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To cite this version:

Joey Allen, Joséphine Leflaive, Charlotte Bringuier, Loïc Ten-Hage, Eric Chauvet, et al.. Allelopathic inhibition of primary producer growth and photosynthesis by aquatic fungi. Fungal Ecology, 2017, 29, pp.133-138. 10.1016/j.funeco.2017.07.001 . hal-01878127

HAL Id: hal-01878127
https://hal.science/hal-01878127v1

Submitted on 20 Sep 2018

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To link to this article: DOI :10.1016/j.funeco.2017.07.001
URL : http://dx.doi.org/10.1016/j.funeco.2017.07.001

To cite this version : Allen, Joey and Leflaive, Joséphine and Bringuier, Charlotte and Ten-Hage, Loïc and Chauvet, Eric and Cornut, Julien and Danger, Michael Allelopathic inhibition of primary producer growth and photosynthesis by aquatic fungi. (2017) Fungal Ecology, 29. 133-138. ISSN 1754-5048

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Short Communication

Allelopathic inhibition of primary producer growth and photosynthesis by aquatic fungi

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Keywords:
Allelopathy
Aquatic hyphomycetes
Chemical interactions
Diatoms
Forest headwater streams

A B S T R A C T

Autochthonous primary production is generally much reduced in forested headwater streams. Several hypotheses have been proposed for explaining this observation, among them, the low light intensity, or the strong constraints exerted by stream current. Allelopathic inhibition of competitors is a common ecological process in aquatic environments. Aquatic hyphomycetes are known to chemically inhibit bacteria and other fungi (including other aquatic hyphomycetes) but a possible allelopathic effect of aquatic hyphomycetes on primary producers has never been tested. The inhibitory effect of twelve aquatic hyphomycete species was tested on three diatom species. Nine aquatic hyphomycete species exhibited anti-diatom activity. Up to 100% diatom growth inhibition was observed. Our study reveals that such allelopathic interactions might be common in streams and probably involve an array of fungal compounds. We propose that the generally reduced primary production observed in forested headwater streams is, among other factors, due to the inhibition of primary producers by allelopathic compounds released by aquatic hyphomycetes.

1. Introduction

Forested headwater streams are generally characterized by low primary production (Fisher and Likens, 1973; Vannote et al., 1980). The low abundance of primary producers (PP) has most often been explained by shading from riparian trees (Vannote et al., 1980; Hill et al., 1995). However, in deciduous forests the incoming solar radiation intensity may be sufficient to sustain PP growth at least from autumn to spring, *i.e.* when there are no leaves on the trees. Alternative hypotheses for slow algal development, at least in some contexts, rely on stream water current (*e.g.* Peterson and Stevenson, 1989). Finally, PP may also be strongly nutrient-limited in these generally oligotrophic streams (Naiman et al., 2000). More recently, competitive exclusion between heterotrophic microbial decomposers and algae has also been proposed to explain the low abundance of PP in forested streams (Danger et al., 2013). Yet, if bacterial decomposers exhibit higher nutrient uptake rates than most PP due to their smaller size (Currie and Kalff, 1984; Danger et al., 2007), this discrepancy is less likely for fungal decomposers because their cells are larger. Indeed, aquatic hyphomycetes, which are among the most important microbial decomposers in headwater streams (Gessner et al., 2007), develop hyphae that are generally larger than most diatom cells occurring in such aquatic ecosystems.

In this study, we propose a supplementary hypothesis that has, to our knowledge, never been tested, for explaining the low amount of primary producers in headwater streams. We hypothesized that, in certain circumstances, primary production could be altered by chemical compounds released from aquatic hyphomycetes. This process, known as allelopathy, is well documented among aquatic microorganisms (Gross, 2003; Allen et al., 2016), but such interactions have never been investigated between aquatic hyphomycetes and PP.

