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A comparative study of ecophysiological traits of the invasive species *Lemna minuta* Kunth and the native *Lemna minor* Linnaeus.

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Declaration

Abstract

Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Simona Paolacci

Abstract

Invasive aquatic plants are a major threat to biodiversity and a considerable amount of money is spent on their management and control. In this study the invasiveness of the alien freshwater plant *Lemna minuta* was investigated. The performance of this species under different environmental conditions was tested and compared with the performance of the native species *Lemna minor*. Physiological and morphological parameters were used to quantify the performance of the two species and interpret the growth strategies adopted. In fully controlled conditions, *L. minuta* and *L. minor* were grown using different nutrient concentrations, different light intensities and in the presence of several physical and chemical stressors. In parallel, the presence and abundance of *L. minuta*, and *L. minor* was monitored for two years in natural freshwater ponds where the two species occur spontaneously. The observation that the water fern *Azolla filiculoides* co-occurs very often with Lemnaceae, led us to include it in monitoring of growth performance. Also investigated was the correlation between the presence and abundance of the three species and environmental factors of the waterbody such as water chemistry and canopy shade. A one year long outdoor experiment was carried out in order to investigate the performance of the three species throughout the seasons. The laboratory experiments showed that *L. minuta* has generally a higher growth rate than *L. minor*. *L. minuta* outgrows the native species at all the nitrate concentrations tested and at all the Ca/Mg ratios and concentrations tested. Only at very low concentrations of phosphate did *L. minor* outgrow the alien species. When exposed to stressors, *L. minor* tolerated low temperatures best, while *L. minuta* tolerated best high aluminium and copper concentrations, high temperatures and drought stress. It was concluded that the commonly accepted believe that competitive species grow faster, but are less able to tolerate stress, is not always correct. The outdoor experiment showed that, in the summer months, *A. filiculoides* and *L. minor* outgrow the native *L. minor*, but the native species is the first one to re-start its growth after the winter in accordance with the tolerance to low temperatures observed under laboratory conditions. The outdoor experiment showed that the fastest growing and most competitive species is *A. filiculoides,* followed by *L. minuta*. Yet, field monitoring showed that these two species were not able to exclude the native species in the wild. Survival of winter conditions and/or re-colonisation, together with the invasibility, need to be considered to explain this apparent discrepancy.

Chapter 1

General introduction

An alien species is a species occurring outside its natural distribution zone. A species is considered alien if it did not reached the new location by its own means, i.e. human activity is involved in dispersing and introducing (accidentally or intentionally) the species concerned in the new location (Shine *et al.*, 2000). Non-native species can be introduced into new locations for several reasons. Often, the intentional introduction of an alien species into a new location is for reasons of economic interest. Plants or animals can be intentionally introduced for their agricultural value, benefitting arable farming, horticulture, forestry and/or aquaculture industries. In many cases alien species are introduced for biological control. For example, invasive cane toads (*Bufo marinus* Linnaeus) were brought to Australia, from Hawaii, in 1935, in an attempt to reduce beetle-caused damage to commercial sugar-cane crops (Dubey and Shine, 2008). Some fast growing plants (e.g. *Alliaria petiolata* Marschall von Bieberstein in Canada, *Pueraria montana* Loureiro in US) were introduced in order to control erosion (Cavers *et al.*, 1979; Blaustein, 2001). In other cases the introduction is unintentional. For example, several species are known to have spread by “hitchhiking” on commercial ships (Bhatt *et al*., 2011). A positive correlation was found between merchandise imports and biological invasions (Hulme, 2009). However, not all alien species become invasive. Indeed, many alien crop plants, including tomatoes and potatoes, are not considered invasive despite being widespread in Europe and other areas outside their natural distribution zone. Williamson (1993) estimated that only 10% of introduced species become established (with a self-sustaining population, naturalized) and of these established species a further 10% become invasive (10:10 rule). The International Union for Conservation of Nature (IUCN) defined invasive species as species which have become established in natural or semi-natural ecosystems or habitats, are agents of change, and threaten biological diversity (Shine *et al*., 2000).

Biological invasions depend on both the invasiveness of the species (ability of an organism to invade a habitat) and on the invasibility of the habitat (susceptibility of a habitat to be invaded) (Alpert, 2000). Characteristics that are often found in invasive species are wide native distribution range, rapid dispersal (Perrins *et al*. 1993; Rejmánek 1995; Goodwin *et al.* 1999), and high phenotypic plasticity (Gray, 1986; Williams *et al.* 1995). Grotkopp and Rejmánek (2007) found that high seedling growth rates and large specific leaf area are traits common in invasive species of woody angiosperms. A comparison between relative growth rate of invasive and non-invasive alien species confirmed that the former tend to grow faster (James and Renovski, 2007). A study carried out in Czech Republic revealed that among the invasive species present in that country 60% were clonal, suggesting that clonal growth may be associated with invasiveness (Pyšek, 1997). However, the traits associated with invasiveness do not always allow prediction of an invasion, probably because the invasiveness of a species depends on the interaction between its characteristics and the characteristics of the new habitats (Alpert, 2000). Habitat characteristics linked with high invasibility are low biodiversity, disturbance events such as fire and grazing and high resource availability. The presence of stressors is often associated with low invasibility of the habitat (Alpert, 2000). According to the theory of fluctuating resources elaborated by Davis *et al.*, (2000), a plant community becomes more susceptible to invasion whenever there is an increase in the amount of unused resources. The amount of unused resources is inversely correlated with the intensity of competition (Davis *et al.*, 1998), and such a lack of competition with native species is an advantage for alien species. If the high availability of resources coincides with availability of invading propagules, the alien species is in a strong position to successfully colonize the habitat (Alpert, 2000).

Freshwater, alien invasive plants

The 10:10 rule on establishment and invasiveness (Williamson, 1993) applies well, for example, to British angiosperms and Pinaceae, pasture plants in Australia's Northern Territory and also several plant and animal species in the US. In other cases, such as British edible crop plants, Hawaiian birds, and insects released for biological control, this rule does not accurately predict invasiveness (Williamson and Fitter, 1996). Another exception observed more recently is represented by invasive aquatic species in Europe. García-Berthou *et al.* (2005) used the Food and Agriculture Organization's Database of Invasive Aquatic Species to study invasion rates in Europe and found that of the 123 aquatic species introduced into six European countries the average percentage established is 63%. This makes aquatic habitats much more vulnerable to invasions. In particular, in freshwater systems, the introduction of invasive species adds to the physical and chemical impacts already exerted by humans. Several authors found that alien species have relatively high chances of becoming invasive in freshwater habitats as this environment is often already modified and degraded due to human activities (Bunn and Arthington, 2002; Koehn, 2004). In Europe there are at least 96 alien aquatic plants, most of which are native to Northern America, followed by Asia and Southern America (Hussner, 2012). The most widespread species is *Elodea canadensis* Michaux, reported in 41 European countries, followed by *Azolla filiculoides* Lamarck (occurring in 25 countries in Europe), *Vallisneria spiralis* Linnaeus (22 countries) and *Elodea nuttallii* Planchon (20 countries). Eighteen out of the 96 alien species reported are considered invasive or potentially invasive within the EPPO region by the European and Mediterranean Plant Protection Organization (EPPO, http://www.eppo.org). Brunel (2009) analysed the route of introduction of alien aquatic plants and from his study it emerged that the ornamental plant trade is the main factor responsible for the introduction of these species. Examples of species that have been increasing in their invasiveness in European freshwaters are *Eichhornia crassipes* Martius, *Hydrocotyle ranunculoides* Linnaeus and *Myriophyllum aquaticum* Velloso. Also species belonging to the genus *Elodea* and *Ludwigia* are considered very invasive in Europe (Sheppard *et al*., 2006; Hussner, 2009). The main problem associated with aquatic invasive plants is their high biomass production. The high amount of biomass produced, can lead to oxygen deficiency during the night and change the hydrochemistry of the waterbody (Laranjeira and Nadais, 2008). Moreover, by reducing light penetration in the water column, invasive alien species can inhibit the growth of the native vegetation and alter the food web structure (Stiers *et al*., 2011). The increase of biomass is associated with the increase of primary production that can lead to an alteration of the biogeochemical cycle in the waterbody (Wigand *et al*., 1997). When an alien invasive species replaces a native species, its nutrient requirements can be different and the nutrients cycle can be altered (Angeloni *et al*., 2006). Often the natural enemies of alien species, that control the population size in the original distribution area are missing in the new habitat and the species can increase its population without limitations (Lowe *et al*., 2000). There are also cases of alien species (e.g. *Nasturtium officinale* Brown) that are able to produce chemicals that are toxic to the native population of herbivores (Newman *et al*., 1996). Hydrogeological issues can also be caused by the presence of invasive alien macrophytes as these species can hinder run-off in rivers. Finally, invasive species may hamper recreational use of waters (e.g. boating, swimming, fishing) (Pot, 2002).

Aquatic plant communities are usually less complex (i.e. lower species diversity) than terrestrial communities and this makes them good study systems to investigate the impact that alien species have on the native community. The present study focused on two free floating macrophytes, the invasive alien *Lemna minuta* Kunth and its co-generic native *Lemna minor* Linnaeus. A comparative approach was followed in order to investigate the ecophysiological differences between the two species, and to identify traits linked to invasiveness. The investigation included laboratory experiments in fully controlled conditions, outdoor experiments in semi-controlled conditions and monitoring of the two species in a natural environment where both species co-occur spontaneously. Field observations highlighted that these two species of Lemnaceae are often associated with another free floating species, the water fern *Azolla filiculoides* (Fig.1). These observations, and the consideration that this species, also alien and invasive, must impact on the presence and abundance of the two Lemnaceae, led to its inclusion in some of the experiments carried out and in the field monitoring.

L. minuta and *L. minor*

L. minuta and *L. minor* belong to the family of the Lemnaceae, a cosmopolitan family that includes five genera and 37 species (Appenroth *et al.*, 2013). Lemnaceae, commonly known as duckweed, are characterized by their extremely reduced size, high growth rate and marked adaptations to the freshwater environment. Due to their high growth rate and their ability to uptake nutrients and metals, Lemnaceae are used for wastewater treatments and bioremediation (Bonomo *et al*., 1997; Alvarado *et al*., 2008). Other applications of these plants include biofuel production (Cui and Cheng, 2015) and production of animal feed (Leng *et al*., 1995). The use of Lemnaceae as a high quality protein source for human production has also been flagged (Appenroth *et al*., 2017). The increasing application of Lemnaceae for different applications, together with their high dispersal ability (Coughlan *et al.*, 2015) emphasises that the potential invasiveness of these species must be investigated.

Lemnaceae have a very simple structure. A true stem or true leaves are not present and the main part of the plant is constituted by a thin frond. In most species, the frond will contain ample aerenchyma which allows the plant to float. *L. minuta* and *L. minor*, like all *Lemna* species, possess one root. In other Lemnaceae genera (i.e. *Landoltia* and *Spirodela*) more roots can be present (Landolt, 1986). In other genera (i.e.

Wolffia) roots can be completely absent (Landolt, 1986). Despite Lemnaceae being flowering plants, sexual reproduction is a rare event and the new fronds are mostly produced asexually. New fronds grow from a meristem located at the base of existing fronds. Under suitable environmental conditions, Lemnaceae are able to duplicate their biomass in less than two days, placing Lemnaceae amongst the fastest growing plants (Sree *et al*., 2015). Occasionally, sexual reproduction occurs and three very small hermaphroditic flowers are produced. The fruit of Lemnaceae is an utricle and the seeds are released in a sac containing air that allows flotation. Self-pollination of *Lemna* flowers is prevented by a delay in the receptivity of the stigma, through which the pollen tube must grow. Some species of Lemnaceae can produce turions under specific unfavourable conditions. Turions are small rootless fronds, which sink to the bottom of the water because of reduced or absent air spaces and high starch content. Several forms of mineral deficiency or imbalance, as well as for the effects of short days, can promote turion formation. *L. minor* can produce turion-like fronds which have a similar functional role as turions. Turions have never been observed in *L. minuta* (Landolt, 1986).

Because of their reduced size, the identification of some species of Lemnaceae can be difficult. Morphologically, *L. minuta* and *L. minor* can be distinguished by the frond shape and size. The fronds of *L. minuta* are 0.8-3 mm long, usually elliptic, with one vein, while those of *L. minor* are 2-5 mm long, usually ovate and they have 3-5 veins (Stace, 2010). Sometimes the morphological identification does not allow to conclusively define the species and a DNA-based molecular identification based on sequence polymorphisms can be required (Wang *et al*., 2010). Moreover, different clones of the same species can differ in their morphological characteristics, the Amplified Fragment Length Polymorphism (AFLP) marker technique is often applied for species identification (Bog *et al*., 2010).

L. minuta and *L. minor* commonly float on the surface of still or slow-moving waterbodies. Like other Lemnaceae, these species tend to be associated with eutrophic waters (Carbiener *et al*., 1990) and can tolerate temperatures between a few degrees below zero and temperatures higher than 35°C (Landolt, 1986). When Lemnaceae cover the entire surface of a waterbody, they may reduce the concentration of dissolved oxygen in the water by blocking the oxygen-transferring interface between the surface of the water body and the atmosphere. Moreover, Lemnaceae also inhibit algal growth by suppressing the natural oxygenation provided by these organisms (Sengupta *et al.*, 2010). Biological control of *L. minuta* has not been well investigated. The shore-fly *Lemnaphila scotlandae* has been reported as attacking some Lemna species in Florida (Buckingham, 1989). Little is known about the natural enemies of the free-floating duckweed *Lemna minuta* in its natural range in North America. The weevil *Tanysphyrus lemnae*, already present in the British Isles, and *L. scotlandae* damage its fronds (Gassmann *et al.,* 2006).

A. filiculoides

A. filiculoides is a small floating freshwater fern belonging to the family of Azollaceae. The body of these plants consist of a main rhizome, branching into secondary rhizomes that hold small leaves. Each leaf consists of an aerial and a ventral lobe, the former contains chlorophyll while the latter, partially submerged, does not contain the pigment but provides buoyancy to the fern (Peters *et al*. 1974). Nutrients from the water are absorbed by numerous adventitious roots that hang down from nodes on the ventral surfaces of the rhizomes (Peters and Meeks, 1989). Each dorsal lobe contains a leaf cavity which houses *Anabaena azollae* (Wagner, 1997), a symbiotic nitrogen-fixing blue-green algae. The fern provides a protected environment for the alga and it also supplies it with a carbon source, while *A. azollae* provides nitrogen for both itself and its host (Peters and Mayne, 1974). *Azolla* sexual reproduction involves a complex cycle and is rare, occurring only under specific environmental conditions (Wagner, 1997). Vegetative reproduction of the fern is the most common propagation strategy. Azollaceae, like Lemnaceae, are very fast growing species, potentially invasive and they are used for bioremediation (Vermaat and Hanif, 1998; Bennicelli *et al.,* 2004). Doe to their high content of nitrogen, Azollaceae are also used as biofertilizer (Kannaiyan, 1993).

Azolla can grow under a diverse range of environmental conditions. Temperature is the most important factor affecting its growth and distribution (Pabby *et al.*, 2004). The optimum air temperature ranges from 18–28°C for *Azolla* (Kösesakal, 2014). Janes (1998) found that *A. filiculoides* can survive encasement in ice and air temperatures of −5°C in outdoor cultures. The same author suggested that natural populations can readily survive air temperatures much lower than −5°C. In adverse conditions such as low temperature and intense light, *A. filiculoides* starts synthetizing anthocyanins and its leaves assume a reddish appearance (Janes, 1998). Anthocyanins may increase the total antioxidant capacity of the plants, resulting in tolerance against biotic and abiotic stress conditions (Kösesakal, 2014). *Azolla filiculoides* forms dense mats (5–20 cm thick) on the surface of the water with consequences similar to those described for Lemnaceae mats (Gratwicke and Marshall, 2001). A combination of a lack of natural enemies, dispersal between water bodies by humans and waterfowl, and phosphorus-enriched waters can increase *A. filiculoides* distribution and establishment with negative consequences for the biodiversity of aquatic ecosystems and all aspects of water utilization (Hill, 1998). Biological control of this water fern using the frond-feeding weevil, *Stenopelmus rufinasus* was found to be successful in South Africa (Hill, 1998) and, in 2002, in the United Kingdom, a massive outbreak of the weevil was correlated to a decline of the fern in southern regions (Gassmann *et al.*, 2006).

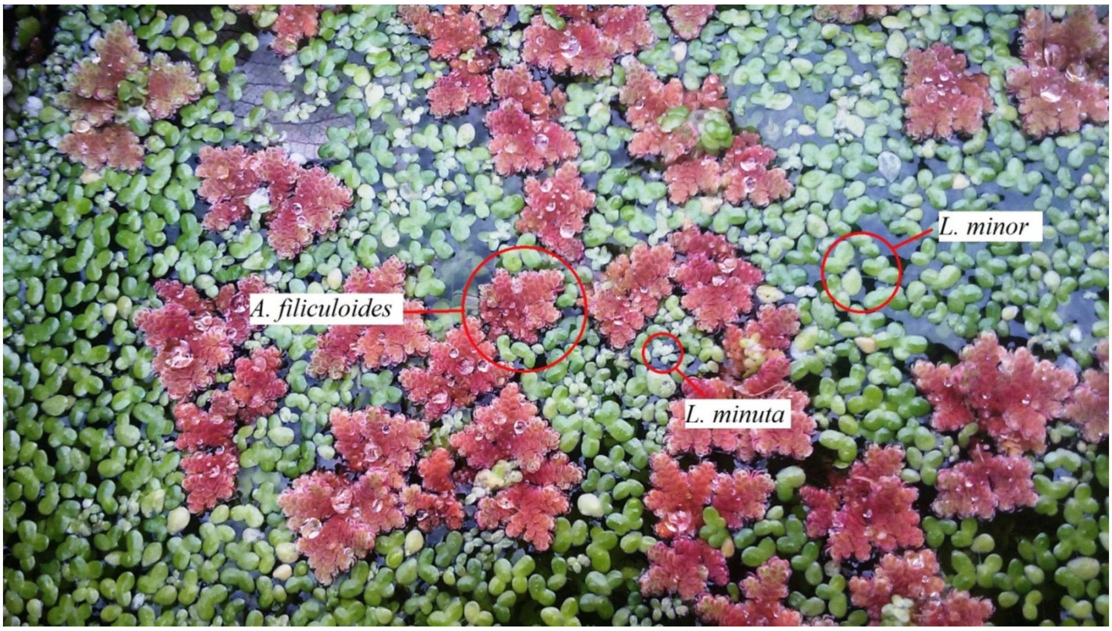

Figure 1. *Lemna minuta*, *Lemna minor* and *Azolla filiculoides* co-occurring in natural freshwaters.

Aims of the study

1. To investigate possible differences, between *L. minuta* and *L. minor,* in resource exploitation. Specifically, to characterise the growth performance of the two species grown at different nitrogen and phosphorus concentrations, and at different concentrations and ratios of calcium and magnesium.

2. To investigate possible differences, between *L. minuta* and *L. minor*, in light utilization strategy. Specifically, to determine growth rate, net assimilation rate, chlorophyll content and photosynthetic efficiency of the two species, grown at different light intensities.
3. To investigate the relative ability of *L. minuta* and *L. minor* to cope with different types of abiotic stress. Specifically, to determine growth rate, chlorophyll content and photosynthetic efficiency of the two species exposed to chemical and physical stressors.
4. To investigate the dynamic changes in abundance of alien and native species in different seasons and years, and in a natural environment.
5. To investigate possible correlations between *L. minuta*, *L. minor* and/or *A. filiculoides* abundance and specific environmental factors, in the natural environment.
6. To investigate the relative growth rates of *L. minuta*, *L. minor* and *A. filiculoides* and to assess their ability to outcompete the other species in different seasons, in outdoor mesocosms.

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Chapter 2

A comparative study of the nutrient responses of the invasive duckweed *Lemna minuta* Kunth, and the native, co-generic species *Lemna minor* Linnaeus.

Abstract

Invasive alien plant species are usually characterized by a growth rate higher than their native competitors, and this higher rate can be achieved through the opportunistic use of plant nutrients. The growth of the invasive alien duckweed *Lemna minuta* Kunth and the co-generic native *Lemna minor* Linnaeus were compared under different conditions of nutrient availability. The two species were grown for one week under fully controlled conditions on medium containing increasing concentrations of NO_3^--N, NH_4^+-N and PO_4^{3-}-P. The effects of different concentrations and ratios of Ca and Mg on growth were also determined. At the end of the experiment the Relative Growth Rate (RGR) of the plants was calculated on the basis of the biomass and number of fronds. The data highlighted that *Lemna minuta* outgrew *L. minor* under conditions of high phosphate supply while, when phosphate concentrations in the medium were low, it grew less than *L. minor*. However, *L. minuta* is not simply an opportunistic species as relative tolerance to imbalances in Ca-Mg ratio in the growth medium was observed. The different responses observed when the *L. minor* and *L. minuta* were grown at specific concentrations of P, Ca and Mg suggest that these elements are potential determinants of the invasibility of freshwaters and that they should be considered in future field studies.

Introduction

Invasive, alien aquatic plants are an important threat to freshwater ecosystems (Dudgeon *et al.*, 2006; Hussner, 2012; Pysek *et al.*, 2012; Gallardo *et al.*, 2015). In Europe alone, 96 alien, aquatic plant species from 30 families are present in at least one country (Hussner, 2012). Alien aquatic plants can have a range of negative impacts on diversity; a recent meta-analysis showed strong negative effects of invasion on the composition of macrophyte, benthic invertebrate and fish communities (Gallardo *et al.*, 2015). Excessive growth of invasive, aquatic species can also have a negative impact upon navigation, recreational activities and water extraction (Hussner, 2012). It was estimated that the annual cost of invasive species to the Irish economy is 203 million euro (Kelly *et al.*, 2013), while the United Kingdom spends every year £1.8 billion (€2.3 billion) on the management of issues related to alien invaders (Williams *et al.*, 2010).

To prevent and manage invasions by alien aquatic species it is vital to identify the factors that promote the distribution of these species and the strategies that allow them to out-compete native species (Blumenthal, 2005; Funk and Vitousek, 2007). Invasiveness has been attributed to intrinsic characteristics of the invading plant (the ideal weed hypothesis) and/or extrinsic factors such as environmental and ecological conditions (Colautti *et al.*, 2014). Already in 1986, Orians hypothesized that newly introduced species become invasive only when new resources are released and/or if they are superior competitors for the available resources. Davis *et al.* (2000) elaborated the theory according to which the invasive species takes advantage of resources not used by natives, i.e. once the difference between gross resource supply and resource uptake increases, so does the communities susceptibility to invasion. The theory by Davis *et al.* does not require the ecology of an invasive species to be distinct from that of the native species. However, increases in the availability of limiting resources, such as phosphorus and nitrogen, will create opportunities for invading species with a higher resource requirement. Indeed, increased nitrogen deposition is referred to as an enabling parameter that facilitates invasion by alien species (Dukes and Mooney 1999). Agricultural, industrial and residential activities can all cause increases in nitrate, ammonium and phosphate content in natural freshwaters, while concentrations of Ca and Mg in the water predominantly reflect the underlying geology of the area.

Many, but not all, studies have linked high Relative Growth Rates (RGR) to competitive success (Larocque and Marshall, 1993; Dawson *et al.*, 2011; Grotkopp *et al*., 2007). "Competitors" have usually a high potential RGR (the maximum RGR they can reach in optimal conditions), while plants adapted to grow under more adverse conditions (stress tolerators) are characterized by a lower potential RGR (Grime and Hunt 1975). Under resource-enriched conditions, interspecific differences in growth, measured as differences in the Relative Growth Rate (RGR), allow some plants to rapidly occupy large spaces. Thus, the ability of native and invasive species to exploit resources for rapid growth is a key aspect in the study of invasive alien species (Funk and Vitousek, 2007; Davis *et al*., 2000; Dawson *et al*., 2011).

In this study the growth performances of alien invasive *Lemna minuta* and the co-generic native species *Lemna minor* were analysed. *L. minuta* is a freshwater floating macrophyte native to temperate areas of North and South America (Stace, 1997). This species has spread extensively outside its natural habitat, and is now present in major parts of America, and Europe. In Europe, *L. minuta* was first recorded in France in 1965 (Jovet and Jovet-Ast, 1966), and it has since spread, and is now present, in most European countries (Hussner, 2012). *L. minuta,* as well as *L. minor,* can form extensive mats of biomass which negatively affect growth of submerged species such as *Potamogeton crispus* and *Elodea nuttallii* Planchon (Janes *et al*., 1996). *L. minuta* shares its habitat with the co-generic *L. minor* (Klein *et al.*, 1995; Sburlino *et al.,* 2004), which is native throughout much of Europe. The two species can often be found closely together in a multi-species floating mat of Lemnaceae, where there will be intense interactions between the species (Gopal and Goel, 1993). While understanding the invasiveness of *L. minuta* is important in its own right, the competitive interaction between *L. minuta* and *L. minor* is also a good model system to study the spread of invasive aquatic plants. Both species grow fast, are easy to manipulate and are morphologically and genetically similar. The comparison of a native species with a highly similar co-generic alien invasive is a very effective approach to understand the mechanisms underlying invasion success (Mack, 1996). Understanding the eco-physiological differences between these two species can provide a model applicable to more harmful species.

Here, the effects of nitrate, ammonium, phosphate and calcium and magnesium on growth of *L. minor* and *L. minuta*, were analysed under *ex situ* conditions. The aim of this study was to determine whether nutrient enrichment can provide a specific growth advantage to *L. minuta* over *L. minor*.

Materials and methods

Plant growth

Lemna minor and *Lemna minuta* strains were collected from the same pond in Blarney (51.940476, -8.563637), Co. Cork, Ireland. The *Lemna minor* strain has since been registered in the RDSC database as strain number 5500 “Blarney”. The plants were cultured under sterile conditions, in glass flasks, on 100 ml of half-strength Hutner's nutrient solution (Hutner, 1953). Plants were kept in a growth room at a constant temperature of 20°C and exposed to a light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$, (cool-white fluorescent tubes) with a light: dark cycle of 16: 8 hours.
To study the effects of varying concentrations of plant nutrients on the growth of *Lemna minor* and *Lemna minuta*, plants were grown in flasks containing either standard half-strenght Hutner’s medium, or variations on this medium. Standard medium was used as a control. In all experiments, the pH was adjusted before autoclaving to 4.5, which is the optimal pH for *L. minuta* and *L. minor* (Landolt, 1986), thus facilitating visualisation of the effects of nutrients on growth.

All the growth experiments lasted one week, such that data show the maximal growth response under particular nutrient conditions (i.e. environmental envelope), and before growth would be slowed down due to nutrient depletion and overcrowding of culture flasks. All the experiments started with 9 fronds (3 colonies) of each species (4.62 mg fresh weight on average for *L. minuta* and 11.32 mg for *L. minor*) and they were replicated 4 times.

Modifications of the growth medium

Nitrogen

To determine the effects of different concentrations of nitrogen on growth, the plants were grown at six concentrations of NO_3^--N and NH_4^+-N ranging from 0 to 1 $g \cdot l^{-1}$ plus the control (standard medium, 0.013 $g \cdot l^{-1}$ of N). To obtain these concentrations, standard half-strength Hutner’s medium was supplemented with potassium nitrate to obtain treatments with an increased concentration of nitrogen. Alternatively, to obtain lower concentrations of nitrogen in the medium, the amounts of calcium and potassium nitrate normally present in half-strength Hutner's were equally reduced. The resulting loss of potassium and calcium was compensated for by adding calcium chloride and potassium sulphate to the medium, such that the concentrations of these cations were similar to those in half-strength Hutner's medium.

To determine the effects of different concentrations of ammonium on growth, medium was modified by leaving out all potassium and calcium nitrate, and substituting these with various concentrations of ammonium sulphate. In order to compensate for the loss of calcium and potassium, calcium chloride and potassium sulphate were added such that the concentrations of these cations were similar to those in standard, half strength Hutner's medium.

Phosphate

Five concentrations of P in form of PO_4^{3-} ranging from 0 to 50 $mg \cdot l^{-1}$ plus the control (standard medium, 93 $mg \cdot l^{-1}$ of P) were tested. To determine the effect of elevated concentrations of phosphate on growth, the amount of potassium phosphate in the medium was increased. To generate low phosphate treatments, the amount of potassium phosphate in the medium was decreased. In this case the decrease in potassium was compensated by adding potassium sulphate to keep the concentration of the cation unchanged. A first attempt to carry out this experiment using plants cultured in standard medium gave no response to P-depletion. The RGR of the species was the same at every P concentration. This happened most likely because the plants stored a certain amount of phosphorus in their fronds that allowed them to keep growing at a normal rate during the subsequent treatment period. To avoid that previous, luxury uptake of phosphate by fronds masking the effects of changed phosphate concentrations in the medium, plants were pre-grown for two weeks in a medium without phosphate. This was not necessary for the other nutrients as a response was observed following omission.

Calcium and Magnesium

Calcium and magnesium were tested together because the optimal amount of Ca for Lemnaceae growth depends on Mg concentration (Zimmermann, 1981). Seven different ratios of the two elements were used, with concentrations ranging between 0 and 200 $mg \cdot l^{-1}$. The standard medium, used as a control, contained these two elements at the ratio Ca:Mg=122:72 $mg \cdot l^{-1}$. The different concentrations of calcium and magnesium in the medium were obtained by increasing or decreasing the amount of calcium nitrate and magnesium sulphate present in half-strength Hutner's medium. In the treatments that required a low concentration of calcium, the decrease in nitrate content was compensated for by adding a solution of potassium nitrate. In the treatments that required a low concentration of magnesium the content of sulphate was compensated for by the sulphate contained in the calcium sulphate.

Measured growth parameters

The relative growth rate (RGR) based on biomass and number of fronds was calculated using the formula by Connolly and Wayne (1996):

$$RGR= \ln (Yf / Yi) / t$$

Where *Yi* is the initial biomass or the initial number of fronds, *Yf* is the final biomass or final number of fronds, *t* is the time in days and *ln* is the natural logarithm. In the present work the word biomass is used to indicate organic matter derived from living, or recently living plants.

The chlorophyll content of fronds was determined at the end of the experiment, according to the method of Inskeep and Bloom (1985). In short, the biomass was suspended in N,N-dimethylformamide, the absorbance was measured using a spectrophotometer Thermo, model Genesys 10-S and the total chlorophyll content was calculated using the formula:

$$\text{Total Chlorophyll} = 17.90 \cdot A647 + 8.08 \cdot A665$$

where A647 and A665 are, respectively, the absorbance at the wavelengths of 647 and 665nm.

Statistical analysis data

Statistical analysis was conducted using IBM- SPSS statistic data editor. The interactions between species and treatments were analysed with 2-way ANOVA. Data are mean ± standard error. Residual analysis was performed to test for the assumptions of the two-way ANOVA. Outliers were assessed by inspection of a boxplot, normality was assessed using Shapiro-Wilk's normality test and homogeneity of variances was assessed by Levene's test. An analysis of the main effect of the treatments applied and the species was performed, All pairwise comparisons were run when the main effect was statistically significant.

Results

Growth responses to a nitrate gradient

The biomass and frond number RGR for both species displayed a similar bell-shaped response curve under different concentrations of nitrate (Fig.1*a* and *b*). The RGR was at its maximum at a concentration of 0.03 $g \cdot l^{-1}$ of N. At this concentration of N, the biomass RGR was 0.258±0.018 day^{-1} (means±SE) for *L. minuta* and 0.17±0.05 day^{-1} for *L. minor,* while the values for the frond number RGR were 0.264±0.011 day^{-1} for *L. minuta* and 0.197±0.023 day^{-1} for *L. minor*. When the plants were grown on medium without nitrate, the biomass RGR dropped to 0.173±0.016 day^{-1} for *L. minuta* and to 0.13±.019 day^{-1} for *L. minor.* At the highest concentration of nitrate, the RGR of *L. minuta* was 0.16±0.02 day^{-1} and 0.073±0.015 day^{-1} for *L minor*.

The interaction effect between species and NO_3-N concentration was not statistically significant neither for the RGR calculated on the basis of the biomass nor for the RGR calculated on the basis of fronds number. The analysis of the main effect for the RGR based on biomass and on fronds number revealed that there was a statistical significant difference between species ($p<0.001$), while difference between treatments was not statistically significant. The results of the pairwise comparisons are showed in fig. 1 *a* and *b.*

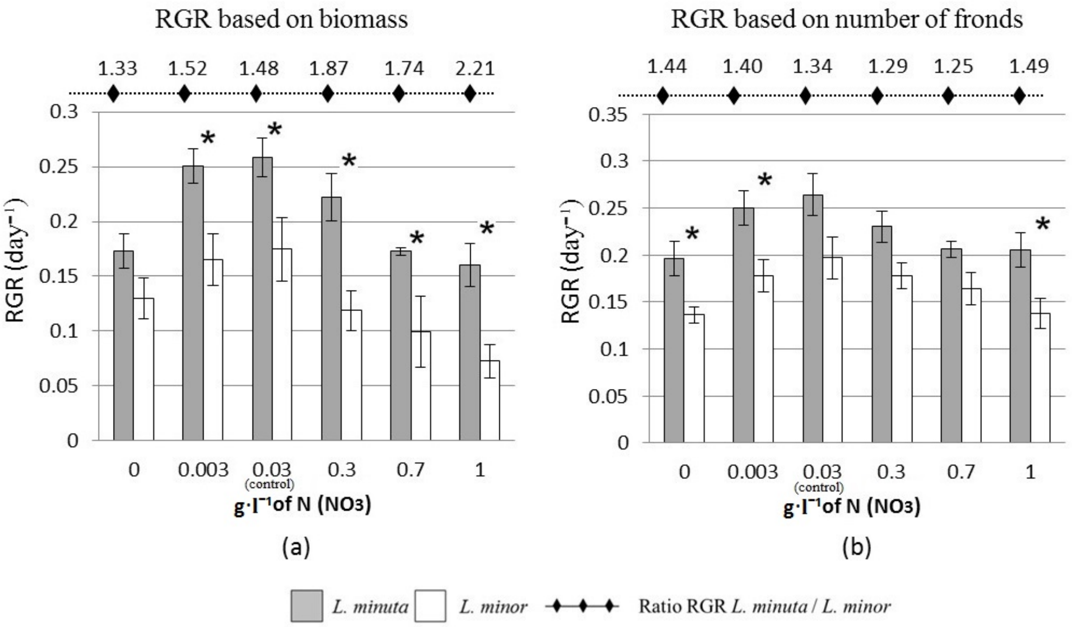

Figure 1. RGR based on biomass accumulated (*a*) or fronds formation (*b*) of the two species grown at different concentration of NO_3^--N. Values are means of 4 replicates and error bars are standard errors. The asterisks indicate a significant difference between the 2 species. The numbers above the graphs indicate the ratio of the RGR of *L. minuta* over the RGR of *L. minor* at each concentration of NO_3^-N. The control in this experiment is included in the N gradient tested.

