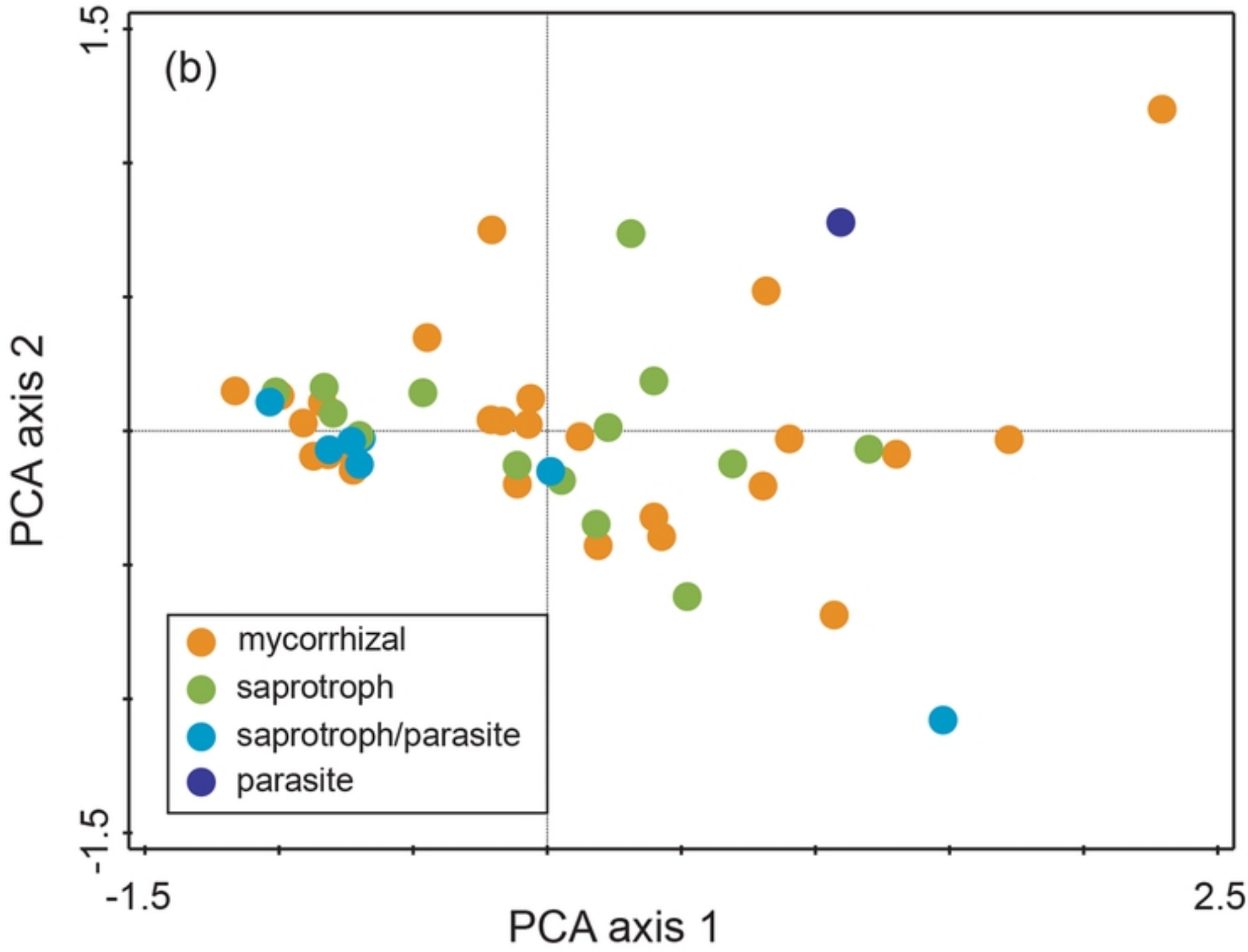

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