2. Material & methods

The aquatic hyphomycete species used in this study were *Alatospora acuminata* (ALAC), *Anguillospora crassa* (ANCR),

Anguillospora filiformis (ANFI), *Arbusculina moniliformis* (ARMO), *Articulospora tetracladia* (ARTE), *Clavariopsis aquatica* (CLAQ), *Flagellospora curvula* (FLCU), *Heliscus lugdunensis* (HULU), *Tetrachaetum elegans* (THEL), *Tetracladium marchalianum* (TEMA), *Tricladium chaetocladium* (TRCH) and *Tricladium splendens* (TRSP) (see Supplementary Material). All strains were isolated from forested headwater streams in south-western France. For Test 1, aquatic hyphomycetes were grown on 2% malt extract agar in Petri dishes incubated in the dark at 15 °C. For Tests 2 and 3, seven aquatic hyphomycete strains (ALAC, ANCR, ARMO, CLAQ, HULU, THEL and TRCH) were cultured in 50 mL of the mineral medium described in Arce Funck et al. (2015) with five alternative sources of organic carbon: glucose (5 g L^{-1}), cotton-strips, or twelve leaf litter discs (ø 12 mm) of alder (*Alnus glutinosa*), maple (*Acer pseudoplatanus*) or oak (*Quercus robur*). To reduce the release of leachates which could also interact with diatom growth, leaf discs and cotton-strips were autoclaved and rinsed in 40 mL of deionized water prior to being placed in sterilized culture medium. For each carbon source tested, a control was prepared, corresponding to sterile culture medium containing the corresponding carbon source but without fungi. Details of each test performed are given below.

The diatom strains used were *Fistulifera saprophila*, *Nitzschia palea*, and *Gomphonema parvulum*. All diatoms were cultured in axenic conditions with COMBO medium (Kilham et al., 1998) in a temperature-controlled chamber at 18 °C with a 16:8 light:dark cycle and 30 μmol m^{-2} s^{-1} light intensity. All experiments were carried out in these conditions (Fig. 1).

Test 1: To determine whether aquatic hyphomycetes produce allelopathic compounds, 6 mm-diameter cores were cut from the active margin of each aquatic hyphomycete solid culture. Cores were placed in 90 mm-diameter Petri dishes on solidified COMBO medium (1% agar) on which diatoms (4×10^6 cells for *F. saprophila* and *G. parvulum* or 2×10^6 cells for *N. palea*) had been homogeneously spread. Algal densities were based on cell size in order to have comparable biomass as described in Leflaive and Ten-Hage (2011). A sterile malt extract agar core, used as a control for culture medium effect, was placed on each Petri dish. The diatom photosynthetic efficiency of photosystem II (PSII yield), which is strongly linked to their physiological state (Baker, 2008), was measured with a Phyto-PAM fluorometer (Walz, Effeltrich, Germany) next to each core after 3 d for *F. saprophila* and *G. parvulum* or 5 d for *N. palea*.

Test 2: To determine whether inhibitory compounds from leaf litter could be released due to its decomposition by aquatic hyphomycetes, liquid cultures of aquatic hyphomycetes were filtered through sterile Polyethersulfone filters (0.22 μm pore size,

