

**EFFECTS OF ELEVATED ATMOSPHERIC CARBON DIOXIDE
CONCENTRATION AND NUTRIENT ENRICHMENT ON
FRESHWATER PLANT SPECIES**

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GENERAL ABSTRACT

Despite being extremely species-rich and highly threatened, freshwater ecosystems have not attracted as much conservation and research efforts as terrestrial or marine ecosystems, specifically in relation to global environmental change. Therefore, the overarching aim of this thesis was to examine the effects of rising atmospheric CO₂ concentration ([CO₂]) and altered nutrient conditions on a select number of invasive exotic freshwater plant species relative to their native and naturalised counterparts in Australia. Firstly, a systematic description to establish the current status of exotic freshwater plant species with reproducing wild populations in Australia was conducted. It was found that there are 63 exotic freshwater species with wild populations including 14 nationally invasive species and 10 species that are invasive in some states (declared weeds). Europe, South America and North America were the main sources of these species and the most important introduction pathway was the ornamental plant trade. The systematic description was then followed by experiments testing the growth, reproduction, functional trait and competitive responses of a range of species with contrasting invasive status to elevated [CO₂] and varied nutrient conditions. In most cases, the results showed that elevated [CO₂] and nutrient enrichment promoted plant growth either independently or in interaction with each other, and did not mediate competition between the tested species. Furthermore, the growth effects were species-specific and not strongly linked to the invasive status of the species. It can therefore be concluded that exotic freshwater plant species are an important component of Australian freshwater systems and their response to global environmental change is largely species-specific. In addition, based on the number of species that showed increased growth under nutrient enrichment, it is likely that it will have a more profound direct effect on the freshwater systems than elevated [CO₂].

CERTIFICATE

This thesis is an original contribution that has not been submitted, in any form, for a degree or diploma in any other university or institution.

Transportation, cultivation and disposal of *Egeria densa* and *Salvinia molesta* for the purpose of this thesis were conducted under New South Wales *Noxious Weeds Act 1993* permit number OUT16/18152. No other form of approval was required for this work.

The work of others has been used to prepare some aspects of the thesis and the extent of their contribution is clearly outlined below. Rachael Gallagher (Macquarie University) provided an initial list of freshwater plant species used in the work described in *Chapter 2*. Anthony Manea (Macquarie University) assisted with running the glasshouse experiment which is presented in *Chapter 3*. All other aspects of the work presented in this thesis were done by me.



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CHAPTER ONE

Introduction

1 **Background**

2 Freshwater ecosystems are experiencing significant biodiversity declines due to a
3 number of interacting human-mediated threats such as pollution, overexploitation, flow
4 modification, habitat degradation and invasive exotic species (Dudgeon *et al.*, 2006).
5 These ecosystems are among the most threatened ecosystems since human-mediated
6 threats are often more severe than in the most degraded terrestrial ecosystems (Sala *et al.*,
7 2000; Saunders, Meeuwig & Vincent, 2002). Despite being extremely species-rich and
8 highly threatened (Abell *et al.*, 2008), freshwater ecosystems remain relatively less studied
9 with regards to global environmental change compared to the terrestrial or marine
10 ecosystems (Brundu, 2015).

11 An important threat to native biodiversity in freshwater systems is the widespread
12 introduction of exotic species usually for ornamental or agricultural purposes (Ricciardi &
13 Kipp, 2008; Hulme, 2009; Keller *et al.*, 2011). In spite of strengthened pre-border
14 biosecurity measures (Weber, Sun & Li, 2008; Dodd *et al.*, 2016), the introduction of
15 exotic plant species is still ongoing as a result of the intensive online plant trade (Kay &
16 Hoyle, 2001) which can inadvertently introduce exotic species as contaminants. For
17 example, 10% of ornamental freshwater plant species ordered by Maki and Galatowitsch
18 (2004) in Minnesota, United States were contaminated with propagules of exotic plants
19 including the highly invasive *Salvinia molesta*. Occasionally, some contaminants prove
20 attractive and easy to grow and may therefore be traded, as was the case with *S. molesta* in
21 Texas, United States for several years (Kay & Hoyle, 2001). *Salvinia molesta* has since
22 become invasive in many freshwater systems across twelve states of the United States
23 (Tipping *et al.*, 2008).

A general paradigm in plant invasion ecology is that only a small proportion of introduced species (~10%) escape cultivation to form reproducing wild populations (Williamson & Fitter, 1996). The majority of these exotic species remain restricted to a few localities and do not exert a negative influence on native biodiversity and are referred to as naturalised species (Richardson *et al.*, 2000). However, approximately 10% of naturalised species spread widely across the landscape and exert a negative influence on local biodiversity (Williamson & Fitter, 1996). This group of exotic species are referred to as invasive and are considered a major threat to native biodiversity and ecosystem function (Richardson *et al.*, 2000). For example, 56% of threatened Australian vascular plant species are estimated to be under threat from invasive exotic plant species (Evans *et al.*, 2011). A comprehensive inventory of all naturalised and invasive plant species in Australia has been established (Randall, 2007). However, a compilation and analysis of the geographical distribution of the exotic freshwater plant species will be a useful step in developing monitoring and management strategies.

There is increasing interest in the effect that ongoing global environmental change may have on the establishment and spread of exotic species. For example, climate change may break some of the ecological barriers that have constrained the spread of naturalised species, allowing more of them to become invasive while simultaneously exacerbating the impacts and spread of the already invasive species (Groves, 2006; Scott *et al.*, 2008; Duursma *et al.*, 2013; Sorte *et al.*, 2013; Leishman & Gallagher, 2015). When this occurs, the highly interconnected nature and dynamic disturbance regimes of freshwater systems may further facilitate their spread through the landscape (Dudgeon *et al.*, 2006).

Perhaps the most prevalent global change environmental factor that impacts freshwater ecosystems by facilitating exotic species invasions is the release of nutrients from anthropogenic sources into water bodies (Sand-Jensen *et al.*, 2000; James *et al.*,

2005; Wersal & Madsen, 2011). Many previous studies have shown that invasive freshwater plant species tend to have a stronger response to increased nutrient availability than their native counterparts (Van, Wheeler & Center, 1999; Hastwell, Daniel & Vivian-Smith, 2008; Hussner, 2009; Liu *et al.*, 2010; Fan *et al.*, 2013). Under eutrophic (high nutrient) conditions some invasive exotic species may partially or wholly displace native species from habitats because of their superior competitive ability, resulting in altered community structure (Njambuya, Stiers & Triest, 2011; Gérard, Brion & Triest, 2014; Ceschin *et al.*, 2018). Consequently, most studies of freshwater plant responses to altered nutrient status have focused on high levels of nutrient additions (e.g. Cary & Weerts, 1983; Al-Hamdani & Sirna, 2008; Yu *et al.*, 2015) and relatively less is known about plant responses to small increases in nutrient levels in oligotrophic (nutrient-poor) systems. The few studies that have examined this have shown that invasive species may also have greater growth rates in low nutrient habitats, possibly due to their high resource use efficiency (Funk & Vitousek, 2007). For example, the invasive *Hydrilla verticillata* (water thyme) had faster growth rates than the confamilial native *Vallisneria americana* (American eelgrass) at low nutrient concentrations of 0.2 mg N-NO₃ L⁻¹ (Kennedy, Horth & Carr, 2009).

Another global change environmental factor that could favour the invasion of exotic species in freshwater systems is the rising concentration of atmospheric carbon dioxide ([CO₂]). It has led to many climate-related changes that affect the physiological performance, phenology, and distributions of many species globally (Walther *et al.*, 2002; Parmesan & Yohe, 2003). In plants, elevated [CO₂] directly affects performance through higher photosynthetic rates, improved water and nitrogen use efficiency, and altered functional traits (Poorter & Navas, 2003; Ainsworth & Rogers, 2007; Leakey *et al.*, 2009). As a result of these changes, plants often experience an increase in growth under

elevated [CO₂] (Poorter & Navas, 2003; Ainsworth & Long, 2005; Stiling & Cornelissen, 2007; Wang *et al.*, 2012). However, the degree of species responsiveness to elevated [CO₂] may depend on various factors including the invasive status of the species. Evidence from terrestrial systems suggests that invasive exotic species tend to be more responsive to CO₂ enrichment than their native counterparts (Dukes & Mooney, 1999; Liu *et al.*, 2017). Although less studied than terrestrial systems, similar trends have been reported for freshwater systems. For example, in an open-top chamber CO₂ enrichment experiment the invasive exotic wetland species *Typha angustifolia* (narrow-leaved cattail) was found to have a stronger growth response compared to the congeneric native species, *T. latifolia* (broadleaf cattail) (Sullivan *et al.*, 2010).

Species of different functional groups have also shown differential growth responses under elevated [CO₂] (Kimball, 1983; Robinson, Ryan & Newman, 2012). For example, terrestrial C₃ and nitrogen-fixing (N-fixing) species tend to respond more strongly to CO₂ enrichment compared to their C₄ and crassulacean acid metabolism (CAM) counterparts (Poorter, 1993; Wang *et al.*, 2012). In C₃ plants, rubisco, the enzyme responsible for photosynthetic carbon fixation also catalyses oxygenation of ribulose-1,5-bisphosphate (the main substrate for photosynthesis), thereby initiating photorespiration. It is estimated that at 25°C, approximately 23% of carbon fixed by photosynthesis is lost due to photorespiration (Ainsworth & Rogers, 2007). Consequently, C₃ plants are carbon limited under the current [CO₂] and would likely be more responsive to elevated [CO₂]. In contrast, the light-dependent reactions and carbon fixation are separated spatially and temporally in C₄ and CAM species respectively, thereby minimising the likelihood of photorespiration. These plants are therefore less carbon limited under the current conditions and are therefore expected to be less responsive to elevated [CO₂]. The N-fixing species on the other hand, represent a large carbon sink due to the presence of the

symbiont and are thus more responsive to elevated $[\text{CO}_2]$ (Poorter, 1993; Wang *et al.*, 2012).

In freshwater systems, it is possible that differences in growth form may also influence species responsiveness to both CO_2 and nutrient enrichment. For example, the emergent and free-floating species utilise atmospheric CO_2 via the C_3 pathway (Bowes, 1993) and are therefore expected to be more responsive to CO_2 and nutrient enrichment. On the other hand, most submerged species are adapted CO_2 limitation occasioned by diffusive resistance of atmospheric CO_2 in water and the large boundary layer by being facultative CO_2 users (Maberly & Madsen, 2002; Hasler *et al.*, 2016). They would thus be less responsive to CO_2 enrichment as long as the HCO_3^- is available (Hasler *et al.*, 2016). In addition, nutrient enrichment would promote increased algal growth resulting in light attenuation that would greatly limit, and even cause disappearance of the submerged species (Scheffer *et al.*, 1993). Therefore, responsiveness of freshwater species to CO_2 and nutrient enrichment would depend on the invasive status of the species, their physiological functional groups, and their growth form.

Species responsiveness to elevated $[\text{CO}_2]$ is dependent on nutrient supply not being limited (Poorter & Navas, 2003; Ainsworth & Long, 2005). For example, the responsiveness of water hyacinth, *Eichhornia crassipes* (an invasive exotic freshwater species) growth under CO_2 enrichment has been shown to be more pronounced in eutrophic conditions compared to oligotrophic conditions (Liu *et al.*, 2010). It has been suggested that rising atmospheric $[\text{CO}_2]$ could interact with other global change environmental factors such as nutrient enrichment to promote exotic plant invasions via changes in competitive outcomes (Leishman & Gallagher, 2015). This could have a major effect on the ecosystem since the dominance of invasive exotic plant species in freshwater communities is often attributed to their superior competitive abilities (Barrat-Segretain &

Elger, 2004). The shift in competitive dominance between native and invasive exotics in response to elevated [CO₂] has been demonstrated for various terrestrial plant species (Poorter & Navas, 2003; Hely & Roxburgh, 2005; Manea & Leishman, 2011; Liu *et al.*, 2017). In contrast, our understanding for aquatic species and in particular freshwater species is much more rudimentary.

Although Australia is generally considered a dry continent (Smith & Morton, 1990), freshwater bodies, mostly wetlands, occur throughout the country (Catford *et al.*, 2017). These include small alpine bogs and fens, artificial reservoirs and farm dams, extensive floodplains of northern and central Australia, coastal wetlands, and large river systems (Finlayson, 2005; Catford *et al.*, 2017). These aquatic systems provide habitat to many native plant and animal species with the dominant plant species ranging from *Oryza* spp., *Eleocharis* spp., *Hymenachne acutigluma*, and *Pseudoraphis spinescens* in the floodplains (Finlayson, 2005), to hydrophytes such as *Azolla* spp., *Lemna* spp., *Hydrilla verticillata*, *Ceratophyllum demersum*, *Nymphoides indica*, and *Potamogeton* spp. in deeper permanent water bodies (Catford *et al.*, 2017). Despite being species-rich and of high conservation value, these freshwater systems are currently among Australia's 10 most vulnerable habitats due to land development, mining, altered hydrology, eutrophication, exotic species invasions and climate change (Laurance *et al.*, 2011). Impacts of altered hydrological regimes on wetland biodiversity have been extensively reviewed (Kingsford, 2000; Bunn & Arthington, 2002). However, there is need for more research to develop better understanding of how eutrophication, invasive exotic species and climate change might interact to alter the structure and functions of these freshwater systems.

Thesis scope and structure

While many studies have compared native and invasive exotic species responses to global environmental change factors in terrestrial systems (e.g. Dukes & Mooney, 1999; Hely & Roxburgh, 2005; Manea & Leishman, 2011; Liu *et al.*, 2017), similar studies in freshwater systems are scarce. The main aim of this thesis was to investigate growth, reproduction, functional trait and competitive responses of native, naturalised and invasive freshwater plant species to different concentrations of CO₂ and nutrients. More specifically, the following questions were addressed in the five data chapters that constitute this thesis: (i) what is the current status of the exotic freshwater plant species in Australia? (ii) do invasive exotic and native freshwater plant species respond similarly to low additional nitrate doses? (iii) what are the effects of elevated [CO₂] on the growth, reproduction and functional trait responses of freshwater plant species? (iv) do the growth and biomass allocation responses of invasive and naturalised emergent freshwater ornamental species to elevated [CO₂] and nutrient enrichment differ? (v) are interactions between the native (i.e. *Azolla filiculoides*) and exotic (i.e. *Salvinia molesta*) species mediated by elevated [CO₂] and nutrient enrichment? Although each data chapter was formatted for submission to different journals, they have been reformatted in a consistent style for this thesis. However, since each data chapter was prepared as a stand-alone manuscript, there is some inevitable repetition, particularly in regards to the Introduction and Methods sections.

Chapter 2 of this thesis is a systematic analysis of the current status of the exotic freshwater plant species in Australia. This analysis identified the exotic species which currently are a part of the Australian flora and described their origin, current distribution in Australia, introduction pathways and reproduction and dispersal mechanisms. This chapter has been published in *Cunninghamia*. The remaining data chapters are all manipulative

glasshouse experiments investigating freshwater plant species responses to global change environmental factors. *Chapter 3* compared the growth responses of two invasive exotic (*Egeria densa*, *Salvinia molesta*) and a native (*Vallisneria spiralis*) freshwater plant species to low levels of N enrichment. This chapter has been published in *Aquatic Botany*. *Chapter 4* investigated the growth, reproduction and functional trait responses of two free-floating ferns (*Azolla filiculoides* – native, *S. molesta* – invasive), and a submerged native angiosperm (*V. spiralis*) to elevated [CO₂]. This chapter has been published in *Aquatic Botany*. *Chapter 5* compared the growth and biomass allocation responses of two invasive (*Mentha pulegium* and *Zantedeschia aethiopica*) and three naturalised (*Ludwigia palustris*, *Mentha aquatica* and *Rorippa nasturtium-aquaticum*) ornamental species to elevated [CO₂] and nutrient enrichment. This chapter has been prepared for publication in *Hydrobiologia*. The final data chapter (*Chapter 6*), assessed whether interactions between the native *A. filiculoides* and the invasive *S. molesta* were mediated by elevated [CO₂] and/or nutrient enrichment. This chapter has been submitted to *Aquatic Sciences*. Finally, *Chapter 7* is the general discussion where the findings of the thesis are consolidated and discussed in a broader context of literature on plant responses to global environmental change.

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372

CHAPTER TWO

The current status of exotic freshwater vascular plants in Australia - a systematic description.

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My contribution to the research and paper: Concept – 90%; Data collection – 90%; Analysis – 100%; Writing – 90%.

1 **Abstract**

2 Freshwater systems are considered particularly vulnerable to human impact,
3 through habitat modification, changes to water regimes and quality, invasion by exotic
4 species and climate change. One major threat to native biodiversity in freshwater systems
5 is the widespread introduction of exotic plant species, some of which have become
6 naturalised or invasive in their introduced range. Using various records, we conducted a
7 descriptive analysis of the naturalised freshwater plant species in Australia. There are 63
8 freshwater plant species belonging to 45 genera and 26 families naturalised in Australia
9 with the dominant families being Cyperaceae, Poaceae and Plantaginaceae. More than
10 40% of these species are categorised as either invasive or declared weeds, the majority of
11 which are perennial species that grow on the margins of wetlands. They originated from
12 all the inhabited continents with most of the species being native to Europe, South
13 America and North America. The greatest number of the species are currently found in
14 New South Wales (90%), Queensland (68%) and Victoria (65%) and ornamental aquarium
15 plant trade was identified as the main pathway of their introduction. Most of the species
16 are clonal plants with flexible modes of reproduction and multiple dispersal vectors. We
17 conclude that exotic plant species are now an important component of Australia's
18 freshwater systems and that ongoing monitoring of their status, distribution and impact
19 should be a high priority in light of the increasing influence of anthropogenic factors
20 including climate change.

21
22 **Key words:** aquatic ecosystem; flora; invasive; native; naturalised; ornamental

25 **Introduction**

26 Freshwater ecosystems are estimated to cover about 3% of the Earth's land surface
27 area (Downing *et al.*, 2006) but they provide habitat to a disproportionately high number
28 of specialised plant and animal species (Balian *et al.*, 2008). Globally, these ecosystems
29 are experiencing severe declines in biodiversity due to a mix of human-mediated threats
30 such as pollution, overexploitation, flow modification, habitat degradation and invasive
31 species (Dudgeon *et al.*, 2006). These declines in many cases are more pronounced
32 compared to terrestrial ecosystems (Sala *et al.*, 2000), thus it has been argued that
33 freshwater ecosystems are the most threatened of global ecosystems (Saunders, Meeuwig
34 & Vincent, 2002; Dudgeon *et al.*, 2006). Despite freshwater ecosystems being extremely
35 species-rich and harbouring many threatened species (Abell *et al.*, 2008), they do not
36 receive the same conservation efforts and research attention as terrestrial ecosystems
37 (Brundu, 2015). For example, there is comparatively little information on freshwater
38 plants, insects, molluscs and crustaceans in most parts of the world (Revenga *et al.*, 2005).
39 This may be due to the difficulty of monitoring freshwater ecosystems (Brundu, 2015)

40 One of the most significant threats to freshwater ecosystems is the widespread
41 introduction of exotic plant species into new areas as a result of increased international
42 human travel and trade (Mack *et al.*, 2000). Most of these species are introduced
43 deliberately for ornamental or agricultural purposes whereas others passively find their
44 way to new regions as contaminants of ballast water or as hitchhikers on other species
45 (Champion, Clayton & Hofstra, 2010). Although strengthened pre-border biosecurity
46 measures have slowed down the rate of species introductions, the process is still ongoing
47 (Weber, Sun & Li, 2008; Dodd *et al.*, 2016) and it is inevitable that a proportion of these
48 introduced species will become naturalised or even a problematic invader.

Naturalised non-invasive species are those that establish self-perpetuating populations in the wild without having profound negative effects on the ecosystem (Richardson *et al.*, 2000). With time, it is estimated that approximately 10% of naturalised species overcome reproductive and dispersal barriers, and become invasive (Williamson & Fitter, 1996; Williams & West, 2000). In future this proportion may increase in some regions as the ongoing global climatic and environmental change may provide ecological opportunities for some of these species to become invasive (Groves, 2006; Scott *et al.*, 2008; Duursma *et al.*, 2013; Sorte *et al.*, 2013; Leishman & Gallagher, 2015). When this occurs, the highly connected nature and dynamic disturbance regimes of freshwater ecosystems would further facilitate the spread of these species through the landscape (Dudgeon *et al.*, 2006).

Similar to their terrestrial counterparts, invasive exotic freshwater plant species can exert dramatic negative impacts on native communities and ecosystems (Evangelista, Thomaz & Umetsu, 2014). For example, a more than 50% decline in species richness of co-occurring native freshwater plant species was observed with increasing abundance of the invasive exotic *Alternanthera philoxeroides* (alligator weed) in natural ponds (Chatterjee & Dewanji, 2014), and *Myriophyllum spicatum* (parrot's feather) in Lake George, New York, USA (Boylen, Eichler & Madsen, 1999). Furthermore, this suppression of native plant communities by exotic plant species may modify trophic interactions (Richardson & van Wilgen, 2004) by simplifying and rendering the native plant communities a poorer food source for herbivores and higher trophic level consumers (Havel *et al.*, 2015). Thus, exotic plant invasions can have detrimental ecosystem-level effects on freshwater systems (Yarrow *et al.*, 2009).

The Australian naturalised flora is considered one of the most species rich in the world (Dodd *et al.*, 2015). Currently, Australia is estimated to have around 2700 naturalised plant species, representing 12% of its total flora (Randall, 2007; Dodd *et al.*, 2015). A large effort has been made to establish a comprehensive inventory of the entire naturalised flora (Randall, 2007). In addition, the Australian Virtual Herbarium (<http://avh.chah.org.au/>) has digitised occurrence records of extant plant species and created a publicly accessible online database (Haque *et al.*, 2017). These records have been useful in assessing patterns of species endemism (Crisp *et al.*, 2001), mapping species threats (Evans *et al.*, 2011), predicting plant invasions (Duursma *et al.*, 2013), analysing drivers responsible for patterns of naturalisation (Dodd *et al.*, 2015), and identifying areas that have high richness of naturalised exotic species (Dodd *et al.*, 2016). However, these outcomes are broad and generalise across different ecosystems. Therefore, it is important for ecosystem-level descriptions of naturalised non-invasive and invasive exotic species to be undertaken so ecosystem-specific monitoring and management practices can be devised. The aim of this study is to provide a systematic description of the distribution, origin and richness of naturalised non-invasive and invasive exotic freshwater plant species in Australia.

Methods

Compilation of species list

We searched ISI Web of Knowledge for information on naturalised plant species in freshwater ecosystems of Australia using the following combinations: (invasi*) OR (invader) OR (non-native) OR (exotic) OR (alien) OR (non-indigenous) OR (introduced) OR (“naturalised species”) OR (“naturalized species”) OR (biological invasion*) AND

96 (plant) OR (macrophyte*) AND (freshwater) OR (aquatic) OR (river*) OR (pond*) OR
 97 (lake*) OR (dam*) OR (“farm dam”) AND (Australia) OR (“New South Wales”) OR
 98 (“NSW”) OR (Queensland) OR (“Northern Territory”) OR (“NT”) OR (“Western
 99 Australia”) OR (“WA”) OR (“South Australia”) OR (“SA”) OR (Victoria) OR (Vic) OR
 100 (Tasmania) OR (“Australian Capital Territory”) OR (“ACT”). In addition, a list of
 101 naturalised freshwater plant species in Australia was compiled from existing inventories
 102 and lists (e.g. Aston, 1973; Sainty & Jacobs, 2003; Randall, 2007) and online databases
 103 (e.g. <http://weeds.dpi.nsw.gov.au/>; <http://plantnet.rbgsyd.nsw.gov.au/>;
 104 <https://keyserver.lucidcentral.org/weeds/data/media/Html/index.htm#A>;
 105 [https://www.business.qld.gov.au/industries/farms-fishing-forestry/agriculture/land-
 106 management/health-pests-weeds-diseases/weeds-diseases](https://www.business.qld.gov.au/industries/farms-fishing-forestry/agriculture/land-management/health-pests-weeds-diseases/weeds-diseases);
 107 <https://nt.gov.au/environment/weeds/weeds-in-the-nt/A-Z-list-of-weeds-in-the-NT>;
 108 <https://florabase.dpaw.wa.gov.au/search/advanced?current=y&alien=y>;
 109 http://www.pir.sa.gov.au/biosecurity/weeds_and_pest_animals/weeds_in_sa;
 110 <http://agriculture.vic.gov.au/agriculture/pests-diseases-and-weeds>;
 111 <https://dpipwe.tas.gov.au/invasive-species/weeds/weeds-index/declared-weeds-index>).

112 We categorised plant species as ‘freshwater’ using the following definition:
 113 “closely bound to freshwater habitats whose vegetative parts actively grow either
 114 permanently or periodically (for at least several weeks each year) submerged below,
 115 floating on, or growing up through the water surface” (Lacoul & Freedman, 2006;
 116 Chambers *et al.*, 2008; Hussner, 2012).

117 Validity of the species names was checked using the Australian Plant Census
 118 website (<https://biodiversity.org.au/nsl/services/APC>) and species not found in the census
 119 or with unresolved nomenclature were excluded. Any species whose status as native or

exotic was unclear according to the Australian Plant Census was also excluded from the analysis. We then checked the naturalisation status of each species using a comprehensive data set of the introduced flora of Australia - an updated version of Randall (2007) containing unpublished data, and excluded any that was not naturalised. We also excluded species that are associated more with saline water than fresh water.

Plant data collation

Data on the native regions of each species, introduction purpose, and their biology (growth habit, longevity and dispersal mechanisms) were compiled from multiple sources including regional floras, published literature and the online databases (e.g. <https://www.cabi.org/ISC/search>; <http://ausgrass2.myspecies.info/content/fact-sheets>; <https://npgsweb.ars-grin.gov/gringlobal/taxon/taxonomysimple.aspx>). Eight broad regions of origin were identified as follows: Europe, North America (including Mexico), Central America (including the Caribbean), South America, Sub-Saharan Africa (including Madagascar), North Africa, temperate Asia (including the Middle East), and southern and south eastern Asia. Multiple sources of origin were assigned where a species had a wide native geographical region. For example, *Alisma lanceolatum* (narrow-leaved water plantain) is native to Europe, North Africa and temperate Asia and was counted as a species of each of these regions. The current economic uses of the species were used to assign their purpose of introduction (Weber, Sun & Li, 2008) where such information was not explicitly available. We also conducted internet searches to determine if each species is currently available for purchase from aquarium suppliers.

The Australian Virtual Herbarium (<http://avh.ala.org.au/>) was used to determine presence or absence of each species in each of the Australian states and territories. Randall's (2007) list was used to categorise the species as naturalised non-invasive,

invasive, or declared weeds. For the purpose of this study, a declared weed was defined as a plant species that is considered noxious only in certain Australian states or territories while invasive species are those considered noxious nationally.