Growth responses to an ammonium gradient

Ammonium is frequently present in eutrophic waters where both species of Lemnaceae may grow. When in our experiments ammonium was used as a nitrogen source neither the biomass RGR nor the frond number RGR did show a strong concentration dependence along the N gradient (Fig. 2 *a* and *b*). *L. minuta* and *L. minor* achieved their highest biomass RGR at 0.3 $g{\cdot}l^{-1}$ of N (0.243±0.007 day^{-1} and 0.174±0.013 day^{-1}, respectively) and their lowest biomass growth rate in the absence of N in the medium (0.173±0.016 day^{-1} and 0.130±0.019 day^{-1}, respectively). The 2-way ANOVA did not highlight any interaction between NH_4^+-N concentraction and species. There was no significant difference in RGR between treatments, while *L. minuta* had an overall RGR significantly higher ($p<0.001$) than *L. minor* (Fig. 2), irrespective of whether biomass or frond number were measured. The results of the pairwise comparisons are showed in figure 2 *a* and *b*.

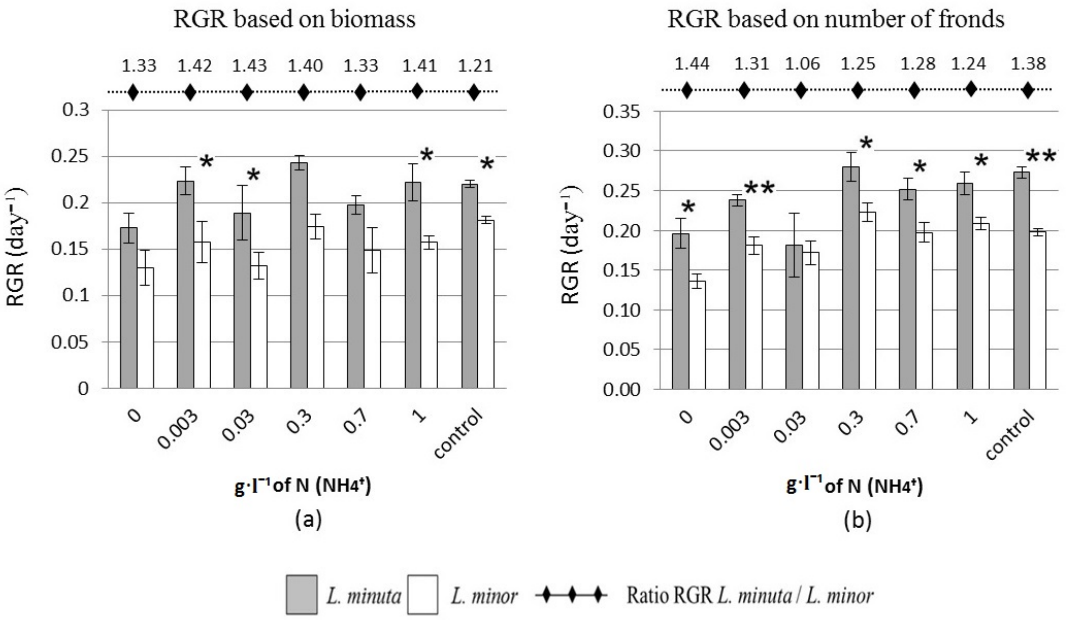

Figure 2. RGR based on biomass accumulated (*a*) or fronds formation (*b*) of the two species grown in allopatric conditions and at different concentration of NH_4^+-N. Values are means of 4 replicates and error bars are standard errors. The asterisks indicate the significance in differences between the two species. * means $p<0.05$, ** means $p<0.01$. The control refers to the standard medium, it does not contain NH_4^+-N, but it contains an optimal concentration of NO_3^--N. The numbers above the graphs indicate the ratio of the RGR of *L. minuta* over the RGR of *L. minor* at each concentration of NH_4^+-N.

Growth responses to a phosphorus gradient

The pilot experiment showed that, without adequate pre-treatment, the RGR of the two species is unaffected by the different P treatments applied (data not shown). The RGR did not vary significantly along the P gradient.

In subsequent experiments, carried out with plants pre-grown for two weeks in the absence of P, the biomass and frond number RGR of the two species increased proportionally with the increase in the concentration of phosphate in the medium (Fig. 3 *a* and *b*). The highest RGR values were found on control medium. Under these conditions, *L. minuta* had a higher biomass RGR than *L. minor* (0.249±0.002 day^{-1} for *L. minuta* and 0.216±0.004 day^{-1} for *L. minor*, respectively). However, at all lower P concentrations *L. minor* had a higher biomass RGR than *L. minuta.* The difference in both biomass and frond number RGR between the two species was greatest at the lowest concentrations of P. For example, in the absence of phosphate in the medium, *L. minor* had a biomass RGR of 0.116±0.004 day^{-1}while the RGR of *L. minuta* was just 0.034±0.006 day^{-1}.

There was a statistically significant interaction between species and treatments on RGR (p=0.02 for RGR based on biomass and p=0.008 for RGR based on fronds number). The pairwise comparisons run for each simple main effect revealed that there was a statistically significant difference in RGR for *L. minuta* and *L. minor* grown at either 50, 50000 or 93000 (control) µg·l^{-1} of P. *L. minor* had a RGR based on biomass significantly higher than *L. minuta* when grown with 50 µg·l^{-1} of P (p=0.004). *L. minuta* had a RGR based on biomass significantly higher than *L. minor* when grown with 50000 µg·l^{-1} of P (p=0.004) and in the control. The results of all pairwise comparisons are shown in fig.3 *a* and *b*.

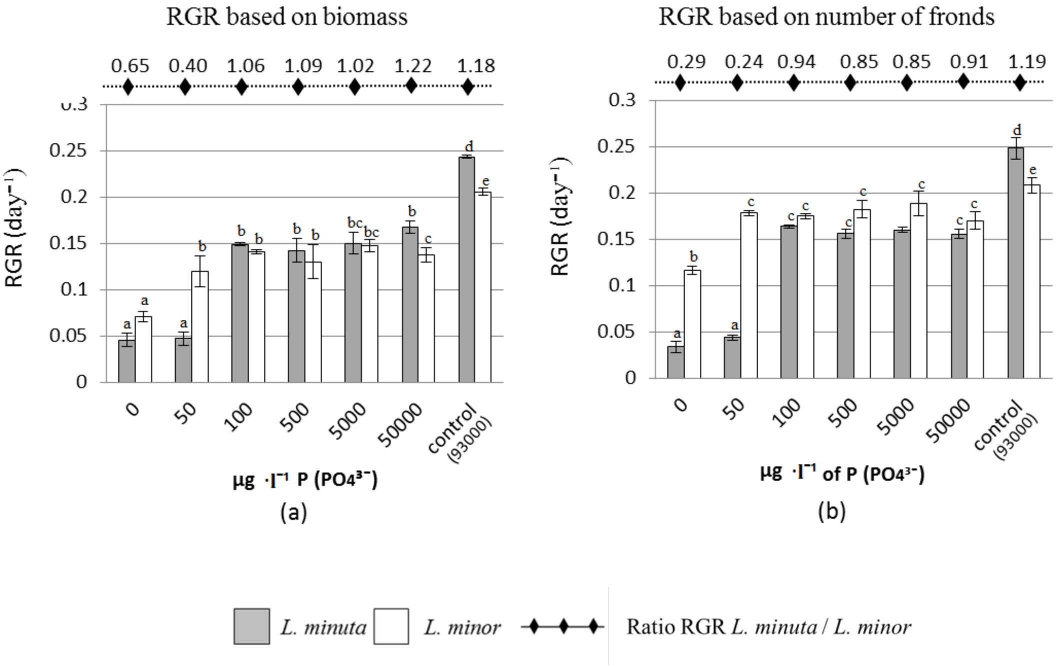

Figure 3. RGR based on the biomass accumulated (*a*) and RGR based on the number of fronds (*b*) of the two species grown at different concentration of PO_4^{3-}-P. Values are means of 4 replicates and error bars are standard errors. Different letters indicate significant differences resulting from the combined effect of species and treatments. The numbers above the graphs indicate the ratio of the RGR of *L. minuta* over the RGR of *L. minor* at each concentration of PO_4^{3-}-P.

Growth responses to variations in calcium and magnesium supply

Plants were grown on modified Hutner's medium with altered calcium and magnesium content. The biomass and frond number RGR values were not strongly affected by variations in calcium or magnesium content, with the exception of medium with very low calcium concentrations and high magnesium concentrations (Fig. 4 *a* and *b*), on which growth was impeded. The biomass RGR of *L. minuta* was 0.087±0.012 day^{-1} at Ca:Mg = 5:100 mg·l^{-1} and only 0.057±0.010 day^{-1} at Ca:Mg = 0:200 mg·l^{-1}, values which are more than 60% smaller than those of plants on control medium. The RGR of *L. minor* was 0.036±0.002 day^{-1} at Ca:Mg = 5:100 $mg \cdot l^{-1}$ and 0.037±0.009 day^{-1} at Ca:Mg = 0:200 $mg \cdot l^{-1}$, versus 0.22±0.002 day^{-1} on control medium.

There was a statistically significant interaction between species and treatments on RGR (p=0.009 for RGR based on biomass and p=0.012 for RGR based on fronds number). *L. minuta* had a higher biomass RGR than *L. minor* at all the ratios of calcium and magnesium, but the difference between the two species was greatest at the treatments with a low concentration of calcium and a high concentration of magnesium, such as at Ca:Mg=5:100 (p<0.01). There was also a significant difference in RGR at Ca:Mg=100:5 (p=0.021). All the pairwise comparisons, run for each simple main effect, are shown in fig.4 *a* and *b*.

Remarkably, distinct visual effects were observed when comparing *L. minor* and *L. minuta* grown on extreme ratios of calcium and magnesium. While *L. minor* displayed reduced RGR values under Ca:Mg 5:100, Ca:Mg 0:200 and Ca:Mg 100:5, it is *L. minuta* which shows strong chlorosis under these conditions, i.e. Ca:Mg 5:100, Ca:Mg 0:200 and Ca:Mg 200:0 (Fig. 4 *c*). The 2 way ANOVA did not revealed a significant interaction between chlorophyll content and calcium and magnesium in the medium. A statistically significant difference between species (p<0.04) was found. In *L. minuta* chlorophyll content ranged between 0.51±0.04 and 0.93±0.06 mg per g of biomass, but it dropped as low as 0.13±0.03 $mg \cdot g^{-1}$ in fronds raised on Ca:Mg=5:100, and 0.26±0.1 $mg \cdot g^{-1}$ at Ca:Mg=0:200. *L. minor* had a content of chlorophyll per g of biomass significantly higher than *L. minuta* at Ca:Mg=200:0 (p=0.001), Ca:Mg=5:100 (p=0.001) and at Ca:Mg=0:100(p=0.042). This highlights a distinct response to imbalances in nutrition.

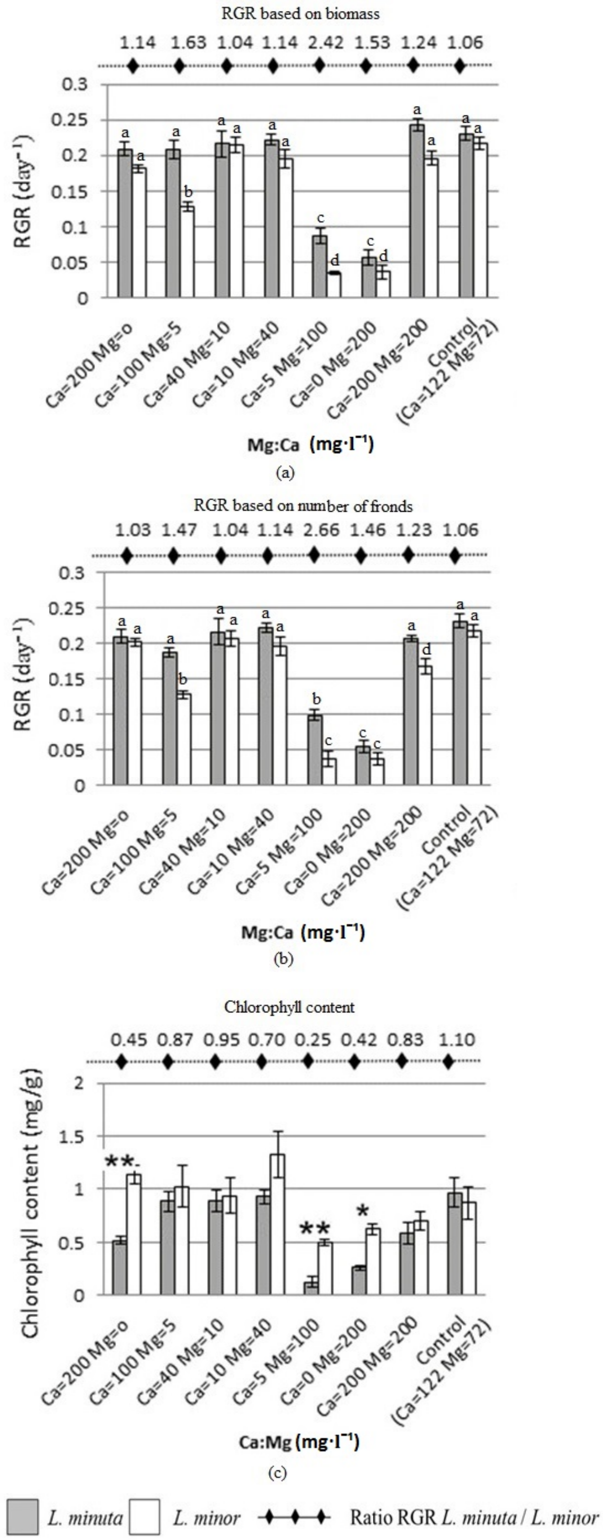

Figure 4 RGR based on the biomass accumulated (*a*), RGR based on the number of fronds (*b*) and chlorophyll content (*c*) of the two species grown at different concentration and ratio of Ca and Mg. Values are means of 4 replicates and error bars are standard errors. In a and *b* different letters indicate significant differences resulting from the combined effect of species and treatments. In *c* the asterisks indicate the significance in differences between species. *means $p<0.05$, ** mean $p<0.01$. The numbers above the graphs indicate the ratio of the RGR of *L. minuta* over the RGR of *L. minor* at each Ca:Mg tested.

Discussion

Nutrient requirements of a number of Lemnaceae species, such as *Lemna minor*, have been extensively researched (Landolt, 1986). The optimum nitrogen concentration for *L. minor* is estimated to be between 2.8 and 350 mg·l^{-1} N (0.2 to 25 mM) depending on the clone, while for *L. minuta* the optimum ranges between 0.56 and 14 mg·l^{-1} (0.04 to 1 mM). These values are summarised by Landolt (1986) and they refer to N without any distinction between the chemical form of the element. The nitrogen data presented in this study are in agreement with published values.

Lemnaceae are among a group of plants that can utilize both ammonium and nitrate as sources of nitrogen (Landolt, 1986). Ammonium is the preferred source of nitrogen for Lemnaceae (Porath and Pollock, 1982). No toxic effects were observed in this study, not even at high concentrations of ammonium. The lack of toxicity is probably due to the pH of the Hutner's medium (4.5). At this pH the NH_4^+ ion is prevalent while the toxic effect is triggered by the ion NH_3 (Caicedo *et al*,. 2000). Körner *et al.* (2003) also concluded that Lemnaceae can tolerate high total ammonia concentrations as long as the pH is less than 7.8.

While no toxic effect was noted for ammonium, a decrease in RGR occurred for both species at high concentrations of nitrate. These results are consistent with the findings of Lasfar *et al.* (2007). These authors observed a growth rate that was slightly inhibited at 120 mg·l^{-1} of N, while, the mathematical model they developed, estimated a saturation concentration at 0.95 mg·l^{-1}of N.

When the performance of *L. minuta* and *L. minor* along the PO_4^{3-}-P gradient was assessed, the highest RGR for both species was reached on medium containing between 50 µg·l^{-1} and 93 mg·l^{-1} of P. This is consistent with the optimum values provided by Landolt (1986) who reported that the optimal P concentrations for *L. minuta* ranged between 86.71 µg·l^{-1} (0.0028 mM) and 10.84 mg·l^{-1} (0.35mM) and for *L. minor* between 0.43 mg·l^{-1} (0.014mM) and 10 mg·l^{-1} (0.35mM) of phosphate.

Ca and Mg are the main contributors to the hardness of the water. Lemnaceae prefer hardwater, although communities dominated by *Lemna* spp., *Spirodela* spp. and *Wolffia* spp. can sporadically be found on softwater (Murphy, 2002). Strauss (1976) found that estimates of the optimum concentration of Ca and Mg for *L. minuta* and *L. minor* vary between 8 and 800 mg·l^{-1} (0.2 – 20 mM) of Ca and 1.21 and 240 mg·l^{-1} (0.05 – 10 mM) of Mg. In this study both species displayed a strong decrease in the

RGR at very low concentrations of Ca when associated with a high concentration of Mg. However, when the same high concentration of Mg was associated with a higher concentration of Ca, the RGR of both species was only slightly slower than that of the control. This is consistent with work by Landolt (1986) who concluded that Ca and Mg behave as antagonists and are able to neutralise the effects of each other. Indeed, very high levels of Ca and Mg (200 $mg{\cdot}l^{-1}$ of each of the two) cause no inhibition of growth, when the two elements are present in equal concentrations. These results are consistent with the findings of Zimmerman (1981) who reported that *Spirodela polyrrhiza* Linnaeus can tolerate high levels (121.55 $mg{\cdot}l^{-1}$; 50 mM) of Mg in the presence of high levels (12 $mg{\cdot}l^{-1}$; 0.3 mM) of Ca.

The degree of invasion of some habitats has been associated with nutrient availability (Alpert *et al.*, 2000). Nevertheless contrasting results are present in literature. For example, James *et al.* (2006) investigated the competition between *Elodea nuttallii*, *E. canadensis* and *Lagarosiphon major* Ridley under different concentrations of NO_3-N, NH_4^+-N and PO_4^{3-} and found that different responses to nutrient enrichment do not explain why native *E. nuttallii* was outcompeted by alien *E. canadensis* under field conditions or why *E. canadensis* was similarly outcompeted by *L. major*. On the contrary, a study by Njambuya *et al.* (2011) showed that growth of *L. minuta* and *L. minor* was differentially affected by nutrient concentrations, and that high levels of nutrients can promote the out-competition of *L. minor* by *L. minuta.* These authors found that growth of *L. minuta* was more reduced under low nutrient concentrations than growth of *L. minor*. In order to pinpoint specific nutrients that are responsible for the faster growth of *L. minuta*, the responses to individual nutrients were explored in this study. When different concentrations of PO_4^{3-} were tested, a clear species-specific response was measured. *L. minuta* achieved a high RGR only on medium containing high concentrations of phosphate. Growth rates of *L. minuta* were dramatically decreased on medium containing very low phosphate levels, although this was significantly less obvious for *L. minor*. Thus, we conclude that *L. minor* tolerated phosphate deficiencies better than *L. minuta*. Previously, it was shown that competition between *L. minuta* and *Landoltia punctata* G. Mayer is PO_4^{3-} concentration dependent (Gérard and Triest 2014). The latter authors observed that at high concentrations of phosphate, *L. minuta* out-competes *L. punctata*, but, when PO_4^{3-} in the medium is reduced, RGR of *L. minuta* decreases significantly and *L.*

punctata outcompetes *L. minuta.* Thus, it appears that both *L. minor* and *L. punctata* out-grow *L. minuta* under low phosphate conditions, suggesting that the latter species is more opportunistic. There is some evidence that phosphorus can be a determinant of invasibility at the ecosystem level. Buchan and Padilla (2000) linked the susceptibility towards invasion of a water body to the amount of phosphorus available. Thus, increased phosphate availability may increase invasibility of ecosystems, especially when there is an alien species that can avail of high nutrient supplies as was demonstrated in this paper.

In this study it was found that at all concentrations of N (low or high, and both NO_3^--N and NH_4^+-N) *L. minuta* was growing faster than *L. minor*, although the dose-response curve was similar. It was concluded that there is no evidence that *L. minuta* achieves a competitive growth advantage depending on the nitrogen status of the medium. A previous study showed that high ammonium concentrations inhibit Lemnaceae growth in a species-specific or strain-specific manner (Zhang *et al.*, 2014), however, in our study*, L. minuta* and *L. minor* showed no differences in their response to high concentrations of ammonium, nor nitrate, suggesting that both species can thrive on ammonium-rich eutrophic waters. A survey of the literature shows that the role of nitrogen in facilitating invasion is not consistent. For example, Green and Galatowitsch (2001) found that *Phalaris arundinacea* Linnaeus, invasive in North American wetlands, suppressed the growth of the native community regardless the concentration of NO_3-N. Conversely, Anderson and Kalff (1986), and Ali and Soltan (2006) concluded that nitrogen additions reinforce the invasive capacity of *Myriophyllum spicatum* Linnaeus.

The difference in phosphate exploitation is not the only disparity between the two species. *L. minuta* tolerated high concentrations of Mg better than *L. minor* when these were associated with low concentrations of calcium. Similarly, *L. minuta* tolerated high concentrations of calcium better than *L. minor* when these were associated with low Mg. Van Dam *et al.* (2010) observed that different species need a different amount of calcium to buffer the toxic effect of magnesium. These authors observed that, at the IC_{50} concentration, magnesium toxicity was eliminated in *Lemna aequinoctalis* Welwitsch and in *Hydra viridissima* Pallas by a magnesium:calcium ratio of ≤10:1 and ≤9:1 respectively. In the case of *Amerianna cumingi*, the toxic magnesium effect was reduced by 70-90% at a magnesium:calcium ratio of ≤9:1. Thus, *L. minuta* tolerates excesses of either calcium or magnesium better than *L. minor*, notwithstanding a disproportionate negative effect on chlorophyll content. A possible correlation between content of calcium and magnesium in the water and susceptibility of natural sites to invasion has not been thoroughly investigated. Ali and Soltan (2006) found that the increase of the content of calcium and magnesium in the Lake Nasser (Egypt) was, along with other factors such as water conductivity and nitrogen concentration, correlated with the invasion by *M. spicatum*. De Winton *et al.* (2012) also found that the water hardness is correlated with the abundance of invasive species in a study in which 195 lakes in New Zealand were investigated.

In the complexity of a natural system it is difficult to identify key factors responsible for the success of a species over another. Conversely, laboratory studies enable the identification of environmental factors that underpin growth-success, but the ecological relevance of data is not immediately evident. Pearson and Dawson (2003) reviewed the literature on climate responses, and concluded that identifying the bioclimate envelope under controlled conditions can provide a useful first approximation of growth in a more complex natural environment. This paper showed that the environmental envelopes of *L. minuta* and *L. minor* differ with respect to magnesium:calcium and phosphorus metabolism. This indicates that these elements are potential determinants of the invasibility of freshwaters and that they should be considered in future field studies.

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Chapter 3

The invasive duckweed *Lemna minuta* Kunth displays a different light utilisation strategy than native *Lemna minor* Linnaeus

Abstract

Lemna minuta Kunth is an invasive, alien duckweed that is present throughout much of Europe, where it competes with native congeneric *Lemna minor* Linnaeus. The aim of this study was to determine whether the ability of the invasive duckweed *Lemna minuta* to outperform native *Lemna minor* is based on differential light utilisation. For this purpose, the growth performance of *Lemna minuta* was compared with that of *L. minor* under a range of different light intensities.

Both physiological and morphological parameters were determined. *L. minuta* showed a higher relative growth rate than *L. minor* when grown under medium and high intensities. Further analysis showed that, at high light intensities, *L. minuta* has a higher net assimilation rate, and displays more photochemical quenching and a higher quantum yield than *L. minor*. In contrast, under low light intensities *L. minor* displayed a marginally higher relative growth rate, due to a greater leaf area ratio, and higher chlorophyll content than *L. minuta.* The results indicate two distinct light utilisation strategies, and reveal that the invasive species *L. minuta* takes more advantage from high intensity light conditions. In turn, this may influence plant distribution, and inform management strategies.

Introduction

Invasive alien species pose a major threat to biodiversity and natural ecosystems worldwide (Chornesky and Randall, 2003). Aquatic ecosystems are particularly at risk from alien invasive plants. These invasive aquatic plants can have substantial negative effects on freshwater communities by decreasing the biodiversity of invertebrate, fish and native plant species in aquatic systems (Zedler and Kercher, 2004), and can affect water quality by altering nutrient cycling and the microclimate of the water body (D’Antonio and Vitousek, 1992). Invasive plants can also negatively affect water-based recreational activities, water extraction and shipping (Hussner 2012), and governments spend a considerable amount of money on aquatic invasive species removal (Baars *et al.*, 2011). Improved understanding of the environmental conditions that facilitate excessive growth of invasive species may help focus management on those ecosystems where a particular invasive species poses the most serious threat to biodiversity.

In this study the role of light in facilitating the growth of two free floating freshwater species belonging to the family of Lemnaceae was explored. *Lemna minuta* Kunth is native in temperate areas of North and South America (Stace, 2010), but alien in much of Europe. In Europe, *L. minuta* was first recorded in 1965 in France (Jovet and Jovet-Ast, 1966). Since then, the species has spread widely and is now considered invasive in northern European countries such as Belgium (Halford *et al*., 2011), and Germany (Hussner *et al*., 2010), in eastern European countries such as Poland (Wójciak and Urban, 2009) and Hungary (Lukács *et al*., 2016), in Mediterranean countries such as Italy (Conti *et al*., 2005) and Malta (Mifsud, 2010), and in western European countries such as Britain (Bramley *et al*., 1995) and Ireland (Lucey 2003). In Europe, *L. minuta* commonly co-occurs with the congeneric species *Lemna minor,* which is native in Europe and Asia. Where *L. minuta* and *L. minor* become dominant, they form floating mats causing a negative impact on wetland ecosystems by suppressing submerged macrophyte species (Janes *et al*., 1996).

Irrespective of the ecological impacts of *L. minuta* on European water bodies, these species can also be exploited as a model species to investigate the competition dynamics between alien and native invasive aquatic plants. Lemnaceae are small, and easy to manipulate. Moreover, comparisons with congeneric species are an effective method to study the invasiveness of an alien species (Mack, 1996). Closely-related species share many traits, and therefore the identification of invasiveness-related traits, not shared between the two species, is possible (Mack, 1996). Nevertheless, it should be appreciated that "invasiveness" traits will not comprehensively explain the success of an invasive species as such success is generally due to the interaction of multiple environmental factors with a range of intrinsic traits (Richardson and Pyšek, 2006).

The focus of this study was to determine if the success of invasive *L. minuta* over native *L. minor* can be explained, in part, by differences in light utilisation. Light is a key-factor for plant growth, and its capture and utilisation play an important role in determining the relative success of one species over another. Different species have evolved different adaptations to optimise growth and photosynthesis in environments with, for example, low or high light availability. Plants more adapted to high levels of direct sunlight are called heliophilous, while plants that thrive at low light levels are called sciophilous. Plants that are adapted to intermediate light levels are called mesic (Hallé, 1978). Sciophilous and heliophilous species achieve the ability to thrive at a particular light level by adopting different light capture and utilisation strategies (Valladares and Niinemets, 2008). For example, plants grown at high light intensities typically have a different leaf morphology than plants grown at low light intensities (Boardman, 1977). Heliophilous plants have usually smaller, but thicker leaves with more palisade and spongy mesophyll layers (Boardman, 1977; Gratani and Ghia, 2002; Zaragoza-Castells *et al*., 2008). In contrast, shade plants often have thin leaves with a lower weight per leaf area. Prevailing light intensities also determine the photosynthetic capacity (Boardman, 1977). For example, the light intensity under which plants are grown influences chloroplast structure, pigment content, photochemical efficiency and stomatal density (Boardman 1977; Demmig and Björkman 1987; Valladares and Niinemets, 2008).

Previous studies demonstrated that light can impact on the ability of invasive species to outcompete native species (e.g. Madsen *et al*., 1991) but the underlying mechanisms have not yet been identified. In the present study the performance of *L. minor* and *L. minuta* at a range of light levels was assessed. The aim of the study was to determine whether the ability of one species to outperform the other is based on differential light utilisation. Both physiological and morphological parameters, such as Relative Growth Rate (RGR), Net Assimilation Rate (NAR), Leaf Area Ratio (LAR), chlorophyll content and photosynthetic efficiency, were measured and analysed.

Material and methods

Cultivation of plant stocks

L. minor and *L. minuta* strains used for this experiment were collected in Blarney (51.940476, -8.563637), Co. Cork, Ireland. The *L. minor* strain has since been registered in the RDSC database as strain number 5500 “Blarney”. The plants were cultured under sterile conditions, in glass flasks, on 100 ml of half-strength Hutner's nutrient solution (Hutner, 1953). Plants were kept in a growth room at a constant temperature of 20°C and exposed to a light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$, (cool-white fluorescent tubes) with a light: dark cycle of 16:8 hours.

Experimental conditions

Plants were grown in Petri dishes without a cover lid containing 50ml of half strength Hutner’s medium. The different light intensities were obtained by placing the plant at different distances from a LED light source (AP67 R-series, Valoya Finland). The experiment was carried out at 20°C with a light: dark cycle of 16: 8 hours. When necessary, distilled water was added to the Petri dishes during the experiment to compensate for evaporation. *L. minuta* and *L. minor* were grown at 6, 10, 20, 30, 42, 93, 150, 250, 400 and 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Each replicate started with nine fronds (4.62 mg fresh weight on average for *L. minuta* and 11.32 mg fresh weight on average for *L. minor*). The experiment lasted one week and each treatment was replicated 4 times. Given the rapid growth of the species, after one week the bulk of the *L. minor* fronds would have developed under the imposed experimental conditions.

Measured end-points

After one week of growth, plants were harvested and the biomass and frond area were measured. The RGR based on biomass and number of fronds was calculated using the formula by Connolly and Wayne (1996):

$$RGR = \ln (Yf / Yi) / t$$

Where *Yi* is the initial biomass or the initial number of fronds, *Yf* is the final biomass or final number of fronds, *t* is the time in days and *ln* is the natural logarithm. In the present work the word biomass is used to indicate organic matter derived from living, or recently living plants.

Frond area was measured using the Image-J software and the LAR and NAR were calculated. The LAR was calculated according to Radford (1967):

$$LAR = \text{Leaf area per plant} / \text{Plant weight}$$

The NAR was calculated according to Williams (1946):

$$NAR = [(W_2 - W_1)/ T] \cdot [(\ln A_2 - \ln A_1)/A_2 - A_1]$$

Where W_2 is the final biomass, W_1 is the initial biomass, T is the time in days, A_2 is the final area and A_1 is the initial area.

Before determination of the biomass, photosynthetic characteristics of fronds grown at different light intensity were analysed using pulse amplitude modulated chlorophyll *a* fluorometry (Schreiber *et al.*, 1986) (WALZ Imaging fluorometer, Effeltrich, Germany). The relative variable fluorescence (Fv/Fm) was measured on dark adapted plants. The steady state yield (YII), photochemical quenching (qP) and non-photochemical quenching (qN) were measured following exposure to different actinic light intensities, ranging between 0 and 701 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

The chlorophyll content of fronds was also determined at the end of the experiment, according to the method of Inskeep and Bloom (1985). In short, the biomass was suspended in N,N-dimethylformamide, the absorbance was measured using a spectrophotometer Thermo, model Genesys 10-S and the total chlorophyll content was calculated using the formula:

$$\text{Total Chlorophyll} = 17.90 \cdot A647 + 8.08 \cdot A665$$

where A647 and A665 are, respectively, the absorbance at the wavelengths of 647 and 665nm. The total chlorophyll content was normalised versus biomass.

Statistical analysis

The statistical analysis was conducted using IBM- SPSS statistic data editor (22). A two-way ANOVA was conducted in order to examine the differences between the two species on RGR, LAR, NAR and chlorophyll content when grown at different light intensities. The differences in Y(II), qP and qN at different actinic light and between species were analysed using a 2-way repeated measures ANOVA. Data are mean ± standard error. Residual analysis was performed to test for the assumptions of the tests run. Outliers were assessed by inspection of a boxplot, normality was assessed using Shapiro-Wilk's normality test for each cell of the design and homogeneity of variances was assessed by Levene's test. When a statistically significant interaction between species and treatments was found, an analysis of simple main effects was performed with statistical significance receiving a Bonferroni adjustment. When the interaction effect was not statistically significant, an analysis of the main effects was performed. All pairwise comparisons were run for each significant simple main effect and main effect.