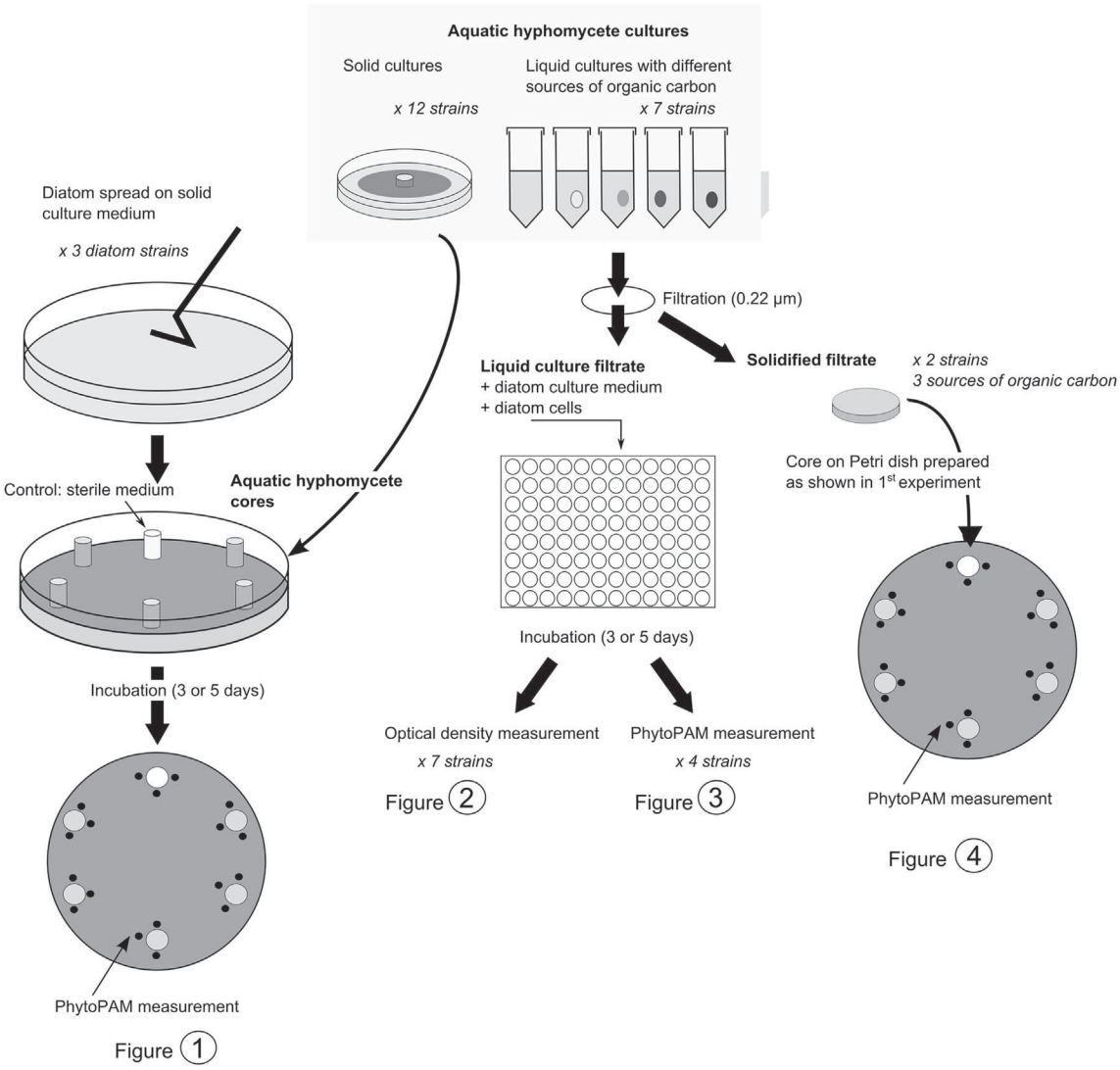

Fig. 1. Schematic representation of the experimental procedures in Test 1 (left panel), Test 2 (medium panel) and Test 3 (right panel).

Nalgene, Rochester, USA). Diatoms were exposed to aquatic hyphomycete filtrates in 96-well plates. Each well was filled with 120 μL of filtrate and 60 μL of twice concentrated COMBO medium. Diatoms were inoculated at densities of either 2×10^5 cell mL^{-1} for *F. saprophila* and *G. parvulum* or 1×10^5 cell mL^{-1} for *N. palea*. After 5 d, optical density (OD, 680 nm, Asys UVM 340, Salzburg, Austria) of diatom cultures exposed to the filtrates of the seven aquatic hyphomycetes and photosynthetic yield of diatoms exposed to four of them (*i.e.* ARMO, CLAQ, THEL and TRCH) were measured.