Results

Taxonomy and status

After screening 255 titles returned by the literature search, 42 papers that were studies of freshwater plants were reviewed for collation of the naturalised species list (Appendix 1), in addition to data derived from existing inventories and online data sources. In total, 63 exotic species of freshwater plants belonging to 45 genera and 26 families were identified as naturalised in Australia (Figure 1; Appendix 2). This represents 29% of all the freshwater plant species in Australia. The plant families with the highest number of naturalised species were Poaceae (nine species), Cyperaceae (nine species) and Plantaginaceae (five species). Fourteen of the 26 families were represented by only one species.

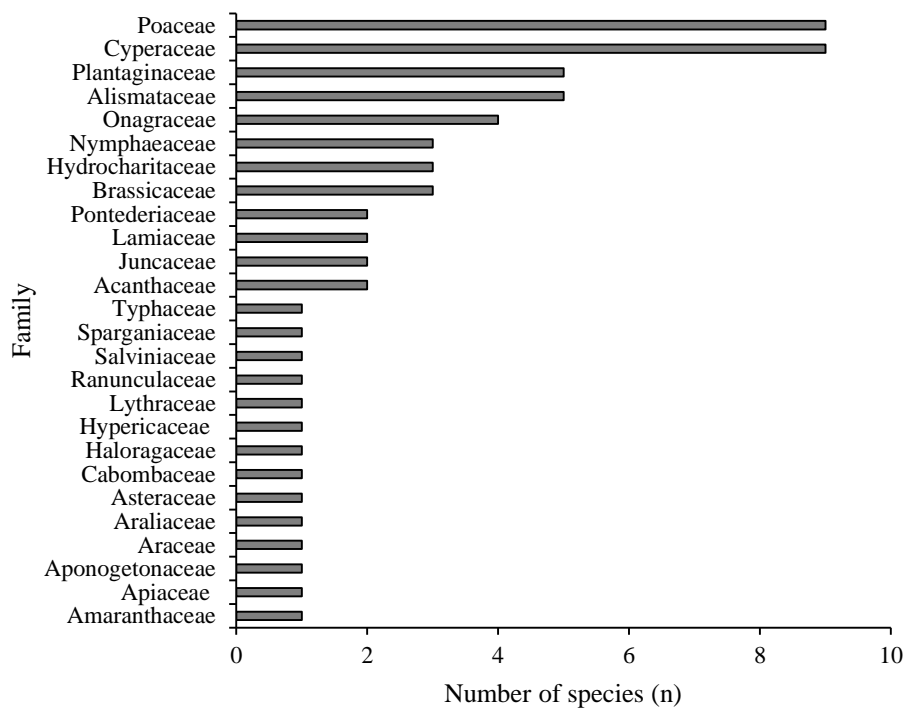


Figure 1: Taxonomic diversity (number) of the naturalised freshwater plant species in Australia.

There were 40 species (63%) that were classified as naturalised non-invasive, 10 (16%) declared weeds and 13 species (21%) that were designated as invasive (Figure 2; Table 1).

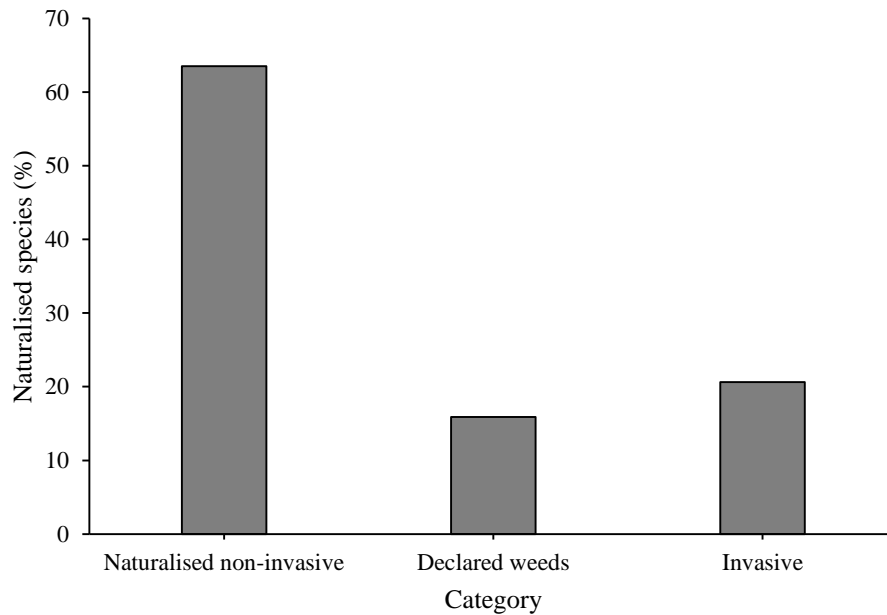


Figure 2: The percentage of naturalised freshwater plant species in different categories of invasive status

Growth habit

The majority of the species (94%) were perennial while three percent were annual and the remaining three percent have annual stems but perennial rhizomes. Most of the species were emergent marginal wetland species (59%). The emergent plants that grow through the water column constituted 24% while the submerged (8%), floating leaved (6%) and free-floating (3%) species made up the remainder.

Table 1: Naturalised freshwater species that are considered invasive in Australia. An asterisk (*) indicates that the species is a Weed of National Significance (WONS) (<http://www.environment.gov.au/biodiversity/invasive/weeds/weeds/lists/wons.html>).

Species	Common name	Family
<i>Alternanthera philoxeroides</i> (Mart.) Griseb.*	Alligator weed	Amaranthaceae
<i>Arundo donax</i> L.	Giant reed	Poaceae
<i>Cabomba caroliniana</i> A.Gray*	Carolina fanwort	Cabombaceae
<i>Egeria densa</i> Planch.	Leafy elodea	Hydrocharitaceae
<i>Eichhornia crassipes</i> (Mart.) Solms*	Water hyacinth	Pontederiaceae
<i>Gymnocoronis spilanthoides</i> (D.Don ex Hook. & Arn.) DC.	Senegal tea	Asteraceae
<i>Hygrophila costata</i> Nees	Glush weed	Acanthaceae
<i>Hymenachne amplexicaulis</i> (Rudge) Nees*	Olive hymenachne	Poaceae
<i>Juncus articulatus</i> L.	Jointed rush	Juncaceae
<i>Ludwigia peruviana</i> (L.) H.Hara	Peruvian primrose	Onagraceae
<i>Myriophyllum aquaticum</i> (Vell.) Verdc.	Parrot's feather	Haloragaceae
<i>Sagittaria platyphilla</i> (Engelm.) J.G.Sm.*	Sagittaria	Alismataceae
<i>Salvinia molesta</i> D.S.Mitch.*	Salvinia	Salviniaceae

Region of origin

Exotic naturalised freshwater species originated from a variety of regions, with Europe, South America and North America being the most widely represented (Figure 3). Species that are native to southern and southeast Asia were the most poorly represented with only two reported as naturalised in Australia. Only eight species (12%) did not have

multiple places of origin. Of the 13 species that are classified as invasive in Australia, 10 are native to South America.



Figure 3: Regions of origin of the naturalised freshwater plant species in Australia.

Distribution in Australia

The largest proportion of the naturalised species (90%) were present in New South Wales (NSW), followed by Queensland (68%) and Victoria (65%) (Figure 4). Northern Territory had the lowest number of naturalised freshwater plant species (14 of the 63 species or 22%). Species that were present in every state include *Cyperus eragrostis* (Cyperaceae), *Arundo donax* (Poaceae), and *Polypogon monspeliensis* (Poaceae).

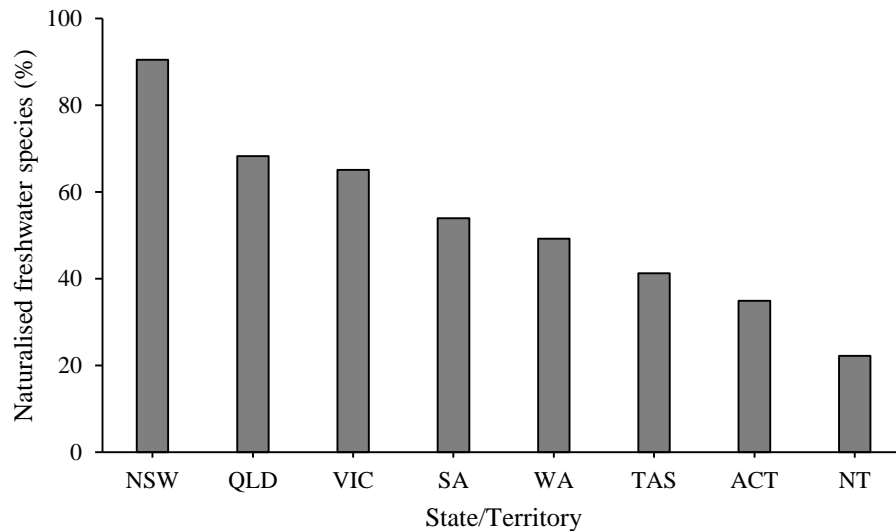


Figure 4: Percentage of introduced freshwater plant species present in the states and territories of Australia.

Introduction pathways

Almost two-thirds of the species (57%) were introduced for aquarium and ornamental water garden purposes while a further 25% were imported for agricultural purposes including as vegetables, for example *Alternanthera philoxeroides* and *Rorippa* spp., and pasture grasses. The remaining 18% of the species may have been introduced accidentally as there was no information available on their known economic use (Figure 5). Thirty-three percent of the species are currently available for sale within Australia either by water garden nurseries or over the internet (Table 2).

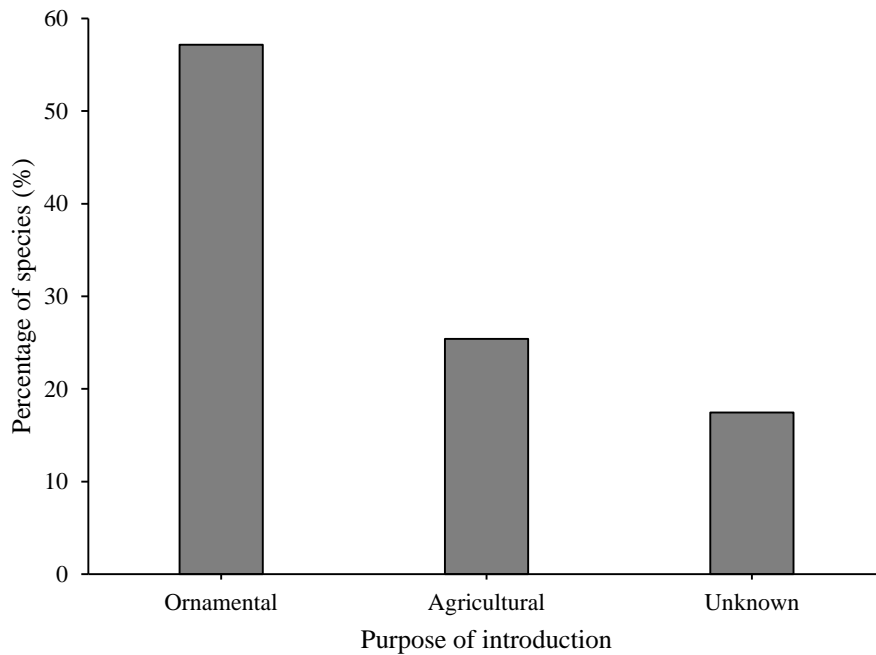


Figure 5: Purpose of introduction of naturalised freshwater plant species.

Reproduction and dispersal

Almost half (49%) of the 63 species reproduce both sexually and vegetatively. Seventeen of these species reproduce by means of both seeds and fragmentation while the other 14 reproduce by means of both seeds and rhizomes. Thirty-three percent of the species (21 out of 63) reproduce exclusively by means of seeds whereas the remaining 17% reproduce exclusively by vegetative means. All the species that reproduce exclusively vegetatively, do so by stem fragmentation. Water currents, waterfowl, flood and watercraft were identified as the main dispersal agents of the seeds and stem fragments.

241 **Table 2:** Naturalised ornamental freshwater plant species available for sale in Australia.

Species	Common name	Family	Origin	Current status	Occurrence
<i>Aponogeton distachyus</i> L.f.	Cape pond weed	Aponogetonaceae	S. Africa	Naturalised	NSW, SA, TAS, VIC
<i>Berula erecta</i> (Huds.) Coville	Water Parsnip	Apiaceae	N. America, Europe, N. Africa, W. Asia	Naturalised	ACT, NSW, QLD, SA, VIC, WA
<i>Bacopa caroliniana</i> (Walter) B.L.Rob.	Lemon bacopa	Plantaginaceae	N. America	Naturalised	NSW, QLD
<i>Cyperus papyrus</i> L.	Papyrus	Cyperaceae	E & S. Africa	Naturalised	NSW, QLD, SA, VIC, WA
<i>Cyperus prolifer</i> Lam.	Dwarf papyrus	Cyperaceae	E & S. Africa	Naturalised	NSW, QLD
<i>Hydrocleys nymphoides</i> (Humb. & Bonpl. ex Willd.) Buchenau	Water poppy	Alismataceae	C & S. America	Naturalised	ACT, NSW, QLD, VIC
<i>Hygrophila polysperma</i> (Roxb.) T.Anderson	East Indian hygrophila	Acanthaceae	Indian subcontinent	Naturalised	NSW, QLD

<i>Hypericum elodes</i> L.	Marsh St. John's wort	Hypericaceae	Europe	Naturalised	NSW
<i>Ludwigia palustris</i> (L.) Elliott	Marsh ludwigia	Onagraceae	N. America, Europe, N & S. Africa, W. Asia	Naturalised	NSW, SA, VIC
<i>Ludwigia repens</i> J.R.Forst.	Red ludwigia	Onagraceae	N & S. America	Naturalised	NSW, QLD, SA, WA
<i>Mentha aquatica</i> L.	Water mint	Lamiaceae	Europe, N. Africa, W. Asia	Naturalised	SA, WA
<i>Mentha pulegium</i> L.	Pennyroyal	Lamiaceae	Europe, N. Africa, W. Asia	Declared	NSW, QLD, SA, TAS, VIC, WA
<i>Nymphaea caerulea</i> Savigny	Blue water lily	Nymphaeaceae	NE & S. Africa, W. Asia	Naturalised	NSW, QLD
<i>Nymphaea mexicana</i> Zucc.	Yellow water lily	Nymphaeaceae	N & C. America	Naturalised	NSW, QLD, SA, VIC, WA

<i>Phalaris arundinacea</i> L.	Reed Canary grass	Poaceae	N. America, Europe, N. Africa, W. Asia	Naturalised	ACT, NSW, SA, TAS, VIC, WA
<i>Pontederia cordata</i> L.	Pickernel weed	Pontederiaceae	America (N, C, & S)	Declared	ACT, NSW, QLD, SA, VIC
<i>Rorippa nasturtium-aquaticum</i> (L.) Hayek	Water cress	Brassicaceae	Europe, N. Africa, W. Asia	Naturalised	ACT, NSW, QLD, SA, TAS, VIC, WA
<i>Rotala rotundifolia</i> (Buch.-Ham. ex Roxb.) Koehne	Roundleaf toothcup	Lythraceae	SE Asia	Naturalised	NSW, QLD, TAS
<i>Typha latifolia</i> L.	Broadleaf cattail	Typhaceae	Europe	Declared	NSW, TAS, VIC
<i>Veronica anagallis-aquatica</i> L.	Blue water speedwell	Plantaginaceae	N. America, Europe	Naturalised	ACT, NSW, QLD, SA, TAS, VIC
<i>Zantedeschia aethiopica</i> (L.) Spreng.	Arum lily	Araceae	S. Africa	Declared	NSW, SA, TAS, VIC, WA

Discussion

Our search identified sixty-three exotic freshwater plant species that have become naturalised in Australia, a large proportion of which are perennial species that grow along the margins of wetlands. They belong to 26 families representing 16% of families of the naturalised flora. The majority of the species originated from Europe, South America and North America and are currently most widely distributed along the eastern coastal fringes of the country. They were mostly introduced for ornamental purposes via the aquarium and water garden plant trade. The majority of the species reproduce both sexually and vegetatively, with water currents, waterfowl and watercraft identified as their main dispersal vectors.

Given that there are 2739 naturalised plant species in Australia (Randall, 2007), freshwater plant species represent a very low proportion (slightly over 2%). However, despite their seemingly small number, they may have disproportionately strong environmental impacts as exemplified by the fact that nearly 20% of the Weeds of National Significance are freshwater species (6 out of 32) (<http://www.environment.gov.au/biodiversity/invasive/weeds/weeds/lists/wons.html>). This may be partly attributed to the widespread geographic distribution of many of these naturalised freshwater species, with most species found in multiple states within Australia. More than 40% of the naturalised freshwater plant species we identified are categorised as either invasive or declared weeds in Australia. This proportion is much greater than for the naturalised terrestrial flora of which around 14% have become invasive (Leishman *et al.*, 2017).

Of the naturalised freshwater species in our analysis, a large majority are in the Poaceae, Cyperaceae and Plantaginaceae families. These families are among the twenty

most commonly represented in the naturalised Australian flora (Dodd *et al.*, 2015) and reflect the Australasian (Jacobs & Wilson, 1996) and worldwide (Chambers *et al.*, 2008) trends where Poaceae and Cyperaceae are the most species-rich freshwater plant families. Many plants belonging to Poaceae and Cyperaceae are important pasture crops on which livestock production in Australia relies heavily (Cook & Dias, 2006), which may further explain their dominance compared to the other families.

The majority of the naturalised species we identified are perennial, clonal plants with the ability to exploit heterogeneous habitats. Clonality may explain the invasion success of some of these species as it enhances persistence and spread of plants at local scales (Santamaría, 2002). The largest proportion (57%) of the species in our analysis were emergent species that grow on the margins of water bodies. This may be due to water margins being suitable for species that can withstand periodic submergence as well as helophytes that can cope with periodic drawdowns (Lacoul & Freedman, 2006). In contrast, open water bodies provide a narrower range of environmental conditions, resulting in fewer species being suited to that habitat. The overrepresentation of the species that grow on the margins of wetlands in our analysis may also have resulted from study biases since these species are conspicuous and easier to sample and identify, in contrast to, for example, the submerged species. In addition, emergent species that occur along the margins of water bodies are able to disperse their propagules not only by water but also by wind, allowing them to colonise widely across the landscape (Soomers *et al.*, 2013).

Many naturalised freshwater plant species in Australia have originated from Europe, South America and North America. This is largely due to historical and trade linkages between Australia and these continents. However, these regions of origin are

likely to have shifted through time, with invasion success of plant species from Europe strongly linked with European settlement in Australia (Phillips *et al.*, 2010) and more recent successful introductions originating from South America now contributing a large proportion of naturalised freshwater plant species. We observed that the majority of the naturalised freshwater plant species in our study had multiple broad regions of origin, probably due to selective advantages provided by asexual reproduction and long distance dispersal of propagules (Santamaría, 2002). Species with large native ranges tend to have broad environmental tolerances and thus may be effectively pre-adapted to their introduced range (Pyšek *et al.*, 2009; Keller *et al.*, 2011). This may explain why the naturalised freshwater species of Australia are small in number but a large proportion have spread extensively across the continent and are now considered as species of concern.

New South Wales, Queensland and Victoria, the most densely populated states (A.B.S., 2018), also have the highest numbers of naturalised freshwater species. This is not surprising as there is a strong correlation between human population density and exotic species richness, due to humans being responsible for the initial deliberate or accidental introduction of exotic species (Weber, Sun & Li, 2008; Dodd *et al.*, 2016; Haque *et al.*, 2017). Furthermore, a higher human population density also means a higher number of potential aquarium keepers, representing a greater propagule pressure (Hussner *et al.*, 2010). Alternatively, biases in herbarium specimen collection may have painted a picture of relatively higher species numbers in the densely populated states than reality (Lavoie *et al.*, 2012; Dodd *et al.*, 2016; Haque *et al.*, 2017). It has been observed that the intensity of herbarium specimen collection in Australia, on which our species regional distribution analysis relied, was higher in the densely populated areas (Dodd *et al.*, 2016).

Our analysis revealed that almost 60% of the freshwater plant species naturalised in Australia were deliberately introduced for ornamental and aquarium purposes. This is consistent with other studies globally reporting that importation and trade in ornamental plants is the most important pathway for freshwater plant introductions (Champion, Clayton & Hofstra, 2010; Strayer, 2010; Keller *et al.*, 2011). In the past it has been suggested that 85% of aquatic weeds in Australia were traded as aquarium or water garden plants (Petroeschovsky & Champion, 2008). We found that a third of Australia's naturalised freshwater plant species are currently available by trade for either ornamental or agricultural purposes. Surprisingly, among these actively traded species are four declared weeds (*Mentha pulegium*, *Pontederia cordata*, *Typha latifolia* and *Zantedeschia aethiopica*). It is also possible that some of these species are traded because of misspelled or incorrect scientific names that mask their exotic status (Brunel, 2009). For example, we found that an aquarium supplier had listed *Eleocharis* for sale without specifying the species. There are also reports of aquarium plant dealers who, mostly due to ignorance, misrepresent exotic plants as similar-appearing native ones (Kay & Hoyle, 2001). A more serious practice that may have contributed to infestation of many waterways of Australia is the deliberate cultivation of exotic ornamental plants in natural waterways by aquarium traders in order to meet customer demands (Petroeschovsky & Champion, 2008).

Twenty-five percent of the naturalised freshwater species of Australia have been introduced deliberately for agricultural purposes. These include traditional vegetable species such as *Alternanthera philoxeroides* and *Rorippa* spp., and garden herbs such as *Mentha aquatica*. However, the majority of the agricultural species are ponded pasture plants that were introduced for livestock grazing. Since commercial livestock production is a major contributor to the Australian economy, many state governments actively promoted introduction of exotic ponded pasture species through much of the 20th century

(Cook & Dias, 2006; Cook & Grice, 2013). These species may have then spread across the broader landscape through natural dispersal mechanisms.

Almost a fifth of the naturalised freshwater plant species in Australia have no known economic uses and may have been introduced inadvertently in ballast water or as contaminants of other deliberately imported species, which is a common occurrence (Kay & Hoyle, 2001). For example, Maki and Galatowitsch (2004) found that ten percent of freshwater plants that they obtained commercially contained exotic plant contaminants. Occasionally, some of these contaminants prove attractive and easy to grow and are therefore placed on the market. A good example is *Salvinia molesta*, which was introduced initially as a contaminant of other plants but was considered sufficiently attractive to be consequently traded as an ornamental species in Texas, USA for several years (Kay & Hoyle, 2001).

Many of the naturalised plants in our analysis reproduce both sexually and vegetatively, and are easily dispersed by water currents and floods, wind, water birds and watercraft (Santamaría, 2002). As vegetative spread and multiple dispersal vectors enhance establishment and therefore naturalisation success (Keller *et al.*, 2011), these factors may also be drivers of invasion success of these naturalised freshwater plants.

From our study, we can conclude that although naturalised freshwater plant species form a very small proportion of the naturalised flora, they nevertheless are an important component of the Australian flora, being widespread across multiple regions. In spite of the existence of many statutory and regulatory measures to control trade in potential weeds in Australia at local, state and federal levels, a few declared weeds continue to be traded. A strict enforcement of these controls is therefore necessary through monitoring of the online aquarium market and periodically assessing compliance by nurseries through site

visits. It is also important that we continue to assess the weed risk of naturalised species in light of ongoing environmental and climatic changes and to monitor potential spread of wild populations constantly (Champion, Clayton & Hofstra, 2010). Finally, accessing information on naturalised freshwater plants ranging from the local to state level is difficult as data are contained within disparate sites. Therefore, a centralised system of storing data on ecology and management of naturalised freshwater plant species would be desirable for better knowledge sharing.

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Supplementary materials

Appendix 1: Titles reviewed for collation of the naturalised species list

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Appendix 2: Link to the species list used in the analysis

https://pirel.files.wordpress.com/2018/11/naturalised_freshwater_macrophytes_au.xlsx

CHAPTER THREE

Responses of two invasive exotic and one native freshwater plant species to low additional nitrate doses

Published as ‘Gufu G.D., Manea A., Vorreiter L. & Leishman M.R. (2018) Do invasive exotic and native freshwater plant species respond similarly to low additional nitrate doses? *Aquatic Botany*, **151**, 1-8.’

My contribution to the research and paper: Concept – 90%; Data collection – 90%;
Analysis – 90%; Writing – 90%

Abstract

Nutrient status of freshwater ecosystems has a significant influence on biological invasions, species richness and community structure. The role of phosphorus in driving these effects has been widely reported while its co-limitation with nitrogen and other elements has received more recent attention. In a greenhouse experiment, we investigated the growth responses of two invasive exotic (*Egeria densa* and *Salvinia molesta*) and one native (*Vallisneria spiralis*) freshwater plant species to additional low concentrations of nitrate nitrogen (N-NO₃). The species were grown at five nitrate concentrations (0.02, 0.05, 0.1, 0.5, and 0.9 mg N-NO₃ L⁻¹). We found that the growth of *E. densa* and *V. spiralis* increased with increasing nitrate concentration. Surprisingly, *S. molesta* had the fastest growth rate at the midrange nitrate concentration of 0.1 mg N-NO₃ L⁻¹ and its leaf production was not affected by nitrate treatment. Irrespective of nitrate concentration, the invasive exotic species, particularly *S. molesta*, showed much greater growth responses than the native *V. spiralis*. We conclude that freshwater plant growth responses to low nitrate concentrations will be species-specific but the faster growth rates of *S. molesta* provide an example of how differences between co-occurring invasive exotic species and native species could have profound effects on the structure and function of freshwater ecosystems under changed environmental conditions.

Key words: *Egeria densa*; eutrophication; growth; nutrient; *Salvinia molesta*; *Vallisneria spiralis*

Introduction

Nutrients from anthropogenic sources are increasingly finding their way into water bodies worldwide (Wersal & Madsen, 2011). This is mainly due to the accelerated conversion of natural areas to grazing, cropping and urban uses resulting in nutrient-enriched runoff (Brodie & Mitchell, 2005). For example, waterways in urban areas often contain higher nutrient concentrations than natural freshwater ecosystems (Moss *et al.*, 2013) and eutrophication has become one of the most frequently observed threats to freshwater ecosystems (Sand-Jensen *et al.*, 2000). The nutrient elements regarded as the most important for primary production in freshwater ecosystems are phosphorus (P) and nitrogen (N) (Bornette & Puijalon, 2011; Bracken *et al.*, 2015). However, their relative importance has been subject to intense debate (Moss *et al.*, 2013) that is still unresolved (Penuelas *et al.*, 2013).