Results

RGR as a function of light intensity

The RGR was calculated from the time dependent increase in biomass. For both species RGR increased with increasing light intensity (Fig.1), with a minimum RGR at the lowest intensity of 6 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (0.03±0.007 day^{-1} for *L. minuta* and 0.041±0.007 day^{-1} for *L. minor*) and a maximum RGR at the highest intensity of 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (0.402±0.013 day^{-1} for *L. minuta* and 0.326±0.008 day^{-1} for *L. minor*). Both species appeared to be very close to light saturation at the highest intensity used. There was a significant interaction effect between species and light intensity ($p=0.01$). A comparison of the two species revealed that *L. minuta* had a significantly higher RGR than *L. minor* at 93, 150, 250, 400 $\mu mol \cdot m^{-2} \cdot s^{-1}$ ($p<0.001$) and at 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ ($p=0.006$). In contrast, at 6, 10, and 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$ *L. minor* grew faster than *L. minuta*, but the differences were not statistically significant.

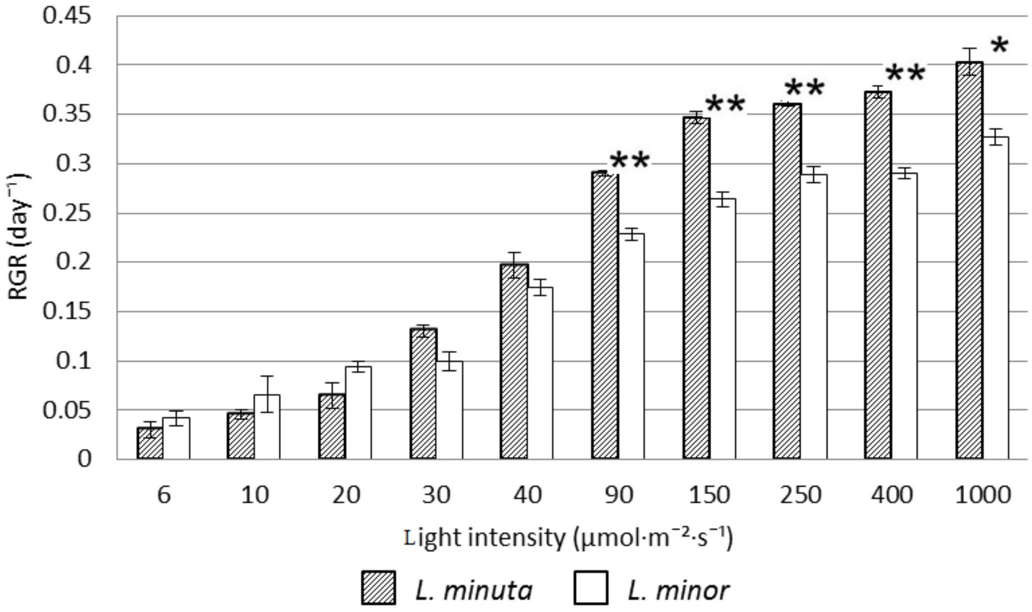

Figure 1. Relative growth rate values for *Lemna minuta* and *Lemna minor*, calculated from the increase in biomass after 7 days of growth at light intensities ranging between 6 and 1000 µmol · m^{-2} · s^{-1}. Values are mean of four replicates and error bars are standard errors. The asterisks indicate the significance in differences between species. * means $p<0.05$, ** means $p<0.01$

Changes in LAR and NAR at different light intensities

The total frond area was measured in order to calculate the LAR and NAR of the two species at all the light intensities tested. There was a significant interaction between species and light intensity in determining both LAR and NAR ($p<0.001$). In general, the LAR decreased with increasing light intensity (Fig. 2*a*). *L. minuta* reached a maximum LAR at a light intensity of 6 µmol·m^{-2}·s^{-1} and displayed a minimum LAR at 1000 µmol·m^{-2}·s^{-1} (70.63±3.66 and 37.51±2.67 cm^{2}·g^{-1} respectively). *L. minor* reached its maximum and minimum LAR at the same light intensities (85.23±0.4 and 36.09±1.66 cm^{2}·g^{-1}, respectively). *L. minor* displayed a significantly higher LAR than *L. minuta* at 6 µmol· m^{-2}·s^{-1} ($p<0.02$), while, at higher light intensities the difference between the LAR of the two species decreased progressively. At the highest light intensities tested the species displayed a very similar LAR.

At low and medium light intensities the two species had a similar, low NAR (Fig. 2*b*). Between 30 and 90 µmol·m^{-2}·s^{-1} an increase in the slope of NAR versus light intensity was observed, while at intensities above 400 µmol·m^{-2}·s^{-1} NAR appeared to have reached saturation. At the highest light intensities, *L. minuta* had a higher NAR than *L. minor*. This difference was significant at 250 µmol·m^{-2}·s^{-1} ($p=0.01$), 400 µmol·m^{-}

$^{2}\cdot s^{-1}$ ($p<0.001$) and at 1000 μmol·$m^{-2}\cdot s^{-1}$ ($p<0.001$). The maximum NAR reached by *L. minuta* was 5.46±0.03 mg·$cm^{-2}\cdot day^{-1}$, while the highest NAR calculated for *L. minor* was 4.19±0.1 mg·$cm^{-2}\cdot day^{-1}$.

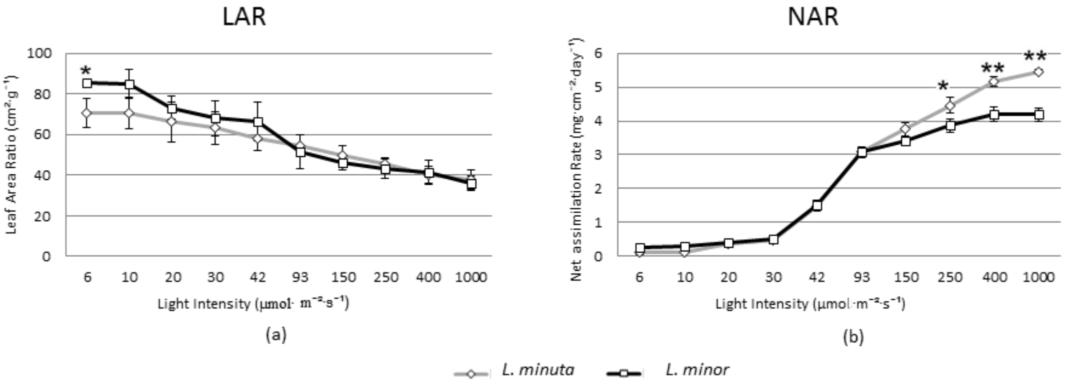

Figure 2. Leaf area ratio (a) and net assimilation rate (b) values for *Lemna minuta* and *Lemna minor*, calculated from the increase in biomass and area after 7 days of growth at light intensities ranging between 6 and 1000 μmol · $m^{-2}\cdot s^{-1}$. Values are mean of four replicates and error bars are standard errors. The asterisks indicate the significance in differences between species. * means $p<0.05$, ** means $p<0.01$

Chlorophyll content as a function of light intensity

The analysis of the total chlorophyll content per unit of biomass showed a decrease of the plant pigment content with increasing light intensity in both species (Fig. 3). The maximum chlorophyll content was reached at the lowest light intensity (1.34±0.024 mg·g^{-1} in *L. minor* and 1.04±0.06 mg·g^{-1} in *L. minuta*) and the minimum content was observed at the highest intensity (0.32±0.03 mg·g^{-1} in *L. minor* and 0.24±0.034 mg·g^{-1} in *L. minuta*). There was a significant interaction between light intensity and chlorophyll content ($p<0.01$). *L. minor* had a higher chlorophyll content than *L. minuta* at every light intensity tested ($p<0.01$ for the overall difference). The results of the pairwise comparison are shown in figure 3.

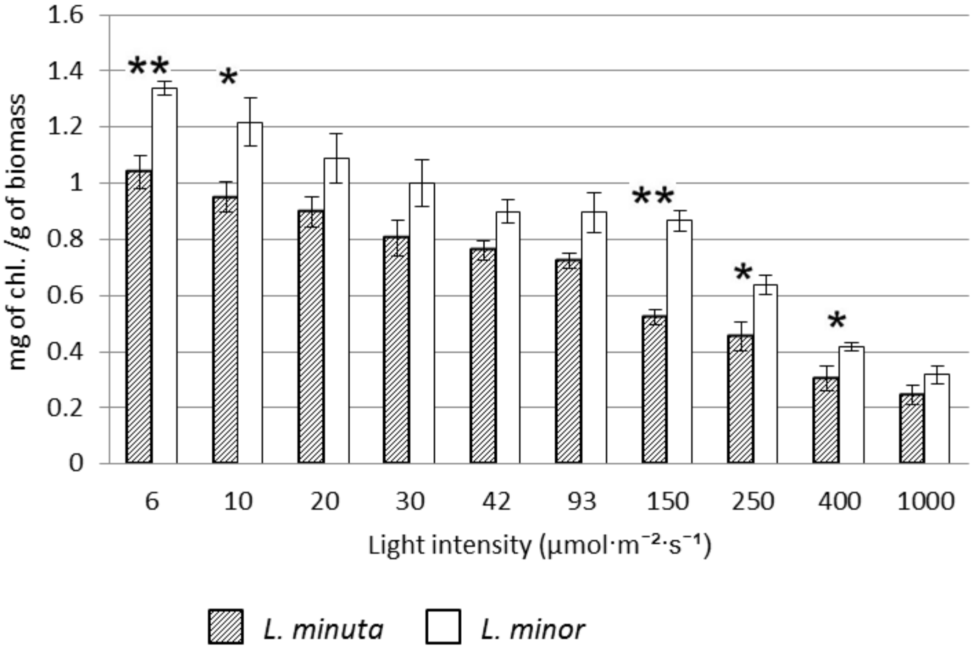

Figure 3. Chlorophyll content values for *Lemna minuta* and *Lemna minor*, measured from the biomass after 7 days of growth at light intensities ranging between 6 and 1000 µmol · m^{-2} · s^{-1}. Values are mean of four replicates and error bars are standard errors. The asterisks indicate the significance in differences between species. * means $p<0.05$, ** means $p<0.01$.

Chlorophyll *a* fluorescence of plants raised under different light intensities

The photosynthetic efficiency of fronds raised under different light intensities was measured using chlorophyll *a* fluorometry. The quantum yield of photosystem II (Y(II)) is a good indicator of the efficiency of the photosynthetic light reactions, under steady-state conditions. Y(II) depended both on the light intensity during growth, as well as the intensity of the actinic light during the measurement. In all intensities tested, Y(II) decreased when the intensity of the actinic light increased (Fig. 4). When the two species were grown at a low light intensity (6, 10 and 20 µmol·m^{-2}·s^{-1}), Y(II) decreased fast with increasing actinic light intensity during the actual measurements. Y(II) reached saturation values close to 0 at an actinic PAR intensity of 186 µmol·m^{-2}·s^{-1} . In the case of *L. minor* and *L. minuta* fronds raised under intermediate light levels, Y(II) decreased less drastically and displayed a long tail that reached saturation only at an actinic light level of 701 µmol·m^{-2}·s^{-1}. In the case of *L. minor* and *L. minuta* raised under the highest light intensities, *L. minuta* still displayed this tail of low Y(II) values, but this was not the case for *L. minor*. When the plants were grown at 6 and 10 µml·m^{-2}·s^{-1}, there was a significant interaction between species and Photosynthetic Available Radiation (PAR) ($p=0.03$). At these intensities, *L. minuta* showed a significantly higher Y(II) than *L. minor* at PAR=0 and 1. The interaction between species and PAR was also significant when the plants were grown at 1000 µmol·m^{-2}·s^{-1}. At this intensity, *L. minuta* showed slightly higher values of Y(II) than *L. minor* although this difference was not significant.

Non-photochemical quenching, (qN), increased following exposure to low and intermediate levels of actinic light and then stabilized under higher actinic light levels (Fig. 5). When the two species had been grown at high light intensities, high qN levels were already induced by relatively low levels of actinic light. However, *L. minuta* displayed a significantly lower qN than *L. minor* ($p<0.001$) when grown at 400 and 1000 µmol·m^{-2}·s^{-1}. When the two species where grown at just 6, 10 or 20 µmol·m^{-2}·s^{-1} *L. minuta* had a higher qN at every actinic light level ($p<0.001$). The results of the pairwise comparison are shown in figure 5.

The curves describing the photochemical quenching qP of the two species show a decrease in qP with increasing intensity of the actinic light during the fluorescence measurements (Fig. 6). Decreases in qP were very similar when the plants were grown at low and medium light intensities (from 6 to 250 µmol·m^{-2}·s^{-1}). Only when plants were grown at 400 and 1000 µmol·m^{-2}·s^{-1}, a significant interaction between species and PAR was found. At these light intensities, *L. minuta* maintained a significantly higher qP ($p<0.001$) than *L. minor* at actinic light intensities above 281 µmol·m^{-2}·s^{-1}. The results of the pairwise comparisons are shown in figure 6.

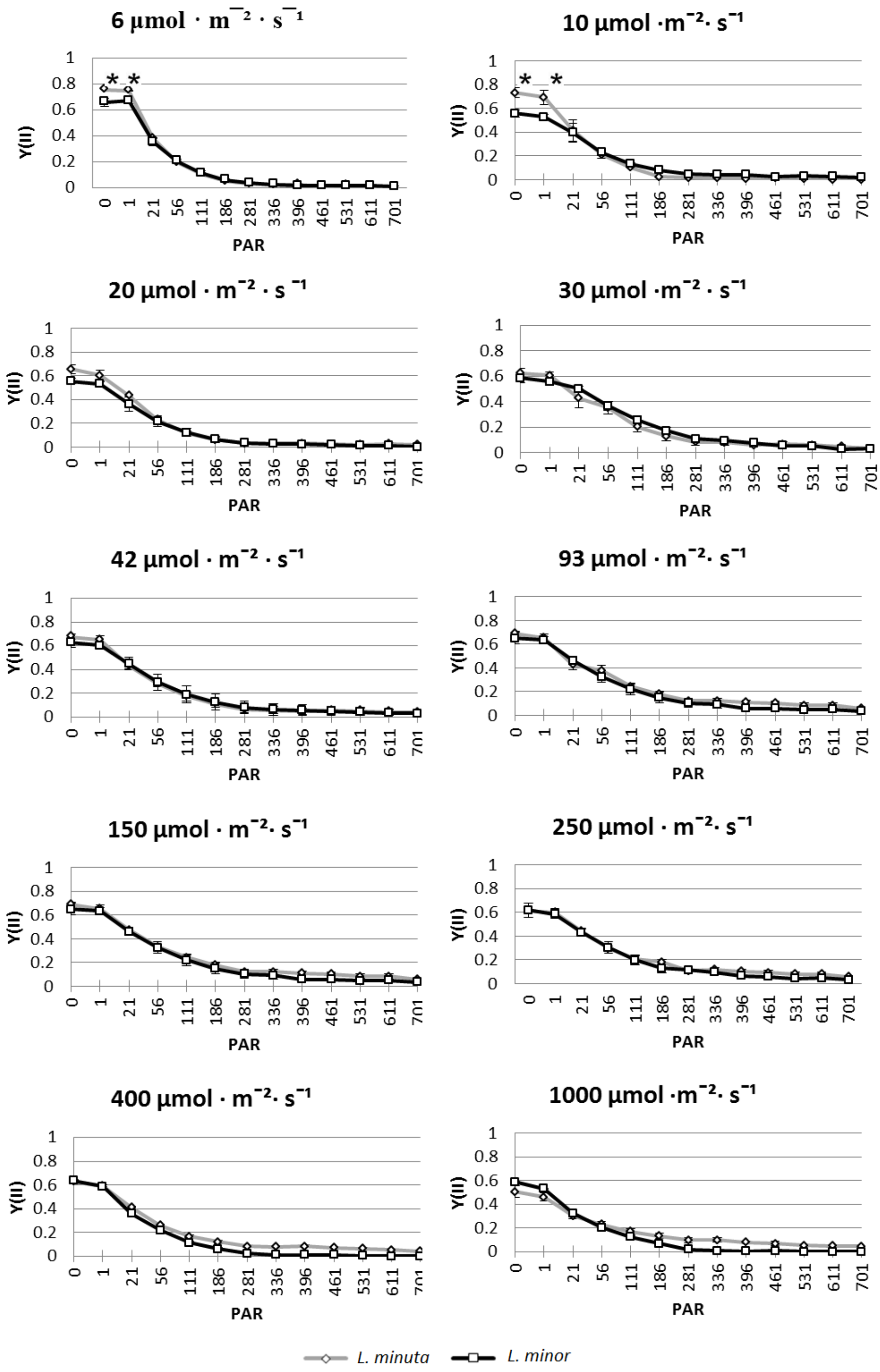

Figure 4. Quantum yield (YII) of the two species along the light intensity gradient, at different actinic light intensity. Values are mean of four replicates and error bars are standard errors. The asterisks indicate the significance in differences between species. * means $p<0.05$, ** means $p<0.01$.

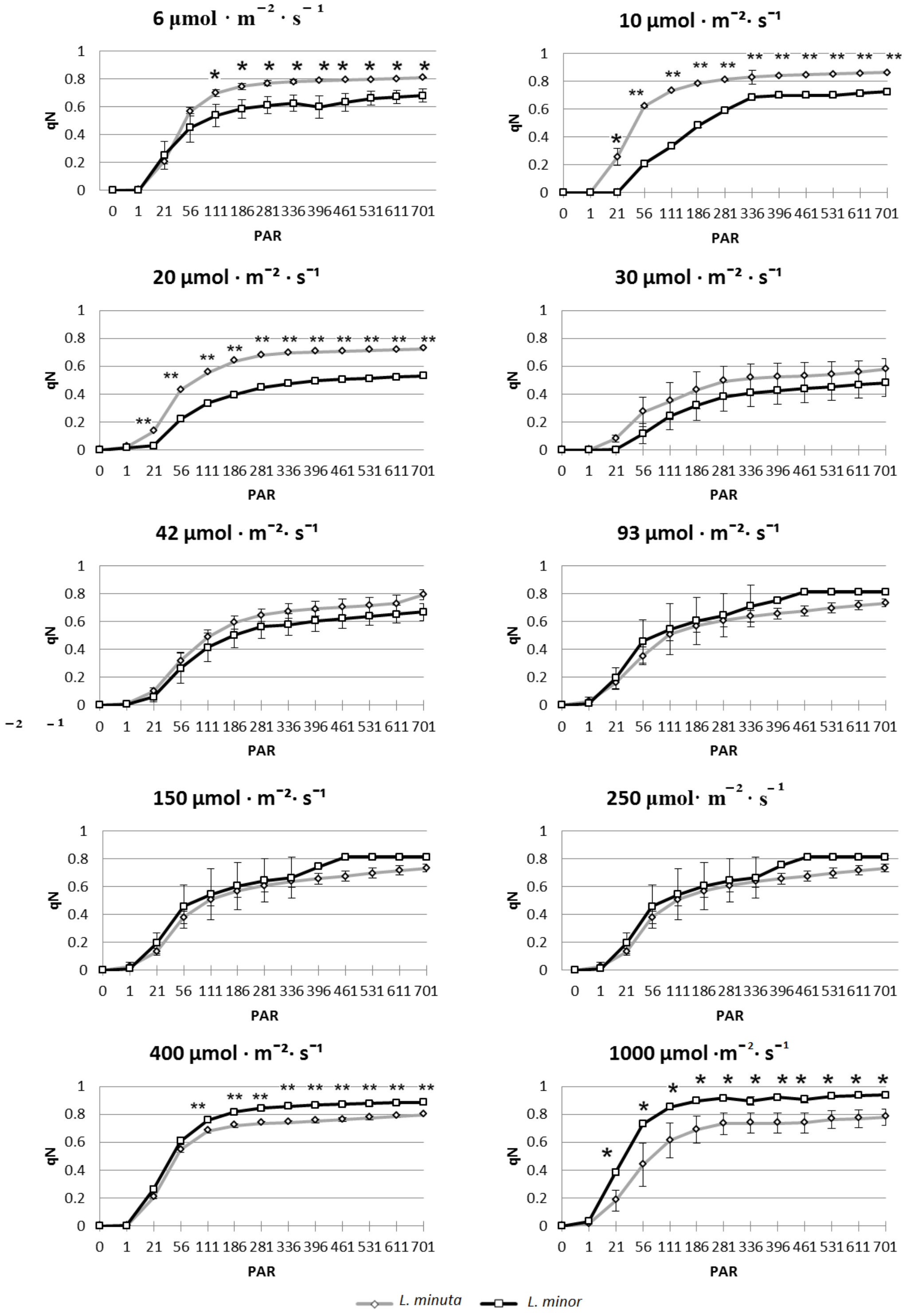

Figure 5. Non-Photochemical Quenching qN of the two species along the light intensity gradient, at different actinic light intensity. Values are mean of four replicates and error bars are standard errors. The asterisks indicate the significance in differences between species. * means $p<0.05$, ** means $p<0.01$.

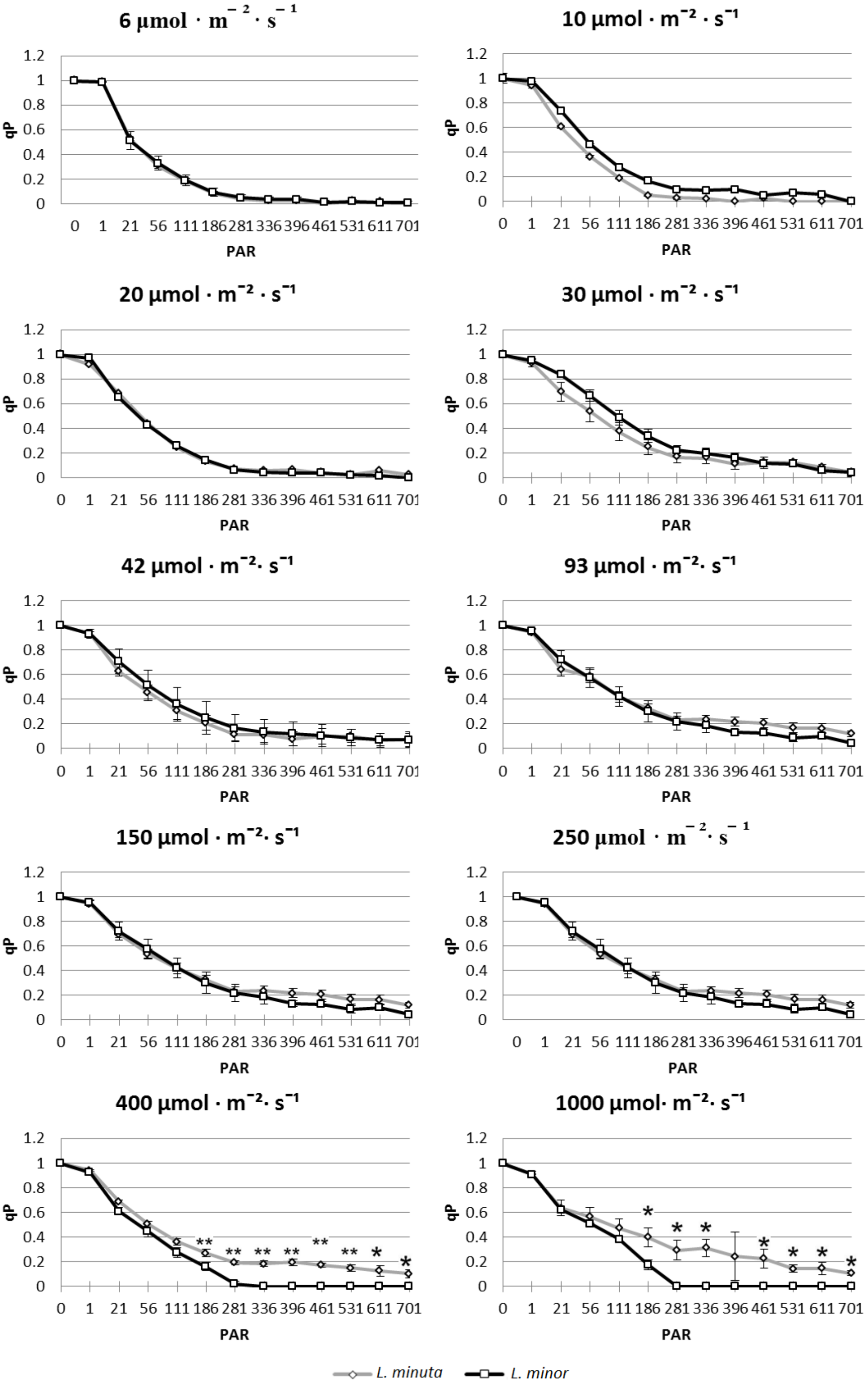

Figure 6. Photochemical Quenching qP of the two species along the light intensity gradient, at different actinic light intensity. Values are mean of four replicates and error bars are standard errors. The asterisks indicate the significance in differences between species. * means p<0.05, ** means p<0.01.

Discussion

Light is a necessity for the autotrophic growth of Lemnaceae. However, the relationship between growth and light-intensity is species, and even clone specific, while environmental factors such as temperature, nutrient and CO_2 supply can also alter this relationship (Landolt, 1986). Wedge and Burris (1982) observed that the light saturation intensity for growth of *L. minor* ranges between 300 and 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$, depending on temperature. For *L. minuta*, the only data available are those of Landolt (1986) who found that at 323 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (published as 17000 lux) light saturation was not yet achieved. In the present study it was found that at a light intensity of 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$ growth of neither *L. minuta*, nor *L. minor*, had reached full light saturation. The somewhat higher saturation light levels observed in this study, might be due to otherwise optimised growth conditions, especially for temperature and nutrient supply. Another explanation could be that the clones used in this study are different from those used by Landolt and Wedge and Burris. This explanation is confirmed by Ziegler *et al.* (2015) who showed that the RGR of Lemnaceae reflects the adaptation of individual clones to specific local conditions.

A comparison of the growth of the two *Lemna* species showed that *L. minuta* had a higher RGR than *L. minor* when grown at medium and high light intensities. In contrast, at lower light intensities, *L. minor* appeared to have a slightly (but not significantly) higher RGR than *L. minuta*. This observation suggests that *L. minor* is better adapted to shade conditions (Givnish, 1988) while *L. minuta* takes more advantage from high light intensities. This hypothesis is supported by the comparison of other parameters. LAR and NAR are often measured to analyse variations in plant growth (Lambers *et al.*, 1989; Poorter and Remkes, 1990). The NAR in this experiment increased with increasing light intensity in both species. However, at high light intensities *L. minuta* has a higher NAR than *L. minor*. This intrinsic ability to exploit high light levels is associated with a higher RGR. Light had a negative effect on LAR, which was higher in plants grown at low light and decreased in plants grown at high light. *L. minor* displayed a higher LAR in shady conditions, while, under high light conditions the two species had similar values of LAR. The latter observation indicates that *L. minor* has a higher morphological plasticity in response to changing light conditions. A more extensive leaf area represents an advantage at low light (Lusk, 2004), thus the observed morphological plasticity is likely to contribute to the slightly higher RGR of *L. minor* in shady conditions. In several studies LAR was recognised as the growth parameter that has the greatest impact on the RGR (e.g. Poorter and Remkes, 1990; Walters *et al.*, 1993; Wright and Westoby, 2000), although, in other studies, NAR was the factor most closely correlated with RGR (e.g. Shipley, 2002). Conflicting literature might depend on several factors such as the species investigated and the experimental conditions. For example Garnier (1991) found that there is a difference in the extent to which NAR impacts on the RGR between monocotyledonous and dicots. Another hypothesis proposed by Poorter (1999) is that LAR and NAR affect the RGR to different extents depending on the light intensity at which the experiment is carried out. In particular, the author hypothesized that, at low light, the scope for variation in photosynthetic activity between species is diminished and therefore LAR plays a relatively important role in determining the RGR, as was observed in this study. Vice versa, at high light intensities, NAR has a relatively greater impact on the plant growth, as was demonstrated in this study by the observed high values for NAR and RGR for *L. minuta*. This explanation is also confirmed by Shipley (2006). The author reviewed 37 studies on 614 different species finding that NAR was the best predictor of variation in RGR in herbaceous species. However, for determining RGR, the importance of NAR decreased with decreasing daily quantum input. Thus, the data in this paper reveal distinct light utilisation strategies for *L. minuta* and *L. minor*, with the latter species performing better at low light, due to its higher LAR, while the former species performs better at high light intensities due a higher NAR.

To further explore the light-intensity dependency of growth, various photosynthetic parameters were measured. This study shows an inverse correlation between light and chlorophyll content. A similar correlation has been observed in numerous studies using a broad range of species (e.g. Eilam and Klein, 1962; Minotta and Pinzauti, 1996; Cao, 2000; Dai *et al.* 2009). Indeed, plant responses to varying light intensities are commonly reported as changes in chlorophyll concentration (Strauss-Debenedetti and Bazzaz, 1991). At high light intensities the reduction in chlorophyll content is considered an acclimation to avoid light damage due to over-excitation (Havaux and Tardy, 1999), and specifically photo-oxidation (Hendry and Price, 1993). Conversely, at low light intensities, the increase in chlorophyll content helps maximise light capture (Kura-Hotta *et al*., 1987, Lei *et al*., 1996). Thus, this study shows that both species of *Lemna* display a "common" light acclimation response by adjusting chlorophyll content in response to altered light availability. The comparison of the chlorophyll content in the two species showed that *L. minor* contains more chlorophyll per unit of biomass than *L. minuta* at all light intensities. A recalculation of chlorophyll on a per leaf area basis gave similar results (data not shown). Higher chlorophyll content is usually associated with shade-tolerance (Valladares and Niinemets, 2008; Lewandowska and Jarvis, 1977; Leverenz, 1987; Thompson *et al.,* 1988; Rijkers *et al*., 2000; Cao, 2000). For example, Murchie and Horton (1997) analysed twenty-two common British angiosperms and the species most adapted to shade contained most chlorophyll when grown at low irradiance. Hence, it was concluded that the higher chlorophyll content in *L. minor* confirms its adaptation to more shady conditions.

Chlorophyll *a* fluorometry was used to explore the mechanisms underlying differences in RGR and NAR. The photosynthetic yield (Y(II)) was measured at a range of actinic light intensities and provides an indication of the photochemical efficiency of photosystem II (Maxwell and Johnson, 2000). When plants were raised under low light conditions (from 6 to 20 $\mu mol \cdot m^{-2} \cdot s^{-1}$), Y(II) displayed a rapid initial decline with increasing actinic light during the measurements. In contrast, in plants raised under intermediate and high light conditions, the decline in Y(II) with increasing actinic light occurred at higher intensities and more gradually. This suggests that plants that had acclimated to higher light levels were able to use a higher portion of the absorbed light for the photosynthetic process. The measurements of the photochemical quenching qP confirmed this ability of plants grown under high light intensities. The photochemical quenching is a measure of the fraction of PSII reaction centres that are in the open state (Krause and Weis, 1991). In this study qP already started to decrease under low intensities of actinic light when plants had been raised under low light conditions, but when plants had been raised under high light the decrease in qP occurred under slightly higher actinic light levels. The comparison of qP and Y(II) between the two species revealed a different ability to cope with both low and high actinic light levels. When fronds were raised under high light intensities *L. minuta* displayed a higher qP suggesting a higher capacity of *L. minuta*'s photosynthetic light reactions to utilise photons at the highest light intensities. This conclusion is reinforced by a slightly higher Y(II) observed in *L. minuta* grown at high light intensities even though in this case the difference between the two species was not statistically significant. The qP data concur with the higher NAR and RGR of *L. minuta* raised under high light intensities, and indicate that at least part of the capacity for growth under high light is associated with adaptive responses at the level of the photosynthetic machinery. Conversely, the data suggest that the performance of *L. minor* in the shade is more dependent on morphological (higher LAR) than on physiological (lower Y(II), qP and NAR) parameters.

Non-photochemical quenching, qN, was also analysed. This parameter refers to the portion of the energy absorbed that the plant dissipates as heat (Müller *et al*., 2001). The qN increased when plants were exposed to higher levels of actinic light during the fluorescence measurements, and it reached its maximum value at the highest actinic light intensity. Thus both species increase the extent of non-photochemical quenching when exposed to higher actinic light levels, demonstrating a capability to adjust photosynthetic performance to prevailing light conditions. A comparison of the two species showed that *L. minor* had a higher qN value than *L. minuta* when the plants were grown at high light intensities. A higher qN might be a necessity for *L. minor* as a result of its relatively high light capture caused by high chlorophyll content. The higher portion of energy dissipated in the form of heat is generally expected to be associated with decreased RGR (Laing *et al*., 1995), as was observed for *L. minor*. In contrast, *L. minuta* has a lower qN value, which is associated with both a higher qP and Y(II), and therefore ultimately a higher NAR.