Test 3: To understand the differences between the results of Test 1 and 2 (hypothesized to be due to different chemical properties of allelochemicals), glucose-, cotton fabric- and alder-grown ARMO and TRCH filtrates were solidified by mixing rapidly 1 mL of filtrate with 0.5 mL of twice concentrated COMBO medium containing 2% low melting point SeaPlaque agarose (Cambrex, Rockland, USA) at 35 °C. Cores of solidified filtrates were used as cores of fungal colonies in Test 1. ARMO and TRCH were used as they have the highest effect in Test 1 and 2 respectively. Only *F. saprophila* was used as a target species.

All experiments were run in triplicate. The effect of each aquatic hyphomycete species was tested against controls using Student *t*-test with log transformed data to fit normal distribution when necessary. A 2-way ANOVA (using carbon source and diatom species as factors) was used to determine the effect of leaf litter alone.

3. Results

Test 1: Of the twelve aquatic hyphomycete species tested, eight had a significant negative effect, on PSII yield for at least one diatom species (Fig. 2). One aquatic hyphomycete (ARMO) inhibited the three diatoms. ARMO had a strong effect on all diatoms (more than 65% of inhibition, $p < 0.001$). Most of the aquatic hyphomycetes specifically inhibited one or two diatoms but not the others (Fig. 2).

Test 2: The carbon source used in liquid culture alone (without aquatic hyphomycetes) had a significant effect ($p < 0.001$) on the measured OD of diatoms. Indeed, it was higher with oak, maple and alder than with glucose (Fig. 3, controls). The most active filtrate ODs were those from TRCH and ANCR, while the filtrates with the lower inhibitory activity were produced by ALAC and ARMO. OD of the three diatoms tested was inhibited by more than 70% by all filtrate types from TRCH except the one produced from cotton fabric. ANCR filtrates also had strong effects, principally on *G. parvulum* and *N. palea*. Stimulatory effects were occasionally observed (Fig. 3). The photosynthetic yield of all diatoms tested was significantly reduced by glucose-, alder-, maple- and oak-grown