Both P and N directly affect growth and development of freshwater plants (Bornette & Puijalon, 2011). P is often considered the most limiting and at the same time the most detrimental nutrient element since excessive P loading has been implicated in dramatic declines in freshwater species diversity and abundance (Sand-Jensen *et al.*, 2000; Hilt *et al.*, 2006). In contrast, N limitation is deemed only transient due to the pervasive occurrence of N-fixers in the environment (Schindler *et al.*, 2008). Moreover, atmospheric reactive N can be distributed by precipitation into areas that are otherwise not directly affected by human mediated eutrophication (Elser *et al.*, 2009). However, effects of increased N input into habitats that are normally nutrient-poor can be quite profound as it may lead to reduced freshwater plant diversity and altered community structure and function (Moss *et al.*, 2013).

A direct consequence of eutrophication in freshwater systems is the promotion of exotic plant invasions since invasive species tend to respond more strongly to increased

nutrient availability than their native counterparts (Van, Wheeler & Center, 1999; Flores-Moreno *et al.*, 2016). This has been shown to be the case across a range of invasive plant species (Funk & Vitousek, 2007; Hastwell, Daniel & Vivian-Smith, 2008; Madsen & Wersal, 2008; Hussner, 2009). Under eutrophic conditions invasive exotic species may partially or wholly displace native species from habitats because of their superior competitive ability, resulting in altered community structure (Njambuya, Stiers & Triest, 2011; Gérard, Brion & Triest, 2014; Ceschin *et al.*, 2017). For example, high nitrate concentrations may lead to proliferation of more competitive free-floating species (including invasive exotic ones) at the expense of native submerged freshwater plants (Barker *et al.*, 2008).

In addition to having enhanced growth in high nutrient conditions, some studies have shown that invasive species may also have greater growth rates in low nutrient habitats due to their high resource use efficiency (Funk & Vitousek, 2007). For instance, the invasive species, *Hydrilla verticillata*, had faster growth rates than the confamilial native species *Vallisneria americana* at low nutrient concentrations of 0.2 mg N-NO₃ L⁻¹ (Kennedy, Horth & Carr, 2009). Most studies of freshwater plant responses to eutrophication (e.g. Cary & Weerts, 1983; Al-Hamdani & Sirna, 2008; Yu *et al.*, 2015) have focused on high levels of nutrient additions. Relatively less is known about freshwater plant responses to small increases in nutrient levels in oligotrophic systems, which presents a knowledge gap that needs to be addressed.

The aim of this study was to assess the growth responses of two invasive exotic (*Egeria densa* and *Salvinia molesta*) and one native (*Vallisneria spiralis*) freshwater plant species to low levels of nitrate addition. *Egeria densa* (Brazilian elodea; Hydrocharitaceae) is a rooted perennial, submerged species native to parts of South America (Uruguay-Paraguay-Brazil) that has been introduced into several water bodies around the world due to its

popularity as an aquaculture species (Thiébaud, Gillard & Deleu, 2016). It has become a nuisance in its introduced range because of its rapid growth and is regarded as one of the most invasive freshwater plant species (Curt *et al.*, 2010). *Vallisneria spiralis* (eelgrass; Hydrocharitaceae) is a widespread rooted submerged Australian native perennial that also occurs in Africa, southern Europe, and southern and eastern Asia (Aston, 1973). *Salvinia molesta* (salvinia; Salviniaceae) on the other hand, is a free-floating fern native to South America that is one of the most destructive invasive species in the lake and river systems of tropical and subtropical habitats (Schooler *et al.*, 2011). The submerged species are capable of taking up nutrients using their leaves as well as roots (Madsen & Cedergreen, 2002). *Salvinia molesta*, in contrast, lacks true roots and utilises its highly dissected submerged leaves and the underside of its floating leaves for nutrient uptake (Julien & Bourne, 1986). *Egeria densa* and other congeners of *V. spiralis* utilise both dissolved CO₂ and bicarbonate ions (HCO₃⁻) for photosynthesis (Pierini & Thomaz, 2004; Yin *et al.*, 2017). However, *E. densa* is a C₄ species while *V. spiralis* is a C₃ species that also fixes carbon via crassulacean acid metabolism (CAM)-like pathway (Webb, Rattray & Brown, 1988; Casati, Lara & Andreo, 2000). Like most free-floating species, *S. molesta* has a C₃ carbon fixation pathway (Longstreth, 1989).

These species have a wide distribution in the lowland freshwater systems of eastern Australia (Roberts, Church & Cummins, 1999). They commonly co-occur in the Hawkesbury-Nepean River system which is a major waterway in the greater Sydney region of New South Wales (NSW), Australia (Rahman & Salbe, 1995; Roberts, Church & Cummins, 1999). The health of this river system is vitally important as it provides 90% of Sydney's drinking water (Rahman & Salbe, 1995) and has a significant conservation and recreation value (Howell & Benson, 2000). Understanding the invasion risk of exotic species into the uninvaded sections of this system at relevant nitrate concentrations should be a high

priority in order to inform future management decisions. We therefore grew the plant species in monocultures in a controlled greenhouse experiment across a range of low nitrate treatments (0.02-0.9 mg N-NO₃ L⁻¹). We hypothesise that:

(i) all species will have greater growth rates and lower foliar C:N ratios in the higher nitrate concentrations due to nitrates benefiting the growth of freshwater plants.

(ii) the invasive exotics growth response will be relatively greater than that of the native *V. spiralis*, particularly at the higher levels of the nitrate concentrations.

Methods

Greenhouse conditions

The experiment was conducted in greenhouses at the Plant Growth Facility of Macquarie University (NSW, Australia; 33.7745° S, 151.1169° E). The ambient temperature of the greenhouses was maintained at 27°C/22°C day/night producing water temperatures of 24°C/19°C day/night. This temperature range is consistent with the mean lower Hawkesbury River summer water temperatures (Sydney Water, unpublished data). The temperature, humidity and photosynthetically active radiation (PAR) of the greenhouses were continuously monitored using a Multi-grow Controller System (Autogrow Systems, Auckland, New Zealand). The average midday PAR at the water surface was 550 (±320) μmol m⁻² s⁻¹. Underwater PAR was not measured due to logistical challenges. The greenhouses received 150 μmol m⁻² s⁻¹ supplemental lighting using LED red and blue Grow lights (Philips, Eindhoven, Netherlands) for two hours per day to ensure a photoperiod of 13/11 day/night hours. The average humidity at midday ranged between 65-75%.

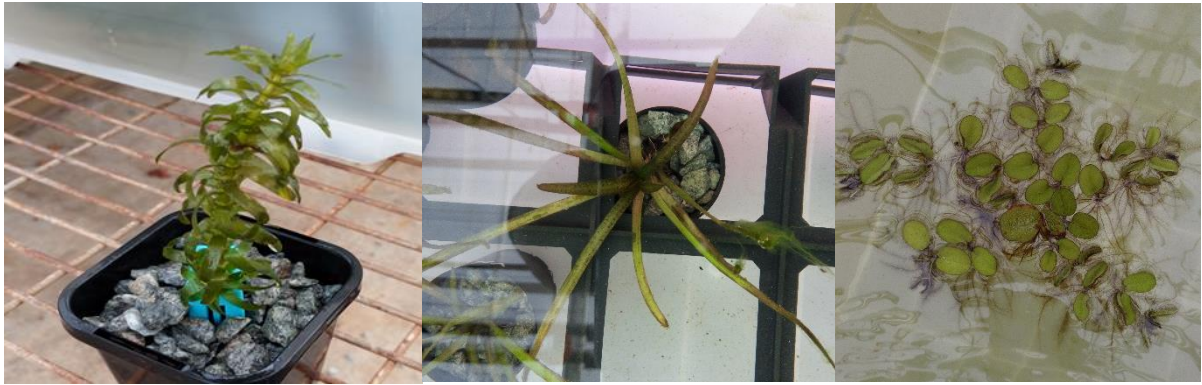


Figure 1: Photos of (a) *E. densa*, (b) *V. spiralis*, and (c) *S. molesta* during harvest after four weeks of growth

Plant preparation

Egeria densa plants (Figure 1a) were obtained from Manly Dam (Warringah, NSW, Australia; 33.7818° S, 151.2556° E), whereas *V. spiralis* (Figure 1b) were obtained from a commercial supplier (Austral Watergardens, Cowan, NSW, Australia; 33.5772° S, 151.1857° E). *Salvinia molesta* plants (Figure 1c) were collected from Lake Munmorah (Wyang, NSW, Australia; 33.1923° S, 151.5749° E). The plants were collected on various dates between 27th June and 15th July 2016 and maintained in tap water until propagation.

Before planting, *E. densa* and *V. spiralis* plants were gently washed under running tap water and a fine paint brush was used to remove periphytes and herbivores. The plants were then trimmed to the following dimensions: 10 cm leaf length for *V. spiralis* (method adopted from Blanch, Ganf & Walker, 1998; Kennedy, Horth & Carr, 2009; Yu *et al.*, 2015), 5 cm stem length with an axillary shoot for *E. densa* and a rhizome section (0.023 ± 0.009 g dry weight) consisting of two fully grown healthy leaves without an apical bud for *S. molesta*. Once prepared, each plant was drained for two minutes on a paper towel and the wet weight measured using an analytical electronic balance (Mettler Toledo, Port Melbourne, VIC, Australia). The number of leaves of each *V. spiralis* plant was also recorded. In order to

obtain the initial dry weights of the experimental plants, we determined the relationship between wet and dry weights of a sub-sample of 20 individual plants of each species. The wet weights of the experimental plants were then used to calculate their initial dry weights using the regression equation calculated from the wet and dry weights of the sub-sample of plants. The sub-sample plants were also used to obtain the initial mean foliar C and N content of each freshwater plant species. This was done by grinding 0.5 g of dry leaf biomass from each plant using a cross beater mill (Glen Creston, Stanmore, UK) and then analysing these samples using combustion with a TruSpec CHN analyser (LECO, St Joseph, MI, USA).

A sub-sample of ten plants from each submerged freshwater plant species (*E. densa* and *V. spiralis*), prepared in the same manner as the experimental plants, were used to obtain chlorophyll-*a* levels as a proxy for the initial periphytic algal load. This was done by shaking each plant by hand in a beaker containing 300 mL tap water for 90 seconds (Zimba & Hopson, 1997; Jones *et al.*, 2002). Chlorophyll-*a* analysis of the wash water from each plant was then conducted by a commercial laboratory following APHA 10200H method (APHA, 1998). This process was repeated for each of the plants harvested at the end of the experiment (after 6 weeks) to determine the amount of periphytic algae accumulated over the growth period. At the end of the experiment samples of the water column were collected and sent to a commercial laboratory for determination of chlorophyll-*a* concentration as a proxy for phytoplankton load. We assumed phytoplankton load was zero at the start of the experiment as we used reverse osmosis (RO) water.

Experimental design

The experiment began on 25th July 2016 when all plants were placed in their respective tubs and ended six weeks later when the final harvest was conducted on 6th September 2016. The experimental plants were grown in monocultures in 86-L tubs

containing 65 L of RO water. 65 mL of nitrate-free modified Hoagland solution (Hoagland & Arnon, 1950) containing calcium carbonate as source of dissolved inorganic carbon, and supplying full strength macro- and micro-nutrients, as well as P ($0.031 \text{ mg P-PO}_4 \text{ L}^{-1}$) and ammonium ($0.03 \text{ mg N-NH}_4 \text{ L}^{-1}$) was added to each tub. This represents a relatively undisturbed lowland freshwater system (Hastwell, Daniel & Vivian-Smith, 2008). Five nitrate treatments ($0.02, 0.05, 0.1, 0.5$ and $0.9 \text{ mg N-NO}_3 \text{ L}^{-1}$) were then established using pre-prepared sodium nitrate stock solutions. Three replicate tubs for each nitrate treatment were made up for each species, giving a total of 15 tubs per species (5 nitrate treatments \times 3 replicates) and 45 tubs in total. Each tub contained 20 uniquely numbered plants of its designated species giving 900 plants in total (15 tubs \times 20 plants = 300 plants/species). *Egeria densa* and *V. spiralis* were planted in individual pots (4.5 cm diameter and 6.5 cm depth) containing near-inert washed gravel to ensure there were no sediment-based additional nutrients. The pots were arranged in the tubs in a checkerboard design using pot trays (Supplementary Figure 1). Similarly, individual *S. molesta* plants were separated by floating pot trays in a checkerboard arrangement. The initial plant spacing for all the species was $7.5 \text{ cm} \times 5 \text{ cm}$. The shoot tips of the submerged species were 15 cm below the water surface.

Each tub was aerated continuously by bubbling using an 11 watt O_2 plus 4000 aquarium air pump (Aqua One, Ingleburn, NSW, Australia) connected to a SMXY 2835 Serenity Aquatics sponge filter (Aqua Blue Distribution, Loganholme, QLD, Australia) by a 2 m air tube. This was done to mimic flowing water and to avoid oxygen or carbon dioxide limitation. Evaporation losses were compensated for daily by the addition of RO water. Twice weekly, nutrient analyses of water samples from each tub were conducted following APHA (1998) protocol and nutrient levels adjusted as necessary to maintain a fairly constant level of nitrates (Supplementary Figure 2), ammonium and P in each treatment/tub. On a weekly basis, each tub was provided with 65 mL of iron as chelated EDTA. Other macro- and

micro-nutrient levels were adjusted once every two weeks based on the results of qualitative analyses. Three times each week, the pH of the nutrient medium in the tubs was checked using a portable HI 9125 pH/ORP pH meter (Hanna Instruments, Woonsocket, RI, USA) and adjusted as required using sodium hydroxide and weak sulphuric acid solutions to maintain it at between seven and eight. The pH of the growth medium on day three of the experiment ranged between 6.16 and 6.72, 5.69 and 5.81, and 6.22 and 6.33 for *E. densa*, *S. molesta* and *V. spiralis* respectively. It mostly remained between 6.51 and 7.56 across the treatments and species throughout the experiment. Once a week, ultraviolet (UV) sterilisation of the nutrient medium in each tub was conducted for approximately four minutes using a ClearTec UV-C sterilization unit (Pond One, Ingleburn, NSW, Australia) to mimic flowing waters that normally experience less phytoplankton build-up.

Plant harvesting and measurement

On a weekly basis, three plants from each tub (nine plants/treatment/species) were randomly selected and harvested until the end of the experiment. The remaining two plants in each tub at the end of the experiment were initially planted to act as insurance against possible mortality and were therefore not included in the final harvest. Once harvested, shoot length of *E. densa* and leaf number of *S. modesta* and *V. spiralis* were measured before the plants were oven-dried at 60°C for 48 hours, and weighed using an analytical electronic balance. The initial and final dry weights were then used to calculate the cumulative relative growth rate (RGR) of each plant as follows:

$$RGR = (\ln W_2 - \ln W_1) / T$$
 where W_1 was the initial dry weight, W_2 was the final dry weight and T was the length of the growth period (days).

The process of obtaining foliar N content described above was repeated for each plant harvested at the final harvest.

Data analyses

We used repeated measures Linear Mixed Models to analyse the effect of nitrate treatment on relative growth rates (RGR) of all the species, percentage increase in *E. densa* shoot length, percentage increase in the number of *V. spiralis* leaves, and the total number of leaves produced by *S. molesta*. Nitrate concentration and time were used as fixed factors with time nested as a repeated co-variable, and tub identity as the random factor. Between-species differences in RGR of plants grown under each N concentration were similarly analysed using the repeated measures Linear Mixed Model with species as a fixed factor, time nested as a repeated co-variable and tub identity as the random factor. Repeated measure covariance type was selected based on the smallest Akaike's Information Criterion. With data split by species, pairwise comparisons of estimated marginal means with Bonferroni adjustment were used to examine within-species differences in RGR response to nitrate treatments. Similarly, between-species RGR responses to each level of nitrate concentration were compared after splitting the data by nitrate treatment. We used the Least Significant Differences (LSD) to compare within-species differences in shoot length change (*E. densa*), leaf number change (*V. spiralis*) and total number of leaves produced (*S. molesta*).

The initial and final foliar C and N content values were converted to molar mass ratios. One-way ANOVAs were then used to analyse the differences in foliar C:N content of the species and chlorophyll-*a* concentration in the wash water and water column across the treatments. Species identity and nitrate concentration were treated as the fixed factors for the initial and final values. When necessary Tukey HSD post-hoc analyses were used to examine differences among species and nitrate concentrations

All statistical analyses were performed using SPSS statistical software, version 25 (IBM, Chicago, IL, USA) with the significance level set at 0.05. When necessary to satisfy

requirements for normality and fulfil the assumptions of models, data were transformed to their \log_{10} .

Results

There was no significant interaction between nitrate treatment and time for any of the species growth traits. As expected, time had a significant effect on all growth traits of each species because the plants increased in size through time (Table 1; Table 2).

Nitrate treatment had a highly significant effect on the RGR of *E. densa* (Figure 2a; Table 1). Pairwise comparisons revealed that the plants grown at the lowest nitrate concentration ($0.02 \text{ mg N-NO}_3 \text{ L}^{-1}$) had significantly lower mean RGR than plants grown in 0.1 , 0.5 , and $0.9 \text{ mg N-NO}_3 \text{ L}^{-1}$ by between 0.012 and $0.016 (\pm 0.001 \text{ SE}) \text{ mg mg}^{-1} \text{ day}^{-1}$ (all $P < 0.037$). There were no significant differences in RGR of the plants grown in the other nitrate treatments.

Similarly to *E. densa*, nitrate treatment had a highly significant effect on the cumulative RGR of *V. spiralis* (Figure 2b; Table 1). The mean RGR of plants grown in 0.5 and $0.9 \text{ mg N-NO}_3 \text{ L}^{-1}$ was $0.019 (\pm 0.005 \text{ SE})$ and $0.016 (\pm 0.005 \text{ SE}) \text{ mg mg}^{-1} \text{ day}^{-1}$ higher than the RGR of plants grown in $0.02 \text{ mg N-NO}_3 \text{ L}^{-1}$ respectively ($P = 0.001$ and 0.01 respectively). However, there were no differences in RGR of the plants grown in the other nitrate concentrations.

Table 1: A summary table of repeated measures mixed effects model showing the effect of nitrate concentration and time on RGR of *E. densa*, *V. spiralis* and *S. molesta*

	<i>E. densa</i>				<i>V. spiralis</i>			<i>S. molesta</i>		
	Numerator <i>df</i>	Denominator <i>df</i>	<i>F</i>	<i>P</i>	Denominator <i>df</i>	<i>F</i>	<i>P</i>	Denominator <i>df</i>	<i>F</i>	<i>P</i>
[Nitrate]	4	49.017	5.328	0.001	57.866	5.280	0.001	49.827	3.824	0.009
Time	5	71.683	36.991	0.001	65.217	15.843	<0.001	61.019	9.852	<0.001
[Nitrate]*Time	20	71.694	1.177	0.299	65.217	1.352	0.179	61.019	1.054	0.418

Table 2: A summary table of repeated measures mixed effects model showing the effect of nitrate concentration and time on change in shoot length of *E. densa*, change in number of leaves produced by *V. spiralis*, and the total number of leaves produced by *S. molesta*

	<i>E. densa</i> shoot length change (%)				<i>V. spiralis</i> leaf No. change (%)			<i>S. molesta</i> leaf No.		
	Numerator <i>df</i>	Denominator <i>df</i>	<i>F</i>	<i>P</i>	Denominator <i>df</i>	<i>F</i>	<i>P</i>	Denominator <i>df</i>	<i>F</i>	<i>P</i>
[Nitrate]	4	15	4.112	0.019	15	7.981	0.001	20.639	2.052	0.124
Time	5	255	38.372	<0.001	255	34.847	<0.001	98.599	144.080	<0.001
[Nitrate]*Time	20	255	1.352	0.147	255	1.558	0.063	98.599	0.979	0.493

The nitrate effect on the cumulative RGR of *S. molesta* was also significant (Figure 2c; Table 1). Pairwise comparisons showed that the RGR of plants grown in 0.1 mg N-NO₃ L⁻¹ was higher than that of plants grown in 0.02 N-NO₃ L⁻¹ by 0.017 (± 0.005 SE) mg mg⁻¹ day⁻¹ ($P = 0.026$). There were no differences in RGR of plants grown in the other treatment combinations.

A comparison of the mean RGR of the three species across the nitrate treatments showed that the mean RGR of *S. molesta* was 287.5% higher than that of *E. densa* and 875% higher than that of *V. spiralis* ($P < 0.001$ at all nitrate levels in both cases). In comparison, the mean RGR of *E. densa* was 300% higher than that of *V. spiralis* ($P < 0.001$ at all nitrate levels).

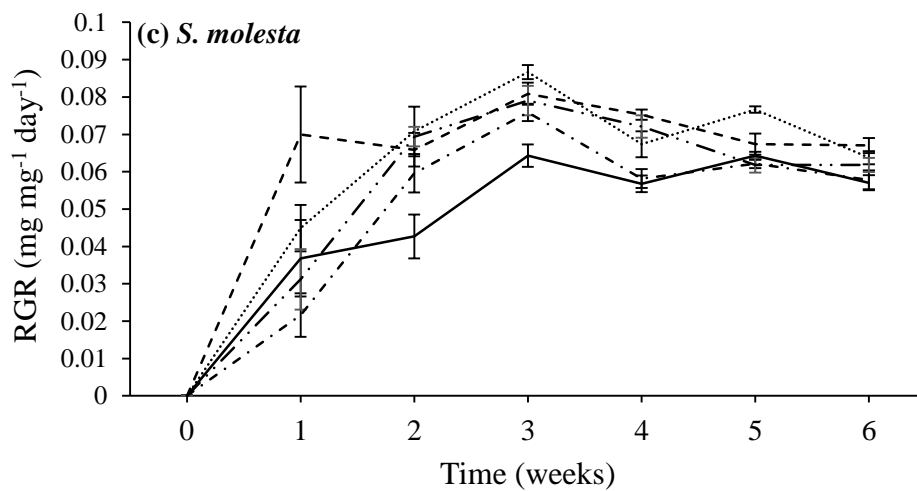
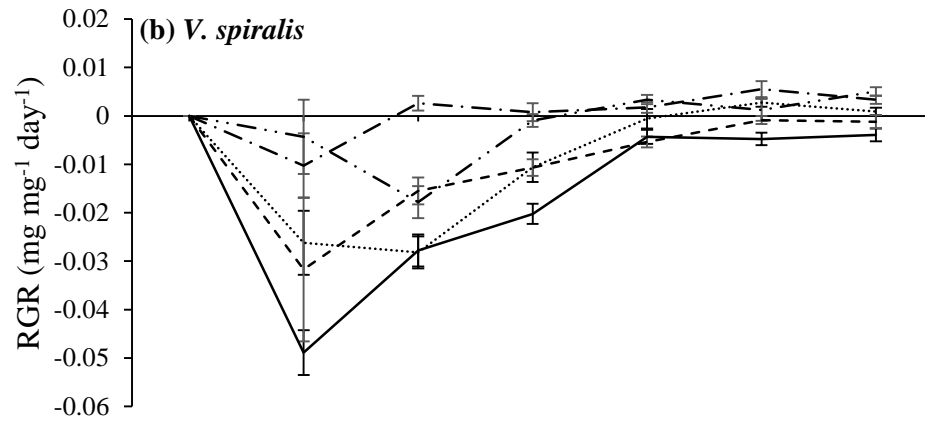
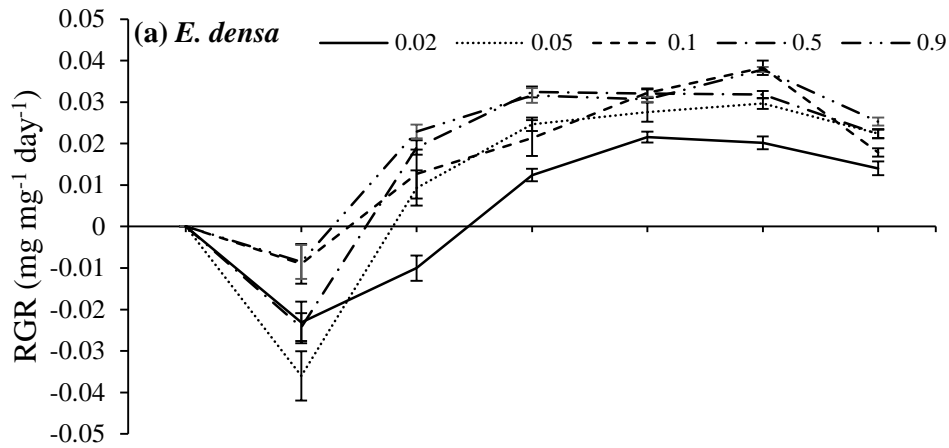


Figure 2: Mean cumulative RGR of (a) *E. densa*, (b) *V. spiralis*, and (c) *S. molesta* plants over time ($n = 9/\text{species}/\text{treatment}/\text{census}$). Error bars represent one standard error, and the y-axis scales are different for each panel.

282 There was a significant effect of nitrate concentration on *E. densa* shoot length over
 283 the growth period (Figure 3a; Table 2), with shoots of plants grown in 0.5 mg N-NO₃ L⁻¹ on
 284 average being 22.2% longer than those of plants grown in 0.02 mg N-NO₃ L⁻¹ ($P = 0.022$).
 285 Similarly, shoot lengths of plants grown in 0.9 mg N-NO₃ L⁻¹ were on average 29.5, 28.1, and
 286 26.8% longer than shoots of plants grown in 0.02, 0.05 and 0.1 mg N-NO₃ L⁻¹ respectively (P
 287 = 0.005, 0.017, and 0.015 respectively). The number of leaves produced by *V. spiralis*
 288 through time was also influenced by nitrate concentration (Figure 3b; Table 2), with plants
 289 grown in 0.05, 0.5 and 0.9 mg N-NO₃ L⁻¹ respectively producing 48.7, 51.4, and 64.2% more
 290 leaves than those grown in 0.02 mg N-NO₃ L⁻¹ ($P = 0.005$, 0.001, and $P < 0.001$
 291 respectively). Furthermore, the number of leaves produced by plants grown in 0.5 and 0.9 mg
 292 N-NO₃ L⁻¹ was larger than the number of leaves produced by plants grown 0.1 mg N-NO₃ L⁻¹
 293 by 32.7 and 45.5% respectively. Nitrate concentration did not have any effect on the number
 294 of leaves produced by *S. molesta* (Figure 3c; Table 2).