The aim of this study was to determine whether light intensity is a factor enabling the invasive duckweed *L. minuta* to outperform the native *L. minor*. The results show that the invasive *species L. minuta* takes better advantage from high intensity light conditions and suggest that this species can potentially out-grow *L. minor* in such conditions. A survey of the literature yields further examples in which the native species copes better with shady conditions while the alien species is more competitive under high light conditions. For example, Madsen *et al*. (1991) studied the photosynthetic rates of seven aquatic macrophytes occurring in Lake George, New York at eight light intensities from 0 to 1000 µmol·m^{-2}·s^{-1}. The species investigated were *Elodea canadensis* Michaux, *Myriophyllum spicatum* Linnaeus, *Potamogeton amplifolius* Tuckerm, *Potamogeton gramineus* Linnaeus, *Potamogeton praelongus* Wulff, *Potamogeton robbinsii* Oakes, and *Vallisneria americana* Michaux. The results showed that changes in light availability resulted in changes in community composition. *M. spicatum* (alien) exhibited a high light requirement in contrast with various native species that exhibited shade-tolerance characteristics. Similarly, Pattison *et al*. (1998) showed that invasive species in Hawaiian rainforest outgrow native species at all tested light intensities, but that invasive species appear to be better suited than native species to high-light environments. A pertinent question is whether the strong growth performance of *L. minor* in the shade and of *L. minuta* in the light, actually leads to competitive success. Laboratory data cannot simply be extrapolated to field conditions, but rather long term mesocosm studies are required that integrate different light responses with other parameters that govern Lemnaceae growth, such as nutrient availability, temperature, wind and rain-exposure, and the presence of stress factors.

Conclusions

This study details the morphological and physiological differences between *L. minuta* and *L. minor* under different light conditions. It is concluded that distinct light utilisation strategies are adopted by the two species. *L. minuta* is a heliophile species which, when grown at high light intensities, maximises its RGR by using a large portion of available light (higher qP and Y(II), and lower qN) to optimise carbon gain (higher NAR). In contrast, native *L. minor* can be classified as sciophilous. When grown at low light intensities, *L. minor* has a higher chlorophyll content and morphological plasticity (higher LAR) that help to limit the reduction of RGR under such growth conditions.

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Chapter 4

A comparative study of the performance of *Lemna minuta* Kunth and *Lemna minor* Linnaeus in stressful environments; an alien species is not necessarily more stress-sensitive

Abstract

It is widely assumed that environmental stressors protect habitats from invasions by alien species, and that native species are better stress-tolerators. To test this assumption, the performance of the invasive alien *Lemna minuta* Kunth was tested under several environmental stressors and compared with the performance of the co-generic native *L. minor* Linnaeus. The effects of temperature and drought, important determinants of the distribution of Lemnaceae, on growth and photosynthesis were explored. Also, tolerance to, and accumulation of aluminium and copper were studied. Finally, tolerance to Reactive Oxygen Species (ROS) was compared by growing the plants at different concentrations of the ROS generator paraquat (methyl-viologen). The present study indicates that the presence of stressors does not simply impede alien invasions. Rather, specific stressors (such as low temperature in this study) can potentially hamper alien invasions. Conversely, this study shows that other stressors can potentially promote alien invasion. Here, four stressors (aluminium, copper, drought and high temperatures), that have all been linked with anthropic activity, can potentially provide a competitive advantage to *L. minuta*. Thus, the role of environmental stressors in facilitating alien invasions is multi-faceted, and stressor-specific.

Introduction

Invasive alien species comprise a major threat to biodiversity across the globe (Gaertner *et al*., 2009). These species are responsible for a decline in native species richness (Galil, 2007), they modify habitats (Didham *et al*., 2007) and can also have a serious economic impact. Pimentel *et al.* (2001) estimated that, overall, 120.000 non-native species are considered invasive in the US, Europe, Australia, South Africa, India, and South America. Many of these invasive species cause economic losses in agriculture and forestry, and it was estimated that non-native species are causing in total more than US$ 314 billion per year in damage in the countries listed above. The estimate includes losses to crops, pastures and forests due to the presence of invasive species, costs associated with livestock and human diseases deriving from invasive alien organisms and costs related with invasive species control.

Invasive, aquatic plants have disproportionate effects on specific ecosystems such as freshwater habitats (Dudgeon *et al*., 2006). The impacts (and associated economic costs) of several aquatic plant species (e.g. *Elodea canadensis* Michaux, *Eichhornia crassipes* Martius, *Ludwigia* spp., *Hydrocotyle ranunculoides* Linnaeus and *Myriophyllum aquaticum* Velloso) have increased in Europe during the last few decades (Sheppard *et al*.; 2006, Hussner, 2009). Aquatic alien species can also out-compete native species leading to biodiversity loss (Stiers *et al*., 2011). Moreover, physico-chemical characteristics of freshwater environments can be altered by invasive, alien macrophytes which may, indirectly result in a further negative impact on native plants, fish and macroinvertebrate communities. For example, when invasive macrophytes form dense floating mats, these species may compromise the natural exchange of oxygen between atmosphere and water column (Masifwa *et al*., 2001; Troutman *et al*., 2007; Villamagna and Murphy, 2010). The majority of aquatic species, both animals and plants, are sensitive to low oxygen concentrations which may cause hypoxia stress. Dense mats of floating plants also shade the submerged environment, limiting algal growth and consequently affecting the aquatic community and food web structure even more (Villamagna and Murphy, 2010).

Aquatic alien species can also impact on human activities such as fisheries, navigation and water-based leisure pursuits (Caffrey, 1993; Caffrey *et al*., 2010).

The dispersal of viable propagules of an alien species in a particular ecosystem does not necessarily result in an actual invasion. Rather, the invasion of particular ecosystems is a consequence of both ecosystem and plant characteristics (Alpert *et al*., 2000). In order to predict the invasiveness of a particular species, plant growth strategies in particular have been scrutinised. Grime (1974) identified three major growth strategies adopted by plants, and used these to divide plant species into three categories: competitive, stress-tolerant and ruderal species. A number of sub-categories cater for plants with intermediate characteristics. Ruderal species are abundant in severely disturbed but potentially productive environments. Competitive species are particularly abundant in environments characterised by low stress and low disturbance. In contrast, stress-tolerant species are more successful in environments in which one or more stressors limit plant growth (Grime, 1974; Alpert *et al*., 2000). Species following a competitive/ruderal strategy are often early successional, short lived, lack mechanical structures, display a high Relative Growth Rate (RGR), and respond strongly to environmental change. Conversely, species following a competitive/stress tolerant strategy are often late successional, long lived, possess mechanical structures, display a low RGR, and do not respond strongly to environmental change (Bussotti, 2008). Thus, knowledge of the growth strategy of a species, not only helps to understand its distribution, but can also help to estimate the invasiveness of a species in a particular habitat. For example, Alpert *et al*. (2000) associated invasive species with ruderal traits. Conversely, the presence of specific stressors in the environment is often associated with low levels of invasibility as invasive species are considered less tolerant to stress than native species (Tillman, 1997; Davis *et al*., 2000; Davis *et al*., 2005, Mac Dougall *et al*., 2006).

Lemna minuta Kunth is a floating freshwater Lemnaceae that originates in temperate areas of North and South America (Stace, 2010). In Europe, the species was first recorded in France in 1965 (Jovet and Jovet-Ast, 1966). Since then, it has progressively spread around Europe becoming naturalised in several countries such as the United Kingdom (Bramley *et al*., 1995), Ireland (Cotton, 1999), Italy (Conti *et al*., 2005), Poland (Wójciak and Urban, 2009), Malta (Misfud, 2010), Germany (Hussner *et al*., 2010), Belgium (Halford *et al*., 2011), and Hungary (Lukács *et al*., 2014). A pertinent question concerns the traits that make *L. minuta* so successful as an invader in Europe. Here, it was used a comparative approach to assess the traits of alien *L. minuta* relative to those of native *L. minor*, a species which commonly shares the same habitat. Such a comparative approach has been used successfully in the past to identify the traits that make some alien species so successful (Mack, 1996). For example, Grotkopp *et al.* (2002) compared 29 species of the genus *Pinus* including species that are either non-invasive or invasive in the Southern Hemisphere. This comparison allowed the authors to identify a difference in Relative Growth Rate (RGR) as the main reason that some species are more invasive than others. This approach is even more effective if the comparison involves co-generic species as the identification of the differences between two closely related, similar species is easier and it is highly likely that observed differences impact on the invasiveness (Daehler, 2003; Lloret *et al*., 2005).

In this study the effect of several stressors on the physiology and morphology of *L. minor* and *L. minuta* was analysed. Plants were exposed to both chemical and physical stressors in the present study. Drought stress tolerance was tested as it is considered an important determinant of the distribution of Lemnaceae (Crawford *et al*., 2006) and also as it plays a role in duckweed dispersal (Coughlan *et al*., 2015). Growth performance of the two species at either low or high temperatures was analysed as this is another major determinant of both distribution as well as competitive abilities (Crawford *et al*., 2006). Lemnaceae have been extensively demonstrated to be strong accumulators of various metals (Axtell *et al*., 2003; Kanoun-Boule *et al.*, 2009). Such metals can have an adverse effect on aquatic animals and plants (Malik, 2004). Therefore, copper and aluminium were tested as stressors. Finally, as the production of Reactive Oxygen Species (ROS) is a common factor in virtually all types of plant stress, the impact of the ROS generator paraquat (methyl-viologen) (Blackburn and Weldon, 1965) was explored on both species of Lemnaceae.

The aim of this study was to assess the growth strategies of native *Lemna minor* and alien *L. minuta*. Previously, it was observed that *L. minuta* displayed characteristics of a ruderal species, such as a high growth rate, and an ability to take advantage of high resource availability (Njambuya *et al.*, 2011; Paolacci *et al*., 2016). For this reason, it was hypothesized that *L. minuta* is less efficient at tolerating stressors than the native

L. minor, and this was tested by assessing growth following exposure to a range of abiotic stressors.

Material and methods

Plant growth

L. minor and *L. minuta* strains were collected from the same pond in Blarney, Co. Cork, Ireland (51.940476, -8.563637). The *L. minor* strain has since been registered in the RDSC database as strain number 5500 "Blarney". The plant material used for all the experiments was cultured under sterile conditions, in glass flasks, on 100 ml of half-strength Hutner's nutrient solution (Hutner, 1953). Plants were kept in a growth room at a constant temperature of 20°C and exposed to a light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$, (cool-white fluorescent tubes) with a light: dark cycle of 16: 8 hours.

All the growth experiments lasted just one week in order to avoid nutrient depletion to affect the results. All the experiments, except the drought exposure experiment, started with nine fronds (3 colonies) of each species (4.62 mg fresh weight on average for *L. minuta* and 11.32 mg for *L. minor*) and were replicated four times.

Drought exposure experiment

L. minuta and *L. minor* desiccation rate, survival rate and ability to recover after drought stress were tested by removing fronds of these two species out of the growth medium and exposing them in a growth room at a constant temperature of 20°C, exposed to a light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and relative humidity of 35% for 10, 20, 30, 40 or 50 minutes.

Ten fronds of *L. minuta* (5 mg on average) and ten fronds of *L. minor* (12 mg on average) were placed in ten Petri dishes on a layer of dry cellulose filter paper (thickness 180µm, pore size 11µm). Every ten minutes fronds where withdrawn from two Petri dishes and weighed in order to measure the loss of weight due to desiccation. The experiment was replicated four times.

The same experimental design was used to determine the survival rate and the ability to restart growth after drought exposure. After exposure to desiccation, fronds were placed in flasks containing 100 ml of growth medium inside a growth room. After one week the green fronds and the fronds that had completely lost their pigments (becoming white) were counted. The white fronds were considered dead and were deducted from the number of fronds initially placed in the medium (ten fronds) to calculate the survival rate. The difference between the initial number of fronds and the white fronds was used to calculate the Relative Growth Rate of the surviving fronds (see paragraph ‘parameters analysed’).

Temperature experiment

The effect of the temperature on the growth of the species was assessed by keeping the medium at temperatures ranging between 0 and 35°C (0, 10, 15, 20, 25, 30, and 35°C). The temperature of the medium under normal growth conditions was 18-20°C. For the lower temperature treatments the flasks containing the plants were placed on a cold plate connected to a thermostat. To test the growth of the plants at temperatures higher than 18°C the flasks were placed in a warm water bath.

Aluminium and Copper exposure experiments

The toxic effect of aluminium on the plants was analysed by growing the two species in 100 ml of growth medium with added $Al_2(SO_4)_3 \cdot H_2O$. Six different concentrations of aluminium were used, ranging between 0.08 to 3 $ng[Al] \cdot l^{-1}$. The standard growth medium does not contain any aluminium and was used as a control. The addition of $Al_2(SO_4)_3 \cdot H_2O$ resulted in an increase in sulfur in the medium, which constituted only 0.3% at the lowest aluminium concentration tested and 30% at 0.08 $ng[Al] \cdot l^{-1}$. At the highest concentration tested (30 $ng[Al] \cdot l^{-1}$) the increase of sulfur in the medium was 100%.

The toxic effect of copper on the plants was analysed by growing the two species in 100 ml of growth medium containing added $CuSO_4 \cdot 5H_2O$ at five different concentrations of copper ranging between 0.01 and 2 $mg \cdot l^{-1}$. The standard medium, used as a control, contains $CuSO_4 \cdot 5H_2O$ at the concentration of 0.03 $mg\ [Cu] \cdot l^{-1}$. The addition of $CuSO_4 \cdot 5H_2O$ resulted in a small increase in sulfur in the medium, which constituted 4.2% at the highest concentration of copper tested (2 $mg \cdot l^{-1}$).

The experiments were separately repeated in order to obtain fronds to be used for the analysis of plant metal accumulation. The plant metal content was measured at three relevant concentrations of aluminium and copper. In order to determine the amount of the metals accumulated in the fronds, the samples (2-3 g fresh weight) were dried at 80 °C (Cedergreen *et al.*, 2007) up to constant weight and transferred to a 100 ml glass digestion tube. Concentrated analytical grade nitric acid (5 ml) was added to the digestion tube. The sample was digested at 125°C for 2 hours in a TECATOR 2040 digester. After cooling the fully digested sample was transfer to a 25 ml volumetric flask and made up to volume with deionised water. Samples were then analysed with the flame method using a Varian SpectrAA 300 Atomic Absorption Spectrophotometer.

Paraquat exposure experiment

In order to assess the toxicity of paraquat on duckweed growth, the compound was added to the standard growth medium. Five different concentrations of paraquat were tested ranging between 0.005 to 1 $mg \cdot l^{-1}$.

Parameters analysed

Relative Growth Rate

After one week of growth, plants were harvested, fronds were counted, and the fresh biomass was determined. The Relative Growth Rate (RGR) based on biomass and number of fronds was calculated using the formula by Connolly and Wayne (1996):

$$RGR = \ln(Yf / Yi) / t$$

where Yi is the initial biomass or the initial number of fronds, Yf is the final biomass or final number of fronds, t is the time in days and ln is the natural logarithm. In the present work the word biomass is used to indicate organic matter derived from living, or recently living plants.

Chlorophyll content

The chlorophyll content was determined according to Inskeep and Bloom (1985). In short, the biomass was suspended in N,N-dimethylformamide, the absorbance was measured using a spectrophotometer Thermo, model Genesys 10-S and the total chlorophyll content was calculated using the formula:

$$\text{Tot. Chlorophyll} = 17.90 \cdot A647 + 8.08 \cdot A665$$

where A647 and A665 are, respectively, the absorbance at the wavelengths of 647 and 665nm.

Chlorophyll *a* fluorescence analysis

Before determination of the biomass, photosynthetic characteristics of fronds grown under the different stressors were analysed using pulse amplitude modulated chlorophyll *a* fluorometry (Schreiber, 1986) (WALZ Imaging fluorometer, Effeltrich, Germany). The relative variable fluorescence (Fv/Fm) was measured on dark adapted plants. The steady state yield (YII), photochemical quenching (qP), and non-photochemical quenching (qN) were measured following exposure to different actinic light intensities, ranging between 0 and 701 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

Statistical analysis

The statistical analysis of the data was conducted using IBM- SPSS statistic data editor. A two-way ANOVA was conducted in order to examine the differences between the two species in RGR, and chlorophyll content. The differences in Y(II), qP and qN at different actinic light and between species were analysed using a 2-way repeated measures ANOVA. Data presented are mean ± standard error. Residual analysis was performed to test for the assumptions of the tests run. Outliers were assessed by inspection of a boxplot, normality was assessed using Shapiro-Wilk's normality test for each cell of the design and homogeneity of variances was assessed by Levene's test. When a statistically significant interaction between species and treatments was found, an analysis of simple main effects was performed with statistical significance receiving a Bonferroni adjustment.

Results

Drought exposure experiment

The fronds that had been exposed to desiccation were weighed in order to assess the water loss. The weight of both species decreased proportionally with increasing desiccation time. For *L. minuta* the weight reduction ranged between 45±5.4 and 90.47±1.1% following 10 and 50 minutes desiccation, respectively. In the case of *L. minor* the weight reduction was less, and ranged between 27.9±3.9 and 86.98 ± 2.2% following 10 and 50 minutes, respectively (overall difference in loss of weight between species $p<0.01$) (Fig.1 *a*). The fronds were moved back to the medium following the desiccation treatments and left to grow for a week on half-strength Hutner's medium. The survival rate was inversely proportional to the desiccation time, ranging between 100% of survival for both species after only 10 minutes spent out of the medium, to 44.7 ± 1.9% of survival in *L. minuta* and 50.4±2.2% in *L. minor* after 50 minutes out of the medium (Fig.1 *b*). The RGR calculated on the basis of the number of surviving fronds was also assessed. Following exposure to desiccation, both species showed a significantly reduced RGR ($p<0.001$) when compared with the RGR of the non-desiccated control (0.23±0.06 day^{-1} for *L. minuta* and 0.198±day^{-1} per *L. minor*). The decrease in RGR was greater as the desiccation time increased. The fronds that spent only 10 minutes out of the medium showed a RGR of 0.16±0.007 day^{-1} and 0.14±0.004 day^{-1} for *L. minuta* and *L. minor,* respectively. The fronds that spent 40 minutes out of the medium showed a RGR of 0.06±0.008 day^{-1} and 0.03±0.01 day^{-1} in *L. minuta* and *L. minor,* respectively. Fronds exposed to desiccation for 50 minutes did not show any growth after a week, irrespective of the species.

Despite *L. minor* showing a lower weight loss than *L. minuta* after the desiccation treatments, the relative decrease of the RGR of the two species was not significantly different when the plants were moved back in the medium after spending from 10 to 30 minutes out of the medium. On the contrary, when the plants were exposed to desiccation for 40 minutes, *L. minuta* showed a slightly higher RGR ($p=0.03$) than *L. minor* (Fig. 1 *c*).

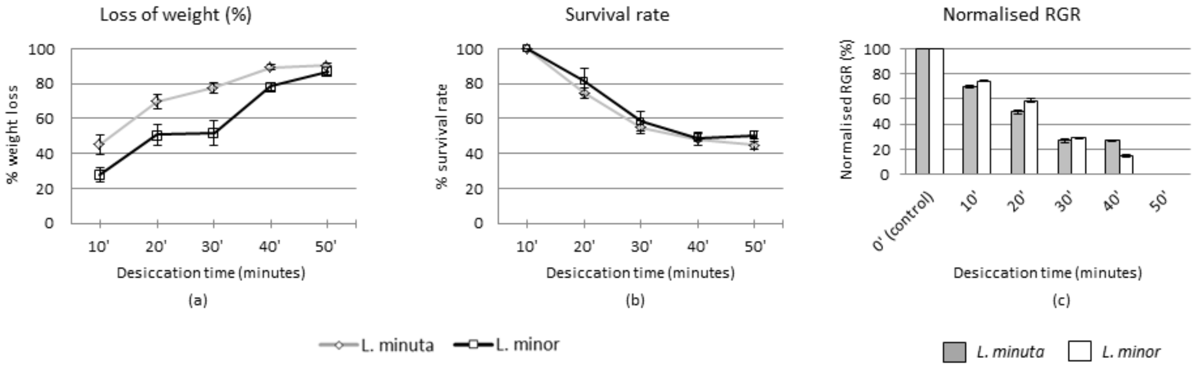

Figure 1. Loss of weight (%) (a), Survival Rate (%) (b) and normalised RGR (c) of *L. minuta* and *L. minor* after having been exposed to desiccation for 10, 20, 30, 40 or 50 minutes. Actual growth rates were normalised versus control growth rates which were 0.235 and 0.198 day $^{-1}$ for *L. minuta* and *L. minor*, respectively. Values are mean of 4 replicates and bars are standard errors.

Temperature exposure experiment

RGR

Plants were grown at different temperatures in order to compare tolerance to low and high temperatures. Analysis of the growth rate showed that the highest RGR was reached by the two species at 20 °C where the RGR of *L. minuta* was 0.2±0.01 day $^{-1}$ and the RGR for *L. minor* was 0.16±0.01 day $^{-1}$. Lowest growth rates were measured when plants were grown at the extreme temperatures of 0°C and 35°C (Fig. 2). The reduction of RGR was more gradual towards low temperatures (0, 10 and 15°C) than high temperatures (35°C). The comparison of the growth rates of the two Lemnaceae showed that at temperatures equal and higher than 20°C *L. minuta* outgrew *L. minor* (overall $p<0.01$), while at low temperatures (15, 10 and 0°C) *L. minor* grew faster than *L. minuta* (overall $p<0.01$). In particular, when the plants were grown at 0°C, The RGR of *L. minor* was twice as high as the RGR of *L. minuta* (0.026±0.005 day $^{-1}$ for *L. minuta* and 0.059±0.001 day $^{-1}$ for *L. minor*).

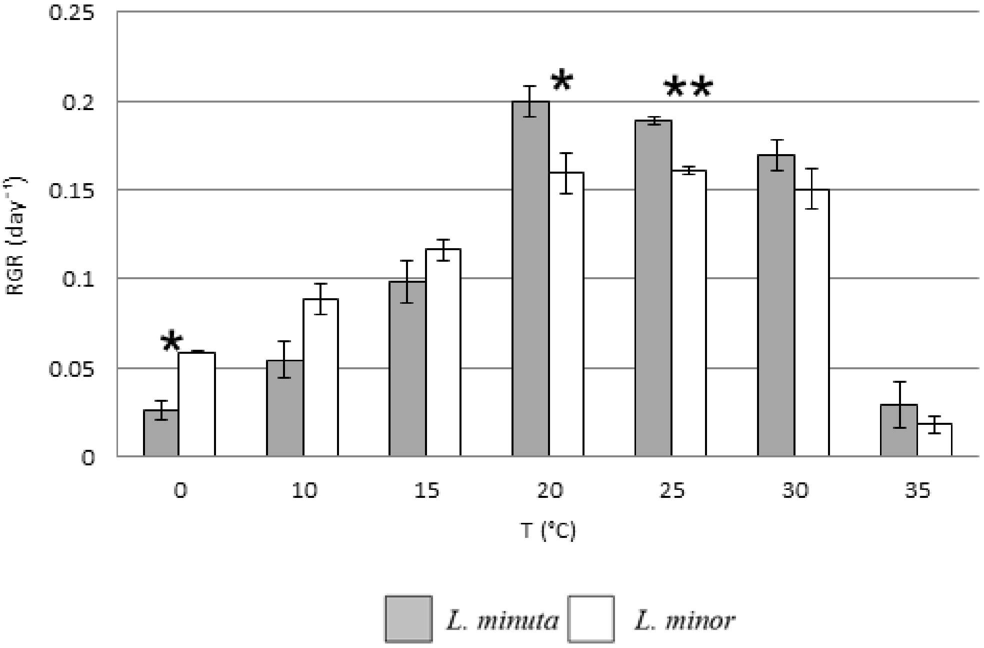

Figure 2. Relative Growth Rate based on the increase in biomass of the two species maintained at different Temperatures. Values are mean of 4 replicates and error bars are standard errors. The asterisks indicate significant differences between the two species. * means $p<0.05$, ** means $p<0.01$.

Chlorophyll content

Frond total chlorophyll content was also analysed for the two species grown at different temperatures. The maximum chlorophyll content was observed for *L. minuta* at 25°C (0.708±0.051 $mg \cdot g^{-1}$) and for *L. minor*, at 20°C (0.873±0.051 $mg \cdot g^{-1}$). As observed for the RGR, the lowest chlorophyll contents were reached at the lowest and highest temperature tested (Fig. 3). At 0°C the reduction in chlorophyll content in the fronds was substantially more severe in *L. minuta*. *L. minor* contained in general more chlorophyll than *L. minuta*, both at low and high temperatures (overall $p<0.01$). This difference was significant at 20°C ($p=0.021$), at 10°C ($p=0.015$) and at 0°C ($p<0.01$).

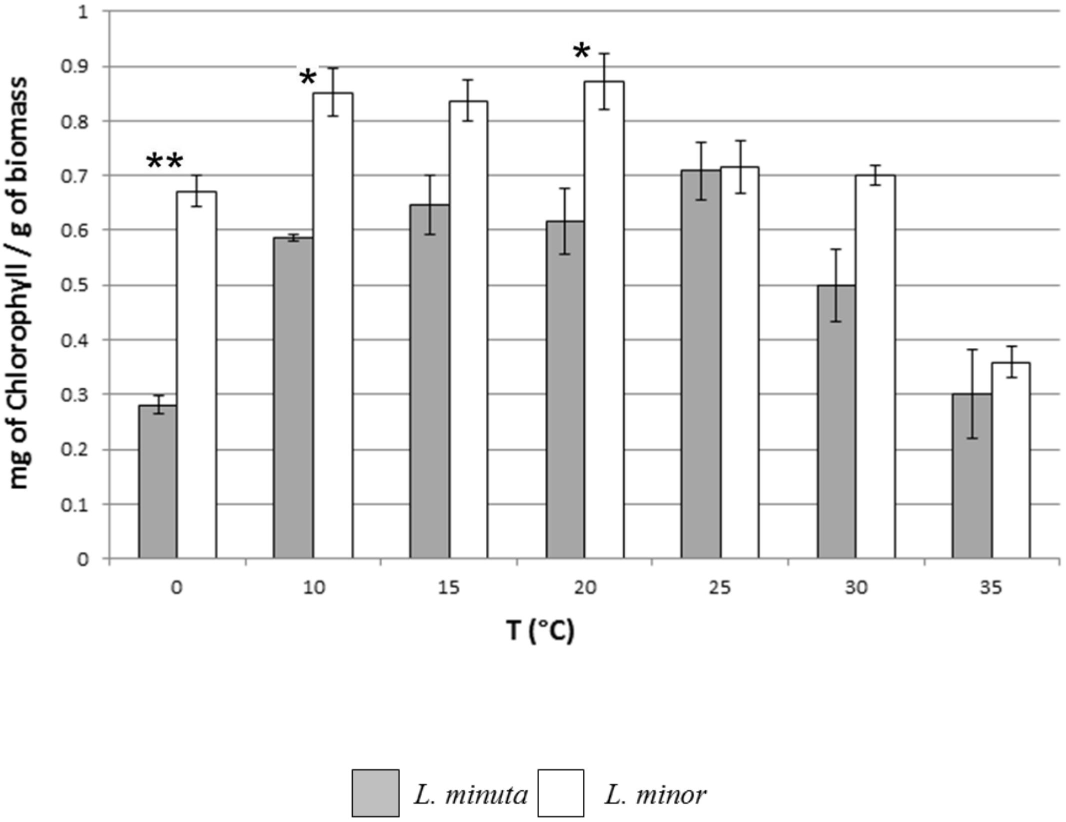

Figure 3. Chlorophyll content of the two species grown at different temperatures. Values are mean of 4 replicates and error bars are standard errors. The stars indicate the significance in differences between species. * means p<0.05, ** means p<0.01.

Chlorophyll *a* Fluorescence analysis

The fluorescence parameters Fv/Fm, Y(II), qN and qP were measured at room temperature on plants grown at 0, 10, 20 and 30°C. The maximal photosynthetic efficiency of PSII (Fv/Fm) decreased with decreasing the temperature. This parameter ranged in *L. minuta* between 0.22±0.002 (when grown at 0°C) and 0.68±0.01 (when grown at 30°C) and, in *L. minor*, between 0.37±0.007 (when grown at 0°C) and 0.58±0.01 (when grown at 30°C). At the lowest temperature tested *L. minuta* showed a significantly lower Fv/Fm (p<0.01) than *L.minor*. At this temperature, *L. minor* maintained a higher Y(II) at low intensities of actinic light (up to 111 $\mu mol\cdot m^{-2}\cdot s^{-1}$) (Fig. 4 *a*). Also qP was higher in plants of *L. minor* grown at this low temperature.

When the two species were grown at 10 and 20°C there were no significant differences in Y(II), qN and qP (Fig. 4 *b*, *c*, *f*, *g*, *j*, *k*) across the various actinic light intensities.

In plants grown at 30°C *L. minor* displayed a higher Fv/Fm ($p<0.01$) (Fig. 4 *d*) and a higher qN ($p<0.01$) (Fig. 4 *h*) while the two species showed a very similar qP at this temperature (Fig. 4 *l*), and across all actinic light intensities.

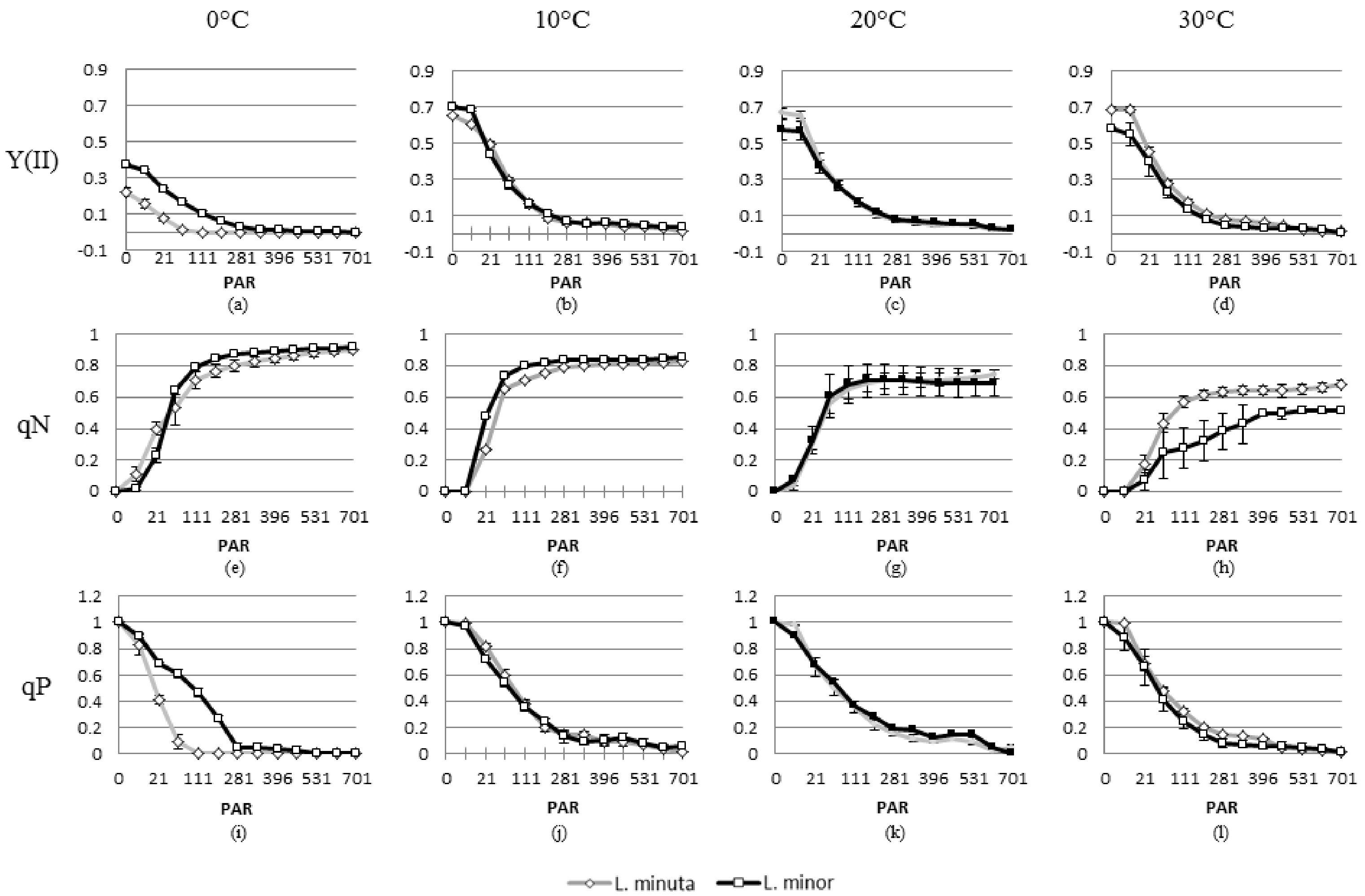

Figure 4. Quantum yield (YII) (*a*, *b*, *c*, *d*), Not-Photochemical Quenching qN (*e*, *f*, *g*, *h*) and Photochemical Quenching qP (*i*, *j*, *k*, *l*) of the two species along the temperature gradient, at different actinic light intensity. Values are mean of four replicates and error bars are standard error

Aluminium exposure experiment

RGR based on biomass

The RGR of the two species did not vary significantly when fronds were exposed to low concentrations of aluminium (0.08, 0.3, 0.8 ng[Al]·l^{-1}). However, RGR dropped substantially when fronds were exposed to 3 and 8 ng[Al]·l^{-1} (In *L. minuta* 0.128±0.014 and 0.126±0.014 day^{-1}, respectively and in *L. minor* 0.124±0.019 and 0.090±0.019 day^{-1}, respectively). The lowest growth rate was observed at a concentration of 30 ng[Al]·l^{-1} (0.052±0.016 day^{-1} in *L. minuta* and 0.041±0.002 in *L. minor*) (Fig. 5). There were no significant differences between the RGR of the two species at any aluminium concentration.