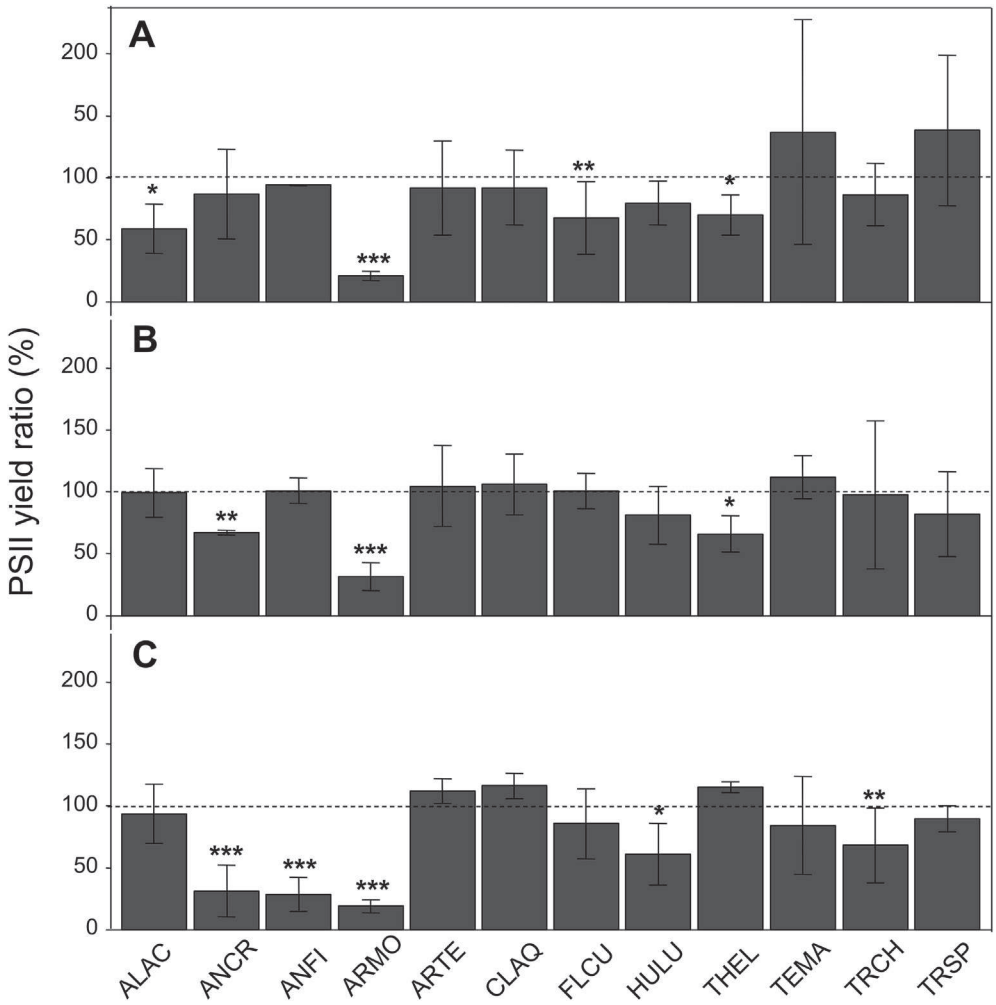

Fig. 2. PSII yield of *F. saprophila* (A), *G. parvulum* (B) and *N. palea* (C) after exposure to twelve aquatic hyphomycete culture cores, measured by PAM fluorimetry and expressed as % of PSII yield in control (Test 1). Mean ± SE ($n = 3$). Dashed line represents control (100%). Asterisk indicates significant difference with the control (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$). ALAC: *Alatospora acuminata*, ANCR: *Anguillospora crassa*, ANFI: *Anguillospora filiformis*, ARMO: *Arbusculina moniliformis*, ARTE: *Articulospora tetracladia*, CLAQ: *Clavariopsis aquatica*, FLCU: *Flagellospora curvula*, HULU: *Heliscus lugdunensis*, THEL: *Tetrachaetum elegans*, TEMA: *Tetracladium marchalianum*, TRCH: *Tricladium chaetocladium*, TRSP: *Tricladium splendens*.