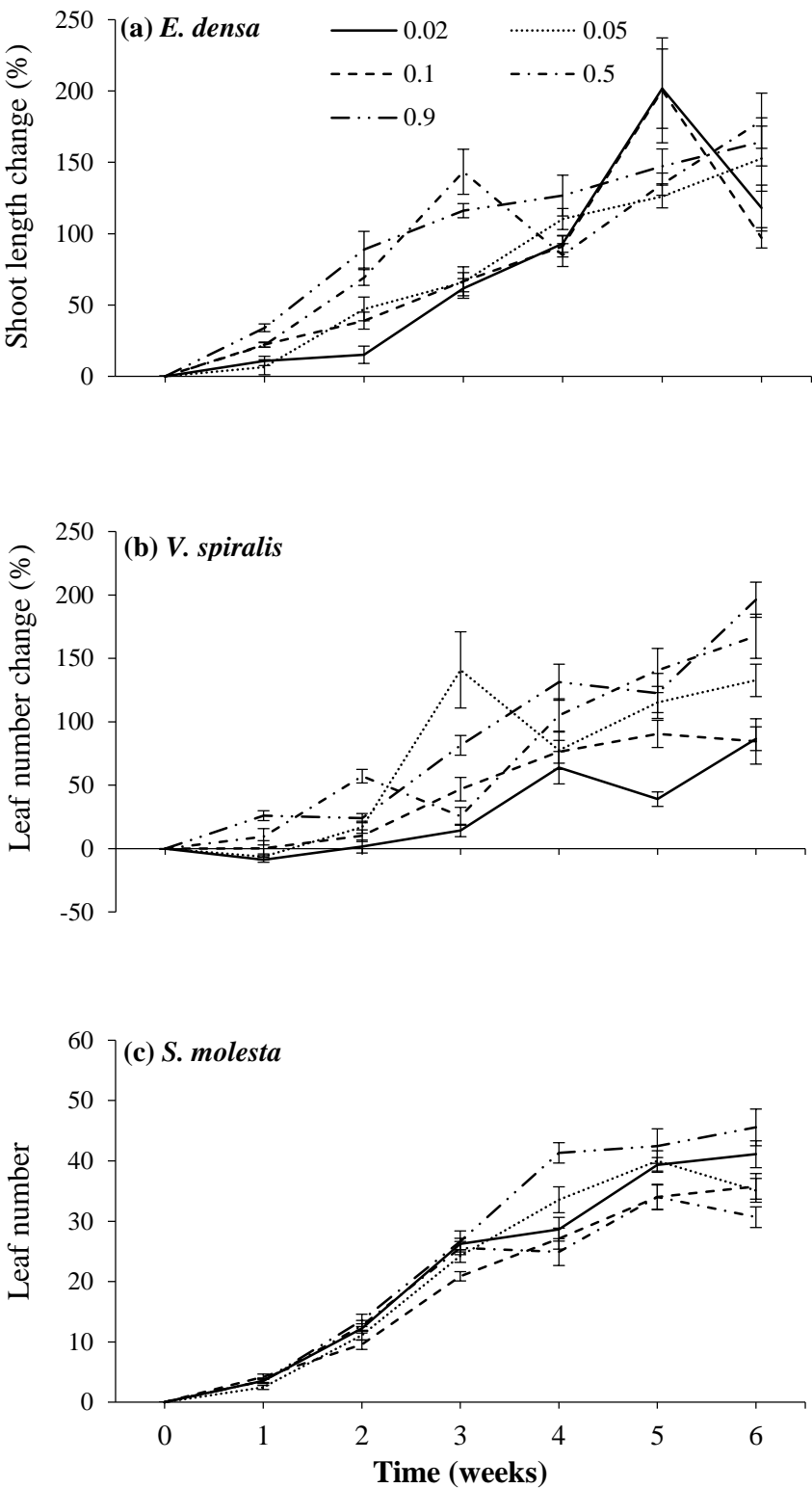


Figure 3: Mean percentage change in (a) *E. densa* shoot length and (b) *V. spiralis* leaf number, and (c) the mean number of *S. molesta* leaves produced over time (n = 9/species/treatment/census). Error bars represent one standard error.

The initial mean foliar C:N of *E. densa* (11.38 ± 0.49 SE) and *V. spiralis* (12.19 ± 0.87 SE) plants were not statistically different. However, the mean initial foliar C:N of *S. molesta* plants (15.55 ± 0.32) was 36.6% higher than that of *E. densa* ($P < 0.001$) and 27.7% higher than that of *V. spiralis* ($P = 0.002$). Nitrate treatment had a significant effect on the final foliar C:N content of *E. densa* ($F_{4, 40} = 23.574$; $P < 0.001$; Figure 4a) and *V. spiralis* ($F_{4, 40} = 6.946$; $P < 0.001$; Figure 4b). However, there was no nitrate effect on foliar C:N of *S. molesta* at the final census ($F_{4, 40} = 2.525$; $P = 0.056$; Figure 4c). Post-hoc tests showed that the foliar C:N of *E. densa* plants grown in 0.5 and 0.9 mg N-NO₃ L⁻¹ were lower than of those plants grown at the lower nitrate concentrations by between 23.3 and 57.4% (all $P < 0.011$). Foliar C:N of the plants grown in 0.02, 0.05 and 0.1 mg N-NO₃ L⁻¹ did not differ. Final foliar C:N of *V. spiralis* plants grown in 0.02 mg N-NO₃ L⁻¹ was 15.7% higher than that of plants grown in 0.9 mg N-NO₃ L⁻¹ ($P = 0.029$). Similarly, the final C:N of plants grown in 0.05 mg N-NO₃ L⁻¹ were 20.4 and 25.4% higher than that of plants grown in 0.5 and 0.9 mg N-NO₃ L⁻¹ respectively ($P = 0.003$ and $P < 0.001$ respectively). The foliar C:N content of the plants grown in the other treatments did not differ. Based on estimated marginal means, the final foliar C:N of *S. molesta* was higher than that of *E. densa* and *V. spiralis* by 37.7 and 73.9% respectively ($P < 0.001$ in both cases). In turn, the final C:N of *E. densa* was 26.3% higher than that of *V. spiralis* ($P < 0.001$).

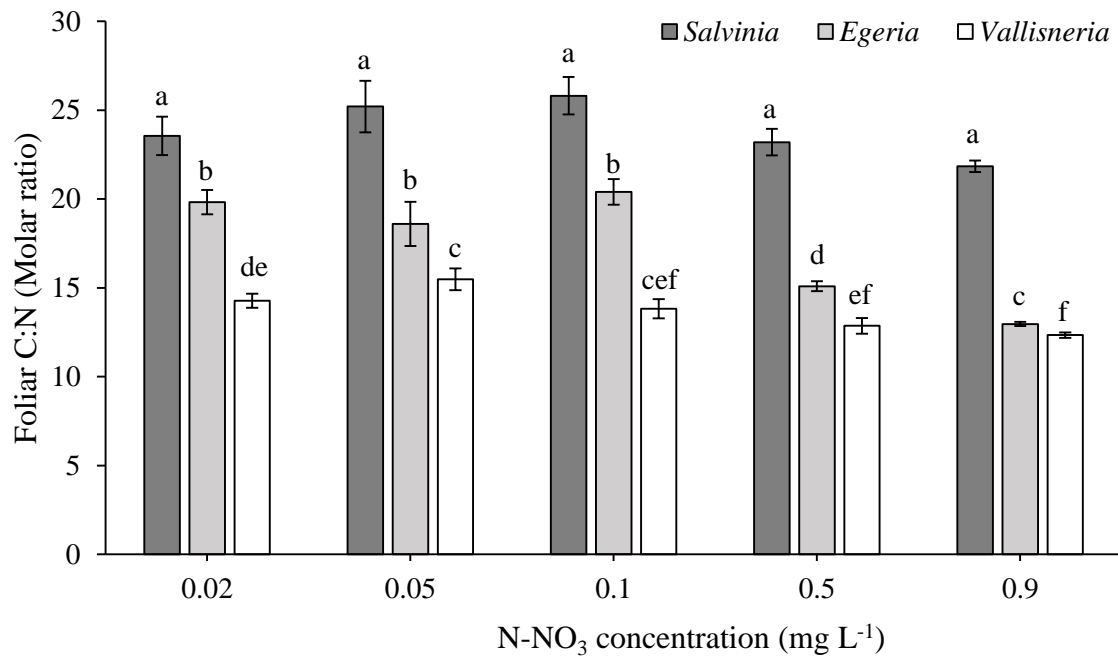


Figure 4: Mean foliar C:N content of *E. densa*, *V. spiralis*, and *S. molesta* plants at the end of the experiment (n = 9/species). Error bars represent one standard error; letters above error bars indicate significant differences between the different treatments and species.

Nitrate treatment did not affect the periphytic algal density on *E. densa* and *V. spiralis* plants (Supplementary Figure 3a and b respectively). However, the amount of phytoplankton in the water column differed with nitrate treatment for *E. densa* ($F_{4,10} = 41.99$; $P < 0.001$; Supplementary Figure 3c) and *V. spiralis* ($F_{4,10} = 4.28$; $P = 0.028$; Supplementary Figure 3d). The higher nitrate treatments had more phytoplankton than the lower treatments.

Discussion

A major threat to the structure and function of freshwater ecosystems globally is eutrophication facilitating the invasion of exotic plants (Van, Wheeler & Center, 1999; Daehler, 2003). The aim of this study was to compare the growth responses of common co-occurring invasive exotic and native freshwater plant species of the Hawkesbury-Nepean

River system, NSW, Australia to a range of low additional nitrate doses. As predicted, growth responses of the invasive exotic *E. densa* (RGR and shoot length) and native *V. spiralis* (RGR and number of leaves) were stimulated by the higher nitrate concentrations. In contrast, *S. molesta* had the highest RGR at mid-range nitrate concentration of 0.1 mg N-NO₃ L⁻¹. As predicted, the two invasive exotic species had at least an order of magnitude faster RGR than the native *V. spiralis*.

The increased growth response of *E. densa* plants (RGR and shoot length) at the higher nitrate concentrations was expected because N is a major requirement for plant growth (Bracken *et al.*, 2015). Stimulation of growth at high N concentrations has been reported for other closely related species *Elodea canadensis* (Canadian waterweed) and *E. nuttalli* (Nuttall's waterweed) (Ozimek, van Donk & Gulati, 1993; Barker *et al.*, 2008). However, *E. densa* has also been shown to grow well irrespective of the amount of nitrate available. For example, Mony *et al.* (2007) observed that biomass production in *E. densa* did not differ at N concentrations ranging from 0 – 0.6 g N kg⁻¹ of sediment. This suggests that *E. densa* has context dependent response to nitrate concentrations and may be competitively superior under oligotrophic conditions.

The RGR and leaf production of the native *V. spiralis* were stimulated by the higher nitrate concentrations, albeit marginally compared to *E. densa*. This finding is consistent with that of Li, Zhang and Jeppesen (2008) who found that *V. spinulosa* biomass increased with increasing nitrate concentrations of up to 7.5 mg N-NO₃ L⁻¹. However, it has been reported that *V. spiralis* typically favours nutrient-poor conditions (Bolpagni *et al.*, 2015). For example, studies of other *Vallisneria* species such as *V. natans* (Yu *et al.*, 2015) and *V. spinulosa* (Zhao *et al.*, 2016) have reported a decline in their growth rates with increasing N concentrations. It should be noted that initially *V. spiralis* plants experienced some leaf

dieback possibly as a result of leaf tip trimming to control for initial plant size and depth of submergence. Although the trimming may have resulted in biomass losses initially, it did not interfere with the meristems that lie in the rosette at the basal area of the leaves enabling the plants to maintain growth. Leaf trimming is a common water garden industry practice for stimulating *V. spiralis* growth (Austral Watergardens, pers. comm). Similar plant preparation methods have been used for *V. americana* (Blanch, Ganf & Walker, 1998; Kennedy, Horth & Carr, 2009) and *V. natans* (Yu *et al.*, 2015). Moreover, it has been shown that *V. spiralis* exhibited compensatory growth and regained up to 108% biomass within four weeks in response to partial leaf removal when supplied with total N of 5 mg N L⁻¹ (Li, Liu & Gu, 2010). Considering the results of RGR, it seemingly took comparatively longer for *V. spiralis* plants in our experiment to recover from the trimming and begin growth.

In contrast to *E. densa* and *V. spiralis*, RGR of *S. molesta* plants was highest under mid-range nitrate concentration of 0.1 mg N-NO₃ L⁻¹ and nitrate treatment did not affect their leaf production. However, irrespective of nitrate concentration, *S. molesta* had the highest RGR of our study species. Studies have shown *S. molesta* to grow well under a range of N concentrations (Toerien *et al.*, 1983; Henry-Silva, Camargo & Pezzato, 2008). For example, previous studies have reported significant increases in *S. molesta* growth rates at N concentrations ranging from 0.02 mg N-NO₃ L⁻¹ (Cary & Weerts, 1983; Room & Gill, 1985) up to 20 mg NH₄-N l⁻¹ (e.g. Cary & Weerts, 1983; Madsen & Wersal, 2008). This prolific growth of *S. molesta* irrespective of N concentration has often been observed when the apical bud is removed which triggers a rapid growth of the other buds (Julien & Bourne, 1986). Since all our plants were obtained from rhizome sections without the apical buds, growth overcompensation may have resulted in the observed rapid growth of the plants. Moreover, *S. molesta* is a known serious invader that grows rapidly to colonise space. While it may be argued that nutrient uptake mechanisms in *S. molesta* may differ from that of the submerged

species due to its free-floating growth form, it has been demonstrated that submerged species are similarly capable of satisfying their mineral requirements by leaf uptake alone (Madsen & Cedergreen, 2002).

The two exotic invasive species had at least an order of magnitude faster RGR than the native *V. spiralis* which could be attributed to differences in CO₂ availability and their photosynthetic pathways. The low diffusive rate of CO₂ in water and the large boundary layer around leaves of submerged freshwater plant species could greatly limit photosynthesis and consequently their growth rates (Maberly & Madsen, 2002). In addition, availability of dissolved inorganic carbon (DIC) depends to a large extent on the pH of the water and CO₂ transfer velocity (Maberly *et al.*, 2015; Hasler *et al.*, 2016). We continuously bubbled atmospheric air into the water column to enhance CO₂ dissolution. We also regularly adjusted the pH to slightly above 7, which may have tipped the dominance of the available DIC species towards HCO₃⁻ (Pedersen, Colmer & Sand-Jensen, 2013). As a species that utilises atmospheric CO₂, *S. molesta* is less affected by DIC content of the water column unlike the submerged species. *Egeria densa* is capable of taking up both CO₂ and HCO₃⁻ in addition to having C₄ carbon concentrating mechanism (CCM) (Casati, Lara & Andreo, 2000; Pierini & Thomaz, 2004). It could therefore be argued that the higher RGR of the exotic invasive species relative to *V. spiralis* was a result of asymmetric access to CO₂ for photosynthesis. Although it is unclear whether *V. spiralis* is capable of utilising HCO₃⁻ similar to other members of the genus, it has been shown to have CAM-like CCM that aids it overcome DIC limitation (Webb, Rattray & Brown, 1988). It is therefore unlikely that the difference between the RGR of *V. spiralis* and those of the exotic invasive species may have been a result of differential CO₂ limitation.

Results of nutrient analyses showed that the initial foliar C:N of *E. densa* and *V. spiralis* did not differ whereas *S. molesta* had a higher foliar C:N ratio. Tissue nutrient content of freshwater plants is generally positively correlated with nutrient availability (Cronin & Lodge, 2003; Bakker & Nolet, 2014; Velthuis *et al.*, 2017). This suggests that the sites from which the experimental *E. densa* and *V. spiralis* plants were obtained had a similar nutrient status. The comparatively higher initial C:N in *S. molesta* could be reflective of the importance of structural C in free-floating species relative to the C associated with metabolic compounds (Duarte, 1992), and may not necessarily suggest that the species was from a comparatively nutrient-poor site. Nevertheless, it is highly likely that the exotic invasive species were not advantaged over the native *V. spiralis* by N stored in their tissue prior to the experiment.

The effect of nitrate treatment on the molar ratio of foliar C and N content of *S. molesta* at the end of the experiment was not significant. This observation supports our earlier argument that *S. molesta* grows rapidly regardless of nutrient status of its growth medium (Toerien *et al.*, 1983; Henry-Silva, Camargo & Pezzato, 2008). Compared to the submerged species, *S. molesta* had the highest C:N across the N treatments. This could also be related to its rapid growth rate that may have led to accelerated conversion of nutrients (including N) into growth (McJannet, Keddy & Pick, 1995). *Egeria densa* and *V. spiralis* plants displayed a pattern of lower foliar C:N with increasing nitrate concentration. This was expected as an increase in foliar N content (reduced C:N ratio) with increasing N availability among freshwater plants has been demonstrated (Cronin & Lodge, 2003; Bakker & Nolet, 2014; Velthuis *et al.*, 2017), although the relationship may be linked more strongly to species identity and less to habitat nutrient status (Demars & Edwards, 2007; Frost & Hicks, 2012). There were marginal increases in the final foliar C:N ratio across all our study species relative to the initial values. This may be a result of suboptimal N supply in our experiment.

Another explanation for the increased foliar C:N ratio through time may be that the trimming of the species meant they had to use stored N to stimulate initial growth (Ferraro & Oosterheld, 2002). However, the mean final foliar N content of all experimental plants across the different nitrate treatments was above the 1.3% suggested critical threshold (Gerloff & Krombholz, 1966) and similar to the 2.4% observed by Duarte (1992).

We found no evidence that periphyton or phytoplankton had a differential suppressing effect on the growth of the plants although the phytoplankton concentration in the higher N treatments of *E. densa* and *V. spiralis* increased during the intervals between UV sterilisations relative to the lower N treatments. It has been shown that some freshwater plant species, such as *E. densa*, can adversely affect phytoplankton by competing for nutrients and producing allelopathic chemicals (Vanderstukken *et al.*, 2011). Therefore, we can conclude that the differences in growth among our species were largely a result of nitrate treatment and not periphyton or phytoplankton load.

Although growth responses to nitrate availability in our study were species-specific, we found that irrespective of nitrate availability, the invasive exotic species had stronger growth responses than the native *V. spiralis* which took longer to recover from the trimming shock. Many studies have shown that invasive exotic freshwater plants perform better than natives under similar environmental conditions (Hastwell, Daniel & Vivian-Smith, 2008) due to their more flexible resource use capabilities (Kennedy, Horth & Carr, 2009). Our results are consistent with the observation that although nutrient-rich habitats may experience more invasions (Daehler, 2003), resource-poor habitats do get invaded as well (Funk & Vitousek, 2007). Therefore, in the short term, we can expect the invasive exotic *E. densa* and *S. molesta* to perform better than *V. spiralis* in natural freshwater ecosystems with N concentrations of between 0.02 - 0.9 mg N-NO₃ L⁻¹ when other resources such as P are not limited. However,

there is a need for more studies with multiple species in the invasive vs native categories to assess the performance of these freshwater plants under oligotrophic conditions in the long-term.

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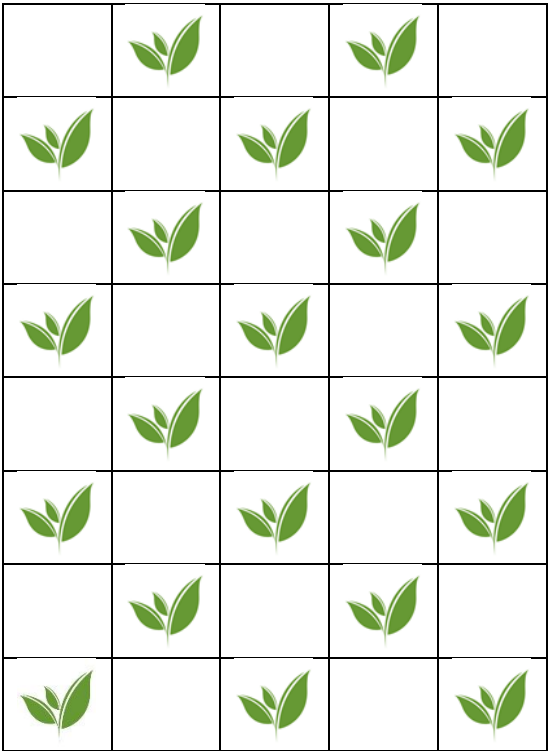
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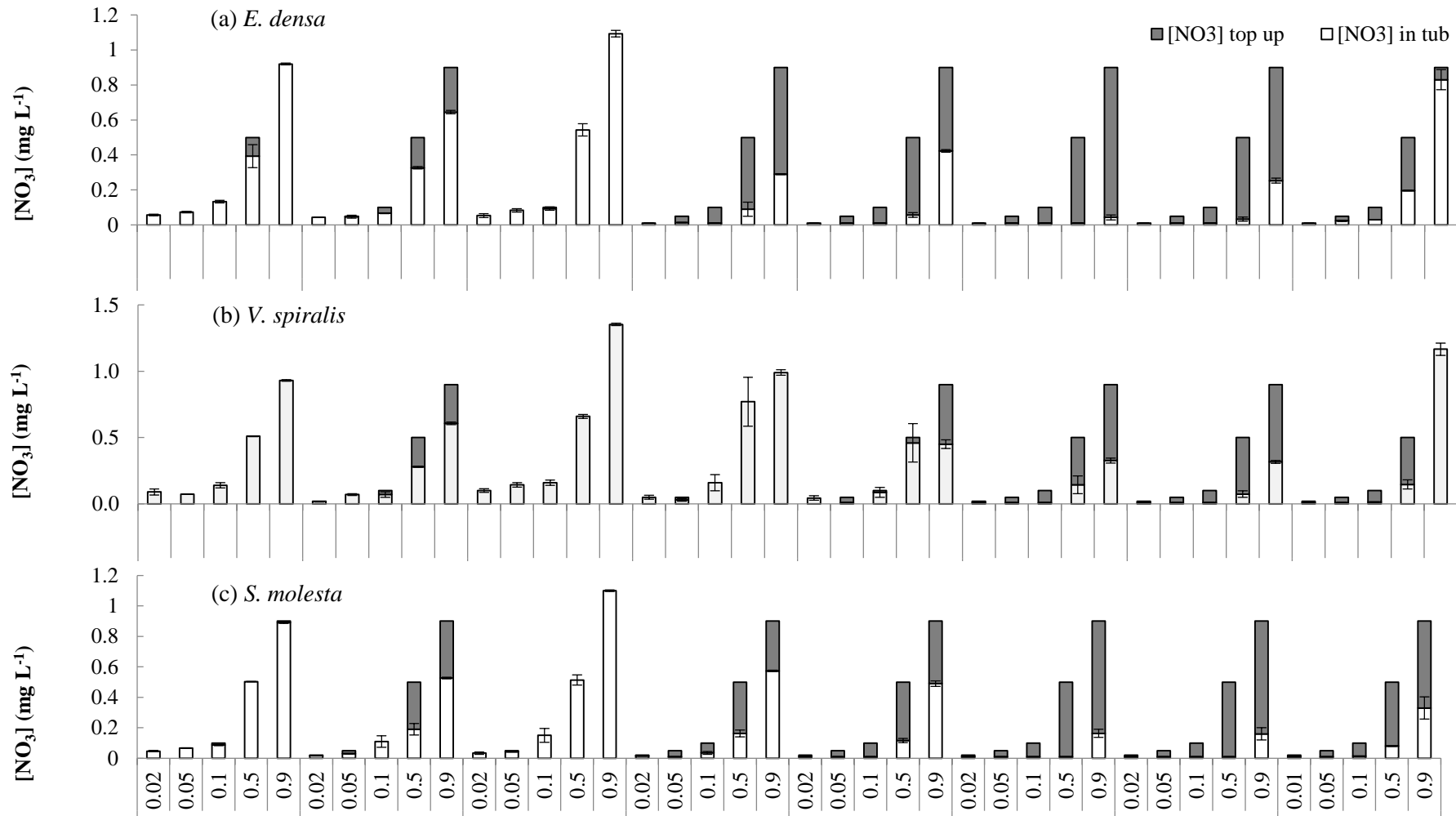
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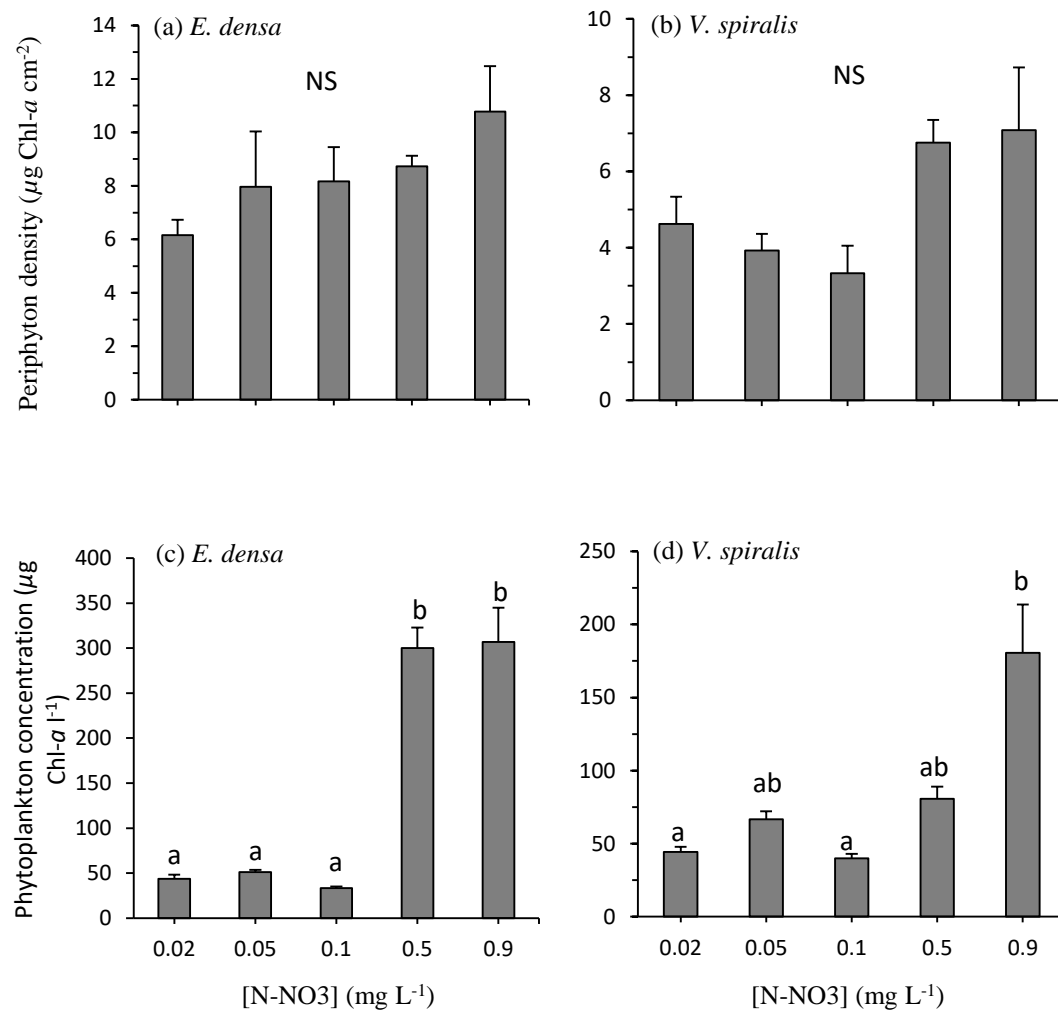
Supplementary materials



Supplementary Figure 1: A schematic diagram illustrating the checkerboard arrangement of the plants in their tubs at the start of the experiment.