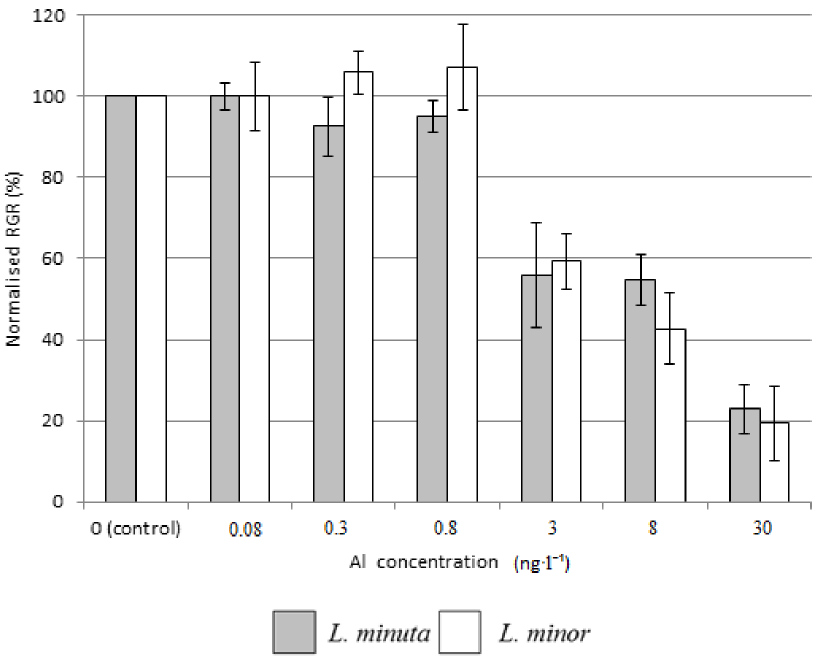

Figure 5. Normalised RGR of *L. minuta* and *L. minor* at different concentrations of Al added to the medium. (100% = RGR of the control, 0.227 and 0.207 day^{-1} respectively for *L. minuta* and *L. minor*). Error bars are standard errors.

Chlorophyll content

The total chlorophyll content was measured in the fronds of *L. minuta* and *L. minor* exposed to various concentration of aluminium. In both species chlorophyll content decreased along the aluminium gradient to reach a minimum at 30 ng[Al]·l^{-1} (0.003±0.002 mg·g^{-1} in *L. minuta* and 0.008±0.002 mg·g^{-1} in *L. minor*) (Fig. 6). At the highest concentration of aluminium the fronds showed advanced symptoms of chlorosis. There were no significant differences in chlorophyll content between *L. minuta* and *L. minor*.

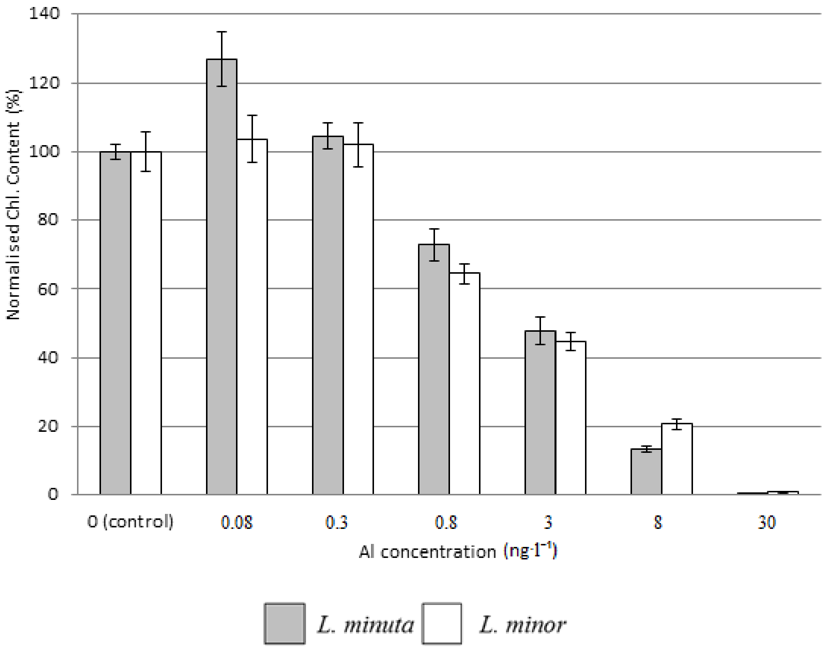

Figure 6. Normalised Chlorophyll content of *L. minuta* and *L. minor* grown with different concentrations of Al added to the medium. (100% = chlorophyll content of the control 0.772 and 0.861 mg·g^{-1} respectively for *L. minuta* and *L. minor*). Error bars are standard errors.

Chlorophyll *a* Fluorescence analysis

The fluorescence parameters Fv/Fm, Y(II), qN and qP were measured at four different concentrations of aluminium (0, 0.8, 8 and 30 ng[Al]·l^{-1}). The maximal photosynthetic efficiency of PSII (Fv/Fm) decreased with increasing aluminium concentration. This parameter ranged in *L. minuta* between 0.76±0.03 (in the control) and 0.507±0.013 (at 30 ng[Al]·l^{-1}) and, in *L. minor*, between 0.78±0.003 (in the control) and 0.55±0.014 (at 30 ng[Al]·l^{-1}). Differences between species were not significant. The Quantum Yield of PSII was measured under a range of actinic light intensities. Typically, Y(II) was very similar in the two species across different aluminium treatments, and at different actinic light intensities (Fig. 7 *a*,*b*, *c*, *d*).

Non-photochemical quenching, qN, ranged in *L. minuta*, between 0.9±0.017 (in the control) and 0.81± 0.011 (at 30 ng[Al]$\cdot$l^{-1}) and, in *L. minor*, between 0.91±0.003 (in the control) and 0.733±0.016 (at 30 ng[Al]$\cdot$l^{-1}). The qN of *L. minuta*, at 0.8 ng[Al]$\cdot$l^{-1} was lower than *L. minor* ($p<0.01$) (Fig. 7 *f*). When the photochemical quenching, qP, was observed in plants grown in medium containing 8 or 30 ng[Al]$\cdot$l^{-1}, the two species showed very similar values across all actinic light intensities (Fig. 7 *i*, *j*, *k*, *l*).

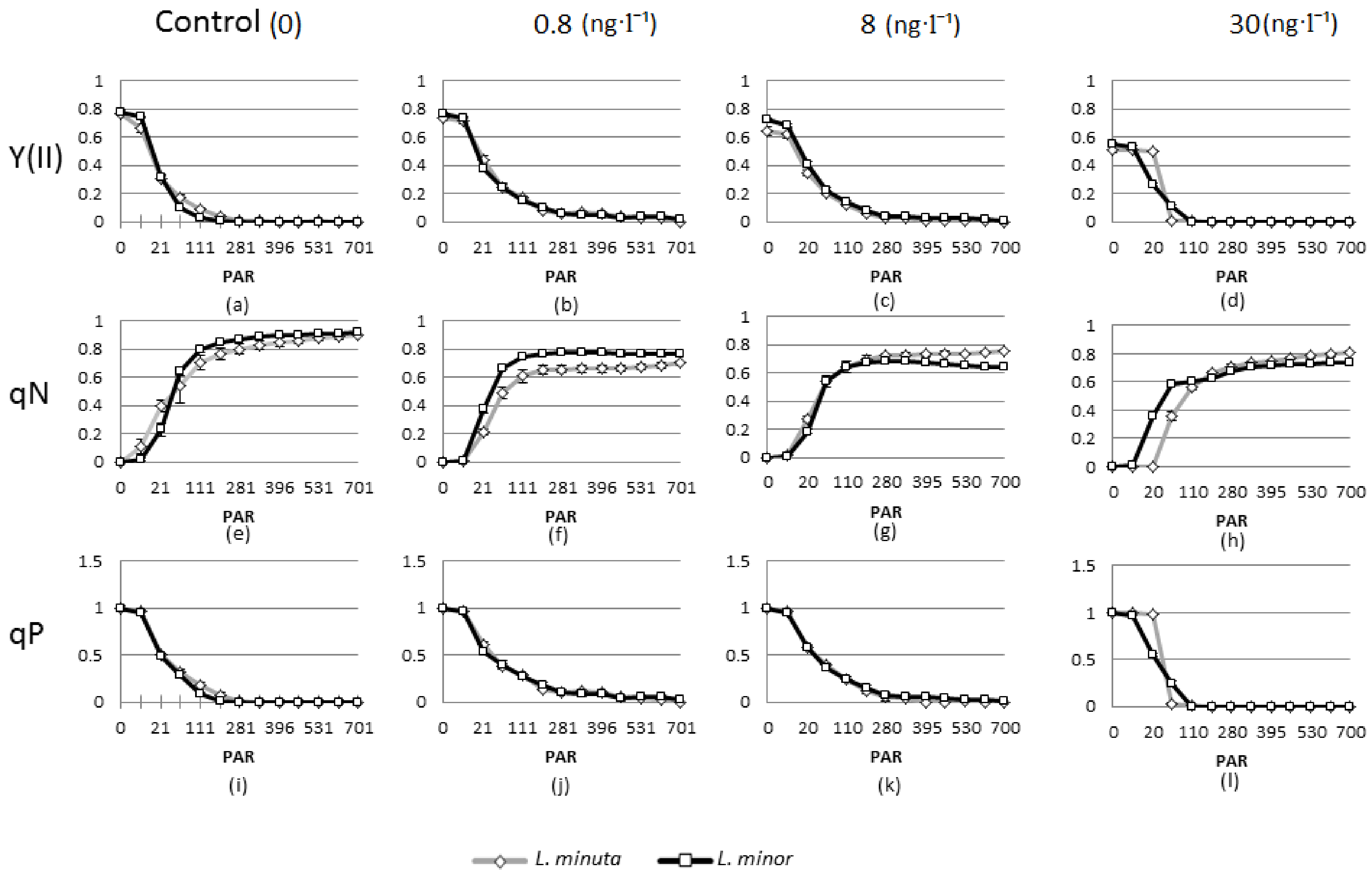

Figure 7. Quantum yield (YII) (*a*, *b*, *c*,*d*), Non-Photochemical Quenching qN (*e*, *f*, *g*, *h*) and Photochemical Quenching qP (*i*,*j*, *k*, *l*) of the two species along the Al gradient, at different actinic light intensities. Values are mean of four replicates and error bars are standard errors.

Aluminium content of the fronds

The aluminium content of the fronds was quantified. When the medium contained 0.08 ng[Al]·l^{-1}, *L. minuta* and *L. minor* contained, respectively, 305±128.67 and 360±54.08 mg [Al] per kg of dry biomass. The Al content in the fronds increased when fronds had been exposed to 8 ng[Al]·l^{-1} in the medium, and reached its maximum at 30 ng[Al]·l^{-1} (6475±257 mg·kg^{-1} in *L. minuta* and 3592.5±254 mg·kg^{-1} in *L. minor*) (Fig. 8). *L. minuta* accumulated more aluminium than *L. minor* at 8 and 30 ng[Al]·l^{-1}, but only at the highest concentration was this difference statistically significant (p<0.01).

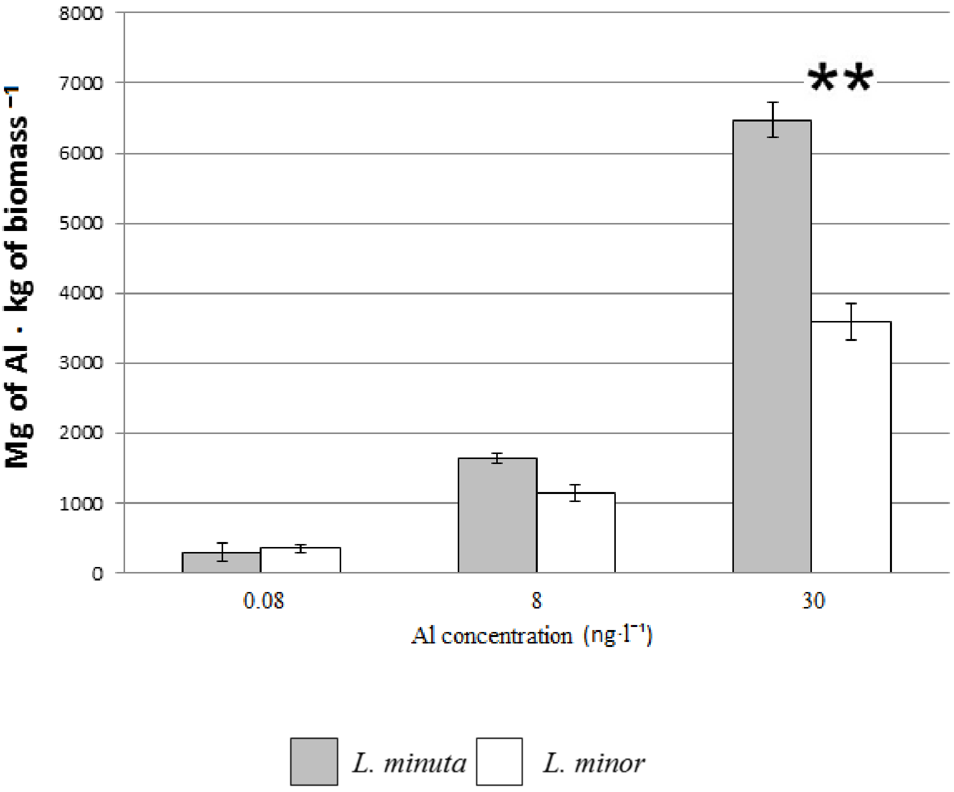

Figure 8. Aluminium accumulated in the fronds of the two species grown at different concentrations of Al in the medium, and normalised versus dry weight. Values are mean of 3 replicates and error bars are standard errors. The stars indicate the significance in differences between species. * means p<0.05, ** means p<0.01.

Copper exposure experiment

RGR based on biomass

When the two species were grown with different concentrations of copper in the medium, their RGR decreased with increasing metal concentration. At 0.01 mg·l^{-1} [Cu], the RGR of both species was only slightly lower than in the control, but, at 0.4 mg·l^{-1} [Cu], the RGR dropped to 0.058±0.008 day^{-1} in *L. minuta* and to 0.046±0.009 day^{-1} in *L. minor*. At the highest concentration (2 mg·l^{-1} [Cu]) the growth was nearly completely inhibited (RGR= 0.001±0.001 in *L. minuta* and 0±0 in *L. minor*). When the RGR of the two species was compered at different copper concentrations, *L. minuta* mostly displayed the smallest decrease of RGR (Fig. 9). The difference was statistically significant at 0.8 (p=0.2) and 1.5 (p=0.4) mg·l^{-1} of copper.

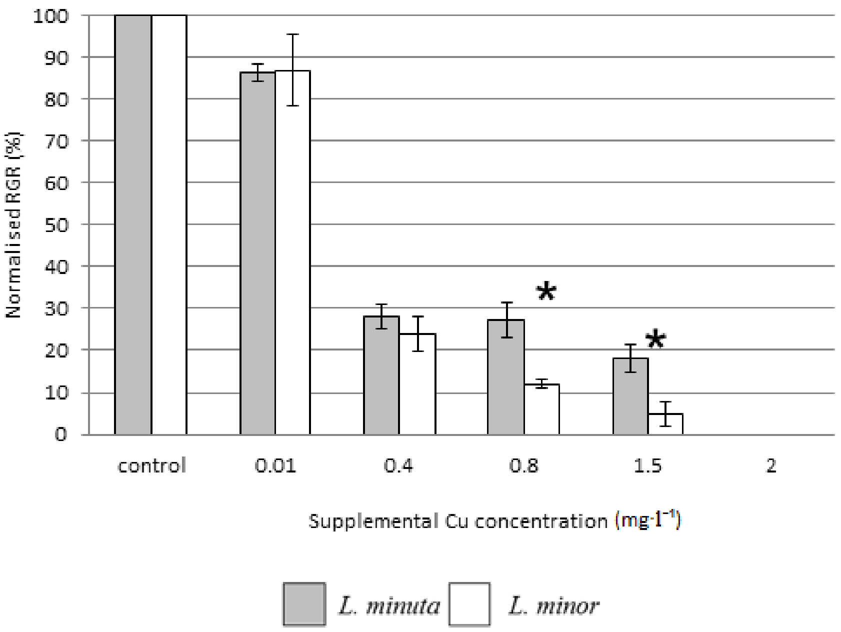

Figure 9. Normalised RGR of *L. minuta* and *L. minor* at different concentrations of Cu provided (100% = RGR of the control, values were 0.204 and 0.189 day^{-1} for *L. minuta* and *L. minor* respectively). Cu concentration in the control = 0.008 mg·l^{-1}. Error bars are standard errors. The asterisks indicate significant differences between species ($p<0.05$).

Chlorophyll content

The frond total chlorophyll content of the two species followed a trend similar to the one observed for the RGR. A notable drop in chlorophyll content was observed at 0.4 mg·l^{-1} [Cu] (0.7±0.173 mg·g^{-1} in *L. minuta* and 0.714±0.288 mg·g^{-1} in *L. minor*). At 1.5 mg·l^{-1} [Cu] a further drop in the chlorophyll content of the two species was observed (0.422±0.078 mg·g^{-1} in *L. minuta* and 0.338±0.084 mg·g^{-1}in *L. minor*). At 2 mg·l^{-1} [Cu] the fronds of both species were in advanced stage of chlorosis and the chlorophyll content was too low to be detected. The comparison of the rate of decrease in chlorophyll content in the two species was similar across all copper concentrations (Fig.10).

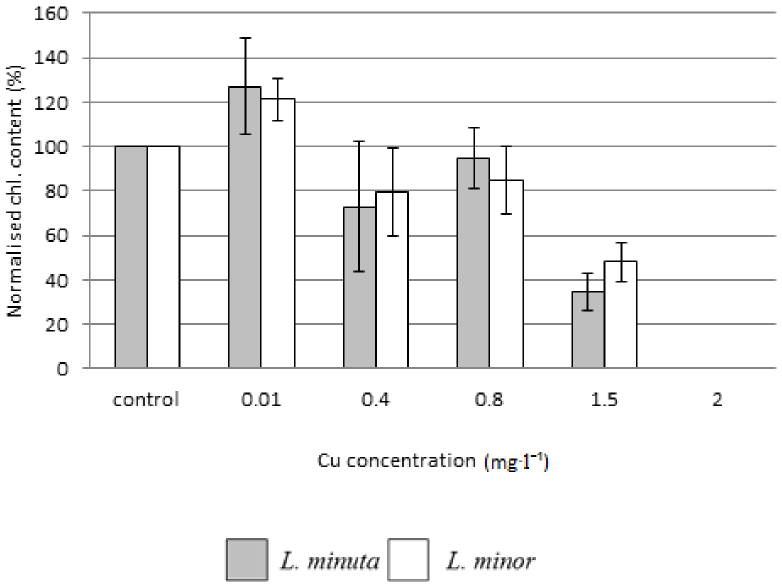

Figure 10. Normalised Chlorophyll content of *L. minuta* and *L. minor* at different concentrations of supplemental copper added to the medium. (100% = chlorophyll content of the control, values were 1.05 and 1.22 mg·g^{-1}for *L. minuta* and *L. minor* respectively). The copper concentration of the control was 0.008 mg·l^{-1}. Error bars are standard errors.

Chlorophyll *a* Fluorescence analysis

The analysis of the chlorophyll *a* fluorescence showed that the efficiency of the photosynthesis was not strongly affected by up to 0.8 mg·l^{-1} [Cu] in the medium. In the treatments containing 0.001, 0.4 and 0.8 mg·l^{-1} of the metal the maximal photochemical efficiency (Fv/Fm) ranged between 0.609±0.012 and 0.554±0.032 in *L. minuta* and between 0.632±0.064 and 0.565±0.047 in *L. minor* (Fig. 11b,c and d). In the control the Fv/Fm was 0.76±0.03 and 0.78±0.003 for *L. minuta* and *L. minor* respectively (Fig. 11a). When the plants were grown in medium containing 1.5 $mg{\cdot}l^{-1}$ [Cu], the maximal photosynthetic efficiency still did not decrease in *L. minor*, while, in *L. minuta*, it was lower than the one observed in the previous treatments with lower copper concentrations (0.445±0.033) (Fig. 11e). The comparison of the quantum yield of the two species did not show significant differences at 0.01, 0.4 and 0.8 $mg{\cdot}l^{-1}$ [Cu], while, at 1.5 $mg{\cdot}l^{-1}$ [Cu], the curve showed significantly higher values for *L. minor* ($p=0.02$).

The non-photochemical quenching of the two species increased with actinic light intensity, reaching a maximum at a PAR of 700 $\mu mol\ m^{-2}{\cdot}s^{-1}$. In *L. minor* the maximal qN did not vary significantly under the lowest three concentrations (0.01, 0.4 and 0.8 $mg{\cdot}l^{-1}$ [Cu]) and ranged between 0.61±0.06 and 0.646±0.018. When grown in medium containing 1.5 $mg{\cdot}l^{-1}$ [Cu], the maximal qN of *L. minor* increased to 0.846±0.08. In *L. minuta* the curve of qN was the same as for *L. minor* in the control and at a concentration of 0.001 $mg{\cdot}l^{-1}$ [Cu] (the two curves are nearly completely overlapping). However, at 0.4 $mg{\cdot}l^{-1}$ [Cu] *L. minuta* showed already increased values of qN. The maximal qN at 0.4, 0.8 and 1.5 $mg{\cdot}l^{-1}$ [Cu] in *L. minuta*, ranged between 0.817±0.051 and 0.922±0.028 (Fig. 11f,g,h,I and j). The statistical analysis showed that *L. minuta* had higher values of qN than *L. minor* at 0.4, 0.8, and 1.5 $mg{\cdot}l^{-1}$ [Cu] ($p<0.01$ in all 3 treatments).

The trend of the photochemical quenching, qP, was very similar, for the two species, when grown at 0.01 $mg{\cdot}l^{-1}$ [Cu] (Fig. 11k, l,m,n and o). At 0.4 and 0.8 $mg{\cdot}l^{-1}$ [Cu], the qP of *L. minor* decreased at lower actinic light intensities than the qP of *L. minuta* ($p<0.01$).

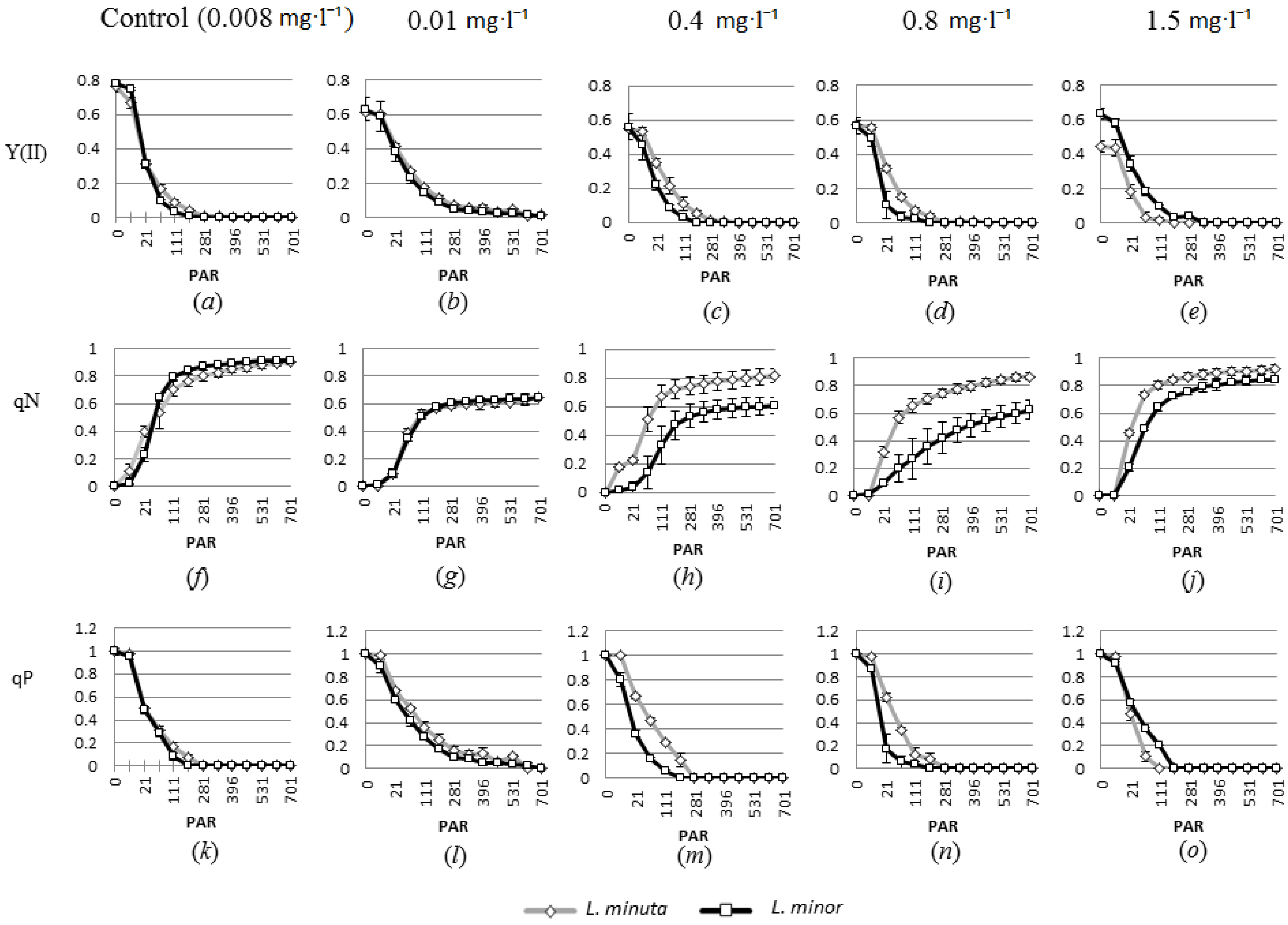

Figure 11. Quantum yield (YII) (*a*, *b*, *c*, *d*), Non-Photochemical Quenching qN (*e*, *f*, *g*, *h*)and Photochemical Quenching qP (*i*, *j*, *k*, *l*) of the two species along a gradient of supplemental Cu, at different actinic light intensities. Values are mean of four replicates and error bars are standard errors.

Copper content

Analysis of the copper accumulated in the fronds of the two species cultured at different concentrations of copper, showed that *L. minuta* accumulated more of this metal. When grown at 0.4 mg·l^{-1} [Cu], *L. minuta* accumulated 1099.67±16.4 mg [Cu] per kg of dry biomass, while *L. minor* accumulated only 728±17.8 mg·kg^{-1}. When the plants were grown at 0.8 mg·l^{-1} [Cu], *L. minuta* accumulated 1843±20.2 mg·kg^{-1} [Cu] and *L. minor* accumulated 1267.67±38.8 mg·kg^{-1} [Cu]. At the highest concentration (1.5 mg·l^{-1} [Cu]) The fronds of *L. minuta* contained 2238.67±81.06 [Cu], while the fronds of *L. minor* contained 1908.67±26.5 [Cu]. The amount of copper in the fronds was higher for *L. minuta* at each copper concentration tested but only at 0.4 and 0.8 mg·l^{-1} [Cu] this difference was significant ($p<0.01$ in both treatments) (Fig.12).

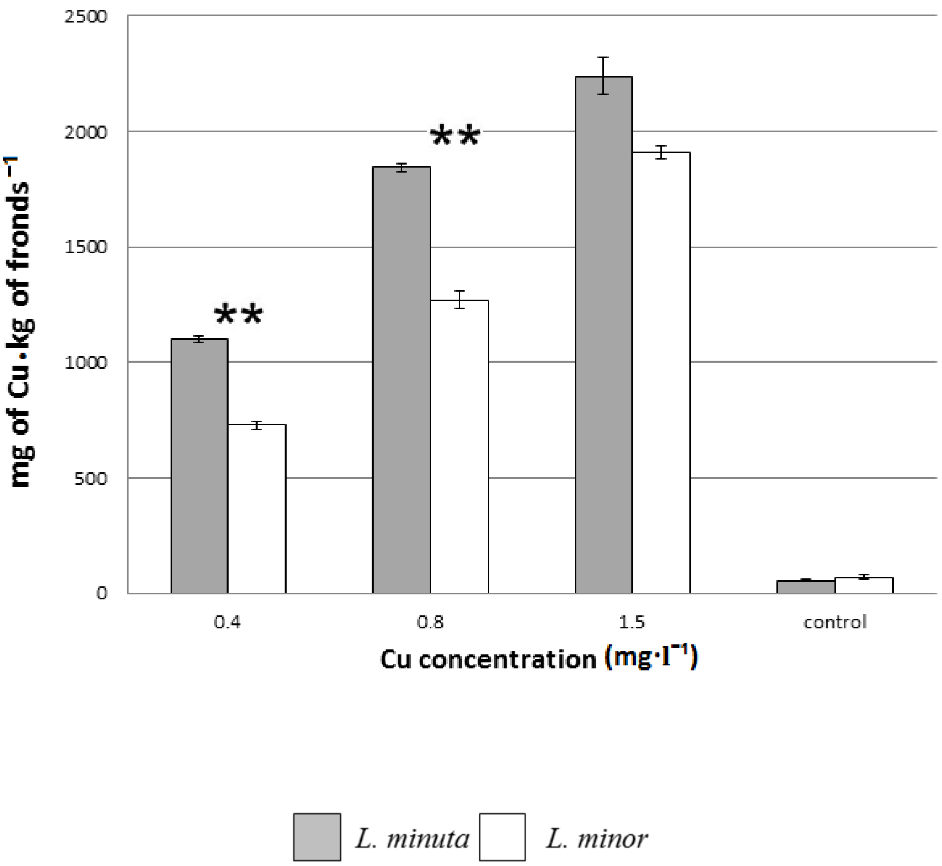

Figure 12. Copper accumulated in the fronds of the two species grown at different concentrations of the metal in the medium. Values are mean of three replicates and error bars are standard errors. The stars indicate the significance in differences between species. * means $p<0.05$, ** means $p<0.01$.

Paraquat exposure experiment

RGR based on biomass

The normalised RGR was not affected when fronds were exposed to 0.005 or 0.01 mg·l^{-1} paraquat (respectively 0.242±0.005 and 0.203±0.008 day^{-1} in *L. minuta*, 0.235±0.005 and 0.228±0.006 day^{-1} in *L. minor*). When 0.1 mg·l^{-1}of paraquat was added to the medium, the RGR was reduced by nearly 50% while, at even higher concentrations, none of the two species of Lemnaceae were able to grow. The reduction in the growth of the two species along the paraquat gradient was very similar, and no significant differences were noted between the two species (Fig. 13).

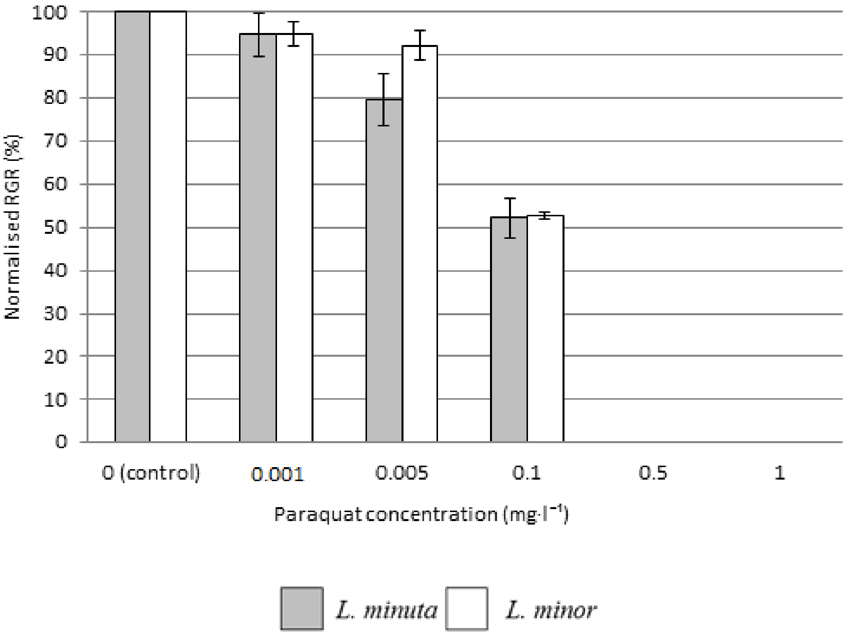

Figure 13. Normalised RGR of *L. minuta* and *L. minor* exposed to different concentrations of paraquat. (100% = RGR of the control, values were 0.25 and 0.24 day^{-1} for *L. minuta* and *L. minor,* respectively). Error bars are standard errors.

Chlorophyll content

The normalised chlorophyll content was measured for the fronds of the two species. In both species a decrease in the pigment concentration was observed with exposure to increasing paraquat concentrations. In the treatments containing 0.005, 0.1 and 0.1 mg·l^{-1} of paraquat, the chlorophyll content ranged for *L. minuta* between 0.648±0.037

and 0.535±0.083 mg·g^{-1} and, for *L. minor* between 0.699±0.043 and 0.385±0.041 mg·g^{-1}. When the plants were grown for a week in medium containing higher concentrations of paraquat, the fronds of both species were chlorotic and it was not possible to detect any chlorophyll. No statistically significant differences were noted between the two species.