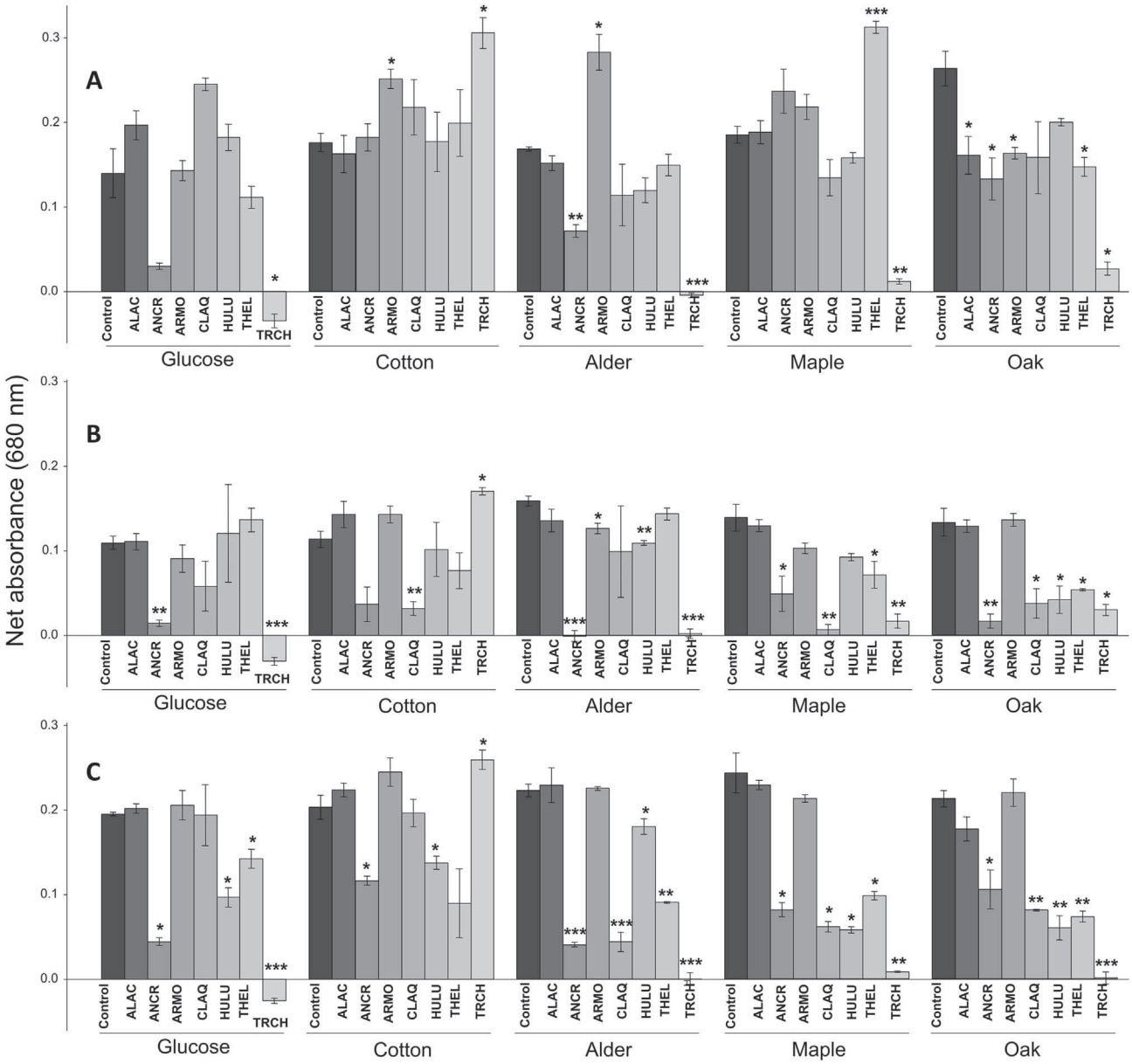

Fig. 3. Net absorbance value (680 nm) of *F. saprophila* (A), *G. parvulum* (B) and *N. palea* (C) after exposure to filtrates of seven aquatic hyphomycete cultures produced from five carbon sources (Test 2). Controls correspond to sterile culture medium containing the corresponding carbon source without fungi. Mean ± SE ($n = 3$). Asterisk indicates significant difference with the control (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

TRCH filtrates but not of cotton fabric-grown TRCH filtrate. Maple- and oak-grown CLAQ filtrates significantly inhibited *G. parvulum* photosynthesis but had no effect on other diatoms. ARMO and THEL filtrates had no effect on diatom photosynthesis (Fig. 4).

Test 3: PSII yield of *F. saprophila* cultured on solid medium and exposed to solidified aquatic hyphomycete filtrates was significantly inhibited by glucose-grown ARMO filtrate (Fig. 5). ARMO filtrate cores had higher inhibitory activity than TRCH filtrate cores ($p = 0.036$).

4. Discussion

While several studies have reported inhibitory effects of aquatic hyphomycetes on bacteria and fungi (*e.g.* Gulis and Stephanovich, 1999; Sati and Arya, 2010) and chemical inhibition among aquatic hyphomycetes (Shearer and Zare-Maivan, 1988; Treton et al., 2004; Ferreira et al., 2010) or between aquatic hyphomycetes and co-occurring bacterial strains (Gulis and Suberkropp, 2003), the allelopathic effect of aquatic hyphomycetes on stream PP has, to our knowledge, never been studied. Our results show that most of the aquatic hyphomycete species tested (8 out of 12 tested in solid cultures) inhibited at least one diatom species. Moreover some aquatic hyphomycete species, ARMO and TRCH particularly, had a strong activity against all the diatoms tested in test 1 and 2, respectively. In their microcosm study of fungal-diatoms interactions on decomposing leaf litter, Danger et al. (2013) showed that algal growth was delayed in the presence of aquatic hyphomycetes, diatom density increasing substantially following a decline in fungal biomass. This result was mainly explained by a release of the competition for nutrients between both functional groups. Considering the results of the present study, the hypothesis that allelopathic interactions may be an important factor that limits