Supplementary Figure 2: Water column nitrate concentration (open bars) and the amount topped-up (shaded bars) in each treatment for each of the three study species on various dates during the course of the experiment.



Supplementary Figure 3: Mean periphytic microalgae density on shoot surfaces of (a) *E. densa* and (b) *V. spiralis*, and phytoplankton concentration in the water columns of (c) *E. densa* and (d) *V. spiralis* tubs a week after UV sterilization. Error bars represent one standard error; letters above error bars indicate significant differences between groups and NS indicates non-significant difference among plants of the different treatments.

CHAPTER FOUR

Growth, reproduction and functional trait responses of three freshwater plant species to elevated carbon dioxide.

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My contribution to the research and paper: Concept – 90%; Data collection – 90%; Analysis – 100%; Writing – 90%

Abstract

Rising atmospheric CO₂ concentration ([CO₂]) is one of the most important ways human activity is contributing to global change. It has led to climate-related changes that affect physiological performance, phenology, and distributions of species globally. Elevated [CO₂] enhances growth in many plant species through increased photosynthetic rates, improved water and nitrogen use efficiency, and altered functional traits. In this study, we investigated the growth, reproduction and functional trait responses to elevated [CO₂] of two free-floating ferns, the native *Azolla filiculoides* (Azollaceae) and the exotic invasive *Salvinia molesta* (Salviniaceae), and a submerged native angiosperm, *Vallisneria spiralis* (Hydrocharitaceae). The species were grown in monocultures under ambient (~400 ppm) and elevated (~600 ppm) [CO₂] in a glasshouse experiment. We found that *A. filiculoides* had enhanced relative growth rate and reproduction under elevated [CO₂]. However, its root to shoot ratio was unaffected while its specific leaf area was reduced under elevated [CO₂]. In contrast, *S. molesta* and *V. spiralis* did not differ in their growth, reproduction or functional trait responses between the CO₂ treatments. We conclude that freshwater plant responses to elevated [CO₂] may vary across species, and that functional differences may play an important role as exemplified by the strong response of the N-fixing *A. filiculoides* compared to the non N-fixing species in this study. Future work should focus on the impact of elevated CO₂ on plant growth in the context of environmental conditions such as pH, temperature and light availability in freshwater systems.

Key words: *Azolla filiculoides*; free-floating species; invasive; *Salvinia molesta*; submerged; *Vallisneria spiralis*

25 **Introduction**

26 Rising atmospheric CO₂ concentration ([CO₂]) is one of the best documented global
 27 changes of the past half century (Prentice *et al.*, 2001). Over the past decade, atmospheric
 28 [CO₂] has been rising steadily by about 2.1 ppm per year, reaching 400 ppm in 2015 (Betts *et*
 29 *al.*, 2016). Rising atmospheric [CO₂] is arguably one of the most important ways human
 30 activity is contributing to global change (Low-Décarie, Fussmann & Bell, 2014). It has led to
 31 climate-related changes such as temperature increases and altered precipitation patterns that
 32 affect physiological performance, phenology, and distributions of many species globally
 33 (Parmesan & Yohe, 2003).

34 In plants, elevated [CO₂] directly affects performance through higher photosynthetic
 35 rates, improved water and nitrogen use efficiency, and altered functional traits (Poorter &
 36 Navas, 2003; Leakey *et al.*, 2009). As a result of these changes, plants often experience an
 37 increase in growth if nutrient supply is not limiting (Poorter & Navas, 2003; Ainsworth &
 38 Long, 2005) although the degree of their responsiveness is often species-specific and depends
 39 largely on their functional group (Kimball, 1983). Despite an increasing number of studies
 40 documenting the effects of CO₂ particularly on submerged plants (Bowes, 1993; Titus &
 41 Andorfer, 1996; Maberly & Madsen, 2002; Hussner *et al.*, 2014; Dülger *et al.*, 2017),
 42 freshwater plant species are still under-represented in this field of research compared to
 43 terrestrial and marine species (Cheng *et al.*, 2010; Cao & Ruan, 2015; Hasler *et al.*, 2016).

44 Freshwater plant species are important sources of food and habitat for many
 45 freshwater animals (Gross, Johnson & Hairston Jr, 2001) and in addition influence hydrology
 46 and sediment dynamics of freshwater ecosystems (Vermaat, Santamaria & Roos, 2000).
 47 Although these species can be indirectly affected by elevated [CO₂] through changes in
 48 climate-associated environmental factors (Carpenter, Stanley & Vander Zanden, 2011), direct

CO₂ effects may be growth form-specific (Bornette & Puijalon, 2011). The growth and functional trait responses of free-floating species are expected to be comparable to those of terrestrial plants as they fix atmospheric CO₂ for photosynthesis (Bowes, 1993). In contrast, there is less agreement on how submerged species might respond to elevated [CO₂]. Some studies suggest that since most freshwater systems are supersaturated with CO₂ (Raymond *et al.*, 2013), submerged freshwater plant species, most of which are not obligate free CO₂ users, will be less responsive to elevated [CO₂] as long as HCO₃⁻ is available (Hasler *et al.*, 2016). A contrary view is that due to the low diffusive rate of CO₂ in water and the large boundary layer around their leaves, submerged freshwater plant species are still carbon-limited even in CO₂-supersaturated waters (Maberly & Madsen, 2002). In agreement with the latter view, a number of experimental studies have shown that submerged freshwater plant species respond similarly to elevated [CO₂] as their terrestrial counterparts. These responses include increased biomass production (Hussner *et al.*, 2016; Dölger *et al.*, 2017), leaf number (Spencer & Bowes, 1986) and root to shoot ratio (Yan, Yu & Li, 2006; Hussner *et al.*, 2014; Cao & Ruan, 2015; Dölger *et al.*, 2017) as well as reduced foliar N content (Yan, Yu & Li, 2006; Hussner *et al.*, 2016). Because of these differences in opinions and empirical observations, it is important that we continue to test how dominant freshwater plants will respond and adapt to changes in [CO₂] at a species-level. This will enable us to gain a better understanding of the full impact that rising atmospheric [CO₂] may have on the structure and function of freshwater ecosystems globally.

In this study, we investigated the growth, reproduction, biomass allocation and functional trait responses of two free-floating ferns, *Azolla filiculoides* (water fern; Azollaceae) and *Salvinia molesta* (salvinia; Salviniaceae), and a submerged angiosperm, *Vallisneria spiralis* (eelgrass; Hydrocharitaceae) to elevated [CO₂]. These species commonly co-occur in freshwater ecosystems in south eastern Australia, with *S. molesta* being an

invasive exotic species while the other two are native. The free-floating ferns both belong to the order Salviniales and fix atmospheric CO₂ via the C₃ pathway (Ray *et al.*, 1979). *Azolla filiculoides* is a mat-forming species that hosts symbiotic N-fixing cyanobacteria *Anabaena azollae* within its leaf cavities and is therefore not nitrogen-limited (Espinar *et al.*, 2015). *Salvinia molesta* lacks true roots and utilises its highly dissected submerged leaves (roots hereafter) and the underside of its floating leaves for nutrient uptake (Julien & Bourne, 1986). *Vallisneria spiralis* on the other hand is considered a C₃ plant that also employs a CAM-like carbon concentrating mechanism (Helder & Van Harmelen, 1982; Webb *et al.*, 1988; Keely, 1998; Yin *et al.*, 2017) and is capable of taking up nutrients using both its leaves and roots (Madsen & Cedergreen, 2002). It is further suggested that *V. spiralis* is capable of utilising both free CO₂ and HCO₃⁻ like most submerged species (Hussner *et al.*, 2016). For all the study species, asexual reproduction is the most common means of reproduction.

We measured growth (relative growth rate - RGR) and reproduction (surface area of *A. filiculoides* mats, number of flowers and ramets produced by *V. spiralis*, and number of buds produced by *S. molesta*), root to shoot ratio (R:S) as well as a range of functional traits including specific leaf area (SLA) and foliar N content for each species grown under ambient and elevated [CO₂]. We hypothesised that under elevated [CO₂] the free-floating species would have enhanced growth and reproduction, greater R:S and reduced SLA and foliar N content. Further, *Azolla* is likely to have a strong response to elevated [CO₂] as it is not nitrogen limited due to its symbiotic cyanobacteria. In contrast, we predicted that the submerged species, *V. spiralis*, would be less responsive to elevated CO₂ due to it partly utilising the CAM photosynthetic pathway and not being an obligate free CO₂ user.

99 **Methods**

100 **Plant collection and preparation**

101 *Salvinia molesta* plants were collected from a wild population at Lake Munmorah,
 102 Wyong, NSW, Australia (33.1923° S, 151.5749° E), *A. filiculoides* plants were collected
 103 from an artificial pond at the Macquarie University Plant Growth Facility and *V. spiralis*
 104 plants were sourced from a commercial supplier (Austral Watergardens, Cowan, NSW,
 105 Australia). The plants were maintained in tubs containing tap water for three weeks before
 106 being prepared for planting. For planting, apical rhizome sections consisting of four leaves
 107 and a terminal bud for *S. molesta* (0.02 ± 0.003 g dry weight) and 1 g fresh weight portions of
 108 *A. filiculoides* (0.04 ± 0.002 g dry weight) were prepared while similar sized *V. spiralis* plants
 109 (0.71 ± 0.3 g dry weight) were selected. A sub-sample of the plants prepared for planting ($n =$
 110 20 per species) was oven-dried at 70°C for 48 hours and weighed using an analytical
 111 electronic balance (Mettler Toledo, Port Melbourne, VIC, Australia) to determine the initial
 112 mean dry weight of the experimental plants.

113 **Experimental design**

114 The experiment was conducted in glasshouses at the Plant Growth Facility of
 115 Macquarie University, North Ryde, NSW, Australia. We prepared 42 86-L transparent plastic
 116 tubs containing 65 L of nutrient-free reverse osmosis (RO) water. Pre-prepared solutions of
 117 sodium nitrate and sodium phosphate were added to each tub to supply 0.8 mg N-NO₃ L⁻¹ and
 118 0.1 mg P-PO₄ L⁻¹. The other nutrients were supplied by adding 15 mL of undiluted Seasol, a
 119 nitrogen- and phosphorus-free organic concentrate (Seasol International Pty Ltd, Bayswater,
 120 VIC, Australia), to each tub. We supplemented dissolved inorganic carbon (DIC) by adding
 121 4.6 g of sodium hydrogen carbonate to each tub, similar to the amounts proposed by Smart
 122 and Barko (1985). It was determined in a preliminary study that these quantities of nutrients

were ideal to promote the growth of the selected species. The tubs were evenly split between two CO₂ treatments: ambient (380 – 420 ppm) and elevated (580 – 620 ppm) across four glasshouses (two ambient and two elevated). We used 99.9% pure CO₂ beverage (BOC, Wetherill Park, NSW, Australia) as the source of CO₂ in the glasshouses where the relevant concentrations were maintained and monitored daily (6 am – 6 pm) by a CO₂ dosing and monitoring system (Canary Company Pty Ltd, Lane Cove, NSW, Australia).

The temperature of the glasshouses was maintained at 27°C/22°C day/night producing water temperatures of 24°C/19°C day/night. This temperature range is consistent with the mean summer water temperatures of the lower Hawkesbury River, NSW, Australia (Sydney Water, unpublished data) which is a freshwater system where the study species commonly co-occur. The temperature, humidity (65-75%) and photosynthetically active radiation (PAR; $283 \pm 102 \mu\text{mol m}^{-2} \text{s}^{-1}$, mean \pm SD at 2 pm) of the glasshouses were continuously monitored using a Multi-grow Controller System (Autogrow Systems, Auckland, New Zealand). We did not supplement the lighting since a pilot study showed that supplemental lighting of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ caused photobleaching in *S. molesta*.

Each of the 42 tubs was designated to one of the three study species (14 tubs per species). Within each of the tubs, two plants of the species designated to that tub were planted (14 plants per species per CO₂ treatment). The free-floating species were kept separated in each tub using a floating polystyrene raft that divided the tub into two equal sectors (Figure 1a and b). Each *V. spiralis* plant was planted in a 25 x 20 x 5 cm porous plastic tray containing near-inert washed gravel (Figure 1c). Prior to planting, their leaves were loosely tagged with paper clips so we could distinguish the old and new leaves.

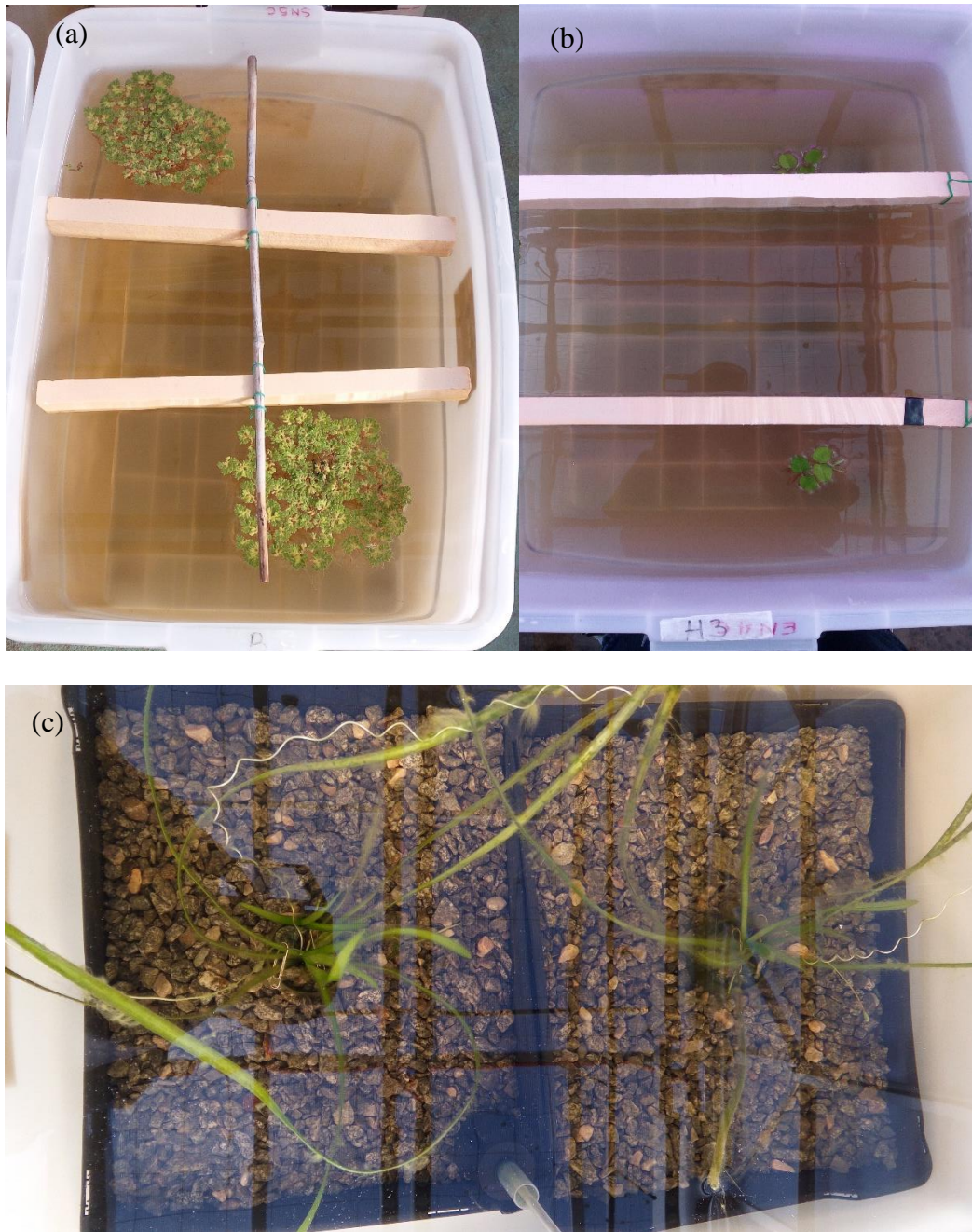


Figure 1: Photos of the experimental (a) *A. filiculoides*, (b) *S. molesta*, and (c) *V. spiralis* plants in their tubs. The photos were taken at different stages of plant growth. Initially, two floating rafts were used to separate the *A. filiculoides* and *S. molesta* plants but these were replaced by one raft each as the plants grew.

The tubs were thoroughly cleaned of periphytic algae and the nutrient medium was replaced with fresh medium on a fortnightly basis to simulate a dynamic system. At the times

of nutrient replacement, the plants were randomly moved between tubs within the same glasshouse to reduce any within glasshouse effects. To mimic flowing waters and to air-equilibrate the tubs containing *V. spiralis* plants we continuously aerated the water using an 11 watt O₂ plus 4000 aquarium air pump (Aqua One, Ingleburn, NSW, Australia) connected to a SMXY 2835 Serenity Aquatics sponge filter (Aqua Blue Distribution, Loganholme, QLD, Australia) by a 2 m air tube. Aeration was not applied to the free-floating species since an initial pilot study showed that bubbling caused *A. filiculoides* plants to disintegrate into tiny fragments. Weekly monitoring of the *V. spiralis* tubs showed that the pH of the water column was 7.4 ± 0.2 and 7.25 ± 0.2 (mean \pm SD) in the ambient and elevated CO₂ treatments respectively.

Trait measurements and harvesting

The experiment was concluded after six weeks as the plant cover in some of the tubs had reached 90%. Before harvesting, we measured vegetative reproduction of each species with plant size (measured as the surface area covered by each plant), number of buds and number of ramets being used for *A. filiculoides*, *S. molesta* and *V. spiralis* respectively. In addition, flower number was used to measure sexual reproduction in *V. spiralis*. Other traits measured included leaf number (*S. molesta* and *V. spiralis*), mean internode length and leaf size (*S. molesta*; for five leaves across the size gradient in the plant), as well as maximum leaf length (*V. spiralis*). Finally, the SLA of a portion of *A. filiculoides* fronds, and three fully expanded *S. molesta* and *V. spiralis* leaves was calculated for each plant as leaf area divided by leaf dry weight. Leaf area was measured using a LI-3100C Area Meter (Li-Cor, Lincoln, NE, United States).

The plants were then harvested and separated into their shoot and root components. The plant components were oven-dried at 70°C for 48 hours, and weighed using an analytical

electronic balance. Foliar N content was then determined after grinding 0.5 g of dry shoot biomass from each plant using a cross beater mill (Glen Creston, Stanmore, UK) and then analysing these samples using combustion with a TruSpec CHN analyser (LECO, St Joseph, MI, USA). R:S was calculated by dividing the dry weight of roots of each plant by the dry weight of its shoot. RGR for each individual plant was then calculated as follows:

$RGR = (\ln W_2 - \ln W_1) / T$, where W_1 was the initial dry weight, W_2 was the final dry weight and T was the length of the growth period (days).

Statistical analysis

The CO₂ effect on the growth, reproduction and functional traits of each species was separately assessed using one-way ANOVAs. When necessary, data were log₁₀-transformed to meet the assumptions of ANOVA. All analyses were performed using SPSS statistical software, version 25 (IBM, Chicago, IL, USA) and significance level set at $P < 0.05$.

Results

The experimental conditions were suitable for the species as even the slowest growing species, *V. spiralis*, experienced a mean biomass gain of at least 118% relative to the initial biomass. The data for the three species were analysed separately, with average trait values for each species provided in Supplementary Table 1.

The RGR of *A. filiculoides* was significantly affected by CO₂ treatment (Figure 2a; Table 1) with plants grown under elevated [CO₂] having about 15% higher RGR than those grown under ambient [CO₂]. However, CO₂ treatment did not affect its R:S (Figure 2b; Table 1). In contrast there was a significant CO₂ effect on its SLA where the plants grown under elevated [CO₂] experienced a 21% reduction in their SLA compared to the plants grown under ambient [CO₂] (Figure 2c; Table 1). The foliar N content of *A. filiculoides* was also

unaffected by CO₂ treatment (Figure 2d; Table 1). There was however a significant CO₂ effect on mat surface area of *A. filiculoides* with plants grown under elevated [CO₂] covering 43.2% more surface area of the tubs than plants grown under ambient [CO₂] (Table 1).

Table 1: A summary ANOVA table of the effect of [CO₂] on the various traits of the study species (RGR = Relative Growth Rate; R:S = Root to shoot ratio; SLA = Specific Leaf Area; DW = Dry weight). Significant effects are shown in bold while the dash symbol (-) indicates where the trait value was not measured.

Trait	<i>Azolla filiculoides</i>			<i>Salvinia molesta</i>		<i>Vallisneria spiralis</i>	
	<i>df</i>	F	<i>P</i>	F	<i>P</i>	F	<i>P</i>
RGR (mg mg ⁻¹ day ⁻¹)	1, 26	27.427	<0.001	1.014	0.323	0.871	0.359
R:S (g/g)	1, 26	0.136	0.715	0.071	0.791	5.555	0.026
SLA (mm ² mg ⁻¹)	1, 26	36.313	<0.001	2.797	0.106	0.260	0.614
N (% of dry mass)	1, 26	0.878	0.357	0.288	0.596	0.326	0.573
Mat Surface Area (cm ²)	1, 26	23.053	<0.001	-	-	-	-
Leaf size (cm ²)	1, 26	-	-	0.405	0.530	-	-
Internode Length (mm)	1, 26	-	-	4.093	0.053	-	-
Bud Number	1, 26	-	-	0.294	0.592	-	-
Leaf Number	1, 26	-	-	1.358	0.255	1.576	0.220
Ramet Number	1, 26	-	-	-	-	0.099	0.756
Ramet DW (g)	1, 26	-	-	-	-	1.180	0.287
Flower Number	1, 26	-	-	-	-	1.029	0.320
Max. Leaf Length (cm)	1, 26	-	-	-	-	0.007	0.935

For *S. molesta*, CO₂ treatment did not have an effect on RGR, R:S, SLA, foliar N content (Figure 2e – h respectively; Table 1) or any of the other traits (Table 1). Similarly,

[CO₂] did not affect the RGR of *V. spiralis* (Figure 2i; Table1) but significantly affected its R:S (Figure 2j; Table 1). The mean R:S of the plants grown under elevated CO₂ was 16.2% lower than that of the plants grown under ambient [CO₂]. There was no CO₂ effect on the SLA, foliar N content (Figure 2k and l respectively; Table 1), the total number of leaves or the maximum leaf length of *V. spiralis* (Table 1).

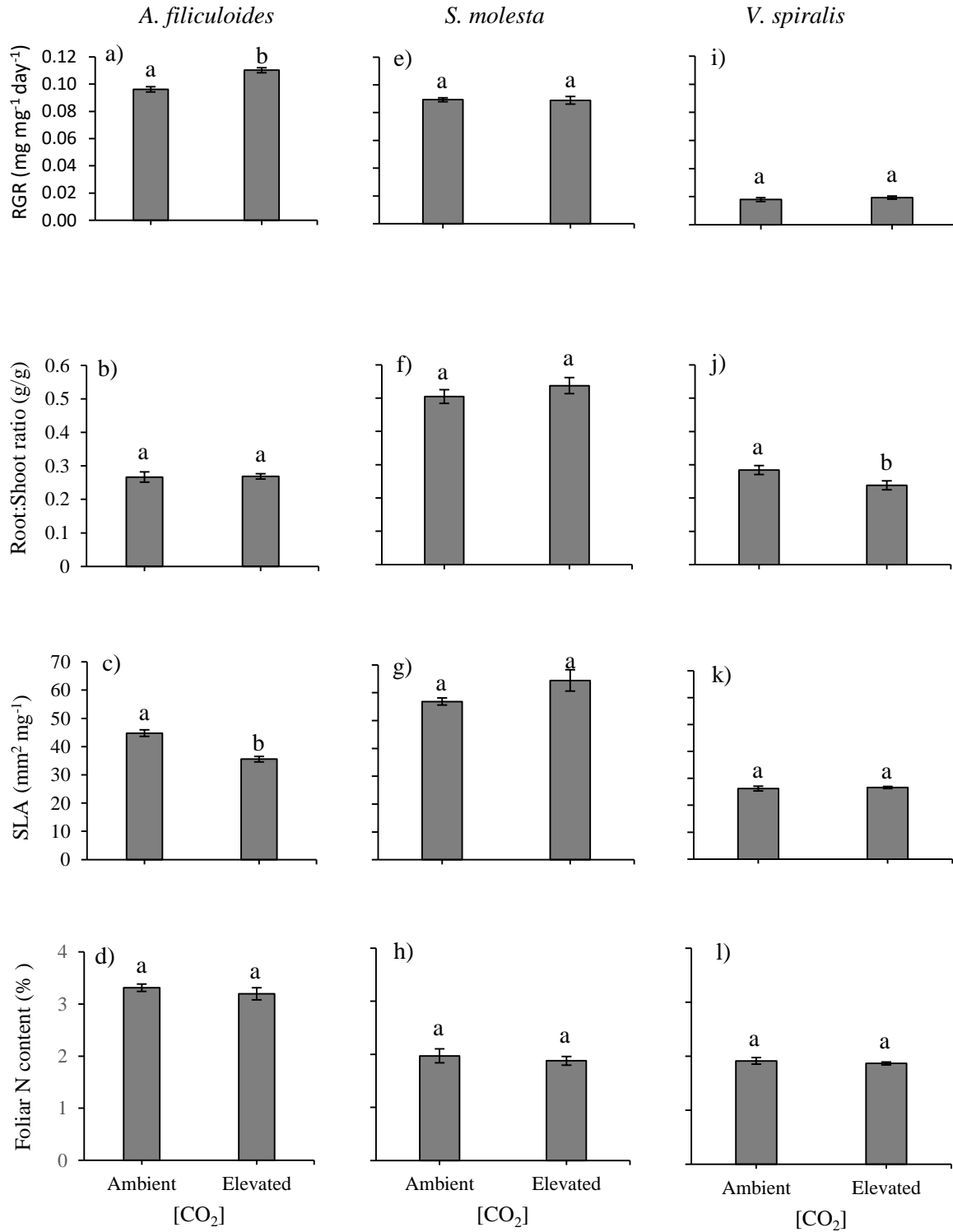


Figure 2: Relative growth rate (RGR), root:shoot ratio (R:S), specific leaf area (SLA), and foliar N content of *A. filiculoides* (a, b, c, and d respectively), *S. molesta* (e, f, g, and h respectively), and *V. spiralis* (i, j, k, and l respectively) grown under ambient and elevated

[CO₂] over 49 days. Error bars represent ± 1 SE and the letters above the error bars indicate where there were significant statistical differences.