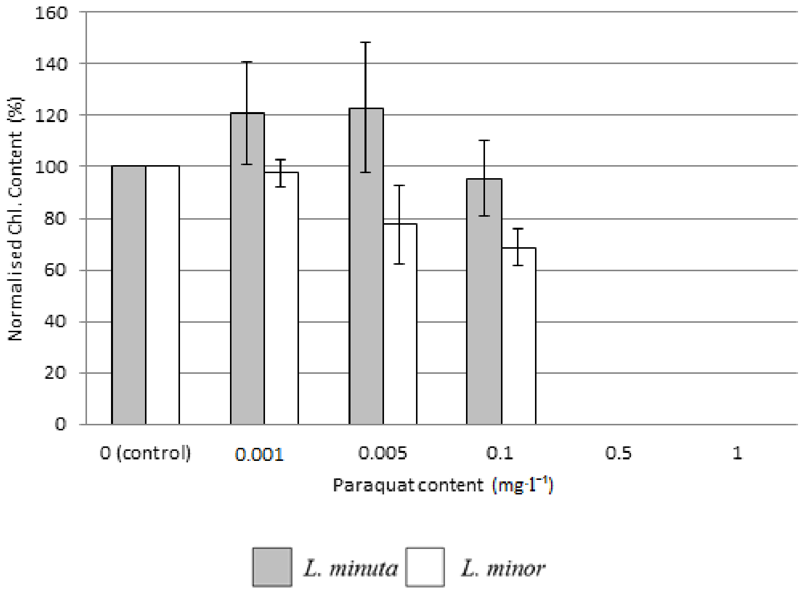

Figure 14. Normalised Chlorophyll content of *L. minuta* and *L. minor* at different concentrations of Paraquat concentrations. (100% = chlorophyll content of the control, 0.56 and 0.71 mg·g^{-1}, respectively, in *L. minuta* and *L. minor*). Error bars are standard errors.

Chlorophyll *a* Fluorescence analysis

The analysis of the chlorophyll *a* fluorescence emitted by the fronds showed that, in *L. minor*, the maximal photochemical efficiency of PSII (Fv/Fm) was not affected by low concentrations of paraquat (0.005 and 0.01 mg·l^{-1}). Only when the fronds were treated with 0.1 mg·l^{-1} of paraquat, did Fv/Fm drop from 0.8 to 0.6. On the contrary, in *L. minuta*, a reduction of Fv/Fm was already observed at the lowest concentration of paraquat tested and a further drop was observed when grown with 0.1 mg·l^{-1} of paraquat (0.3±0.06) (Fig. 15 *a*, *b*, *c*, *d*). The comparison of Fv/Fm in the two species showed that *L. minor* had a higher maximal photosynthetic efficiency at 0.005, 0.01 and 0.1 mg·l^{-1}of paraquat ($p=0.036$ in the first treatment and $p<0.01$ in the other two). The differences between species were mainly restricted to Fv/Fm values. When the curves of Y(II) of the two species were compared, a significant difference was only observed at the highest paraquat concentration tested (0.1 mg·l^{-1}), when *L. minuta* showed lower yield values than *L. minor* ($p<0.01$).

In *L. minor*, qN followed a very similar trend during the actinic period in all the three treatments tested, irrespective of paraquat exposure. The maximal qN increased slightly with increasing paraquat concentration in the medium, varying between 0.828±0.005 and 0.899±0.005. The maximal qN of *L. minuta*, at 0.005 mg·l^{-1}of paraquat, was 0.758±0.036. Also in this species qN did not change significantly with increasing the paraquat concentration in the medium (Fig. 15 *e*, *f*, *g*, *h*). The fronds of *L. minor* showed a slightly higher qN than *L. minuta* at every paraquat concentration tested, but only at 0.01 and 0.1 of paraquat was this difference significant ($p<0.01$ at 0.01 mg·l^{-1} and $p=0.03$ at 0.01 mg·l^{-1}).

When the qP was measured in the plants grown with 0.005 mg·l^{-1} of paraquat, no significant differences were observed between the two species. At 0.01 mg·l^{-1} of paraquat, *L. minuta* showed a higher qP than *L. minor* ($p<0.01$), while, at the highest paraquat concentration tested (0.1 mg·l^{-1}), *L. minuta* showed a qP lower than *L. minor* ($p=0.02$) (Fig. 15 *i*, *j*, *k*, *l*).

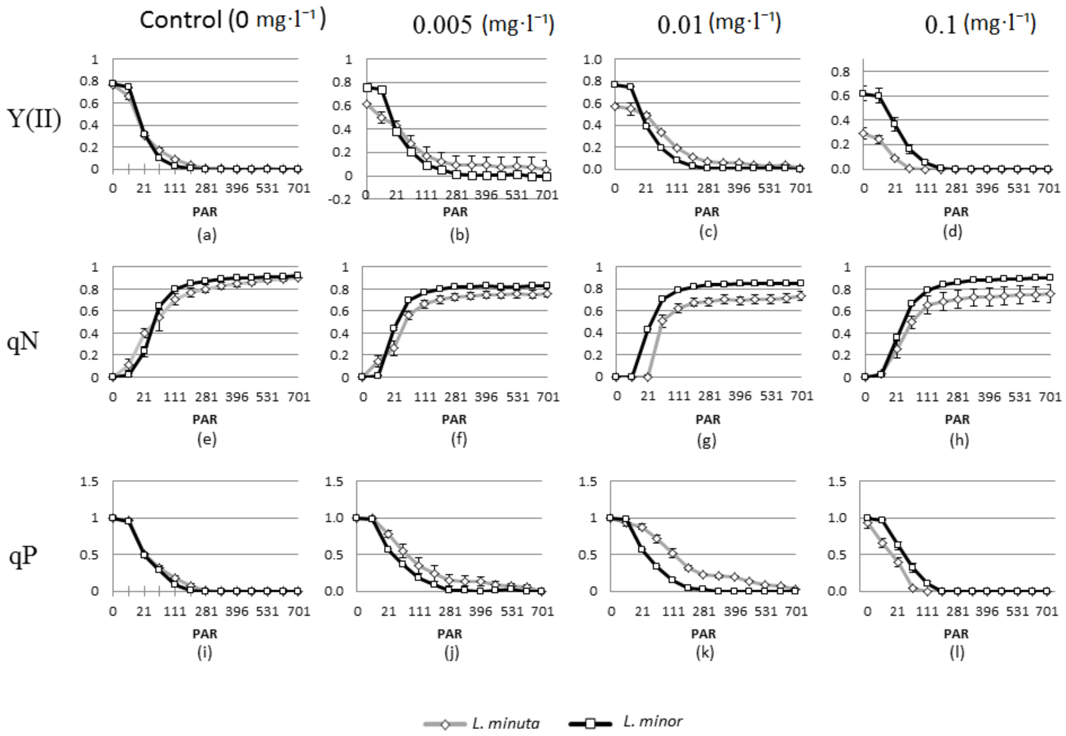

Figure 15. Quantum yield (YII) (*a, b, c, d*), Non-Photochemical Quenching qN (e, *f, g, h*) and Photochemical Quenching qP (*I, j, k, l*) of the two species along the paraquat gradient, at different actinic light intensity. Values are mean of four replicates and error bars are standard errors.

Discussion

Stress response in *L. minuta* and *L. minor*

Different stressors interact with different components of cellular metabolism. However, a common component of the damage caused by diverse biotic and abiotic stressors in plants is oxidative stress due to the production of Reactive Oxygen Species (ROS) (Apel and Hirt, 2004). If the accumulation of ROS is higher than the capacity of the antioxidant system, oxidative damage occurs. However, accumulation of ROS in the cytoplasm cannot be simply equated with damage, but also works as a signal to activate metabolic pathways for defence and/or repair (Neill *et al*., 2002). Thus, plants with upregulated antioxidant defence systems possess a degree of cross-tolerance to many stressors (Perez and Brown, 2014). Here it was explored whether *L. minor* is inherently more protected from ROS, compared to *L. minuta*. Paraquat was used to generate production of superoxide radicals in the plants. This molecule is the main active ingredient of the bipyridillium herbicides that were once commonly used.

Paraquat catalyses the transfer of electrons from photosystem I to oxygen, resulting in the production of superoxide radicals (Preston *et al.*, 1991). In the present study, paraquat decreased the RGR and chlorophyll content at the concentration of both species. Frankart *et al.* (2003) observed a decrease in growth rate and chlorophyll content in *L. minor* exposed to 0.001 $mg \cdot l^{-1}$ of paraquat in the medium. In this study higher concentrations of paraquat were required to impede growth, and this is likely due to the relative low light intensity in this study (40 versus 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in the study by Frankart *et al*. High intensities of light are known to enhance the toxicity of the herbicide (Blackburn and Weldon, 1965). Both species seem to be equally affected by the paraquat in term of RGR, chlorophyll content. The absence of a difference between the two species implies that the plants investigated do not substantially differ in their overall anti-oxidant stress defences.

Response to aluminium and copper

Lemnaceae are known for their high level of tolerance toward metals. Lemnaceae are also known to accumulate high amount of metals in their biomass. For these reasons Lemnaceae species are often used in ecotoxicology studies (Lakatos *et al*., 1993) and in phytoremediation (Appenroth *et al.*, 2010). However not all species of duckweeds have the same level of tolerance to metals and the same ability to bio-accumulate (Landolt, 1986, Wang *et al*., 2013). In this study both species displayed a similar decrease in growth and chlorophyll content, and similar alterations in chlorophyll fluorescence parameters. Previously, Radic *et al*. (2010) reported toxic effects of aluminium on *L. minor* at slightly higher concentrations, while Khellaf and Zerdaoui (2010) reported copper toxicity to *L. gibba* at similar concentrations as in this study.

In this study it was found that aluminium and copper had similar effects on growth and photosynthesis in *L. minor* and *L. minuta*. Yet, *L. minuta* accumulated a higher concentration of metals in its fronds than *L. minor*. Despite accumulating a greater amount of both metals tested, *L. minuta* maintained an RGR equal or greater than *L. minor*. Thus, *L. minuta* is a better tolerator of high tissue-concentrations of the two metals tested. Paraquat exposure studies do not reveal enhanced antioxidant defences. Therefore, it is highly likely that enhanced metal-tolerance in *L. minuta* is related to effective sequestration of the metals, a trait that typically incurs a fitness cost (Maestri *et al*., 2010).

Response to temperature

Temperatures can vary considerably in ponds and lakes according to the season. Low or high temperatures can be a limiting factor for aquatic species (Carr *et al*., 1997). The ability of duckweeds to deal with different temperatures depends on the species and, often, on the clones as well (Landolt, 1986). In this study *L. minor* tolerated low temperatures better than *L. minuta*. The native species grew faster than the alien species at 0, 10 and 15°C at 20°C, and this was associated with a higher yield (Y(II)) and qP. In contrast, *L. minuta* grew faster than *L. minor* between 20 and 35°C, and this was associated with a more efficient energy dissipation (qN). The comparison of these results with previous studies can be difficult as strains of the same species, collected in different climates, can considerably vary their optimum temperatures and their ability to survive extreme conditions (Crawford *et al*., 2006). However, the growth data observed fall within the range identified by Landolt (1986), according to which, the optimum temperature for duckweeds growth is between 20 and 30°C. Moreover, in the same review, Landolt (1986) mentioned studies in which several clones of *L. minuta* (reported as *L. minuscula*) are subject to growth inhibition below 13°C. For *L. minor* Landolt (1986) stated that the minimum temperature required for growth is as low as 8°C.

Response to drought stress

Ponds and streams that provide a habitat to duckweeds may dry out, completely or partially, during the dry season. Some species of Lemnaceae deal with drought stress by closing their stomata and consequently limiting the water loss for transpiration (Landolt, 1986). In the present study drought tolerance was assessed in terms of loss of weight, survival rate and ability to re-establish growth after different periods of time spent out of the growth medium. Both species displayed increasing loss of weight as the desiccation time increased. The reduced water loss in *L. minor* is probably due to morphological characteristics, this species having thicker (i.e. lower surface to volume ratio) fronds than the alien species. The survival rate was inversely proportional to the time spent out of the water. In both species RGR was also inversely proportional to the desiccation time. No survival was recorded when species had been in excess of 50 minutes out of the water, a finding consistent with a previous experiment by Coughlan *et al.* (2015). Both species grow equally well following exposure to drought stress. Yet, *L. minuta* had lost considerably more weight (i.e. water) in the desiccation phase. Thus, *L. minuta* possesses a higher tolerance to cellular drought stress. This result is in agreement a report by Crawford *et al*. (2006). In a comparison between *L. minuta* and *Lemna valdiviana* Philippi, the authors report a more frequent occurrence of *L. minuta* in both drier and humid areas of Central and South America, while, *L. valdiviana*, only occupies more humid habitats.

Conclusions about stress response in *L. minuta* and *L. minor*

The present study showed that *L. minuta* was more tolerant to aluminium, copper, drought and high temperatures than *L. minor*. *L. minor* tolerated better low temperatures. As indicated by the paraquat experiment, these differences do not depend on differences in ROS tolerance. Thus differences must depend on stressor-specific mechanisms developed by the species. For example, the better metal tolerance of *L. minuta* can be speculated to be related to more efficient production of phytochelatin. Phytochelatins act as metal chelators, and the chelated metal is typically moved to the vacuole.

Considerations over *L. minuta* strategy and its invasiveness in stressed environments

It is widely accepted that invasive species take advantage of disturbances, both in the initial stage of colonization (Davis *et al*., 2000) and during the established stage. Plants adapted to disturbance are classified as ‘ruderal’ by Grime (1974) and typical characteristics of these species are, among the others, ability to multiply vegetatively and a fast growth rate. Some of these characteristics represent a disadvantage in environments characterized by the presence of stressors (Grime, 1977). Ruderal invasive species often lack adaptations to deal with environmental stressors (Caño *et al*., 2008). In this study, the stress tolerance of *L. minuta* was compared with that of *L. minor* in order to learn if the high growth rate of the former species is due to a lack of ability to tolerate stress.

The original hypothesis of this study that *L. minuta* is less efficient at tolerating stressors than *L. minor*, was disproved by the results. The majority of environmental stressors tested (with the exception of low temperature) did not have a disproportional effect on the alien species. Rather, this study generated evidence that the alien species is in some cases more stress-tolerant. Our findings are not in accordance with the conclusion of Alpert *et al*. (2000). These authors concluded that the presence of environmental stressors gives a competitive advantage to native species over invasive aliens. The generalization according to which environmental stress protects from biological invasions is often based on the assumption that native species are more adapted to local stresses. For example, Konisky and Burdick, (2004) observed that *Phragmites australis* Cavanilles, *Lythrum salicaria* Linnaeus and *Typha angustifolia* Linnaeus, invasive aliens in New England (USA) salt marshes, displace the natives *Spartina alterniflora* Loisel, *Spartina patens* Muhlenberg and *Juncus gerardii* Loiseleur only in brackish environment, while, at higher salinity levels, the native species are more competitive because they are more adapted to tolerate salinity stress. However, in other studies different conclusions emerged. For example, Ewel (1986) found that high salinity did not protect mangrove swamps from invasion. Baars *et al*. (1998) found that a non-native liana in rainforest is more tolerant to high pH and Perrins *et al.* (1993) showed that three invasive species of the genus *Impatiens* can tolerate frost better than native species.

The present study indicates that the presence of stressors does not simply impede alien invasions. Rather, specific stressors (such as low temperature in this study) can potentially hamper alien invasions. Conversely, this study shows that other stressors can potentially promote alien invasion. Here, four stressors (aluminium, copper, drought and high temperatures), that have all been linked with anthropic activity, can potentially provide a competitive advantage to *L. minuta*. This scenario is supported by other studies. In particular, in terrestrial environments pollution has been linked with higher invasibility (Tyser and Worley, 1992). Thus, the role of environmental stressors in facilitating alien invasions is multi-faceted, and stressor-specific.

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Chapter 5

Seasonal fluctuations, in the invasiveness of *Lemna minuta* Kunth and *Azolla filiculoides* Lamarck, relative to native *L. minor* Linnaeus, and their consequences for abundance in natural water bodies.

Abstract

Lemna minuta Kunth and *Azolla filiculoides* Lamarck are two free-floating freshwater species considered invasive in several European countries and often found together. These two species share a habitat with native *Lemna minor* Linnaeus. In this study an area in the South of Ireland was monitored in order to assess the presence of the three species and identify possible differences in their environmental preference. For each selected waterbody in the study area, presence and abundance of the three species were observed several times over a period of 19 months. Water analysis was carried out for each site and the canopy cover was estimated. A field experiment was also carried out in order to determine if growth rates varied between species and waterbodies. To do this, three ponds dominated by *L. minuta*, three ponds dominated by *L. minor* and three ponds in which none of the three species was present were selected. In each selected waterbody all possible combinations of the three species (the three species alone, *L. minuta* with *L. minor*, *L. minuta* with *A. filiculoides*, *L. minor* with *A. filiculoides* and the three species all together) were grown inside plastic floating enclosures. Finally, dynamic changes in abundance and growth-rate of three species of floating macrophytes were analysed in mesocosms. All the possible combinations of the three species were grown in outdoor mesocosms for one year and growth rates and the relative area occupied by the three species were measured each month. Field monitoring showed that the presence and distribution of the three species changed with season and year. *A. filiculoides* was the least abundant species. Growth rates of the three species did not vary between waterbodies, irrespective of whether the species were naturally present in a particular waterbody. Both the field and the mesocosms experiment showed that *A. filiculoides* has the highest growth rate, followed by *L. minuta*. *L. minor* had the lowest growth rate. *A. filiculoides* also outcompeted the other two species when they were grown together during the summer. In contrast, the mesocosm experiment revealed that, in winter months, *A. filiculoides* and *L. minuta* lose their ability to outperform the native species. Thus, it was also highlighted that the ability of *L. minor* to restart its growth earlier in the year might provide it with a competitive advantage despite the fact that it has a lower growth rate during the summer. Furthermore, it is speculated that a major role in determining the distribution and relative abundance of the three species is played by winter floods that wash away

the floating species. Waterbodies need to be re-colonised every year and this might be an advantage for *L. minor* over *L. minuta* and *A. filiculoides* as the native species is characterized by a higher dispersal rate. Thus, disturbances of the habitat (winter floods, summer drought) may favour the native species and thus decrease invasibility of the habitat.

Introduction

Biological invasions have been increasing in the past 50 years (Levine and D'Antonio, 2003) and they are a source of concern because of their negative effects on native species, habitats and biodiversity (McGeoch *et al.*, 2010). Alien aquatic plants can have a negative impact on ponds, streams, rivers and wetlands. The dense growth of some alien aquatic plants can reduce flora richness and structural diversity and cause alterations in ecosystem functions such as nutrient cycling (Zedler and Kercher 2004). Invasions can also have serious economic implications, particularly if they affect food production, shipping, water-extraction, fisheries, tourism and/or recreation. Across all ecosystems, there are estimated to be more than 1000 alien species in Europe that have been shown to cause an ecological or economic impact (Vilá *et al.*, 2009).

Understanding the factors that promote the invasiveness of alien species is fundamental in order to prevent invasions and restore invaded habitats (Byers *et al*., 2002). The ability of plants to invade a habitat is called invasiveness while, the susceptibility of an environment to the colonization and establishment by species not currently part of the resident community, is called invasibility (Davis *et al.*, 2005). A biological invasion depends on both the invasiveness of the alien species and the invasibility of the habitat (Alpert *et al*., 2000). The degree of invasibility of a habitat depends on many factors including the species richness and the strength of interactions between species (Case, 1990). Resource availability, disturbance and environmental stressors have also been demonstrated to have an impact on the invasibility of habitats (Davis *et al*., 2000). Among the traits that seem to be correlated with a high invasiveness of a species are a broad native distribution range (Goodwin *et al*., 1999), rapid population growth (Rejmánek and Richardson, 1996), ability to deal with stress and disturbance and rapid dispersal (Alpert *et al*., 2000). The competitive strength of an alien species, relative to native species, also impacts on the success of an alien invasion (Alpert *et al*., 2000). Furthermore, it was observed that the performance of alien species varies with the season. For example, Myers and Anderson (2003) found that *Alliaria petiolata* Marschall von Bieberstein, invasive in the US, optimizes its growth rate by producing more chlorophyll when irradiance is high, and temperature and moisture conditions are favourable for the species.

In this study, the abundance, growth-rate and distribution pattern of two alien freshwater plants (*Lemna minuta* and *Azolla filiculoides*) were analysed along with several environmental parameters, in order to identify factors that determine the colonisation of habitats by these two alien species and to develop management approaches. A co-occurring native species (*Lemna minor*) that shares the same habitat as the two alien species was also included in the study. *L. minuta* (Least Duckweed) is a small monocotyledon native to temperate regions of North and South America (Stace, 2010). This duckweed occurs in a wide range of habitats from mountains regions, up to 4000 m of altitude, to temperate and tropical regions (Landolt, 1986). Invasive, alien *L. minuta* has been spreading in Europe for the last 40 years (Gassmann *et al.* 2006). It is widespread in Germany (Hussner *et al*., 2010), Belgium (Halford *et al.*, 2011), Poland (Wójciak and Urban, 2009), Hungary (Lukács *et al.*, 2014), France (Jovet and Jovet-Ast, 1966), Italy (Conti *et al*., 2005) and Malta (Misfud, 2010). In England *L. minuta* is becoming more prevalent, since being discovered in 1977 (Bramley *et al*., 1995). *L. minuta* was first found in Ireland in Co. Cork in 1993. Since, it has been reported at 133 lowland sites and is now considered an established species (Lucey, 2003).

A. filiculoides is a freshwater fern that grows in symbiotic association with the blue-green alga *Anabaena azollae*. The alga fixes atmospheric nitrogen enabling the fern to live and grow in nitrogen deficient waters (Ashton and Walmsley, 1976). This species is originally from North and South America where it is widespread from Patagonia to Alaska, including the Caribbean Islands (Wagner, 1997). *A. filiculoides* was native in Europe in previous interglacials, but in the present interglacial it occurs only because it has been re-introduced (Preston and Croft, 1997). The species has now been recorded in 19 European countries. The European and Mediterranean Plant Protection Organization (EPPO) included it in the EPPO List of Invasive Alien Plants (IAP) (Hussner, 2012). *A. filiculoides* was introduced in the British islands at the end of the

19th century as an ornamental plant (Simonsen, 1968). It rapidly spread and it is now considered invasive (Preston and Croft, 1997). This water fern can cause severe problems by impeding navigation, water flow and angling and by causing fish kills and damage to wetland biodiversity (Janes, 1998).

L. minuta and *A. filiculoides* often co-occur with the native *L. minor* (Preston and Croft, 1997) and appear to compete for the same habitat (Dickinson and Miller, 1998; Ceschin *et al*., 2016). A comparative approach with native species has often been used in studies of invasive species (Daehler, 2003; Bossdorf *et al*., 2005; Funk, 2008). This approach consists of a comparison between alien and native species, and attempts to identify traits associated with invasiveness such as biomass allocation, growth rate, size and fitness. Comparative studies are particularly meaningful when comparing species that occupy the same ecological niche, and/or species that are closely related, as this facilitates the identification of differences that may be responsible for invasiveness (Mack, 1996).

The aims of this study are:

- To quantify the presence and abundance of *L. minuta*, *L. minor* and *A. filiculoides* in the study area and assess their dynamic changes in different seasons and years.
- To observe whether the availability of resources or other environmental factors can promote the presence of *L. minuta* and *A. filiculoides.*
- To assess the growth rate of the three species and the ability to outcompete each other in different seasons.

Material and methods

This study was composed of three parts. In the first part, the abundance of the three species (*L. minuta*, *L. minor* and *A. filiculoides*) was quantified in a series of water bodies in the south of Ireland. The spatial distribution of the three species was determined together with physico-chemical factors of individual ponds. In the second part, a manipulative field experiment was performed in order to explore whether spatial distribution patterns can be linked to growth and competitiveness. The third part of the study consisted of an outdoor mesocosm experiment in which the growth and competitiveness of the three species was measured across four seasons.

1-Monitoring the presence and abundance of three species of floating macrophytes in natural water bodies

Area investigated

The water bodies investigated are situated along the north and south banks of the River Lee in southern Ireland, a few kilometres west of Cork City. The area includes a range of small still- and slow-flowing water bodies that provide a suitable habitat for the three floating plant species. A total of 24 water bodies were selected for further study (Tab.1). The sites selected included water bodies with heterogeneous characteristics (e.g. different aspect, canopy-cover, proximity to farms and/or houses). Most ponds are less than 100m^2, and the depth generally varies between 50 and 150 cm (Fig.1). The bedrock in the area is composed of Devonian sandstone, covered by Carboniferous limestone. Brown podzolic topsoil originated from glacial drift of sandstone-limestone mix is present. The area is used for agricultural and recreational activities. In winter, the entire area is subjected to inundation. Occasionally, some of the water bodies dry out, completely or partially, in summer.

The climate of Ireland is classified as cfb (temperate/mesothermal climate with significant precipitation in all seasons, temperature in warmest month averaging below 22°C, and temperature in at least 4 months averaging above 10°C) by Köppen and Geiger (Peel *et al.*, 2007). In Cork, the average annual temperature is 10.4°C. The average rainfall is 1055 mm per year (Walsh, 2012). The monthly average temperature during the period of field monitoring is shown in figure 2.

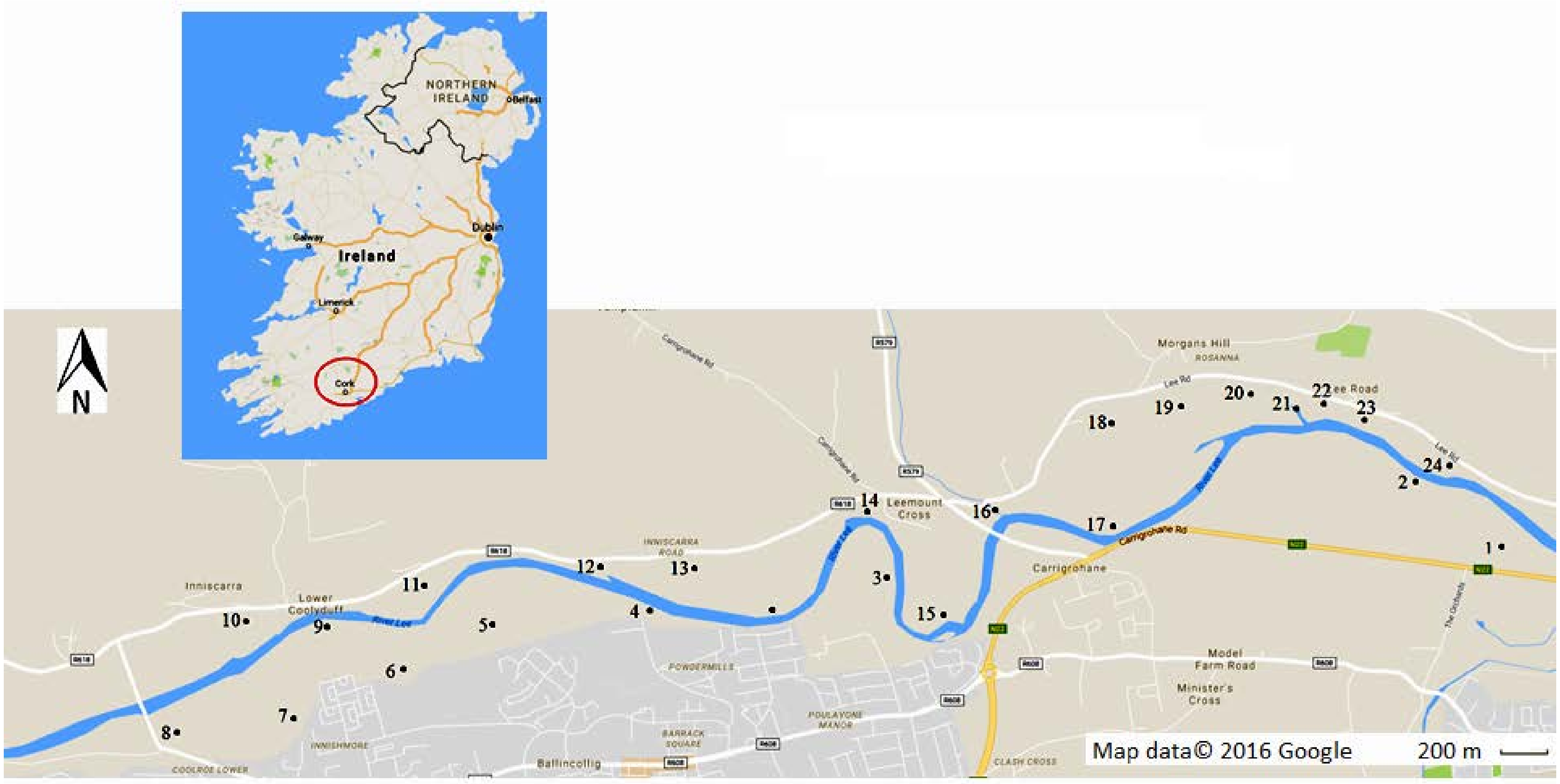

Figure 1. Map of the monitored area. Numbers indicate the sites investigated. Insert shows the location within Ireland.

Waterbody	Latitude	Longitude
1	51.892843	-8.531734
2	51.895062	-8.525105
3	51.895225	-8.574605
4	51.893905	-8.589299
5	51.893072	-8.599597
6	51.89229	-8.605667
7	51.891575	-8.612367
8	51.891741	-8.614858
9	51.893527	-8.610699
10	51.893897	-8.615992
11	51.894662	-8.603649
12	51.895326	-8.59256
13	51.895898	-8.583327
14	51.897965	-8.576325
15	51.893601	-8.571032
16	51.897624	-8.568047
17	51.897519	-8.559594
18	51.899935	-8.559874
19	51.901459	-8.557579
20	51.90226	-8.552388
21	51.901587	-8.548277
22	51.901591	-8.546123
23	51.901102	-8.543642
24	51.896009	-8.52175

Table 1. Coordinates of the waterbodies monitored.

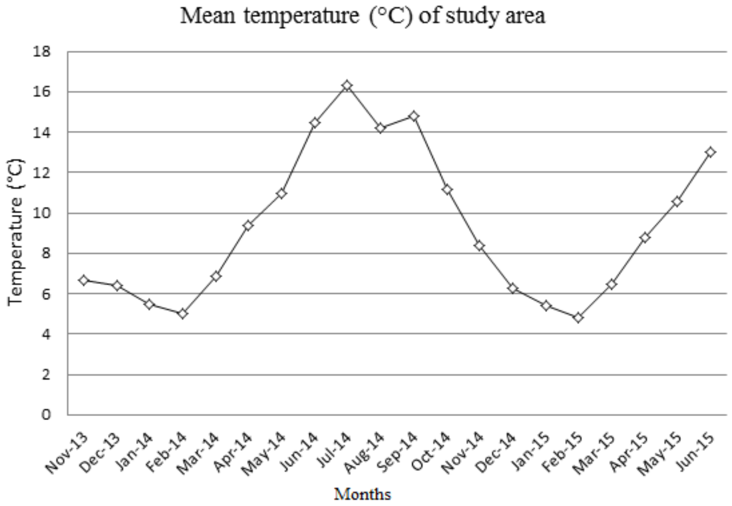

Figure 2. Average monthly temperature during the time of this study. Data were provided by the Irish Meteorological Service, and measured at Cork airport meteorological station (51°50'50" N 8°29'10" W).

Monitoring approach

Field monitoring started in November 2013 and lasted till June 2015. A total of six observations were made of plant abundance (November 2013, April, June and November 2014, May and June 2015), one of shading (in June 2014) and two of water nutrient content (April and June 2014).

The presence and abundance of three species of floating macrophytes was quantified for each of 24 still- and slow-flowing water bodies in the study area. A 50x50 cm floating quadrat was used to estimate the percent cover of each species in each quadrat. Four random throws of quadrats were carried out in each water body and the mean of the four quadrats calculated. It was assumed that the percent coverage of the quadrats reflected the percent coverage of the water body. The values estimated were translated into the following classes:

- 1– absent
- 2 – present (1 - 25% of the surface of the water body covered)
- 3 – abundant (26 - 75% of the surface of the water body covered)
- 4 – dominant (76% - 100% of the surface of the water body covered)

The canopy produced by other plants (trees, bushes and reeds growing around and inside the ponds) on the whole surface of the water body was visually estimated. Each site was classified according to the following scale:

- not shaded (0% canopy)
- partially shaded (up to 25% of canopy)
- mostly shaded (25-75 % of canopy)
- completely shaded (75-100% of canopy)

Total Oxidised Nitrogen (TON) and Total Phosphorus (TP) concentrations were quantified in each water body in early spring (April 2014) and in early summer (June 2014). The content of TP in the water was determined using the ascorbic acid method

(Murphy and Riley, 1962), while the TON content was measured using a DR 2800 Spectrophotometer following the cadmium reduction method (Koroleff, 1972).

2-Quantifying growth-rates of three species of floating macrophytes in natural water bodies

To add to the data on presence and abundance of three species of macrophytes, growth rates were determined in a subset of nine ponds. In three selected ponds *L. minuta* was more abundant than the other species investigated, while in a further three ponds *L. minor* was most abundant. In the final three ponds none of the three species was present. The experimental design would also have required the inclusion of three ponds in which *A. filiculoides* was the abundant species, but at the time of this experiment none of the sites presented this characteristic. Four plastic, floating enclosures were placed in each of the selected ponds. Each enclosure was divided into 7 circular sub-units (short 12-cm lengths of plastic piping of 10.5 cm diameter, perpendicular to the water surface), inside which all possible combinations of the three species (the three species alone, *L. minuta* with *L. minor*, *L. minuta* with *A. filiculoides*, *L. minor* with *A. filiculoides* and the three species all together) were grown (licence IAS 4/2013 by the Department of Arts, Heritage and Gaeltacht). The design of the enclosures is shown in figure 3. The four enclosures were bound together and tied with a rope to the edge of the water body so that they had a certain degree of freedom, but they could not be dragged too far by the current or the wind. A net was placed over the enclosures to prevent birds from accessing the sub-units and to prevent leaves and other material entering. Growth was quantified by placing biomass of each of the three species in the appropriate 10.5 cm diameter sub-unit (30 mg of *L. minuta*, 50 mg of *L. minor* and 80 mg of *A. filiculoides* starting biomass). Plants were then allowed to grow for four weeks. At the end of the four week period the plant material was collected, the different species were separated, weighted and the RGR was calculated according to the formula of Connolly and Wayne (1996):

$$RGR = \ln (Yf / Yi) / t$$

Where Yi is the initial biomass or the initial number of fronds, Yf is the final biomass or final number of fronds, t is the time in days and ln is the natural logarithm. In the present work the word biomass is used to indicate organic matter derived from living, or recently living plants.