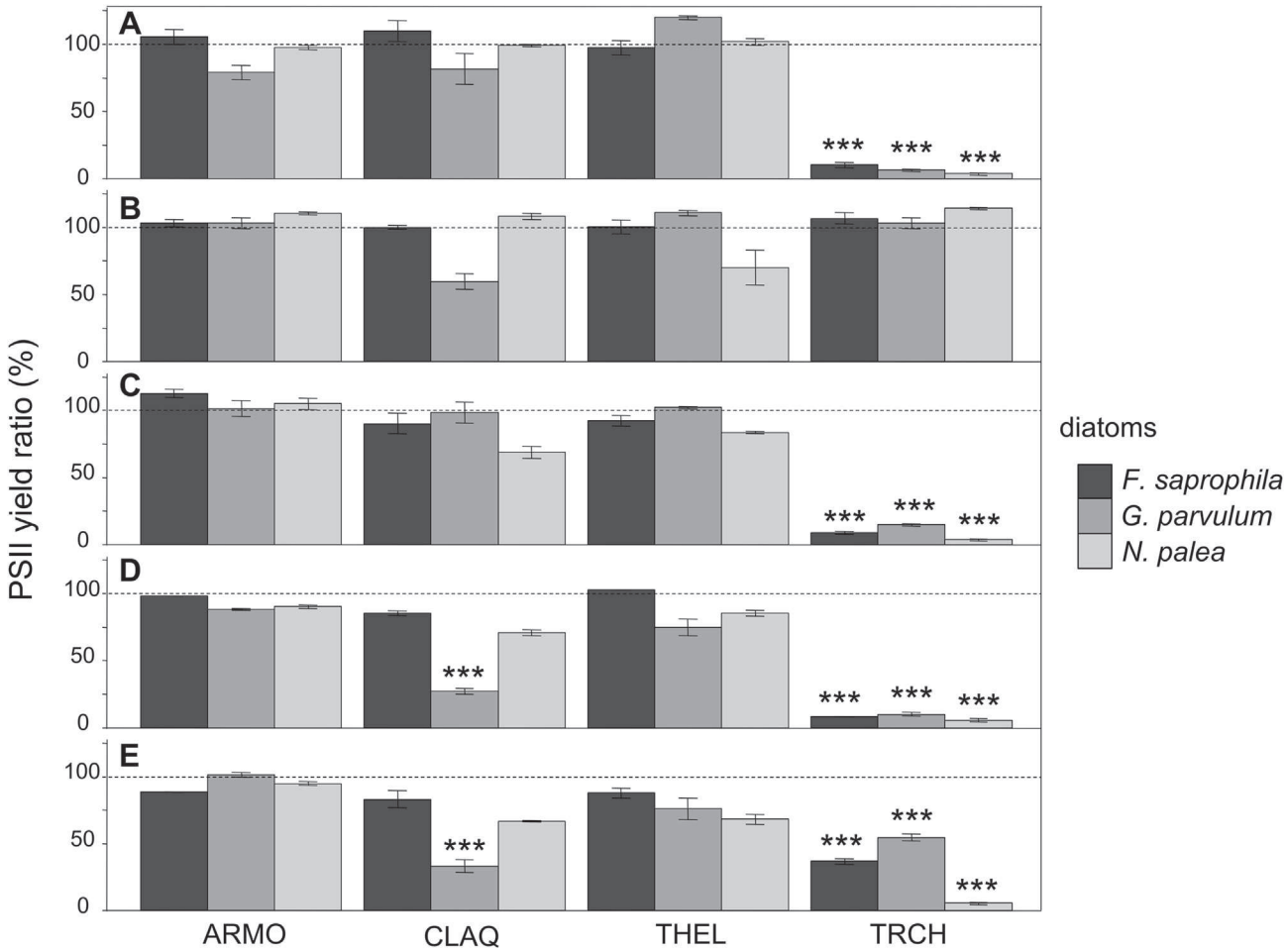

Fig. 4. PSII yield of *F. saprophila* (black), *G. parvulum* (grey) and *N. palea* (light grey) measured by PAM fluorimetry and expressed as % of PSII yield in control, after exposure to filtrates of four aquatic hyphomycete cultures produced from five carbon sources (Test 2): Glucose (A), cotton fabric (B), and leaf litter of alder (C), maple (D) and oak (E). Controls correspond to sterile culture medium containing the corresponding carbon source without fungi. Dashed line represents control (100%). Mean ± SE ($n = 3$). Asterisk indicates significant difference with the control (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Fig. 5. PSII yield of *F. saprophila* after exposure to solidified aquatic hyphomycete culture filtrates, measured by PAM fluorimetry and expressed as % of PSII yield in control (Test 3). Dashed line represents control (100%). Mean ± SE ($n = 3$). Asterisk indicates significant difference with the control (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

PP in headwater streams seems to be supported, and could also partly explain the results of Danger et al. (2013). Indeed, the inhibition of PP could help fungi in reducing the competition for limiting nutrients with PP in the generally oligotrophic forested headwater streams. We cannot exclude that the observed inhibition could also be due to parallel allelopathy (Sinkkonen, 2006), *i.e.* a side effect of allelochemicals produced to inhibit other organisms, *e.g.* other aquatic microbial decomposers.

The activity of the aquatic hyphomycetes tested varied among target diatom species. While ALAC and FLCU only inhibited *F. saprophila*, ANCR only inhibited the two other diatoms, suggesting the involvement of different compounds. Similarly, the inhibitory activity depended on whether diatoms were in liquid or solid cultures, but this varied among species. For instance, ARMO allelopathic compounds inhibited diatoms in solid cultures, when diatoms were exposed to solid culture cores or to solidified filtrates,

while TRCH inhibited diatoms in liquid cultures, but solidified filtrates had no effect. This might be due to different chemical properties of compounds affecting their diffusion within the agar, suggesting that various compounds with distinct chemical properties are involved.