Discussion

In this study, we investigated the growth, reproduction, biomass allocation and functional trait responses of two free-floating ferns, *Azolla filiculoides* (Azollaceae) and *Salvinia molesta* (Salviniaceae), and a submerged angiosperm, *Vallisneria spiralis* (Hydrocharitaceae) to elevated [CO₂]. We first hypothesised that *A. filiculoides* and *S. molesta* would have greater growth and reproduction under elevated [CO₂] because they are fast-growing species that photosynthesise via the C₃ pathway (Ray *et al.*, 1979), which are typical characteristics of CO₂ responsive species. We found that the native *A. filiculoides* responded as predicted, with plants grown under elevated [CO₂] having faster RGR and greater mat size than those grown under ambient [CO₂]. Previous studies have reported similar findings for *A. filiculoides* under high [CO₂] (Cheng *et al.*, 2010; van Kempen *et al.*, 2016). It has often been shown that CO₂ responsiveness in plants depends on nutrient availability, suggesting that N-fixing species should respond strongly to elevated [CO₂] (Poorter, 1993). The *Azolla-Anabaena* complex is reported to have the highest rates of nitrogen fixation among N-fixing organisms (Wagner, 1997) and it has been shown that even in nitrogen-free growth media *A. filiculoides* still had a strong growth response to elevated [CO₂] (Idso *et al.*, 1987). Therefore, it is likely that the strong growth response of *A. filiculoides* under elevated [CO₂] in our experiment was a result of it having ample N supply due to the presence of the N-fixing symbiont so it was able to utilise the additional carbon that was available.

In contrast to *A. filiculoides*, *S. molesta* did not show differential response in growth or reproduction (bud number) to CO₂ treatment, despite also having the C₃ photosynthetic system. A possible explanation for this lack of responsiveness is that *S. molesta* in the present study was neither N- nor carbon-limited. With regards to nutrients, *S. molesta* has been shown to grow well at nitrate concentrations 0.02 – 0.9 mg N-NO₃ L⁻¹, with the highest growth rate being at a mid-range concentration of 0.1 mg N-NO₃ L⁻¹ (Gufu *et al.*, 2018). In addition, simultaneous elevation of both [CO₂] and nutrient supply was found to result in marginal growth increases in *S. molesta* compared to when only one of the resources was enriched (chapter 6 of this thesis). Therefore, *S. molesta* in the present study might have already been growing at close to its full capacity under ambient [CO₂] such that an increase in [CO₂] would have had little effect. Alternatively, the dense hydrophobic hairs that cover the upper leaf surface of *S. molesta* (Barthlott *et al.*, 2009) might have reflected light and in turn diminished the photosynthetic capacity of the species, as has been shown in the desert shrub, *Encelia farinosa* (brittlebush) (Ehleringer & Mooney, 1978).

As predicted, growth and reproduction of *V. spiralis* was unaffected by elevated [CO₂]. This prediction was based on the fact that *V. spiralis* has a carbon concentrating mechanism so that it is capable of overcoming CO₂ limitation under current ambient [CO₂]. Furthermore, it has been suggested that most species that switch between the use of free CO₂ and HCO₃⁻ such as *V. spiralis* show only small growth changes in response to elevated [CO₂] (Hasler *et al.*, 2016). However, previous studies have reported dramatic increases in both growth and reproduction for a number of *Vallisneria* spp. under elevated [CO₂] (Titus & Andorfer, 1996; Yan, Yu & Li, 2006; Cao & Ruan, 2015). The disparity between our results and those obtained by these studies may be related to the respective concentrations of both CO₂ and nutrients applied, as well as the pH of the growth medium. For example, while our elevated [CO₂] concentration was approximately 600 ppm, these studies used [CO₂] of up to

22-times the ambient [CO₂]. In addition, while our nitrogen and phosphorus concentrations were 0.8 mg N-NO₃ L⁻¹ and 0.1 mg P-PO₄ L⁻¹ respectively, Titus and Andorfer (1996) used up to 10.4 mg N L⁻¹ and 1.3 mg P L⁻¹. In general, submerged freshwater plant species have been shown to vary widely in their growth and reproduction responses to CO₂ enrichment (Madsen & Sand-Jensen, 1994; Pagano & Titus, 2004; Hussner *et al.*, 2014). It is also possible that the diffusive resistance of CO₂ in water as well as outgassing created by turbulence (Maberly & Madsen, 2002) meant that a 200 ppm increase in [CO₂] as applied in our experiment was not large enough to appreciably alter the dissolved carbon content of the water column and consequently stimulate growth and reproduction of species that possess carbon concentrating mechanisms. Alternatively, the relatively low light levels during the growth period might have limited *V. spiralis* growth response to elevated CO₂.

We then hypothesised that the study species would allocate more biomass to roots under elevated [CO₂]. This is based on the fact that plants tend to differentially allocate biomass to their structures in order to optimise resource acquisition and minimise imbalance (Bloom, Chapin III & Mooney, 1985). Therefore, under elevated [CO₂], carbon becomes less limiting so most plants tend to allocate more biomass to their roots to increase their nutrient acquisition potential in order to maximise their carbon fixation capacity (Arp, 1991). However at the 600 ppm treatment we applied there was no difference in root:shoot ratio compared to the ambient 400 ppm treatment for any of the species studied. This result was predicted for *A. filiculoides* because its symbiotic association with N-fixing *A. azollae* was expected to provide the plants with sufficient nitrogen so that further allocation to nutrient acquisition was not required. For *S. molesta* and *V. spiralis*, biomass production was not affected by CO₂ enrichment, suggesting that they were either not carbon limited under ambient [CO₂] or that the CO₂ increase was insufficient to have an effect. As both species utilise leaves as well as roots for nutrient uptake (Julien & Bourne, 1986; Madsen &

Cedergreen, 2002), there is less need for a shift to allocate resources for nutrient acquisition towards the roots, especially when nutrient solutions are directly added to the water column. Interestingly, the ramets of *V. spiralis* plants grown under elevated CO₂ allocated comparatively more biomass to their shoots than roots, suggesting that the ramets, being shorter in stature, may have been more light limited than carbon limited (Poorter & Nagel, 2000).

Further, we examined how the key functional traits (SLA, foliar N content) of the study species are altered by elevated [CO₂]. We first predicted that the SLA of both *A. filiculoides* and *S. molesta* will be reduced under elevated [CO₂] due to an increase in non-structural carbohydrates and leaf thickness which is a common response in C₃ terrestrial species (Poorter & Navas, 2003; Ainsworth & Long, 2005). We found this was the case for *A. filiculoides* and not for *S. molesta*. It is important to note that the strategy of *S. molesta* is to invest in colonising space rapidly and it may not store non-structural compounds in its tissues until third growth phase is reached when leaves become crowded and start expanding in size (Mitchell & Tur, 1975). Our *S. molesta* plants were harvested before reaching the third growth phase which may explain the lack of the CO₂ effect on their SLA. However, the SLA of *S. molesta* was significantly higher than the SLA of the two native species, irrespective of CO₂ treatment, which is consistent with its rapid growth capacity and success as an invasive species (Baruch & Goldstein, 1999; Leishman *et al.*, 2007; Leishman, Thomson & Cooke, 2010). As predicted, SLA of *V. spiralis* was unaffected by [CO₂].

Finally, we predicted that foliar N content of the C₃ species (*A. filiculoides* and *S. molesta*) would be reduced under elevated [CO₂] due to increased accumulation of C resulting from increased growth diluting the amount of N in the plant tissue (Titus & Andorfer, 1996). This trend has been reported in previous studies for *A. filiculoides* and *Vallisneria* spp. (Yan, Yu & Li, 2006; Cheng *et al.*, 2010; Hussner *et al.*, 2016). In contrast

to our predictions, we found that foliar N content did not differ between CO₂ treatments for any of our study species. Despite being contrary to our hypothesis, this result is unsurprising for *S. molesta* and *V. spiralis* because their growth did not differ between CO₂ treatments, and therefore there was no differential growth dilution of foliar N. However, it is surprising that despite increased growth response under elevated [CO₂], *A. filiculoides* did not exhibit growth dilution of foliar N.

In summary, the observed differences in responses of our study species suggest that freshwater plant responses to elevated [CO₂] are likely to be species-specific, but that N-fixing species may show the strongest responses. In addition, a modest increase in atmospheric [CO₂] under moderately eutrophic and low light conditions is likely to have little effect on growth of the submerged species. This is also partly due to the diffusive resistance of atmospheric CO₂ in water and the large boundary layer (Maberly & Madsen, 2002; Hasler *et al.*, 2016). One question that has largely remained unexplored is the potential weak acidification of freshwater systems induced by elevated [CO₂] and how this might affect organisms and ecosystem processes (Phillips *et al.*, 2015; Hasler *et al.*, 2016; Hasler *et al.*, 2018) by altering dissolved inorganic carbon and nutrient dynamics in fresh waters as well as photosynthetic rates in freshwater plants (Santamaría, 2002). For example, a dramatic increase in *Vallisneria* spp. growth rates in response to elevated [CO₂] has been observed when the pH is low relative to when it is high (Titus, Feldman & Grise, 1990; Titus & Andorfer, 1996; Hussner *et al.*, 2016). There are many factors in the freshwater system that could mediate plant responses to elevated [CO₂], including pH changes, eutrophication, turbidity, altered thermal and hydrological regimes, and biotic interactions (Dudgeon *et al.*, 2006). Therefore, it is important to investigate how the interactions between CO₂ and other global and local environmental change factors will influence performance and biotic

interactions among freshwater plant species, in order to better understand global change impacts on freshwater systems.

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- 508
- 509 **Supplementary materials**

510 **Supplementary Table 1:** Summary descriptive statistics of CO₂ effect on the traits values of the study species (RGR = Relative Growth Rate;
 511 R:S = Root to shoot ratio; SLA = Specific Leaf Area; DW = Dry weight). The dash symbol (-) indicates that a trait value was not measured.

Trait	[CO ₂]	<i>Azolla filiculoides</i>			<i>Salvinia molesta</i>			<i>Vallisneria spiralis</i>		
		Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
RGR (g g ⁻¹ day ⁻¹)	Ambient	0.096	0.007	0.002	0.089	0.005	0.001	0.018	0.006	0.001
	Elevated	0.110	0.007	0.002	0.089	0.010	0.003	0.019	0.004	0.001
R:S (g/g)	Ambient	0.267	0.058	0.015	0.505	0.077	0.021	0.284	0.051	0.014
	Elevated	0.269	0.030	0.008	0.538	0.089	0.024	0.238	0.051	0.014
SLA (mm ² mg ⁻¹)	Ambient	44.768	4.320	1.155	56.867	4.867	1.301	26.213	3.355	0.897
	Elevated	35.555	3.655	0.977	64.441	14.326	3.829	26.566	1.532	0.409
N (% of dry weight)	Ambient	3.313	0.262	0.070	1.972	0.495	0.132	1.916	0.225	0.060
	Elevated	3.196	0.441	0.118	1.878	0.307	0.082	1.868	0.102	0.027
Mat Surface Area (cm ²)	Ambient	347.079	74.773	19.984	-	-	-	-	-	-
	Elevated	496.929	83.069	22.201	-	-	-	-	-	-
Leaf size (cm ²)	Ambient	-	-	-	1.020	0.118	0.032	-	-	-
	Elevated	-	-	-	0.993	0.162	0.043	-	-	-

512 **Supplementary Table 1 continued.**

Trait	[CO ₂]	<i>Azolla filiculoides</i>			<i>Salvinia molesta</i>			<i>Vallisneria spiralis</i>		
		Mean	SD	SE	Mean	SD	SE	Mean	SD	SE
Internode Length (mm)	Ambient	-	-	-	15.762	0.956	0.255	-	-	-
	Elevated	-	-	-	16.476	0.903	0.241	-	-	-
Bud Number	Ambient	-	-	-	70.786	25.223	6.741	-	-	-
	Elevated	-	-	-	65.929	21.840	5.837	-	-	-
Leaf Number	Ambient	-	-	-	229.857	56.500	15.100	37.000	7.348	1.964
	Elevated	-	-	-	206.714	76.039	20.322	40.000	6.679	1.785
Ramet Number	Ambient	-	-	-	-	-	-	5.429	1.505	0.402
	Elevated	-	-	-	-	-	-	5.643	1.550	0.414
Ramet DW (g)	Ambient	-	-	-	-	-	-	0.259	0.094	0.025
	Elevated	-	-	-	-	-	-	0.311	0.117	0.031
Flower Number	Ambient	-	-	-	-	-	-	5.286	1.684	0.450
	Elevated	-	-	-	-	-	-	6.857	2.878	0.769
Max. Leaf Length (cm)	Ambient	-	-	-	-	-	-	51.100	7.209	1.927
	Elevated	-	-	-	-	-	-	50.779	5.772	1.543

513

CHAPTER FIVE

Responses of invasive and naturalised ornamental freshwater plant species to elevated carbon dioxide concentration and nutrient enrichment.

This chapter has been prepared for publication in *Hydrobiologia*.

My contribution to the research and paper: Concept – 90%; Data collection – 100%; Analysis
– 100%; Writing – 90%

Abstract

Global freshwater systems are currently experiencing significant threats as a result of human activities. One such threat is the invasion of exotic species which is thought to be facilitated in some parts by ongoing global environmental changes such as rising atmospheric CO₂ concentration ([CO₂]) and eutrophication. Although an increasing number of studies have documented the impacts of global change environmental factors on native and invasive freshwater plant species, relatively little is known about how factors such as elevated CO₂ and nutrient enrichment might interact to impact invasive exotic species relative to their naturalised or native counterparts in these systems. In this study we investigated the growth and biomass allocation responses of two invasive (*Mentha pulegium* and *Zanthesdeschia aethiopica*) and three naturalised ornamental freshwater plant species (*Ludwigia palustris*, *Mentha aquatica* and *Rorippa nasturtium-aquaticum*) to varied levels of CO₂ (ambient and elevated), and nutrient (low and high) concentrations. We found that relative growth rate (RGR) of the naturalised *L. palustris* was enhanced under elevated [CO₂] while RGR of the naturalised *R. nasturtium-aquaticum* and invasive *M. pulegium* was greater in the high nutrient treatment. The combination of elevated CO₂ and higher nutrient availability increased RGR of only the invasive *Z. aethiopica*. Overall, we found no evidence for consistent growth and biomass allocation differences between the invasive and naturalised species. However, it is necessary that future research assesses potential shifts in the competitive interactions between native and exotic (naturalised and invasive) freshwater species in response to global environmental changes in order to determine community-level consequences of these changes.

Key words: emergent; glasshouse; growth; RGR; root; shoot

25 **Introduction**

26 Globally, freshwater ecosystems are under threat from a variety of factors including
 27 loss of habitat, eutrophication, climate change and invasive species (Dudgeon et al. 2006).
 28 Among these threats, the widespread introduction of exotic species, usually through
 29 ornamental and horticultural plant trade, is thought to be one of the most important (Ricciardi
 30 & Kipp, 2008; Hulme, 2009; Keller *et al.*, 2011). This is because the majority of freshwater
 31 systems are highly modified by human activity (Geist, 2011), making them particularly
 32 vulnerable to establishment of exotic species which tend to be favoured by such disturbances
 33 (Alpert et al., 2000). However, only a small proportion of the introduced plant species
 34 (~10%) tend to establish and become naturalised, remaining localised within a few areas
 35 without exerting negative impacts on the native community (Williamson & Fitter, 1996). A
 36 subset of these naturalised species (~10%) become invasive by spreading widely and
 37 simultaneously exerting negative influence on the ecosystem (Richardson *et al.*, 2000).
 38 Consequently, invasive exotic species have been implicated as the major cause of severe
 39 native biodiversity declines in freshwater systems (Riis *et al.*, 2012; Gallardo *et al.*, 2016).
 40 An important applied question is whether ongoing global environmental change will favour
 41 more of the naturalised species to become invasive while simultaneously amplifying the
 42 effects of the already invasive species (Groves, 2006; Scott *et al.*, 2008; Duursma *et al.*,
 43 2013; Sorte *et al.*, 2013).

44 One of the best documented global change environmental factors that could favour the
 45 invasion of exotic species into freshwater systems is the rise in atmospheric carbon dioxide
 46 concentration ([CO₂]). A number of experimental studies of submerged freshwater plant
 47 species have shown that these species respond to elevated [CO₂] by having increased growth
 48 rates (Hussner *et al.*, 2016; Dülger *et al.*, 2017), increased root to shoot ratio (Yan, Yu & Li,

2006; Hussner *et al.*, 2014; Cao & Ruan, 2015; Dölger *et al.*, 2017) and reduced foliar N content (Yan, Yu & Li, 2006; Hussner *et al.*, 2016). However, the few studies that have compared the responses of freshwater plant species with different invasion status to elevated [CO₂] have revealed no consistent patterns (Hussner *et al.*, 2014; Hussner & Jahns, 2015).

Another environmental factor that has been shown to promote exotic species invasions in freshwater ecosystems is the addition of nutrients from anthropogenic sources into water bodies (Carpenter, Stanley & Vander Zanden, 2011; Wersal & Madsen, 2011). This is based on the evidence that invasive freshwater plant species tend to have a stronger growth response to increased nutrient availability than their native counterparts (Van, Wheeler & Center, 1999; Hastwell, Daniel & Vivian-Smith, 2008; Fan *et al.*, 2013). Furthermore, nutrient enrichment may interact with other global change environmental factors to modify plant growth responses. For example, it often acts synergistically with elevated [CO₂] to promote plant growth (Poorter & Navas, 2003; Reich, Hungate & Luo, 2006). This is evident in the responsiveness of *Eichhornia crassipes* (an invasive exotic freshwater species) growth to CO₂ enrichment which has been shown to be more pronounced under eutrophic conditions compared to oligotrophic conditions (Liu *et al.*, 2010). In addition to influencing growth responses, both [CO₂] and nutrient concentration may interact to affect leaf- and plant-level traits such as specific leaf area (SLA) and root to shoot ratio (R:S). For example, terrestrial plants tend to allocate more biomass to their roots when grown under CO₂ enriched and nutrient-poor conditions to optimise acquisition of the most limiting resource (Arp, 1991; Craine, 2006; Nie *et al.*, 2013). Despite a number of studies documenting the effects of either elevated [CO₂] (Hussner *et al.*, 2014; Hussner & Jahns, 2015) or nutrient enrichment (Hastwell, Daniel & Vivian-Smith, 2008; Silveira & Thomaz, 2015) on native and invasive exotic freshwater plant species, little is known about how in

combination, these factors might affect invasive exotic species relative to their naturalised counterparts.

In this study we investigated the responses of five exotic ornamental freshwater plant species to varied CO₂ and nutrient concentrations. All five study species are readily available for purchase from water garden nurseries in Australia. They all have an emergent growth form and fix atmospheric CO₂ via the C₃ pathway and are therefore likely to be comparable to terrestrial plants in their response to elevated [CO₂] (Bowes, 1993). Two of the selected exotic species, *Mentha pulegium* (pennyroyal) and *Zantedescia aethiopica* (arum lily), are considered invasive in some Australian states and territories while the remaining three, *Mentha aquatica* (water mint), *Ludwigia palustris* (marsh ludwigia), and *Rorippa nasturtium-aquaticum* (water cress), are naturalised but not invasive (Randall, 2007). *Zantedescia aethiopica* is a rhizomatous species while the rest may be classified as creeping emergent (Rejmánková, 1992). These species have wide native distributions and are therefore likely to have wide environmental tolerances which may have contributed to their successful establishment in their introduced ranges.

We grew the species in a controlled glasshouse experiment under ambient and elevated [CO₂] and low and high nutrient concentrations and then measured their growth and allocation traits including relative growth rate (RGR), root to shoot ratio (R:S), and specific leaf area (SLA). We hypothesised that:

- (i) all the species, particularly the invasive ones, will have the greatest growth responses under resource enriched conditions (elevated [CO₂] × high nutrient concentration).

- (ii) all species will allocate more biomass to their roots when grown under elevated [CO₂], and low nutrient concentration compared to ambient [CO₂] and high nutrient concentration respectively.
- (iii) all species will have reduced SLA when grown under elevated [CO₂] and low nutrient concentration compared to ambient [CO₂] and high nutrient concentration respectively but the invasive species will have comparatively higher SLA values.

Methods

Plant collection and preparation

Similar sized seedlings of each of the species (Figure 1; Table 1) were purchased from a commercial supplier (Watergarten Paradise, Bass Hill, NSW, Australia). Prior to the start of the experiment, a sub-sample of 20 individual seedlings of each species with known sizes (based on shoot length and fresh weight) were randomly selected, oven-dried at 70°C for 48 hours and weighed using an analytical electronic balance (Mettler Toledo, Port Melbourne, VIC, Australia). Using this biomass data, we obtained a regression equation for the relationship between the fresh and dry weights of the sub-sample seedlings for each species. These regression equations were later used to calculate the initial dry weight of each experimental seedling from its initial fresh weight.

119 **Table 1:** Details of the exotic freshwater plant species selected for the experiment

Species	Family	Native range	Naturalised range	Status in Australia
<i>Ludwigia palustris</i> (L.) Elliott	Onagraceae	Europe, North America, Northern and southern Africa, South America, western Asia	Australia, Hawaii, New Zealand	Naturalised
<i>Mentha aquatica</i> L.	Lamiaceae	Europe, North Africa, western Asia	Argentina, Australia, Bolivia, Brazil, Chile, Iceland, New Zealand	Naturalised
<i>Mentha pulegium</i> L.	Lamiaceae	Europe, North Africa, western Asia	Australia, Hawaii, New Zealand, southern South America, mainland USA	Invasive
<i>Rorippa nasturtium-aquaticum</i> (L.) Hayek	Brassicaceae	Europe, North Africa, western Asia	Australia, New Zealand, southern Africa, USA	Naturalised
<i>Zantedeschia aethiopica</i> (L.) Spreng.	Araceae	Southern Africa	Australia, Hawaii, New Zealand, south-western mainland USA, UK	Invasive

121



122

123 **Figure 1:** Photos of the experimental plants after (a) one week, and (b) nine weeks of
 124 growth taken in different glasshouses.

125 **Experimental design**

126 The experiment was conducted in climate controlled glasshouses at the Plant
 127 Growth Facility of Macquarie University, North Ryde, NSW, Australia. We used a two-
 128 way factorial design with two levels each of [CO₂] and nutrient concentration as the
 129 treatments. The [CO₂] was set and continuously maintained at 380–420 ppm (ambient) and
 130 580–620 ppm (elevated), by a CO₂ dosing and monitoring system (Canary Company Pty
 131 Ltd, Lane Cove, NSW, Australia). Nutrients were supplied using complete slow release
 132 water garden fertiliser pellets (10N:15P:10K; Manutec Pty Ltd, Cavan, SA, Australia).
 133 The low nutrient treatment received one 10 g fertiliser tablet while the high nutrient
 134 treatment received two tablets (20 g). Between 14th - 16th February 2018, we selected
 135 similar sized pre-weighed seedlings of each species and transplanted one seedling into a

10-L transparent plastic tub (34×25×14cm) containing 5 L of washed river sand. Each treatment combination was replicated eight times resulting in a total of 160 tubs, which were evenly split across four glasshouses (two ambient and two elevated [CO₂]). The substrate (i.e. river sand) was saturated with tap water on a daily basis and the tubs randomly reassigned new positions within the same glasshouse every three to four days to reduce any within-glasshouse effects.

The temperature of the glasshouses was maintained at 27°C/22°C day/night which is consistent with the mean summer temperatures experienced by the major freshwater systems in Sydney region, NSW, Australia (Sydney Water, unpublished data). In addition to temperature, the humidity (65-75% at midday) and photosynthetically active radiation ($358 \pm 163 \mu\text{mol m}^{-2} \text{s}^{-1}$, mean \pm SD at 2 pm) of the glasshouses were continuously monitored using a Multi-grow Controller System (Autogrow Systems, Auckland, New Zealand). After six weeks of growth, each plant received the same amount of fertiliser as at the beginning of the experiment.

Measurements and harvesting

After a three-month growth period, three fully expanded leaves from each plant were harvested and their area was measured using a LI-3100C Area Meter (Li-Cor, Lincoln, NE, United States). The leaves were then oven-dried at 70°C for 48 hours before being weighed. The specific leaf area (SLA) of each plant was then calculated as the area divided by dry mass of the selected leaves. The remaining material of each plant was then harvested and separated into its shoot and root biomass before being oven-dried at 70°C for 48 hours and weighed as described above. We then calculated the RGR and R:S (root dry weight/shoot dry weight) of each plant. The RGR of each plant was calculated as follows:

RGR = $(\ln W_2 - \ln W_1)/T$, where W_2 = final dry weight, W_1 = initial dry weight, and
 T = duration of experiment (days).

Statistical analysis

We initially analysed the growth and allocation data using a linear mixed effects model with [CO₂], nutrient concentration and invasive status (invasive, naturalised) as fixed factors, and species nested within invasive status as the random factor. We found that there was a significant interaction between [CO₂] and species for RGR, R:S and SLA (all $P < 0.05$). We therefore opted to analyse the data at a species-level using two-way ANOVAs with each value treated as the dependent variable, and [CO₂] and nutrient concentration as the predictor variables. Where necessary, data were log₁₀- transformed to meet the assumptions of ANOVA. Tukey HSD post-hoc analyses were used to determine the differences between treatment combinations when an interaction existed. We then used a one-way ANOVA with species as the independent variable to make between-subjects comparisons of the mean RGR and SLA values of the invasive species with each of the naturalised species under the different levels of each treatment.

All analyses were performed using SPSS statistical software, version 25 (IBM, Chicago, IL, USA), with significance level set at $P < 0.05$.

Results

Relative growth rate

A significant interaction between [CO₂] and nutrient concentration affected only the RGR of *Z. aethiopica* (Figure 2; Table 2) where the plants grown under ambient [CO₂] in the low nutrient treatment had 25-31% lower RGR compared to the plants grown under

the other CO₂ × nutrient treatment combinations. The RGR of *Z. aethiopica* did not differ between any of the other CO₂ × nutrient treatment combinations.

Significant effect of [CO₂] on RGR was observed only in *L. palustris* where plants grown under elevated [CO₂] had 15% greater RGR compared to the plants grown under ambient [CO₂] (Figure 2; Table 2). Similarly, the effect of nutrient concentration on RGR was only evident in *M. pulegium* and *R. nasturtium-aquaticum* (Figure 2; Table 2) with the plants grown under high nutrient concentration having 10% and 7% greater RGR respectively compared to their counterparts grown in low nutrient concentration.