The experiment started the 20th of May 2014 and it ended the 20th of June 2014. In the three weeks of the experiment the average maximum temperature registered in the Cork meteorological station was 15°C, while the average minimum temperature was 8.6°C.

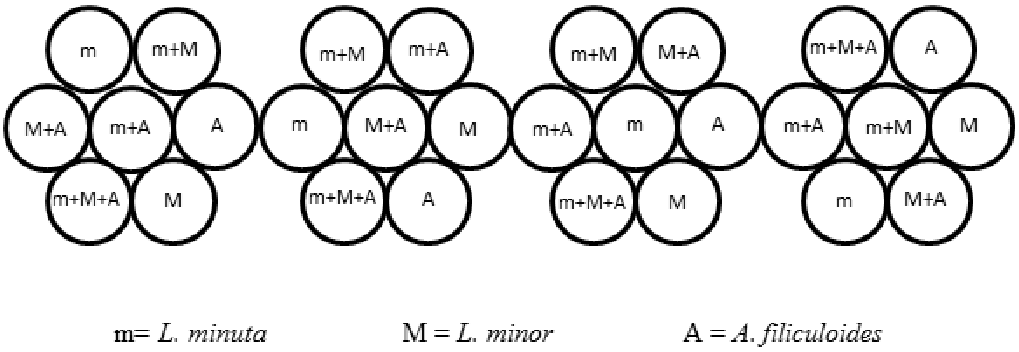

Figure 3 Design of the floating enclosures for the field experiment. Four composite enclosures (each of which composed of 7 sub-units) where placed in each of the 9 ponds selected for the experiment. The 4-times replicated enclosures contained all the possible combinations of the 3 species.

3-Analysing dynamic changes in abundance and growth-rate of three species of floating macrophytes in mesocosms

Plant material

All plant material used for the experiments was collected from a pond where all the three species are present. The pond was located in the valley of the river Lee, inside the area monitored for the first part of this study (Site number 20 in figure 1). The mesocosm experiment started in November 2013 and finished in November 2014, and was designed to identify dynamic differences in abundance and growth rate across the different seasons. Twenty-eight mesocosms were constructed by sinking plastic containers 31 cm deep into the ground. Containers had a surface area of 1753 cm^2. Sediments were gathered from the pond from which all the three species were collected, and 750 g sediment was added to each mesocosm. Rain water (15 l) was added to each container. The mesocosms were left plant-free for four days in order to allow the sediment to settle, and for some of the nutrients contained in the sediments to dissolve in the water. When the experiment started the concentration of soluble orthophosphate (SRP) in the water was 0.03±0.001 $mg \cdot l^{-1}$ and the concentration of nitrate was 4.1±0.3 $mg \cdot l^{-1}$. These concentrations were similar to those observed in several water bodies along the river Lee where the three species were naturally present.

Each mesocosm consisted of the main tank and a small floating enclosure used to measure growth rates (Fig. 3). Mesocosms contained either single species, combinations of two species, or a mixture of three species. In November 2013, 5 cm^2 of all the possible combinations of the three species were placed in main-part of each of the mesocosms. Each combination was replicated four times, with replicate mesocosms located at random within the experimental array, to avoid spatial confounding. The mesocosms were covered with wide-mesh netting to prevent birds interfering with the experiment. The relative area occupied by each species in each mesocosm was estimated every month with the method of the point intercept (Floyd and Anderson 1987). Monitoring was done over a full calendar year.

The floating enclosures present in each mesocosm measured 14.3 cm diameter (Fig. 4). Fifty mg of each species (50 + 50 mg in the enclosures containing a combination of two species and 50 + 50 + 50 mg in the enclosures containing a combination of three species) were added to the floating enclosure every month, removed after 2 weeks and the weight of each species was measured. The RGR was calculated according to the formula described in the previous paragraph. The parallel observations of biomass growing in the main ring (standing stock) and growth rate allowed the monitoring of the performance of the species across different seasons. The monthly mean temperature for the experimental time frame is shown in figure 5.

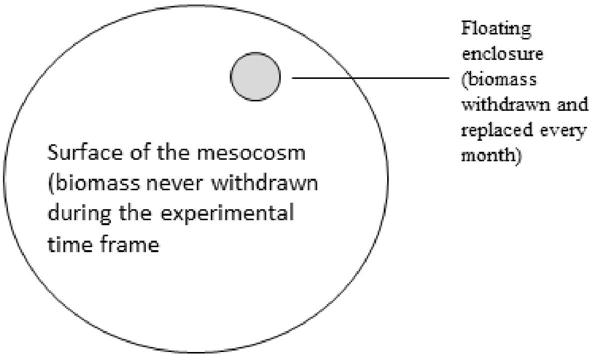

Figure 4 Design of the mesocosm. A main compartment was used to monitor standing stock, while the enclosure was used to determine growth rates.

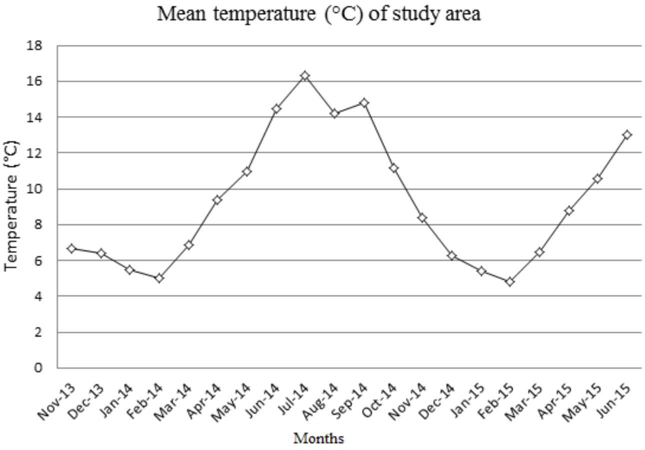

Figure 5. Monthly temperature average during the time frame of the mesocosm experiment. Data provided by the Irish Meteorological Service, and measured at Cork airport meteorological station (51°50'50" N 8°29'10" W).

Data analysis

All the statistic tests were performed using IBM SPSS Statistic 22.

Field monitoring. The relation between the presence/abundance of the three species and TON, TP and canopy cover was investigated by carrying out Kendall's Tau b test. The correlation tests between the percentage coverage of *L. minuta*, *L. minor* and *A. filiculoides* with TON, TP and Canopy refer to the data collected in April and June 2014 for TON and TP and June 2014 for the canopy cover. The data of percentage surface cover by aquatic macrophytes refer to the same months of 2014.

Field experiment. A 2-way ANOVA was run in order to identify differences in RGR between the three species grown in allopatric conditions in the different types of pond. For each of the three species, another 2-way ANOVA was run to analyse the differences in RGR for plants grown in different categories of ponds, and in allopatric or sympatric conditions.

Mesocosm experiment. A 2-way repeated measures ANOVA was used to analyse the differences in RGR and in percentage of surface cover between the three species, grown in allopatric conditions, in the different months of the year. For each of the three species, another 2-way repeated measures ANOVA was run in order to analyse the differences, in RGR or % of surface cover, between the species grown in allopatric or sympatric conditions.

Outliers were assessed by visual inspection of boxplots. Normality and homogeneity of variances were assessed by Shapiro-Wilk's normality test and Levene's test respectively. Sphericity was assessed with Mauchly's test. Tukey post hoc analysis was used to identify the significant differences between different species. The p values for main effects and simple main effects received a Bonferroni adjustment. For all the tests run the interval of confidence assumed was 95%.

Results

1-Monitoring the presence and abundance of three species of floating macrophytes in natural water bodies

Of the three investigated species, *L. minor* and *L. minuta* were the most abundant in the 24 water bodies monitored during the two years of the study. *L. minor* was found in twelve waterbodies, *L. minuta* in eleven water bodies and *A. filiculoides* only in three waterbodies. In five waterbodies, both *L. minuta and L. minor* were found, in three of them co-occurring at the same time, in the other two the two species were present at different times. In only one pond all three species were found together. In two other ponds *A. filiculoides* co-occurred with *L. minuta*. Thus, *A. filiculoides* never occurred in the absence of at least one *Lemna* species. No floating plants were observed in five of the 24 ponds (Tab. 2).

Analysis of water nutrient content revealed that Total Oxidised Nitrogen (TON) ranged between 0.01 and 5.207 mgl^{-1} (mean 1.798 $mg{\cdot}l^{-1}$) across all 24 waterbodies in April 2014 and between 0.01 and 3.807 $mg{\cdot}l^{-1}$ (mean 0.88 $mg{\cdot}l^{-1}$) in June 2014. Total Phosphorus (TP) ranged between 0.001 and 0.118 $mg{\cdot}l^{-1}$ (mean 0.0132 $mg{\cdot}l^{-1}$) across the 24 waterbodies in April 2014 and between 0.001 and 0.06 $mg{\cdot}l^{-1}$ (mean 0.0062 $mg{\cdot}l^{-1}$) in June 2014.

	Canopy
	not shaded
░	partially shaded
▒	mostly shaded
█	completely shaded

L. minuta	*L. minor*	*A. filiculoides*	% coverage
			Absent
•	▴	▪	1-25%
●	▲	■	26-75%
⬤	⯅	⬛	76-100%

Site no.	TON (average April/June)	TP (average April/June)	Canopy shade in June	Species	Nov. 2013	Apr. 2014	Jun. 2014	Nov. 2014	May. 2015	Jun. 2015
1	0.01	0.005	░	*L. minuta*	•	●	⬤			
				L. minor						
				A. filiculoides						
2	0.99	0.006	▒	*L. minuta*						
				L. minor		▴	▴			
				A. filiculoides						
3	2.507	0.004	█	*L. minuta*		•	•			
				L. minor					▴	
				A. filiculoides						
4	1.169	0.007	█	*L. minuta*						
				L. minor	⯅	▴	▲	▲	▴	▴
				A. filiculoides						
5	1.252	0.002	█	*L. minuta*	•	●	●			•
				L. minor						
				A. filiculoides						
6	1.139	0.011		*L. minuta*		•	•			
				L. minor					▴	▴
				A. filiculoides						
7	0.01	0.06		*L. minuta*	•	•	●	•	•	●
				L. minor			▴			
				A. filiculoides						
8	1.623	0.013		*L. minuta*	•	●	●		•	●
				L. minor						
				A. filiculoides	▪	▪	■		▪	▪
9	2.566	0.019	█	*L. minuta*						
				L. minor		▲	▲		▲	▲
				A. filiculoides						
10	0.02	0.008	█	*L. minuta*						
				L. minor						
				A. filiculoides						
11	0.935	0.004		*L. minuta*						
				L. minor						
				A. filiculoides						
12	0.01	0.004		*L. minuta*		•	•	•	•	•
				L. minor						
				A. filiculoides						
13	0.537	0.004	▒	*L. minuta*						
				L. minor						
				A. filiculoides						
14	0.91	0.004	▒	*L. minuta*						
				L. minor						
				A. filiculoides						
15	4.204	0.006	▒	*L. minuta*						
				L. minor						
				A. filiculoides						
16	0.01	0.03	█	*L. minuta*	•	⬤	⬤		•	•
				L. minor	▴		▲			
				A. filiculoides			■			
17	2.95	0.002	█	*L. minuta*						
				L. minor	⯅	▲	▲		▴	▴
				A. filiculoides						
18	0.01	0.001		*L. minuta*	●	●	⬤		•	•
				L. minor						
				A. filiculoides	▪	■	■			
19	1.167	0.002		*L. minuta*	●	⬤	⬤	•	⬤	⬤
				L. minor		▴	▲		▴	▴
				A. filiculoides						
20	0.785	0.006		*L. minuta*						
				L. minor	▴	▴	▴	▴	▴	▴
				A. filiculoides						
21	3.336	0.005	█	*L. minuta*						
				L. minor	▴	▴	▴	▴	▴	▴
				A. filiculoides						
22	1.814	0.002	█	*L. minuta*						
				L. minor						
				A. filiculoides						
23	3.807	0.002	█	*L. minuta*	•	⬤	⬤	⬤	⬤	•
				L. minor						
				A. filiculoides						
24	0.38	0.002	▒	*L. minuta*						
				L. minor			▲		▴	▲
				A. filiculoides						

Table 2. Presence and abundance of *L. minuta*, *L. minor* and *A. filiculoides* in 24 sites monitored at 6 different time points. Total Oxidised Oxygen (TON), Total Phosphorus (TP), and canopy cover are also shown.

There was no clear relationship (Tab. 3) between the percentage surface cover of the three species and TON and TP concentrations. Also the analysis of canopy cover did not reveal any significant correlation with the occurrence of the three species.

	Correlation coefficient		
	L. minuta	*L. minor*	*A. filiculoides*
TON	-0.193	0.044	-0.195
TP	-0.054	0.268	0.183
Canopy	-0.219	-0.174	-0.163

Table 3. Correlation coefficient (Kendall's Tau b test) between the percentage surface cover of *L. minuta*, *L. minor* and *A. filiculoides* with Total Oxidised Nitrogen (TON), Total Phosphorus (TP) and Canopy cover. Correlation refers to the data collected in April and June 2014 for TON and TP and only June 2014 for canopy cover. The data of percentage coverage by aquatic macrophytes refer to the same months of 2014.

The occurrence of the three species in the same month (June), but in subsequent years was analysed. In general, the three species were more widespread in the first year of monitoring. The comparison of June 2014 with June 2015 (months with the highest presence of the three species), showed that the number of ponds in which *L. minuta* was present decreased from eleven (in 2014) to eight (in 2015). The eight water bodies that contained *L. minuta* in 2015 also contained *L. minuta* in 2014. The number of sites in which *L. minor* was present decreased from ten to eight. One of the ponds that contained *L. minor* in 2015 did not contain *L. minor* in 2014. The number of sites in which *A. filiculoides* was present decreased from three to one from 2014 to 2015 (Fig. 6).

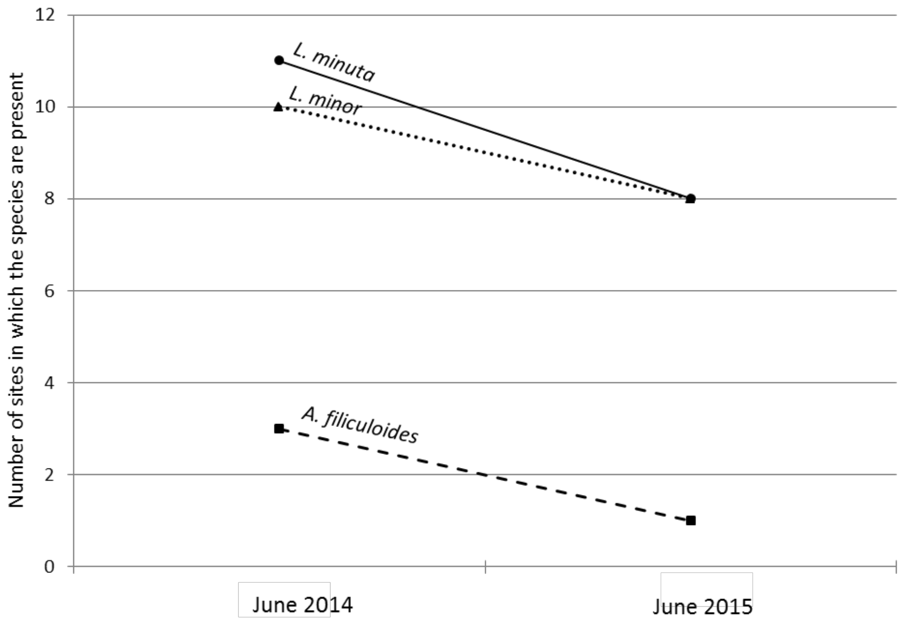

Figure 6 Decrease of the occurrence of the three species from 2014 to 2015. A total of 24 ponds were monitored. The graph shows the total number of sites in which the species were found in June 2014 and 2015.

2-Quantifying growth-rates of floating macrophytes in natural water bodies that differ in species composition and dominance

(a) Growth of three floating macrophytes under allopatric conditions

A total of nine ponds were selected to monitor in more detail growth rates (RGR). The nine ponds comprised three categories of ponds; three in which *L. minuta* was abundant, three in which *L. minor* was abundant and three in which none of the three species was present. RGR varied between 0.013 and 0.075 day^{-1} over the period from 20th of May to 20th of June 2014 (Fig. 4). *A. filiculoides* displayed the highest RGR and *L. minor* the lowest RGR in each pond category, over the course of the experiment (Fig. 7). There was a significant difference in the RGR of the three species, but no significant effect of pond category, nor was there any significant interaction between species or pond category (Fig. 7; Tab. 4). Tukey post-hoc tests revealed that the RGR of *A. filiculoides* was significantly greater (P=0.003) than of *L. minor*.

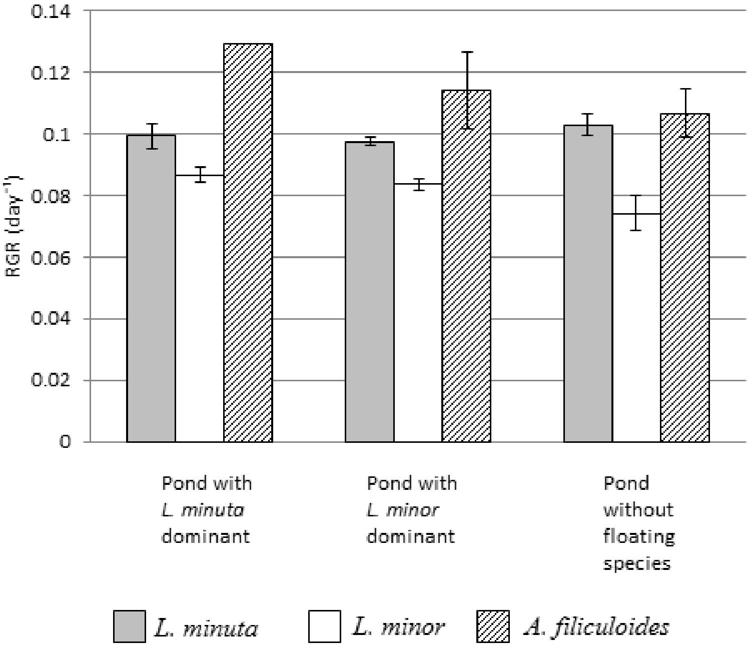

Figure 7. Mean (+/- 1 S.E.) RGR of *L. minuta*, *L. minor* and *A. filiculoides*, grown in allopatric conditions, in the 3 different categories of ponds (ponds dominated by *L. minuta*, ponds dominated by *L. minor* and ponds with floating species absent) over the period from 20th of May to 20th of June 2014.

Tests of Between-Subjects Effects

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
species	.005	2	.003	7.865	.004	.466
pond	.001	2	.000	1.306	.295	.127
species * pond	.000	4	5.415E-05	.170	.951	.036
Error	.006	18	.000			
Total	.272	27				
Corrected Total	.012	26				

a. R Squared = .514 (Adjusted R Squared = .298)

Table 4. Results of the 2-way ANOVA. Interaction between species (*L. minuta, L. minor* and *A. filiculoides*) and categories of pond (dominated by *L. minuta*, dominated by *L. minor* or without floating species) in the comparison of the RGR of the three species grown in allopatric conditions.

(b) Growth of three floating macrophytes under sympatric conditions

When *L. minuta* and *L. minor* were grown in the presence of *A. filiculoides* the RGR of the Lemnaceae was significantly reduced. *A. filiculoides* appeared to prevent the spread of the other two species by taking over most of the space available (Fig. 8). For *L. minuta*, RGR was significantly affected by both other species, but there was no significant effect of pond category, nor was there a significant interaction between the two factors (Fig. 9 *a*; Tab. 5). Tukey post-hoc tests revealed that RGR of *L. minuta* grown alone was significantly greater than when grown with *L. minor* (p=0.002), with *A. filiculoides* (p<0.001) and with both species (p<0.001).

Also for *L. minor* RGR was significantly affected by the presence of the other species but the different categories of pond did not affect the growth. There was no significant interaction between the two factors (Fig. 9 *b*; Table 5). Tukey post-hoc tests revealed that RGR of *L. minor* grown alone was significantly greater than when grown with *A. filiculoides* (p=0.003) and with both species (p<0.001).

For *A. filiculoides*, RGR was not affected by the category of pond, but it was affected by the presence of other species (Fig. 9 *c*; Tab. 5). This species showed a significantly different RGR when grown in the presence of *L. minuta* (p=0.027).

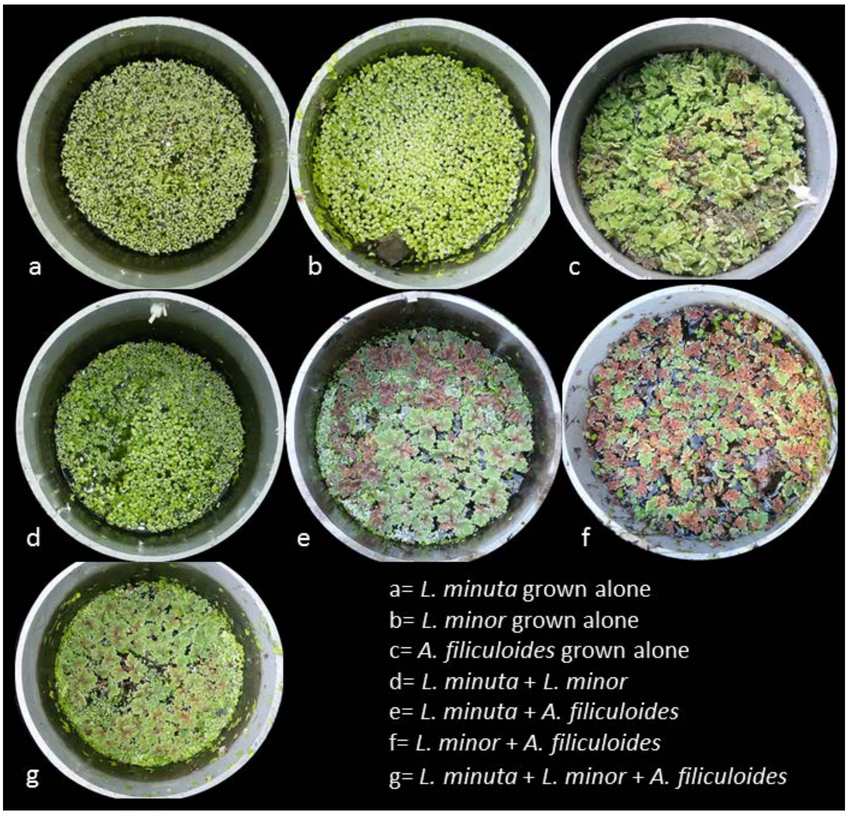

Figure 8. Example of final species composition and abundance at the end of the fourth week of experiment that took place between May and June 2015 in natural ponds along the river Lee in Ireland.

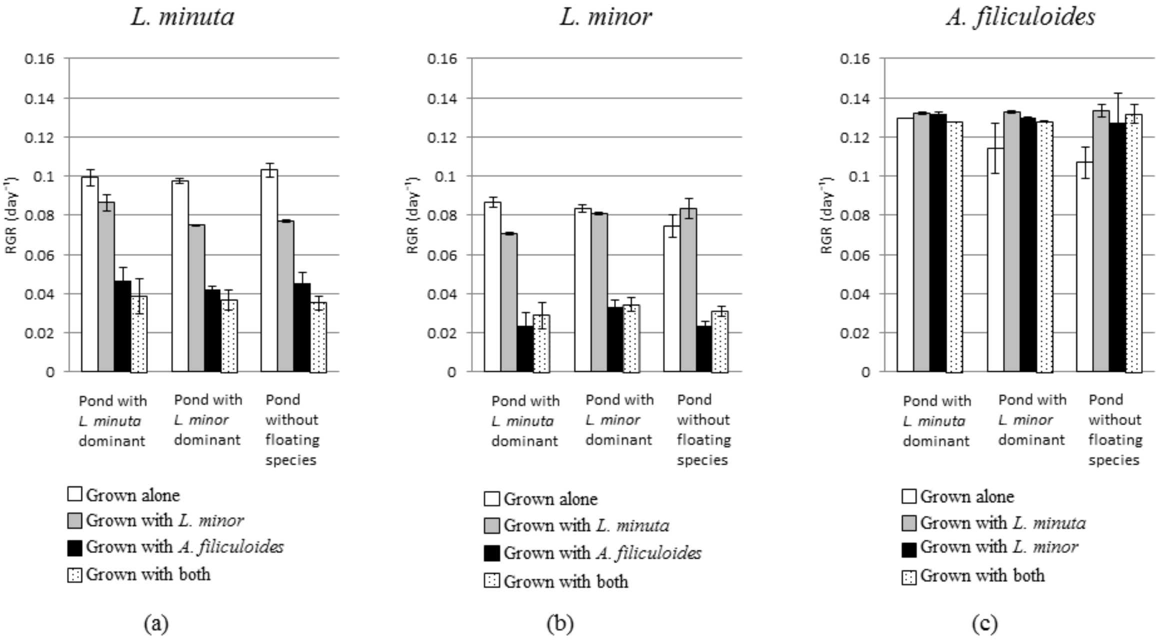

Figure 9. Mean (+/- 1 S.E.) RGR of *L. minuta*, *L. minor* and *A. filiculoides* grown in different mixtures of species (alone, with one of the other two species and with both), in the three different categories of ponds (ponds dominated by *L. minuta*, ponds dominated by *L. minor* or ponds with floating species absent).

Tests of Between-Subjects Effects

Species	Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
L. minuta	mix	.023	3	.008	137.944	.000	.945
	pond	.000	2	7.059E-05	1.244	.306	.094
	mix * pond	.000	6	2.874E-05	.507	.797	.112
	Error	.001	24	5.674E-05			
	Total	.179	36				
	Corrected Total	.025	35				
L. minor	mix	.023	3	.008	165.625	.000	.954
	pond	.000	2	.000	2.221	.130	.156
	mix * pond	.000	6	8.161E-05	1.729	.157	.302
	Error	.001	24	4.719E-05			
	Total	.133	36				
	Corrected Total	.025	35				
A. filiculoides	mix	.001	3	.000	3.579	.029	.309
	pond	.000	2	8.412E-05	.727	.493	.057
	mix * pond	.001	6	.000	.867	.533	.178
	Error	.003	24	.000			
	Total	.587	36				
	Corrected Total	.005	35				

Table 5. Summary of 2-way ANOVAs for each species, of the effects of Species Mix (alone, with one of the other two species and with both) and pond category (ponds dominated by *L. minuta*, ponds dominated by *L. minor* or ponds with floating species absent) on RGR.

3-Quantifying dynamic changes in growth-rate and competition between the three species of floating macrophytes in mesocosms

a) Analysis of the RGR of the three species across the seasons under allopatric conditions in mesocosms.

Mesocosms were constructed to facilitate the study of growth throughout the four seasons. The analysis of growth under allopatric conditions showed that the three species had a reduced RGR in the colder months (From November to January), while their RGR increased from spring onwards. Growth of *L. minuta*, *L. minor* and *A. filiculoides* peaked in the summer period between May and September. For *L. minuta* the highest RGR was 0.077±0.015 day^{-1} in July. For *A. filiculoides* the highest RGR

(0.12±0.02 day^{-1}) was obtained in July. For *L. minor* RGR peaked in September (0.087±0.007day^{-1}). Lowest growth rates were measured in January, when none of the three species grew. Both in December and February, only *L. minor* and *A. filiculoides* displayed growth, while *L. minuta* only displayed substantial growth from March (Fig. 10).

Analysis using 2-way repeated measures ANOVA highlighted that there was a significant difference in RGR, both between species and between months. The interaction between the two factors was also significant (Tab. 6). In the colder months (from December from February) *A. filiculoides* did not significantly outgrow the other two species, but in March the RGR was higher than for *L. minuta* (p=0.042) and *L. minor* (p=0.02). The water fern continued to grow faster than *L. minuta* and *L. minor* in all the following months until September (although not always significantly, see Fig. 10).

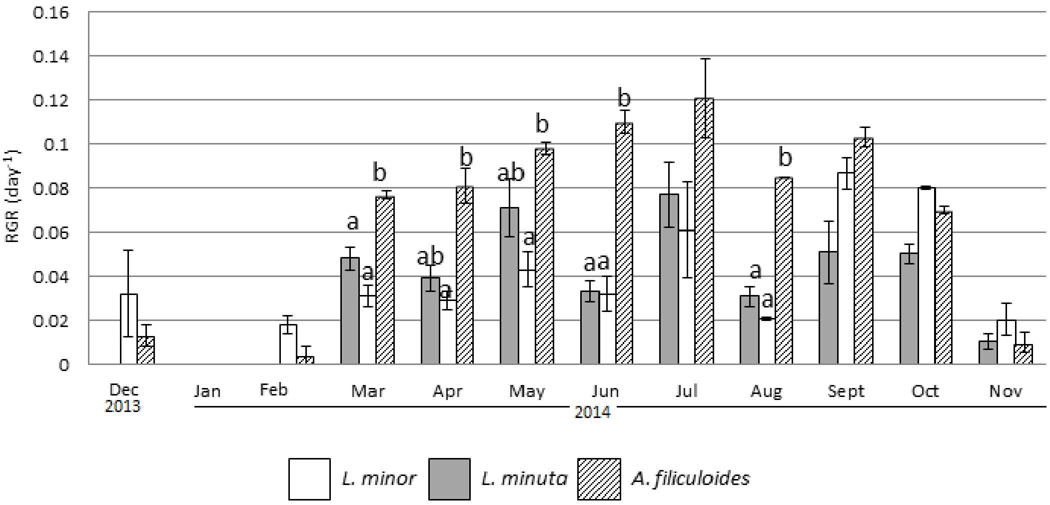

Figure 10. Mean (+/- 1 S.E.) RGR of *L. minuta*, *L. minor* and *A. filiculoides*, grown in allopatric conditions, in outdoor mesocosms, from December 2013 to November 2014.

Tests of Within-Subjects Effects						
		Type III Sum of Squares	df	Mean Square	F	Sig.
species	Greenhouse-Geisser	.030	1.282	.023	22.444	.009
	Huynh-Feldt	.030	1.821	.017	22.444	.002
	Lower-bound	.030	1.000	.030	22.444	.018
time	Greenhouse-Geisser	.116	1.584	.074	35.030	.002
	Huynh-Feldt	.116	3.064	.038	35.030	.000
	Lower-bound	.116	1.000	.116	35.030	.010
species * time	Greenhouse-Geisser	.036	2.290	.016	6.868	.021
	Huynh-Feldt	.036	10.084	.004	6.868	.000
	Lower-bound	.036	1.000	.036	6.868	.079

Table 6. Results of the 2-way repeated measures ANOVA. Interaction between species (*L. minuta*, *L. minor* and *A. filiculoides*) and time in the comparison of the RGR of the three species, grown in allopatric conditions in mesocosms.

b) Analysis of the surface cover (%) of the three species across the seasons and under allopatric conditions.

The percentage of surface cover was measured every month for each of the three species grown in allopatric conditions. In the period December through to February, none of the species covered more than 2% of the surface area. From March to May only *L. minor* slightly increased its percentage of surface cover. *A. filiculoides* increased its percentage of coverage only from May, and *L. minuta* only from June. In the period July to November *A. filiculoides* covered up to the 100% of the mesocosm surface. The highest percent cover reached by *L. minuta* was 74.9±13.4 % in August. For *L. minor*, the highest percentage of cover was 42.64±9.17 % in July (Fig. 11).

A 2-way repeated measures ANOVA showed that there was a significant difference in surface cover between species and between months. The interaction between the two factors was also significant (Tab. 7). From November to March 2015 there was no significant difference in surface cover between the three species, while, in April, *L. minor* surface cover was significantly higher than that by both *L. minuta* (p=0.024) and *A. filiculoides* (p=0.046). From June to November *A. filiculoides* always covered an area significantly greater than both *L. minuta* and *L. minor*. In these summer months, *L. minuta* covered an area greater than *L. minor*, but only in October was this difference significant (p= 0.006).