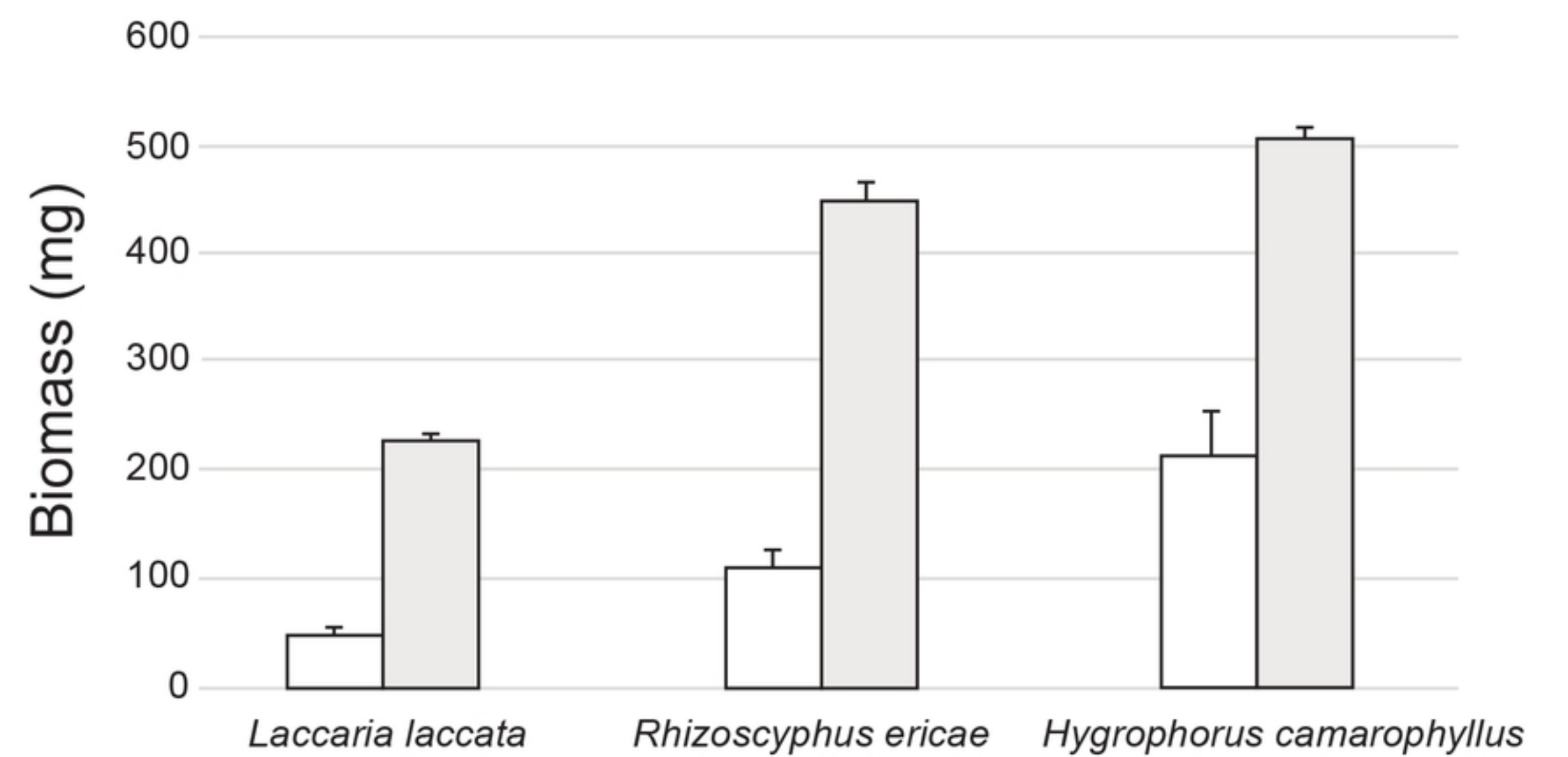

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