The mean RGR of the invasive *M. pulegium* was comparable to that of each of the naturalised species irrespective of treatment except for *M. aquatica* which had a higher RGR under ambient [CO₂] ($P = 0.024$). In contrast, the invasive *Z. aethiopica* had significantly lower mean RGR compared to each of the naturalised species irrespective of treatment (all $P < 0.05$).

197 **Table 2:** Summary ANOVA of the effect of [CO₂] and nutrient concentration on RGR, R:S, and SLA of the study species. Significant effects are
 198 shown in bold.

Species	Status	Source	df	RGR		R:S		SLA	
				F	P	F	P	F	P
<i>L. palustris</i>	Naturalised	[CO ₂]	1, 27	4.468	0.044	8.448	0.007	16.982	<0.001
		Nutrient	1, 27	0.006	0.937	7.388	0.011	0.915	0.347
		[CO ₂]×Nutrient	1, 27	2.013	0.167	2.964	0.097	0.001	0.970
<i>M. aquatica</i>	Naturalised	[CO ₂]	1, 27	0.076	0.785	0.431	0.517	2.598	0.119
		Nutrient	1, 27	0.181	0.674	0.321	0.576	2.678	0.113
		[CO ₂]×Nutrient	1, 27	0.050	0.825	0.637	0.432	0.003	0.957
<i>R. nasturtium-aquaticum</i>	Naturalised	[CO ₂]	1, 27	3.571	0.070	12.394	0.002	10.547	0.003
		Nutrient	1, 27	6.295	0.018	0.375	0.545	1.159	0.291
		[CO ₂]×Nutrient	1, 27	1.093	0.305	0.971	0.333	0.534	0.471
<i>M. pulegium</i>	Invasive	[CO ₂]	1, 22	0.580	0.454	0.169	0.685	0.002	0.962
		Nutrient	1, 22	1.250	0.004	5.084	0.034	2.215	0.150
		[CO ₂]×Nutrient	1, 22	0.460	0.505	0.254	0.620	7.086	0.014

<i>Z. aethiopica</i>	Invasive	[CO ₂]	1, 27	6.554	0.016	0.976	0.332	4.917	0.035
		Nutrient	1, 27	3.008	0.094	9.525	0.005	0.001	0.980
		[CO ₂]×Nutrient	1, 27	7.617	0.010	0.616	0.439	0.316	0.439

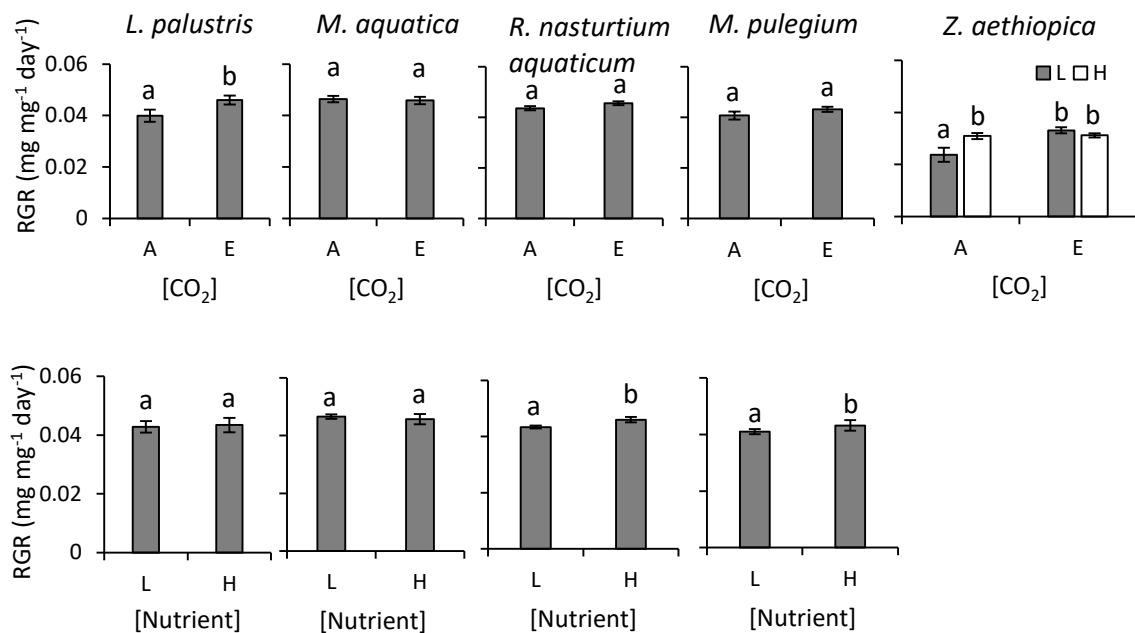


Figure 2: Effect of [CO₂] (top row; A = Ambient; E = Elevated) and nutrient concentration (bottom row; L = Low; H = High) on RGR of each species. There was a significant CO₂ × nutrient effect on the RGR of *Z. aethiopica* as shown in the figure (top row). Error bars represent ± 1 SE and letters above them indicate where there were significant within-species differences.

Root to shoot ratio

There were no significant interactions between [CO₂] and nutrient concentration for R:S for any of the species. [CO₂] had a significant effect on R:S of only *L. palustris* and *R. nasturtium-aquaticum* with the plants grown under elevated [CO₂] having 32% and 54% higher R:S respectively compared to their counterparts grown under ambient [CO₂] (Figure 3; Table 2). Similarly, nutrient concentration significantly affected the R:S of only *L. palustris*, *M. pulegium* and *Z. aethiopica* (Figure 3; Table 2). *Ludwigia palustris* and *M. pulegium* plants grown under low nutrient concentration allocated 30% and 53% more

biomass respectively to their roots compared to their counterparts grown in high nutrient concentration. In contrast, *Z. aethiopica* plants grown under high nutrient concentration allocated 49% more biomass to their roots compared to their counterparts grown in low nutrient concentration.

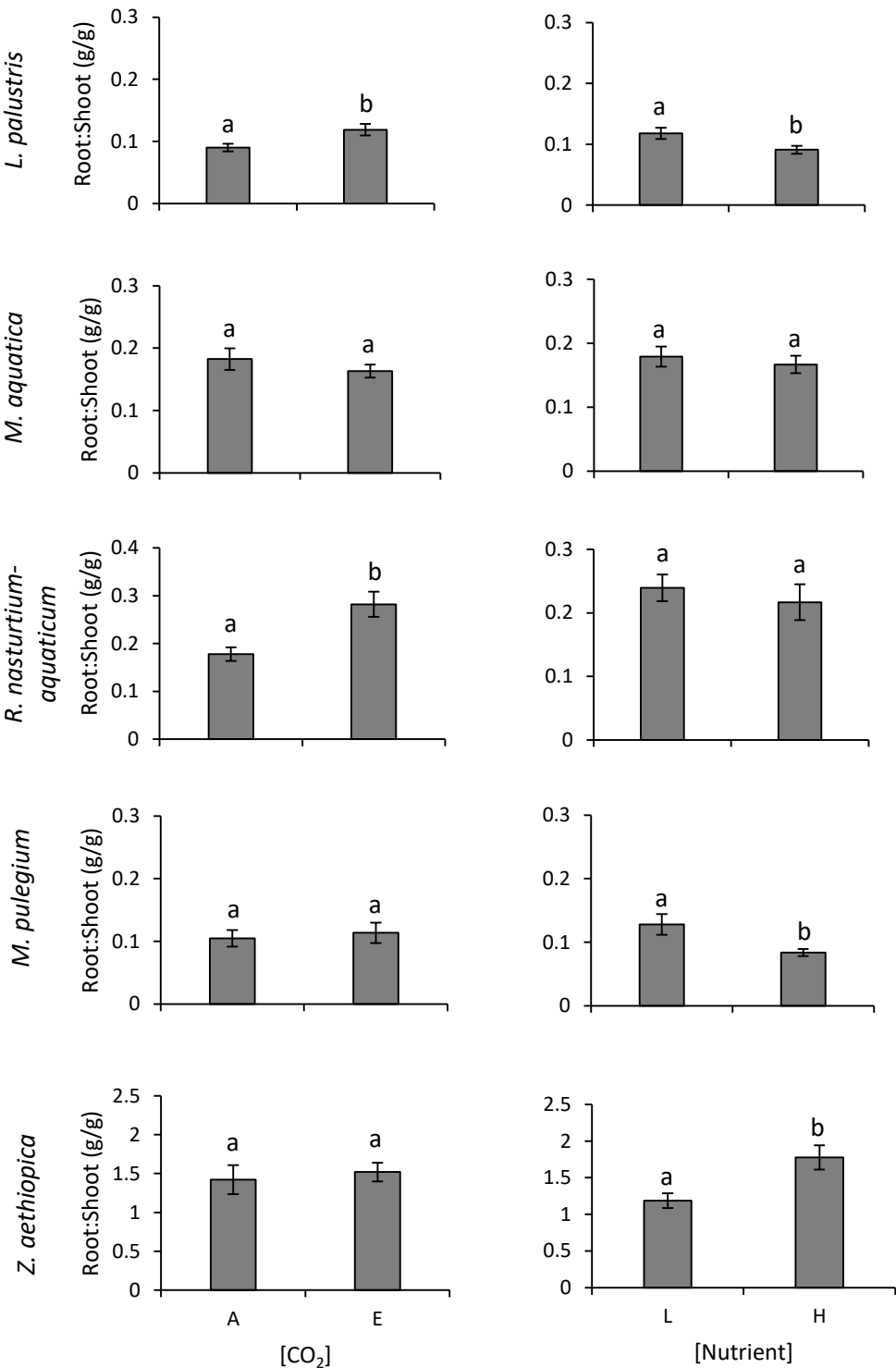


Figure 3: Effect of [CO₂] (left column; A = Ambient; E = Elevated) and nutrient concentration (right column; L = Low; H = High) on R:S of each species. Error bars represent ± 1 SE and letters above them indicate where there were significant within-species differences.

Specific leaf area (SLA)

There was a significant interaction between [CO₂] and nutrient concentration for SLA of *M. pulegium* (Figure 4; Table 2) where the plants grown under ambient [CO₂] in the high nutrient treatment had 20% higher SLA compared to the plants grown under the other CO₂ × nutrient treatment combinations. The SLA of *M. pulegium* did not differ between any of the other CO₂ × nutrient treatment combinations.

There were no significant interactions between [CO₂] and nutrient concentration for SLA of any of the other species. In contrast, there was a significant [CO₂] effect on the SLA of *L. palustris*, *R. nasturtium-aquaticum*, and *Z. aethiopica* (Figure 4; Table 2) with the plants grown under elevated [CO₂] having 35%, 24% and 12% lower SLAs respectively than the plants grown under ambient [CO₂]. However, there was no CO₂ effect on the SLA of *M. aquatica* while nutrient concentration did not affect the SLA of any of the species (Figure 4; Table 2).

Irrespective of treatment, the mean SLA of the invasive *M. pulegium* was significantly higher than that of the naturalised species (all $P < 0.001$) except for *R. nasturtium-aquaticum* while the mean SLA of the invasive *Z. aethiopica* was significantly lower than that of the naturalised species (all $P < 0.001$).

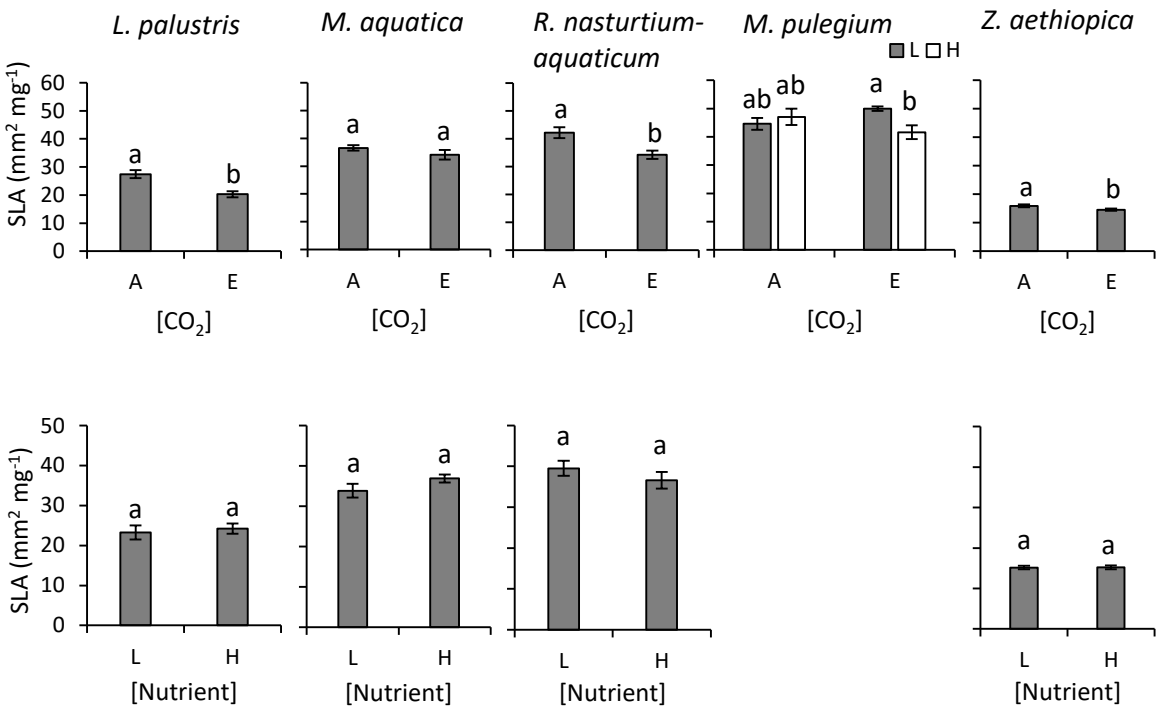


Figure 4: Effect of [CO₂] (top row; A = Ambient; E = Elevated) and nutrient concentration (bottom row; L = Low; H = High) on SLA of each species. There was a CO₂ × nutrient effect on SLA of *M. pulegium* as shown in the figure (top row). Error bars represent ± 1 SE and letters above them indicate where there were significant within-species differences.

Discussion

In this study we grew two invasive exotic and three naturalised emergent freshwater plant species under two levels of [CO₂] and two levels of nutrient concentration. We first hypothesised that [CO₂] and nutrient concentration would have an interactive effect on the relative growth rates of the species, with highest RGR expected under elevated [CO₂] and nutrient enrichment. The CO₂ × nutrient interaction had an effect only for the invasive species *Z. aethiopica*, although simultaneous enrichment of

both CO₂ and nutrients did not result in further growth enhancement relative to when only one of the factors was enriched. Similar findings where simultaneous elevation of [CO₂] and temperature resulted in marginal growth increases compared to when only one condition was elevated has been observed in terrestrial grass species *Austrodanthonia eriantha* and *Vulpia myuros* (Hely & Roxburgh, 2005). Three of the other four species (*M. aquatica* being the exception) had enhanced growth in response to [CO₂] or nutrient enrichment individually. This is consistent with the response of most C₃ species to CO₂ and nutrient enrichment in terrestrial systems (Poorter & Navas, 2003; Reich *et al.*, 2006). The growth of the naturalised species *L. palustris* was enhanced under elevated [CO₂]. This positive response has also been found for its congener *L. repens* [CO₂] (Lytle, 2003). However, *L. palustris* growth was unaffected by nutrient treatment, consistent with findings of a previous study of this species, where nutrient enrichment was up to 0.8 mg N-NO₃ L⁻¹ (Hastwell, Daniel & Vivian-Smith, 2008). In contrast, the growth of the invasive *M. pulegium* and naturalised *R. nasturtium-aquaticum* was enhanced only by nutrient enrichment. Although we did not find comparable studies for *M. pulegium*, previous studies have shown that the RGR of *R. nasturtium-aquaticum* (syn. *R. officinale*) increased with increasing nutrient concentration (Fernandez-Going, Even & Simpson, 2013). The naturalised *M. aquatica* on the other hand, was not responsive to either CO₂ or nutrient enrichment. While unexpected, our results are consistent with an earlier study by Pedersen and Sand-Jensen (1997) who found that the growth of *M. aquatica* was unresponsive to varied levels of sediment fertility.

We further predicted that the invasive exotics *M. pulegium* and *Z. aethiopica* would have stronger growth responses to both elevated [CO₂] and nutrient enrichment compared to the three naturalised species. This was because invasive exotic terrestrial species tend to be more responsive to resource enrichment than their naturalised

counterparts (Leishman & Thomson, 2005; Schlaepfer *et al.*, 2010; van Kleunen, Weber & Fischer, 2010). For example, a comparison of 14 pairs of invasive and naturalised herbaceous species showed that the invasive species had faster germination and RGR compared to their naturalised congeners (Schlaepfer *et al.*, 2010). Our results did not support the hypothesis since the RGR of *M. pulegium* was comparable to the RGR of the naturalised species whereas the RGR of *Z. aethiopica* was significantly lower than that of the naturalised species regardless of the treatment. The relatively poor responsiveness of the invasive *Z. aethiopica* to enriched resources may be due to its rhizomatous nature where the active growth sites are in the substrate unlike the other species that have creeping stolons. Although the belowground structures rarely covered more than 80% of the substrate volume, it is possible that *Z. aethiopica* growth may have been constrained to some extent by the tub size relative to the plant size as has been demonstrated for many species in similar experiments (Poorter *et al.*, 2012a). For instance, the CO₂ effect on biomass production of *Vallisneria americana*, a submerged species with its meristem in the substrate, has been shown to be significantly influenced by pot size (Titus & Wendlberger, 2016). Alternatively, the *Z. aethiopica* plants in our experiment could be hybrids or slow-growing cultivars that have been artificially selected and bred by the nurseries.

We then hypothesised that the plants grown under elevated [CO₂] and/or low nutrient concentration would allocate more biomass to their roots compared to their counterparts grown under ambient [CO₂] or high nutrient concentration respectively. The R:S of *M. aquatica* was not affected by either CO₂ or nutrient enrichment while that of *Z. aethiopica* increased under nutrient enrichment but was unaffected by [CO₂]. Since rhizomes serve as storage and reproductive organs, it is not surprising that belowground biomass of *Z. aethiopica* was higher under enriched nutrient conditions compared to under

low nutrient availability. Consistent with findings for congeners *L. grandiflora* and *L. peploides*, the R:S of *L. palustris* increased in response to low nutrient concentration (Hussner, 2010) as well as elevated [CO₂]. In contrast, the R:S of *R. nasturtium-aquaticum* increased under elevated [CO₂] but not under low nutrient concentration while the that of *M. pulegium* increased under low nutrient concentration but not under elevated [CO₂]. These findings suggest that plant species tend to allocate more biomass to the acquisition of the most limiting resource (Arp, 1991), but not invariably. The lack of R:S responsiveness to elevated CO₂ by a majority of our study species is consistent with the findings for most terrestrial plant species (Poorter *et al.*, 2012b).

We hypothesised that elevated [CO₂] and low nutrient concentration would result in reduced SLA in the study species compared to under ambient [CO₂] and high nutrient concentration respectively. In terms of [CO₂], our findings supported this hypothesis with three of the study species (exceptions being the two *Mentha* spp.) having reduced SLAs under elevated [CO₂]. This reduction in SLA under elevated [CO₂] is normally attributed to increased leaf density per unit leaf area due to accumulation of non-structural compounds such as starch and increased leaf thickness (Poorter & Navas, 2003; Ainsworth & Long, 2005). However, we found that nutrient concentration did not affect the SLA of any of the study species, contrary to observations from an analysis of 22 herbaceous terrestrial species (Meziane & Shipley, 1999). It has been suggested that nutrient effect on SLA is strongly dependent on other environmental factors (Meziane & Shipley, 1999; Anacker *et al.*, 2011). Since there was no interaction between [CO₂] and nutrient concentration on the growth and R:S allocation of most of our study species, it is therefore not surprising there was no SLA response to nutrient treatment.

Further, we predicted that the invasive species would have higher SLA values compared to naturalised species. However, our results did not follow this trend with the invasive *Z. aethiopica* having the lowest SLA of all the study species, consistent with its relatively slow RGR. In addition, the lack of aboveground stem for support makes it necessary for *Z. aethiopica* leaves to be structurally strong thereby contributing to the low SLA values (Leishman *et al.*, 2007).

In conclusion, the effects of [CO₂] and nutrient enrichment on the study species was species-specific and less linked to their invasive status. Surprisingly, growth responses to elevated [CO₂] were not enhanced by increased nutrient availability, suggesting that other factors such as light may be limiting. We acknowledge that in a comparative study with relatively few species, species selection could have a strong influence on overall findings. In the case of this study, we were constrained in our species selection by the availability of the species. However, we tried to select species with similar growth habits (emergent) that also utilise a similar photosynthetic pathway (C₃), thus reducing between-species variation. Nonetheless, *Z. aethiopica*, the invasive rhizomatous species, showed a markedly lower RGR relative to the creeping emergent species, suggesting that it may not have been suitable for comparison purposes in this study. The invasive and naturalised creeping emergent species on the other hand, had similar RGR regardless of [CO₂] or nutrient concentration, suggesting that they are likely to respond similarly to global environmental change. While this study contributes to our species-level understanding of how emergent freshwater may respond to ongoing global environmental change, we propose future research efforts should concentrate on determining the consequences of these responses on competitive interactions and community-level processes in freshwater systems.

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CHAPTER SIX

Are interactions between the native *Azolla filiculoides* and exotic *Salvinia molesta* mediated by elevated CO₂ and nutrient enrichment?

This chapter has been submitted to *Aquatic Sciences*.

My contribution to the research and paper: Concept – 90%; Data collection – 100%; Analysis – 100%; Writing – 90?%

1 **Abstract**

2 Ongoing global environmental changes such as rising atmospheric CO₂
3 concentration ([CO₂]) and nutrient enrichment may modify the competitive interactions
4 between co-occurring plant species. In this study we used a glasshouse experiment to test
5 the effect of elevated [CO₂] and nutrient enrichment on competition between two free-
6 floating fern species that co-occur in south-eastern Australia - native *Azolla filiculoides*
7 and invasive exotic *Salvinia molesta*. We grew the species in monoculture and
8 competition, and replaced the nutrient medium weekly to simulate a dynamic system. We
9 found that the relative growth rate (RGR) of both species was greater under high resource
10 conditions (elevated [CO₂], high nutrients) with neither having a decrease in RGR when
11 grown in competition. On the contrary, *A. filiculoides* had a facilitative effect on *S.*
12 *molesta*. In addition, *A. filiculoides* gained more biomass under high resource conditions
13 relative to *S. molesta* and the opposite was true under low resource conditions. We
14 conclude that [CO₂] and nutrient concentration did not mediate competition between the
15 species but instead influenced RGR independent of competition. These findings suggest
16 that species composition in dynamic water bodies will in the future likely be determined
17 by the species' responses to environmental changes rather than by changes in competitive
18 interactions.

19 **Key words:** competitive interactions, free-floating species, freshwater system, global
20 change, invasive, native

Introduction

Invasive exotic plant species constitute a major problem in many freshwater systems as they affect both the economic and recreational value of these systems (Dudgeon *et al.*, 2006; Riis *et al.*, 2012), as well as causing a decrease in the abundance and diversity of co-occurring native species (Gallardo *et al.*, 2016). These decreases can happen indirectly due to the invasive exotic species altering the physical and chemical properties of the water thereby creating novel environmental conditions that the native species are less well adapted to (Strayer, 2010; Gallardo *et al.*, 2016), and/or directly through competition for resources (Agami & Reddy, 1990). Direct competition for resources is considered the most important form of plant interactions (Gopal & Goel, 1993) as it has the strongest influence on the abundance, diversity and distribution of species (Tilman, 2000). This is particularly relevant to freshwater communities where the dominance of invasive exotic plant species is often attributed to their superior competitive abilities that suppress native species (Barrat-Segretain & Elger, 2004). For example, the invasive exotic *Myriophyllum spicatum* (parrot's feather) has competitively displaced 13 of the 20 co-occurring native species and significantly reduced the abundance of the remaining native species in Lake George, New York, United States (Boylen, Eichler & Madsen, 1999).

Currently, the earth is experiencing an unprecedented level of change in a range of environmental factors including rising atmospheric CO₂ concentration ([CO₂]) and nutrient enrichment (Walther *et al.*, 2002). Consequently, an important applied question in ecological research is whether the competitive interactions between invasive exotic and native plant species will be affected by these changes (Manea & Leishman, 2011). Many studies have shown that plants tend to have faster growth rates in response to elevated

[CO₂] (Poorter, 1993). However, the degree of the response is often species-specific and depends largely on nutrient supply not being limited (Oren *et al.*, 2001; Poorter & Navas, 2003; Reich *et al.*, 2006), as well as the functional group of the plants (Robinson, Ryan & Newman, 2012). For example, nitrogen (N)-fixing species tend to respond more strongly to CO₂ enrichment compared to other species that are not capable of fixing N since their nodules represent additional carbon sinks (Poorter, 1993; Wang *et al.*, 2012). Responsiveness to CO₂ enrichment may also depend on the origin of the species. For example, it has been demonstrated in an open-top chamber CO₂ enrichment experiment that the invasive exotic wetland species *Typha angustifolia* (narrow-leaved cattail) had a stronger growth response compared to the congeneric native species, *T. latifolia* (broadleaf cattail) (Sullivan *et al.*, 2010). By differentially stimulating species' growth rates, elevated [CO₂] may modify competitive interactions between native and exotic invasive plant species. For terrestrial species, the shift in competitive interactions between native and invasive exotics in response to elevated [CO₂] has received some attention (Poorter & Navas, 2003; Hely & Roxburgh, 2005; Manea & Leishman, 2011; Liu *et al.*, 2017). In contrast, our understanding for aquatic species and in particular freshwater species is much more limited.

In freshwater systems, nutrients are the most important limiting resource for which plants compete (Gopal & Goel, 1993). Over the last half a century, freshwater systems have experienced significant nutrient enrichment as a result of human activities (Carpenter, Stanley & Vander Zanden, 2011; Wersal & Madsen, 2011). A consequence of this nutrient enrichment is that it makes these systems more vulnerable to exotic plant invasions (Chytrý *et al.*, 2008) due to many exotic species having enhanced competitive ability relative to native species under these conditions (Daehler, 2003). For example, it has been experimentally demonstrated that stronger growth response to nutrient

enrichment allowed the invasive exotic *Hydrilla verticillata* (water thyme) to have a competitive edge over the native *Vallisneria americana* (American eelgrass) (Van, Wheeler & Center, 1999). Similarly, the invasive exotic *Lemna minuta* (least duckweed) was shown to be a better competitor against the native *L. minor* (common duckweed) in eutrophic as opposed to mesotrophic conditions (Gérard & Triest, 2018). This can subsequently alter the structure and function of these systems at a broader scale (Scheffer *et al.*, 2003) and therefore potentially constitutes a major threat to these systems (Njambuya, Stiers & Triest, 2011).