Leaf litter leachates, especially labile phenolic compounds, are considered as potential inhibitors of aquatic PP (Tsuda et al., 2005; Bährs et al., 2012). We hypothesized that additional inhibitory compounds originating from the leaf litter could also be released during microbial decomposition. To test this hypothesis, the leaf discs were initially autoclaved in deionized water to remove labile compounds. As a result, no inhibitory effect of leaf litter alone was observed, suggesting that most inhibitory compounds were lost with the leachates (Danger et al., 2013). The slight stimulation effect of leaf litter on algal growth may be due to a supply of growth factors by leaf litter. No leaf litter effects were observed: glucose-grown aquatic hyphomycetes inhibited diatoms as much as leaf litter-grown ones. While fungal biomass was not estimated, the lower effect of cotton fabric-grown aquatic hyphomycetes is likely due to their lower and delayed growth on this substratum (Cornut et al., unpublished results).

Our study highlights that aquatic hyphomycetes commonly produce diverse allelochemicals that inhibit PP. The aquatic hyphomycete and diatom species used are quite common in streams, and tests performed were not likely to overestimate the effect of aquatic hyphomycetes therefore this inhibitory effect could occur in streams. Whether, and in which proportion, these compounds participate to reduce PP in forested headwater streams remains to be determined. Our study was performed on single species, but allelopathic interactions in diversified communities, with several fungal and algal species interacting, might be more complex. Nevertheless, such a process certainly adds to the hypotheses currently proposed to explain the reduced development of algal biofilms in forested headwater streams.

Acknowledgments

This work was supported to JA by a grant from the Scientific Council of the University Paul Sabatier. This work is part of the ANR *FunctionalStream* to EC (ANR-14-CE01-0009-01). JC gratefully acknowledges funding by the Portuguese Foundation for Science and Technology (FCT-SFRH/BPD/108779/2015). We also thank three anonymous reviewers and Professor Lynne Boddy for their constructive comments.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funeco.2017.07.001.

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