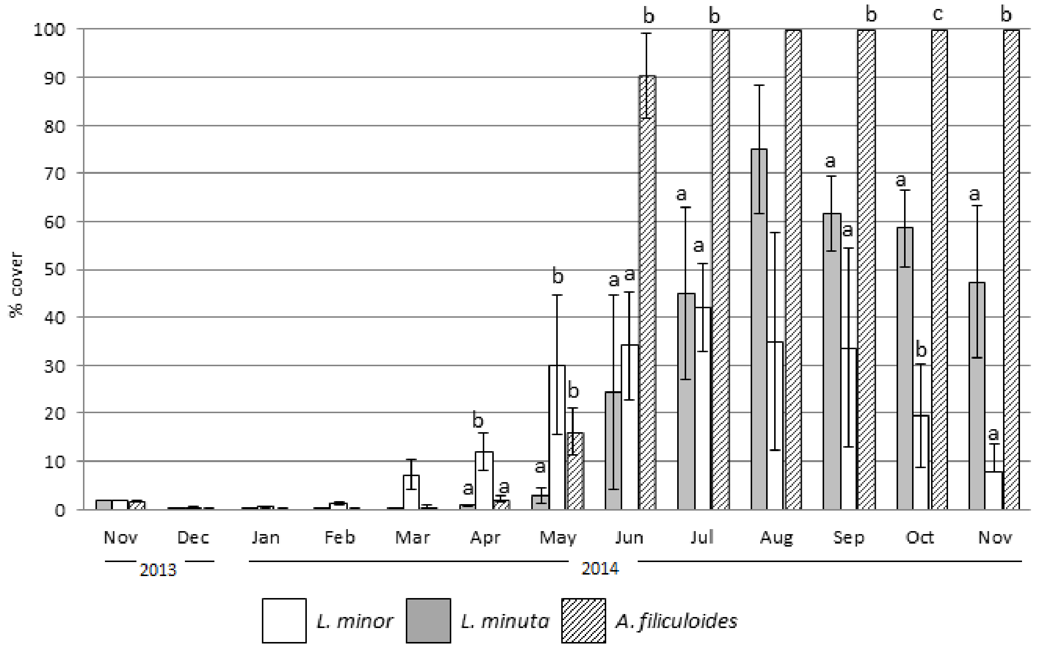

Figure 11. Mean (+/- 1 S.E.) percentage surface cover by *L. minuta*, *L. minor* and *A. filiculoides*, grown in allopatric conditions, in outdoor mesocosms from December 2013 to November 2014. The total surface area (100%) was 1753 cm^2.

Tests of Within-Subjects Effects						
		Type III Sum of Squares	df	Mean Square	F	Sig.
species	Greenhouse-Geisser	28118.260	1.069	26312.858	13.462	.031
	Huynh-Feldt	28118.260	1.178	23877.070	13.462	.025
	Lower-bound	28118.260	1.000	28118.260	13.462	.035
time	Greenhouse-Geisser	115234.953	1.355	85037.560	55.056	.001
	Huynh-Feldt	115234.953	2.079	55416.844	55.056	.000
	Lower-bound	115234.953	1.000	115234.953	55.056	.005
species * time	Greenhouse-Geisser	39591.563	1.700	23286.138	9.429	.021
	Huynh-Feldt	39591.563	3.694	10718.931	9.429	.002
	Lower-bound	39591.563	1.000	39591.563	9.429	.055

Table 7. Results of the 2-way repeated measures ANOVA. Interaction between species (*L. minuta*, *L. minor* and *A. filiculoides*) and time in the comparison of the surface cover by the three species grown in allopatric conditions.

c) Analysis of the RGR of the three species grown in different species mixtures (alone, with one of the other two species or with both).

Data analysis by 2-way repeated measures ANOVA was separately run for each species. For *L. minuta*, the RGR of allopatric cultures was compared with the RGR of sympatric mixtures in which *L. minuta* was grown together with *L. minor*, and/or *A. filiculoides*. Similarly, the RGR of the other species was compared between allopatric and sympatric conditions. Overall, the data show a similar seasonality as was observed under allopatric conditions (Fig. 12).

For *L. minuta* and *A. filiculoides* significant differences in RGR occurred across different months, while for *L. minor* RGR did not vary significantly with time. There was no significant effect of mixing species on RGR. For none of the three species a significant interaction between culture mix and time was found (Tab. 8).

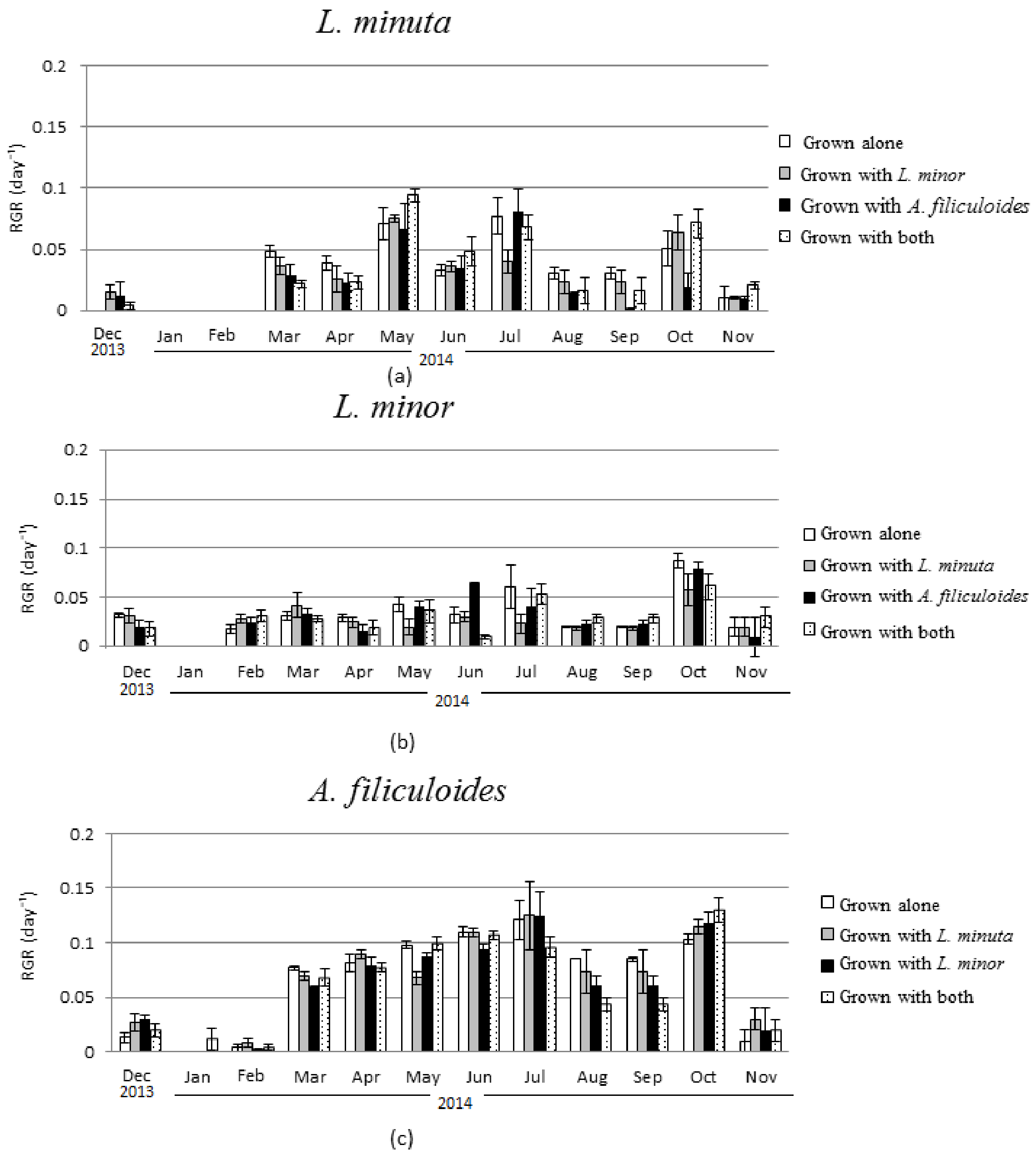

Figure 12. Mean (+/- 1 S.E.) RGR of *L. minuta*, *L. minor* and *A. filiculoides*, grown in different mixtures of species (alone, with one of the other two species and with both), in outdoor mesocosms from December 2013 to November 2014.

Tests of Within-Subjects Effects

			Type III Sum of Squares	df	Mean Square	F	Sig.
L. minuta	mix	Greenhouse-Geisser	.002	1.462	.001	1.286	.343
		Huynh-Feldt	.002	2.501	.001	1.286	.341
		Lower-bound	.002	1.000	.002	1.286	.339
	time	Greenhouse-Geisser	.047	1.950	.024	15.058	.005
		Huynh-Feldt	.047	5.526	.008	15.058	.000
		Lower-bound	.047	1.000	.047	15.058	.030
	mix * time	Greenhouse-Geisser	.029	2.417	.012	3.340	.089
		Huynh-Feldt	.029	13.163	.002	3.340	.002
		Lower-bound	.029	1.000	.029	3.340	.165
L. minor	mix	Greenhouse-Geisser	.011	1.147	.009	.476	.559
		Huynh-Feldt	.011	1.396	.008	.476	.588
		Lower-bound	.011	1.000	.011	.476	.540
	time	Greenhouse-Geisser	.220	1.142	.193	4.875	.103
		Huynh-Feldt	.220	1.383	.159	4.875	.086
		Lower-bound	.220	1.000	.220	4.875	.114
	mix * time	Greenhouse-Geisser	.329	1.571	.209	1.294	.342
		Huynh-Feldt	.329	2.997	.110	1.294	.335
		Lower-bound	.329	1.000	.329	1.294	.338
A. filiculoides	mix	Greenhouse-Geisser	.001	1.692	.000	.248	.756
		Huynh-Feldt	.001	3.000	.000	.248	.861
		Lower-bound	.001	1.000	.001	.248	.653
	time	Greenhouse-Geisser	.285	1.295	.220	23.970	.008
		Huynh-Feldt	.285	1.866	.153	23.970	.002
		Lower-bound	.285	1.000	.285	23.970	.016
	mix * time	Greenhouse-Geisser	.031	2.122	.015	1.119	.387
		Huynh-Feldt	.031	7.393	.004	1.119	.387
		Lower-bound	.031	1.000	.031	1.119	.368

Table 8. Results of a 2-way repeated measures ANOVA. Interactions between different species mixtures and time in the comparison of the RGR of the three species.

d) Analysis of surface cover of the three species grown in different species mixtures (alone, with one of the other two species or with both).

The monthly surface cover by each species grown alone was compared with the cover of that same species grown with one of the other two species or with both. Data analysis by 2-way repeated measures ANOVA was separately run for each species.

Surface cover by floating macrophytes strongly depended on the season, as was also observed for allopatric conditions (Fig. 13). For all the 3 species there was a significant interaction between species mixture and time (Tab. 9).

The surface area occupied by *L. minuta* was significantly affected by both the species mixture and the month of the year (Tab. 9). Surface cover was strongly reduced when this species was cultured in the presence of the other two species, but only from July to November (Fig. 13 *a*). In July, *L. minuta* surface cover was reduced when grown together with *L. minor* and surface cover was nearly completely suppressed when *A. filiculoides* was present in the culture mix. A similar situation was observed also in the following months.

The surface area occupied by *L. minor* was also significantly affected by the month of the year, while the effect of the species mixture was not statistically significant (Tab. 9). The percentage surface cover of this species was reduced by the presence of *A. filiculoides*, in the period from July to November, but not significantly. The presence of *L. minuta* had no effect on *L. minor* surface area (Fig. 13 *b*).

The surface area occupied by *A. filiculoides* was significantly affected by both the species mixture and the month of the year (Tab. 8). The most evident difference between species mixes occurred in May and June, when *A. filiculoides* surface cover was reduced by the presence of the other species. From July to November there was a smaller difference between the surface coverage for this species grown alone and in sympatric conditions (Fig. 13 *c*).

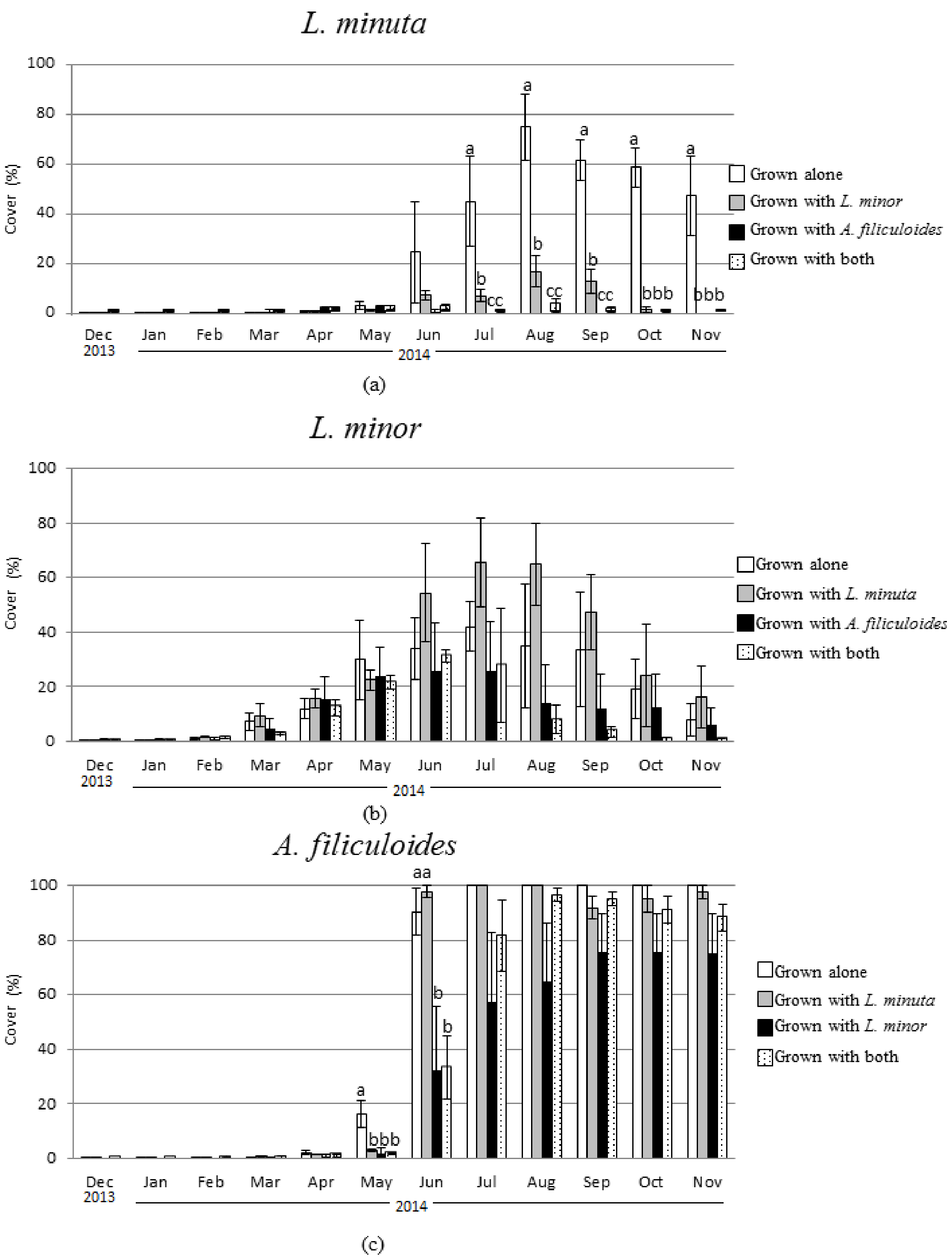

Figure 13. Mean (+/- 1 S.E.) Percent surface cover of *L. minuta*, *L. minor* and *A. filiculoides*, grown in different mixes (alone, with one of the other two species or with both), in outdoor mesocosms from December 2013 to November 2014. Different letters indicate significant differences between species in each month. The total surface area (100%) was 1753 cm^2.

Tests of Within-Subjects Effects

			Type III Sum of Squares	df	Mean Square	F	Sig.
L. minuta	mix	Greenhouse-Geisser	21169.910	1.231	17201.353	69.012	.001
		Huynh-Feldt	21169.910	1.652	12814.787	69.012	.000
		Lower-bound	21169.910	1.000	21169.910	69.012	.004
	time	Greenhouse-Geisser	11512.566	1.439	7999.795	17.413	.010
		Huynh-Feldt	11512.566	2.407	4783.764	17.413	.001
		Lower-bound	11512.566	1.000	11512.566	17.413	.025
	mix*time	Greenhouse-Geisser	24376.574	1.472	16560.542	10.882	.022
		Huynh-Feldt	24376.574	2.544	9580.624	10.882	.005
		Lower-bound	24376.574	1.000	24376.574	10.882	.046
L. minor	mix	Greenhouse-Geisser	9772.164	1.715	5697.963	1.346	.331
		Huynh-Feldt	9772.164	3.000	3257.388	1.346	.320
		Lower-bound	9772.164	1.000	9772.164	1.346	.330
	time	Greenhouse-Geisser	33933.470	1.414	24001.549	13.787	.016
		Huynh-Feldt	33933.470	2.304	14725.586	13.787	.003
		Lower-bound	33933.470	1.000	33933.470	13.787	.034
	mix*time	Greenhouse-Geisser	12316.381	1.868	6594.991	1.722	.260
		Huynh-Feldt	12316.381	4.830	2549.817	1.722	.193
		Lower-bound	12316.381	1.000	12316.381	1.722	.281
A. filiculoides	mix	Greenhouse-Geisser	10883.911	1.174	9269.019	5.053	.096
		Huynh-Feldt	10883.911	1.477	7368.305	5.053	.076
		Lower-bound	10883.911	1.000	10883.911	5.053	.110
	time	Greenhouse-Geisser	351738.347	1.603	219371.285	215.791	.000
		Huynh-Feldt	351738.347	3.160	111302.221	215.791	.000
		Lower-bound	351738.347	1.000	351738.347	215.791	.001
	mix*time	Greenhouse-Geisser	16870.522	2.185	7722.444	2.485	.156
		Huynh-Feldt	16870.522	8.264	2041.433	2.485	.038
		Lower-bound	16870.522	1.000	16870.522	2.485	.213

Table 9. Results of a 2-way repeated measures ANOVA. Interactions between different species mixtures and time in the comparison of the surface cover of the three species.

Discussion

Invasiveness and invasibility have been studied for years as they are considered the key for predicting biological invasions and for restoring invaded habitats (Alpert *et al.*, 2000). Factors thought to increase habitats invasibility are, for example, low competition with other species, altered disturbance regimes, absence of environmental stressors, and high resource availability (Alpert, 2000). Several studies tried to define the most common factors that promote invasions (e.g. Beerling, 1991; Rouget and Richardson, 2003; Rejmanek *et al*. 2005) and many of them used a comparative approach (e.g. Mack, 1996; Grotkopp *et al*., 2002). In this study growth rates in allopatric and sympatric conditions, and occurrence in a natural environment were compared for two invasive and a native species, using a combination of field monitoring and mesocosm experiments.

Plant resources and distribution of floating aquatic species

L. minor was the most abundant species in the study area, closely followed by *L. minuta*. In comparison, *A. filiculoides* was relatively rare. A substantial gradient of nutrient and light conditions was found to occur in natural ponds. Several papers reported that the presence of free floating species is associated with high levels of nutrients (Carbiener *et al*., 1990; Morris *et al*. 2003). In our area of study no correlation was observed between degree of eutrophication and the presence and/or abundance of the three species (Tab. 1). The three species were present both in eutrophic and in oligotrophic waters. Conversely, some water bodies rich in nutrients did not contain any floating species. It was expected to find more *A. filiculoides* in oligotrophic waters as it is well known that this species can grow in waters low, or even devoid of nitrogen. Yet, the data show that *A. filiculoides* was actually absent in most of the oligotrophic ponds and that the sites where it was abundant contained substantial levels of TON. Also the advantage of *L. minor* over *L. minuta* in oligotrophic waters, hypothesized by Njambuya *et al*. (2011) after observing a better performance of the native species under low nutrients conditions in a lab experiment, was not confirmed by our field monitoring of presence and/or abundance of these species. The lack of agreement between laboratory and field studies is not very surprising considering that, in a complex natural system, there are multiple chemical and physical factors interacting. The effect of nutrients on the presence of free-floating plants can be masked by other factors. A similar observation was made by Makkay *et al*., (2008). These authors tested the importance of physical and chemical factors in explaining aquatic plant community composition and the authors observed that single physical and chemical variables failed to explain the variation in community composition. Similar considerations apply to the lack of correlation between distribution of the three plant species and light levels. Although low light conditions have been associated with a lower invasibility of the habitat (Daehler, 2003), this factor alone does not determine the distribution pattern.

Consistent with the lack of a major role for environmental factors in determining the dominance of one of the three floating macrophytes, the reciprocal transplanting experiments demonstrated that all species are perfectly able to grow in ponds, even ponds were they do not naturally occur. As shown in figures 7 and 9, all species are able to grow in all the tested waterbodies, irrespective the specific characteristics. These data strongly emphasise that the distribution of the three species is not limited by the prevailing physico-chemical characteristics of the studied water bodies during the summer period. A similar observation was made by Ceschin *et al.* (2016). These authors observed a progressive replacement of *L. minor* with *L. minuta* in central Italy and concluded that the out-competition of the alien species is not explained by different ecological requirements.

RGR, seasonality and competition between the three species

The field experiment highlights the high RGR of *A. filiculoides* compared to the other two species. Moreover, *L. minuta* and *L. minor* had a reduced RGR when grown in close vicinity to the water fern. Thus, in the field *A. filiculoides* suppressed the growth of the other two species, possibly by taking over the space available and consequently reducing the surface necessary for the other species to expand. This explanation is confirmed by the mesocosm experiment. In the mesocosm experiment plants in the enclosures were harvested before the available surface was completely covered. Under these conditions, the RGR of *L. minuta* and *L. minor* were not suppressed by the presence of *A. filiculoides*. On the contrary, in the main area of the mesocosms, where the plants were never harvested, *A. filiculoides* was able to reduce the coverage of *L. minuta* and *L. minor*, confirming the results of the field experiment. The analysis of the areas covered by the three species in the different species mixtures clearly highlighted that *L. minuta* and *L. minor* can easily grow and cover all the available surface, unless *A. filiculoides* prevents them from growing by taking over the space available.

It is possible to conclude that *A. filiculoides* is able to out-compete the two duckweeds due to a higher RGR and these findings are consistent with the results of Filizadeh (2002). However, these conclusions are valid only in the warmer months. The mesocosm experiment showed that *L. minor* displayed a significantly higher RGR than the other two species in winter (December – February). Thus, the data show differences in phenology, with *L. minor* being able to restart its growth earlier in the year. Reddy and DeBusk (1985) also concluded that the growth of the water fern (A*zolla caroliniana* Willdenow) was more influenced by seasonal changes than *L. minor*. This suggests that *L. minor* is better adapted to lower temperatures, a suggestion that is consistent with work by Paolacci (chapter 3) showing a relatively strong tolerance of this species to low temperatures under laboratory conditions.

What drives presence, abundance and dominance of the three species?

Based on high RGR and surface cover, it can be argued that *A. filiculoides* was the species with the highest degree of invasiveness. Remarkably, despite its invasive character, *A. filiculoides* was relatively rare in the study area. Indeed, the species most present throughout the study area was *L. minor* which has the lowest RGR, under field conditions in summer.

Before discussing this conundrum, it is worth pointing out variations in the distribution of floating aquatic plants changes between years. For example, substantial levels of *L. minuta* were found in pond 1 up to the summer of 2014, but the species was absent in 2015. Similarly, while *L. minor* was highly abundant in pond 17 in 2014, the species was relative rare in 2015. Shifts in floating plants composition were earlier observed by O'Farrell *et al*. (2011) who observed cyclic alternation of free-floating plants with phytoplankton in response to seasonal flooding and extreme drought and flood events. Flooding and drought are also considered responsible for changes in aquatic plants distribution by Bornette and Puijalon (2011), along with other factors such as fluctuation in nutrient content and temperature. As stated before, the area investigated is subjected to both cold and flooding in winter and drought in summer. Based on literature analysis, the field observations and the two experiments performed in this study, two mechanisms can be proposed to explain the variations in species composition between years.

(1) It can be argued that re-colonisation of these ponds after winter flooding and/or summer drought is a determinant of vegetation composition and, therefore, that dispersal pathways need to be considered when analysing vegetation dynamics. Chance dispersal from a source has often been implicated in explaining the "spotty" distribution of floating plants (Wolek, 1983; Kline and McCune, 1987). The area examined in the present study is rich of waterfowl and the small size of the two Lemnaceae investigated in this work enables them to stick to the birds feathers and be transported from a pond to another relatively easily. This mechanism is well described for short distance dispersal (Jacobs 1947, Reynold *et. al.*, 2015) and it was recently argued that waterfowl can be also responsible long-distance dispersal of small aquatic plants (Coughlan *et al.*, 2015 *a* and *b*). Given the proximity of one water body to another (less than 50 m in some cases), it is reasonable to assume that both *L. minuta* and *L. minor* were effectively dispersed between water bodies. Different considerations apply to *A. filiculoides*. While the literature contains numerous studies about Lemnaceae dispersion, there is a knowledge gap in the study of *Azolla* dispersion. Yet, an experiment carried out in proximity of the study area, showed how short distance dispersal (3 m) mediated by waterfowl is much more frequent for Lemnaceae than for *A. filiculoides* (unpublished data). Thus, the difficulty of dispersal can, at least in part, explain the reduced presence of *A. filiculoides* in the field despite of the species out-competing the Lemnaceae both in the field and in mesocosms.

(2) In this study it has been found that *L. minuta* and *A. filiculoides* need higher temperatures to re-start their growth. In comparison, native *L. minor* displays growth throughout the year. Thus, *L. minor* can potentially have a competitive advantage over the other species due to an earlier restart of growth after the winter. It could be argued, that this may result in outcompeting of the other two species. Once the temperature increases, *L. minuta* and *A. filiculoides* become more competitive. Thus, the balance between species will depend on the seasonal pattern of temperatures. A long cool spring is likely to cause dominance by *L. minor,* while a long hot summer would favour the other two species. Such weather dependent shifts in balance between species may

> explain the observed differences in macrophyte distribution between the two years of study (Fig. 6). This scenario resembles observations by Peeters *et al*. (2013). These authors observed that milder winters are correlated with a higher abundance of free-floating plants and a reduced presence of submerged plants. It was argued that a mild winter prevents the floating plants from dying and enables them to form a mat on the water surface that inhibits the growth of submerged plants. The study of Peeters *et al*. (2013) did not analyse differences and competition between free-floating plants, but it is reasonable to think that the composition of the mats can reflect differences in phenology and different annual temperature trends. Also the studies of Hussner and Losch (2005) and Sajna *et al*. (2007), who concluded that invasive species can be favoured by the increase of winter temperatures, support the thesis according to which the distribution pattern observed in this study can depend, at least in part, on the annual pattern of temperature.

Both scenarios described above can affect the spread and abundance of the species and they can also co-occur and interact. The necessity of the species to re-colonize the water bodies every year (because of cold winters and/or floods), along with the ability of the native *L. minor* to start growing earlier in the year protects the habitat from more extensive invasion by *A. filiculoides*. This explanation implies that the disturbance of the habitat (winter floods, summer drought), defined as an event that kills or removes their biomass, plays in favour of the native species and thus would decrease invasibility of the habitat. In contrast, many authors have found that disturbance increases invasibility (Hobbs and Huenneke, 1992; Smith and Knapp, 1999;) as it provides a gap for colonisation by invasive species. However, there are other cases in literature in which disturbance events impeded a particular invasion and the suppression of the disturbance led actually to a higher invasibility. For example, Milchunas *et al*. (1989) found that in North American grassland, grazing decreased invasion by alien species.

It is concluded that, based on growth performance, *A. filiculoides* is the most competitive of the three species during the summer. Thus, this species possesses a high degree of invasiveness. *L. minuta* is less invasive than *A. filiculoides.* This study shows that the invasiveness of the species during the summer months is not reflected in the actual distribution pattern in a series of natural water bodies. In fact, alien species are under-represented in the monitored area, probably because of the interaction of several factors related to maintaining growth under winter-conditions and/or dispersal after that bottleneck season.

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Chapter 6

Discussion

Invasive alien plants are considered one of the primary threats to biodiversity as well as to the integrity and function of ecosystems (Pimentel *et al*., 2001; Semenya *et al*., 2012). Pimentel *et al*., (2001) estimated that invasive, non-native species are causing financial damage in excess of US$ 314 billion per year in the United States, United Kingdom, Australia, South Africa, India, and Brazil. These costs include loss of crops, impediments to recreational activities such as fisheries and navigation, and costs related to the application of eradication and control programmes. Preventive management actions, such as eradication of newly arrived invasive alien species before establishment, is often the most realistic approach from an economic point of view. However, it is unrealistic to manage all alien species colonizing a new environment. Thus, it is necessary to predict which alien species can potentially become invasive in order to avoid investing money on species that will have little environmental impact. Any prediction of the threat of invasion must evaluate both the invasiveness of the species and the invasibility of the habitat that it is colonized. Indeed, some species are known to be very invasive in one particular environment, but not in a habitat with different characteristics (Daehler, 2003; Richardson and Rejmanek, 2004).

Invasive species are often associated with a high growth rate (Cronk and Fuller, 1995), production of large numbers of seeds with high germination rates (Callaway and Josselyn, 1992; Honig *et al*., 1992) and high phenotypic plasticity (which allows invaders to succeed in a wider range of environments) (Williams and Black, 1994; Pattison *et al*., 1998). The ability to disperse is another characteristic often associated with invasive species (Rejmanek, 2000). Habitats that are particularly at risk of invasions are, for example, habitats with low biodiversity or habitats with a high availability of nutrients (Milbau and Nijs, 2004). On the other hand, Davis *et al.* (2000) associated the presence of specific environmental stressors with a lower invasibility of the habitat. According to these authors, specific stressors can protect the habitat from invasion by giving the native species, supposedly adapted to the stressor, a competitive advantage over the generalist alien species. The theory of fluctuating resources of Alpert (2000) identifies invasive species with opportunistic species able to maximize their growth and performance under extremely favourable resources. According to this theory, random or periodic events of disturbance can disrupt and/or remove a native plant community, leaving unused resources that can promote the invasion by an alien, competitive/ruderal species.

Comparative studies of native and closely related invasive alien species have been successfully used to identify traits associated with invasiveness, and such knowledge can be used to inform management policies. The present case study investigated how several factors influence the physiological response and the performance of the alien species *Lemna minuta*, and the co-generic native *Lemna minor*. The effect of several nutrients on *L. minuta* and *L. minor* was analysed. The experiments showed a better performance of both species at high nutrients availability, but while high concentrations of nitrates did not have a disproportionate effect on either species, *L. minuta* took more advantage from the abundance of phosphates. In contrast, when the plants were grown on low concentrations of phosphate, the negative effect on the growth rate in *L. minor*, was not as strong as in *L. minuta*. Thus, these data show that an alien species can not necessarily take advantage of any form of eutrophication, but rather might respond to enrichment by a single plant nutrient.

Another difference between *L. minuta* and *L. minor* was found in the light utilization strategy. *L. minuta* is a heliophile species which, when grown at high light intensities, maximises its RGR by using a large portion of available light (higher qP and Y(II), and lower qN) to optimise carbon gain (higher NAR). In contrast, native *L. minor* can be classified as sciophilous. When grown at low light intensities, *L. minor* has a higher chlorophyll content and morphological plasticity (higher LAR) that help to limit the reduction of RGR under such growth conditions. Thus, the two closely related species show distinct light utilisation strategies, and this knowledge can be exploited to predict which water bodies are most at risk from invasion by *L. minuta*.

This study did not confirm that alien species are more sensitive to stress. In fact, *L. minuta* showed a higher tolerance to aluminium, copper, drought and high temperatures than *L. minor*. The alien species presents both characteristics typical in opportunistic species (such as a high growth rate and the ability to maximize the growth in conditions of high availability of resources) and an ability to tolerate a variety of stressors.

Based on laboratory experiments, the only stressor that *L. minor* seems to tolerate better than *L. minuta* is exposure to a low temperature. Consistently, in outdoor experiments the native species showed a higher growth rate than the aliens in winter. *L. minor* was also the species that restarted its growth earliest in the year. Although the better performance in colder months is not necessarily due to a better tolerance of low temperatures, it is possible to speculate that cold winters represent an advantage for the native species. This would also support the hypothesis of Hellmann *et al*. (2008), according to which, alien species that are presently unsuccessful out of their native range, will become more likely to succeed and become invasive if the average temperature rises.

Another fact which emerged from this study is that the potential invasiveness of *L. minuta* and *A. filiculoides* is not really reflected on the occurrence and abundance of these two species, observed in the field. Thus, high growth rates over the summer do not automatically result in high abundance. Daehler (2003) reviewed 79 papers in order to identify factors that promote biological invasions. He highlighted that, in studies that compared alien with native plants, only a small percentage of invasive species out-competed the native ones across all the growing conditions tested. In most cases, the performance was context-dependent. Daehler (2003) found that the most common growing conditions favouring natives over invaders were environments with low resource availability (nutrients, water, or light). In our study area no correlation between water chemistry, canopy cover and presence of the alien species emerged. Thus, other factors, related to the habitat or climate, prevent abundance of the alien species, it is speculated that one of these factors is a better ability of *L. minor* to survive the winter period.

Key findings

- *L. minuta* has characteristics typical of an opportunistic species such as the ability to maximize the growth when resources are abundant.
- Invasive, alien *L. minuta* is a good stress tolerator.
- *L. minor* can outperform *L. minuta* in conditions of low light, low phosphorus availability and low temperatures.
- *L. minuta* and *A. filiculoides* are characterised by a high degree of invasiveness, but the low invasibility of the studied habitat impedes invasion.

Conclusions

Foreseeing an invasion by an alien species is necessary in order to inform preventive management. This study shows that several widely accepted general assumption on invasiveness do not always apply. For example, the presence of a stressor does not necessarily disproportionally affect the alien species. Thus, the threat caused by the establishment of an alien species needs to be considered on a case-by-case basis, whereby the best approach comprises the parallel and integrated analysis of species and habitat characteristics.

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