It is often the case that multiple global change environmental factors interact with one another to modify competitive interactions between native and invasive exotic plant species (Netten *et al.*, 2010; Stiers, Njambuya & Triest, 2011). For example, increased temperature has been shown to favour the invasive exotic *Salvinia natans* under enriched nutrient conditions at the expense of native submerged species (Netten *et al.*, 2010). One of the most well documented interactions between global change environmental factors is that between rising atmospheric [CO₂] and nutrient enrichment as they can have a strong synergistic effect on plant growth (Bornette & Puijalon, 2011; Low-Décarie, Bell & Fussmann, 2015). For example, the responsiveness of water hyacinth, *Eichhornia crassipes* (an invasive exotic freshwater species) growth to CO₂ enrichment has been shown to be more pronounced under eutrophic conditions compared to oligotrophic conditions (Liu *et al.*, 2010). It is therefore likely that elevated [CO₂] coupled with nutrient enrichment will modify the competitive interactions between species with contrasting origins.

In aquatic systems, competition is believed to be most intense between species with similar growth forms that occupy similar positions in the water column (Gopal &

Goel, 1993). Among the various growth forms of freshwater plants, free-floating species have the greatest detrimental impact on freshwater systems as they significantly alter habitat structure and create more anoxic environments which in turn has a cascading effect on animal diversity (Scheffer *et al.*, 2003; Villamagna & Murphy, 2010). Free-floating species have also been shown to be extremely responsive to [CO₂] and nutrient enrichment (Scheffer *et al.*, 2003; Feuchtmayr *et al.*, 2009; Netten *et al.*, 2010). Despite this, there are only a few studies that have tested how global change environmental factors have altered the competitive interactions between native and invasive exotic free-floating freshwater species. Furthermore, the majority of these studies have focused on competition between native and invasive species from the genus *Lemna* (Njambuya, Stiers & Triest, 2011; Ceschin *et al.*, 2018; Gérard & Triest, 2018; Paolacci, Jansen & Harrison, 2018). Therefore, the aim of this study was to test the effect of [CO₂] and nutrient concentration on the growth, biomass allocation and competitive ability of two free-floating ferns, the native *Azolla filiculoides* (water fern; Azollaceae) and the invasive exotic *Salvinia molesta* (salvinia; Salviniaceae), which commonly co-occur in freshwater systems in south eastern Australia. We did this by growing the species in a three-way factorial glasshouse experiment with two [CO₂] (ambient and elevated), two nutrient concentrations (low and high), and three competition treatments (monocultures of each species and both species in competition). We then measured the relative growth rates (RGR), root to shoot ratio (R:S) and maximum root length of the species in each treatment combination. We calculated the RGR instead of the analysing the final biomass because the initial biomass of a species in the competition treatment was 50% of its initial biomass in the monoculture treatment. These traits were selected as they have important effects on resource exploitation and tend to be influenced by elevated [CO₂], nutrient availability and competition. For example, plants tend to allocate more biomass to their roots when grown in CO₂ enriched and

nutrient-poor conditions in order to optimise acquisition of the most limiting resource (Arp, 1991; Craine, 2006; Nie *et al.*, 2013). Furthermore, both [CO₂] and nutrient concentration affect root length which influences the ability of a species to pre-empt nutrient supply in the presence of competitors (Craine, 2006; Nie *et al.*, 2013).

Both study species belong to the order Salviniales and fix atmospheric CO₂ via the C₃ pathway and are thus expected to respond positively to elevated [CO₂], assuming nutrient supply is not limited (Ray *et al.*, 1979; Longstreth, 1989). *Azolla filiculoides* hosts symbiotic N-fixing cyanobacteria *Anabaena azollae* within its leaf cavities (Wagner, 1997) and is therefore not N-limited (Espinar *et al.*, 2015). This means that irrespective of the nutrient supply in the water column, it should respond positively to elevated [CO₂]. In contrast, *S. molesta* does not fix N but rather absorbs nutrients from the water column through the underside of its floating leaves and the highly dissected submerged leaves (roots hereafter) (Julien & Bourne, 1986). This means that *S. molesta*'s response to elevated [CO₂] may be mediated by the nutrient supply in the water column.

Thus we predicted that:

- (i) in the absence of competition, both species will have highest RGR under elevated [CO₂] in the high nutrient treatment.
- (ii) RGR of both species will be suppressed in the competition treatment but *S. molesta* (exotic) will have a higher RGR relative to *A. filiculoides* (native) under high resource conditions and the opposite will be true for low resource conditions.
- (iii) regardless of competition, elevated [CO₂] and low nutrient concentration will each result in increased biomass allocation to roots, and relatively longer roots in both species.

Methods

Plant collection and preparation

Salvinia molesta plants were collected from a wild population at Lake Munmorah, Wyong, New South Wales (NSW), Australia (33.1923°S, 151.5749°E) and *A. filiculoides* plants were collected from an artificial pond at Macquarie University Plant Growth Facility, North Ryde, NSW, Australia (33.7745° S, 151.1169° E). The plants were maintained in tubs containing tap water for three weeks prior to being prepared for planting.

Prior to the start of the experiment, similar sized *S. molesta* rhizome sections (fresh weight: 0.25 g \pm 0.05 SD) each consisting of four leaves and an apical bud were selected. A sub-sample of 20 plantlets were randomly selected, oven-dried at 70°C for 48 hours and weighed using an analytical electronic balance (Mettler Toledo, Port Melbourne, VIC, Australia). Similarly, 20 frond portions of *A. filiculoides* of similar fresh weights (0.9 g \pm 0.05 SD) were oven-dried and weighed as described above. In order to ensure equivalent biomass of the species at the start of the experiment, we calculated the ratio of the mean fresh weight to the mean dry weight of *A. filiculoides* frond portions to determine the fresh weight of the species that would be equivalent in dry weight to one *S. molesta* plantlet. It was thus determined that in order to obtain one *S. molesta* dry weight (0.021 g \pm 0.003 SD) worth of *A. filiculoides*, 0.3 g fresh weight of *Azolla* fronds were required.

Experimental design

The experiment was conducted in climate controlled glasshouses at the Plant Growth Facility of Macquarie University beginning on 5-6th September 2017. The temperature of the glasshouses was maintained at 25°C/20°C day/night producing water

temperatures of 24°C/19°C day/night. This temperature range is consistent with the mean summer water temperatures of the lower Hawkesbury River, NSW, Australia (Sydney Water, unpublished data) which is one of the major freshwater systems in south eastern Australia where the study species commonly co-occur. In addition to temperature, humidity (65-75%) and photosynthetically active radiation (230-870 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 2 pm) of the glasshouses were continuously monitored using a Multi-grow Controller System (Autogrow Systems, Auckland, New Zealand).

For the experiment, we used 10-L transparent plastic tubs each containing 8 L of reverse osmosis water to which 2 mL of undiluted Seasol, a nitrogen- and phosphorus-free organic concentrate (Seasol International Pty Ltd, Bayswater, VIC, Australia), was added to supply potassium and micro-nutrients. The experiment utilised a three-way factorial design with two [CO₂] (ambient: 380–420 ppm, elevated: 580–620 ppm), two nutrient concentrations (low: 0.3 mg N-NO₃ L⁻¹, 0.02 mg P-PO₄ L⁻¹, high: 1.5 mg N-NO₃ L⁻¹, 0.1 mg P-PO₄ L⁻¹), and three competition treatments (*A. filiculoides* monoculture, *S. molesta* monoculture and both species in competition). Pre-prepared solutions of sodium nitrate and sodium phosphate were used to supply nitrogen and phosphorus at concentrations specified by the different nutrient treatments. These nutrient concentrations are representative of the levels found at fairly undisturbed (low nutrient) and disturbed (high nutrient) sites in the Hawkesbury-Nepean river system of Sydney region (Pinto, Maheshwari & Ollerton, 2013) where the study species co-occur. Each treatment combination was replicated ten times resulting in a total of 120 tubs, which were evenly split across four glasshouses (two ambient and two elevated [CO₂]). These CO₂ concentrations were maintained and monitored daily by a CO₂ dosing and monitoring system (Canary Company Pty Ltd, Lane Cove, NSW, Australia).

Within each tub, the same fresh weight of each species equivalent to the dry weight of one *S. molesta* plantlet was used as the starting biomass (Figure 1). This meant that $0.3 \text{ g} \pm 0.05 \text{ SD}$ and $0.25 \text{ g} \pm 0.05 \text{ SD}$ fresh weights of *A. filiculoides* and *S. molesta* respectively were placed in the monoculture tubs at the start of the experiment. For the competition tubs, half of these fresh weights of each species were placed in each tub. The plants were grown for seven weeks at which point some tubs had 90% coverage of the water surface. Throughout the duration of the experiment, the nutrient medium was replaced with fresh medium on a weekly basis after cleaning the tubs thoroughly to remove any periphytic algae. In addition to controlling competition from algae, nutrient replacement was also meant to simulate a dynamic system (e.g. rivers, creeks) where water residence time is relatively short. The tubs were also randomly reassigned a new position within the same glasshouse at these times to reduce any within-glasshouse effects.

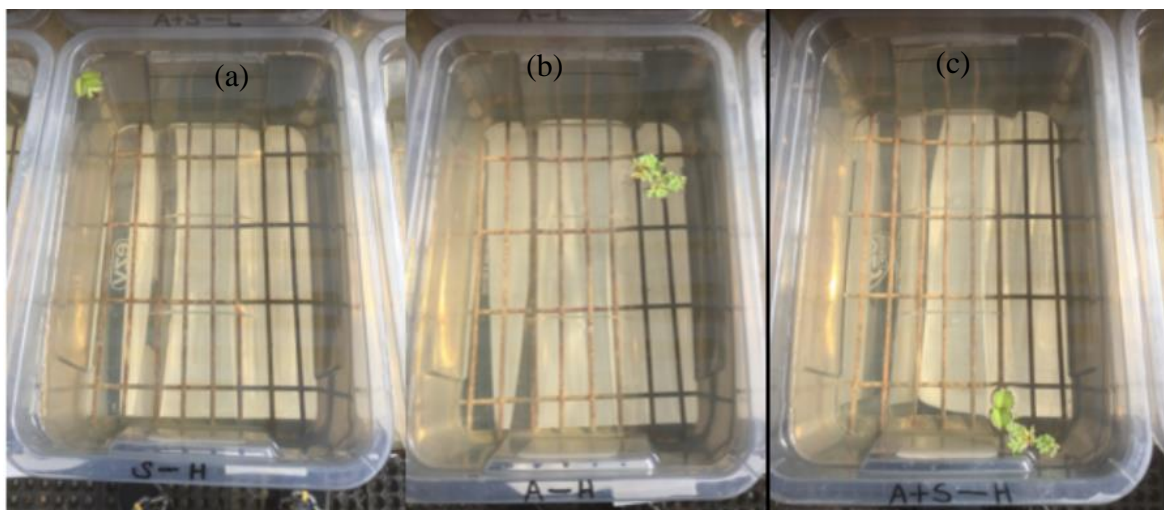


Figure 1: Photos showing (a) *S. molesta* monoculture, (b) *A. filiculoides* monoculture, and (c) both species in competition at the start of the experiment.

Measurements and harvesting

After the seven week growth period, the relative growth rate (RGR), root to shoot ratio (R:S-root dry weight/shoot dry weight) and maximum root length were measured for each plant. To do this plants were harvested and separated into their shoot and root biomass. The biomass components were then oven-dried and weighed as described above. RGR of each plant was then calculated as follows:

$$\text{RGR} = (\ln W_2 - \ln W_1)/T, \text{ where } W_2 = \text{final dry weight, } W_1 = \text{initial dry weight, and } T = \text{duration of experiment (days).}$$

To determine species' competitive responses in each of the competition tubs, we calculated the difference in the relative growth rates between the two species (RGRD) (Connolly & Wayne, 2005) using the following equation: $\text{RGRD} = \text{RGR}_{\text{Salvinia}} - \text{RGR}_{\text{Azolla}}$, where a value > 0 indicates that *S. molesta* gained comparatively more biomass than *A. filiculoides* and vice versa for a value < 0 .

Statistical analysis

The effect of [CO₂], nutrient concentration, and competition on the RGR and root length of the species were assessed using a three-way ANOVA for each species. The RGR data were log₁₀-square root-transformed to meet the assumptions of ANOVA while the raw root length data were normally distributed and did not require any transformation. Where an interaction was detected, Tukey HSD post-hoc analysis was used to determine the differences between treatment combinations. The R:S data were not normally distributed even when transformed so non-parametric Kruskal-Wallis analyses were used to analyse each factor ([CO₂], nutrients, competition) individually for each species. Finally, the effect of [CO₂] and nutrient concentration on the RGRD of the competition

tubs was assessed using a two-way ANOVA. The RGRD data were normally distributed and hence did not need transformation.

It should be noted that plants that died during the course of the experiment (one *A. filiculoides* and eight *S. molesta*) and their corresponding competition treatment pair were excluded from the analyses. All analyses were performed using SPSS statistical software, version 25 (IBM, Chicago, IL, USA), with significance level set at $P < 0.05$.

Results

Azolla filiculoides

The RGR of *A. filiculoides* was 41% higher under elevated [CO₂] compared to ambient [CO₂] ($F_{1, 71} = 69.036$, $P < 0.001$; Figure 2a), and 59% greater in the high nutrient treatment compared to the low nutrient treatment ($F_{1, 71} = 119.139$, $P < 0.001$; Figure 2a). In contrast, competition did not influence the RGR of *A. filiculoides* ($F_{1, 71} = 0$, $P = 0.985$; Figure 2a). There were no significant interactions between any of the treatments for the RGR of *A. filiculoides* (Supplementary Table 1).

The R:S of *A. filiculoides* was reduced by 12% under elevated [CO₂] compared with ambient [CO₂] ($H_{1, N=72} = 4.612$, $P = 0.032$; Figure 2b), and by 26% in the high nutrient treatment compared to low nutrient treatment ($H_{1, N=72} = 14.476$, $P < 0.001$; Figure 2b). Similarly to RGR, competition did not influence the R:S of *A. filiculoides* (Supplementary Table 2).

Maximum root length of *A. filiculoides* was significantly affected by an interaction between the [CO₂] and nutrient concentration ($F_{1, 71} = 8.318$; $P = 0.006$; Supplementary Table 1). Post-hoc analyses revealed that the maximum root length of plants grown in the

257 high nutrient treatment under elevated [CO₂] was significantly longer than that of the
258 plants grown under the other treatment combinations (all $P \leq 0.013$; Figure 2c). Maximum
259 root length of *A. filiculoides* was also affected by the competition treatment ($F_{1, 71} =$
260 6.048; $P = 0.018$) with the maximum root length of the plants grown in monocultures
261 being 14% longer than that of the plants grown in competition.
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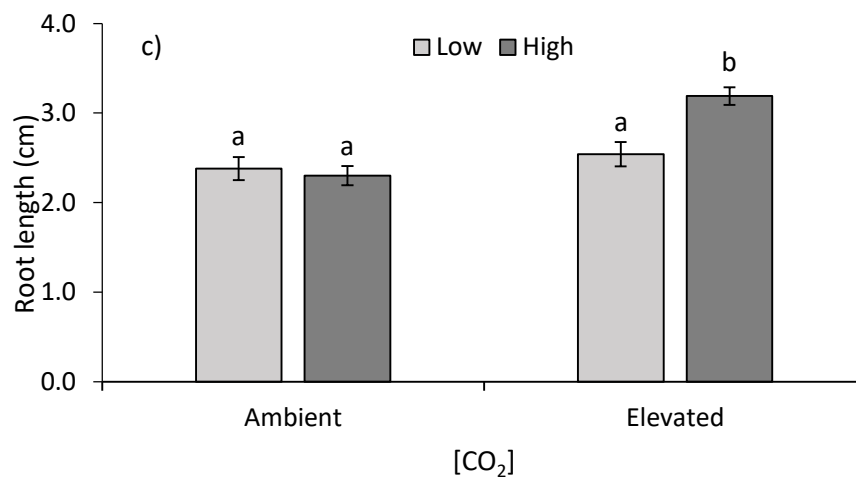
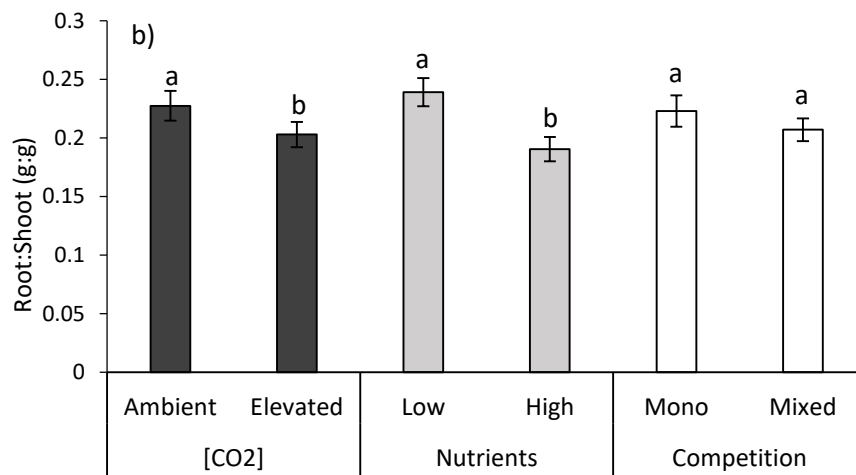
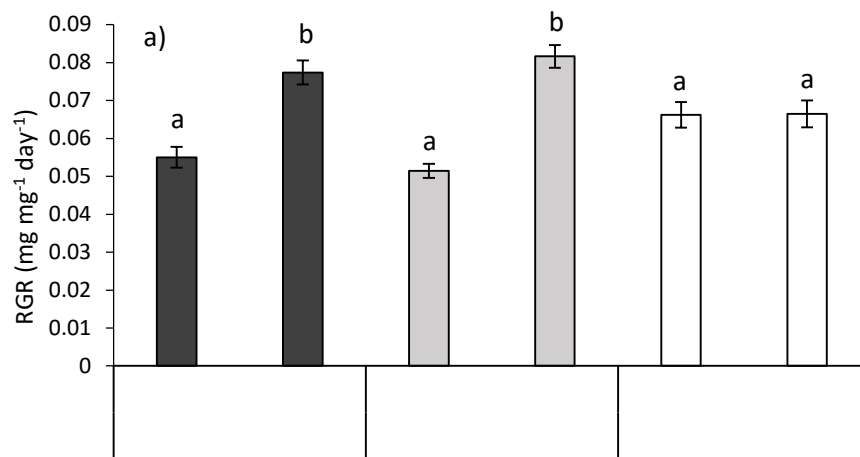


Figure 2: Effect of [CO₂], nutrient concentration, and competition on the mean (a) RGR and (b) R:S; and the effect of (c) [CO₂] × nutrient concentration on maximum root length,

for *A. filiculoides*. Error bars represent ± 1 SE and the letters above the error bars indicate significant differences between treatments ($P < 0.05$).

Salvinia molesta

The RGR of *S. molesta* was significantly affected by an interaction between [CO₂] and nutrient concentration ($F_{1, 64} = 14.418$; $P < 0.001$; Supplementary Table 1). Post-hoc analyses revealed that the plants grown under ambient [CO₂] in low nutrient conditions had significantly lower RGR than plants grown in the other treatment combinations (all $P < 0.001$; Figure 3a). Furthermore, the plants grown under elevated [CO₂] in the low nutrient treatment had significantly lower RGR than the plants grown under elevated [CO₂] in the high nutrient treatment ($P = 0.023$; Figure 3a). The RGR of *S. molesta* was also affected by the competition treatment ($F_{1, 64} = 11.28$; $P = 0.001$) with plants grown in competition having 8% higher RGR than their counterparts grown in monoculture.

The R:S of *S. molesta* was significantly affected by nutrient treatment ($H_{1, N=72} = 52.604$, $P < 0.001$) where the plants grown in the low nutrient treatment had 176% greater R:S compared to the plants grown in the high nutrient treatment (Figure 3b). In contrast, the R:S of *S. molesta* was not affected by the [CO₂] or competition (Supplementary Table 2).

The maximum root length of *S. molesta* was 37% greater under elevated [CO₂] ($F_{1, 64} = 27.95$; $P < 0.001$; Figure 3c) compared to ambient [CO₂]. In contrast, the nutrient and competition treatments did not affect maximum root length of *S. molesta* (Figure 3c; Supplementary Table 1).

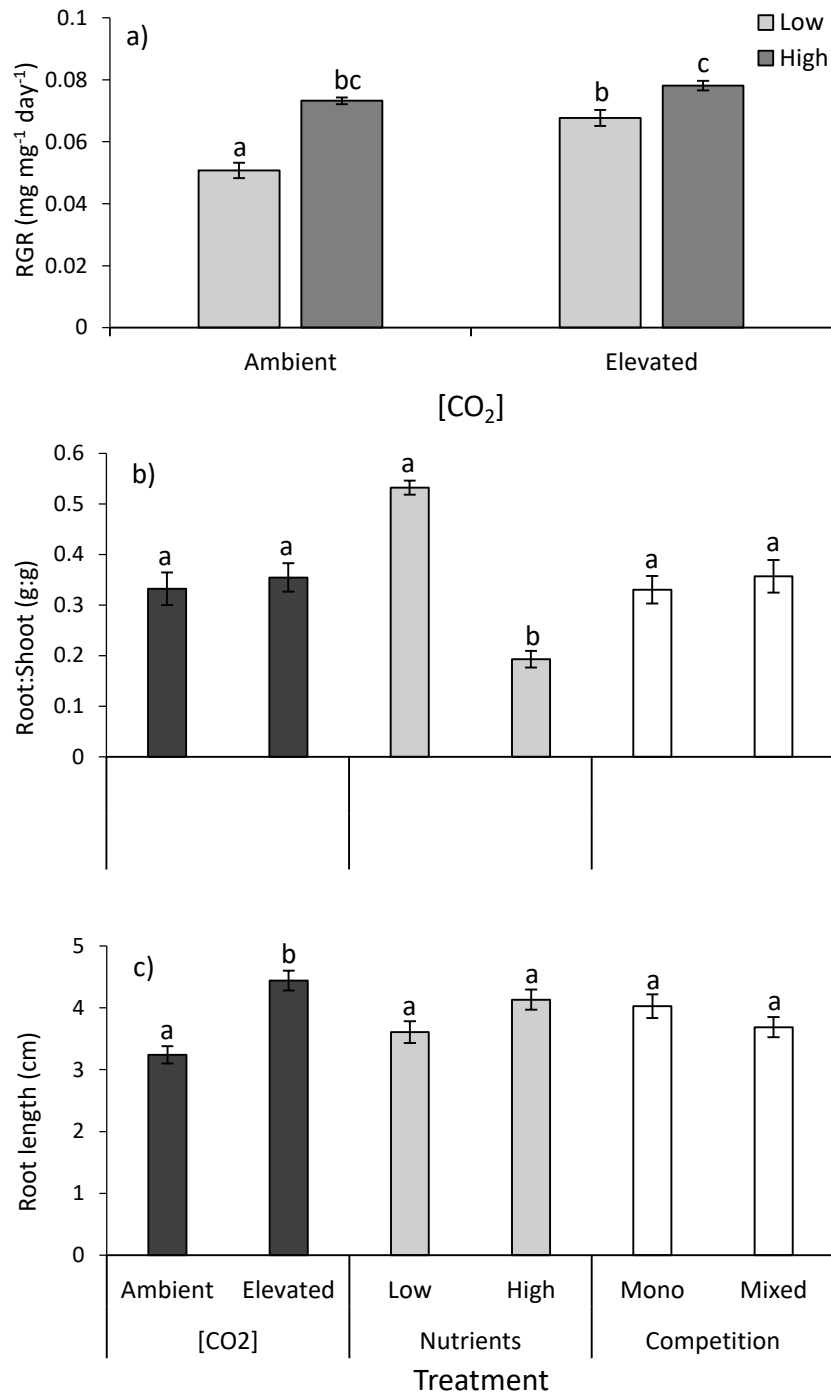


Figure 3: Effect of (a) [CO₂] × nutrient concentration on the mean RGR; and the effect of [CO₂], nutrient concentration, and competition on (b) R:S, and (c) maximum root length of *S. molesta*. Error bars represent ± 1 SE and the letters above the error bars indicate significant differences between treatments ($P < 0.05$).

Species competitive response

The change in relative biomass composition, measured as the difference in RGR of the competing species (RGRD) in response to [CO₂] × nutrient interaction was significantly different from zero ($F_{1,31} = 6.60$; $P = 0.015$; Figure 4). The RGRD value in the low nutrient treatment under ambient and elevated [CO₂] was 0.012 ± 0.002 (mean \pm SE) and 0.01 ± 0.002 (mean \pm SE) respectively corresponding to *S. molesta* having 27% and 13% greater RGR respectively than *A. filiculoides*. However, the RGRD value under ambient [CO₂] in the high nutrient treatment (0.014 ± 0.008 , mean \pm SE) was not significantly different from zero. In contrast, the RGRD value under elevated [CO₂] when the nutrient concentration was high was -0.016 ± 0.002 (mean \pm SE) corresponding to *A. filiculoides* having 20% greater RGR compared to *S. molesta*.

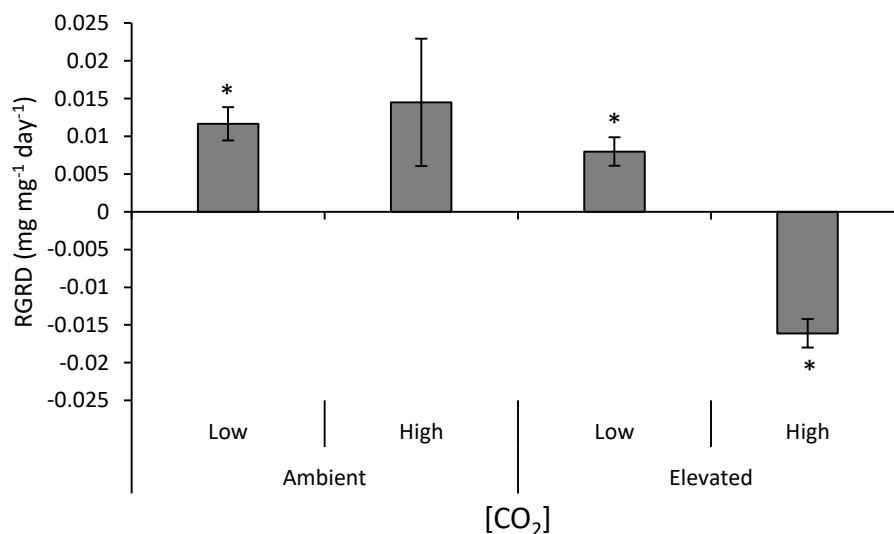


Figure 4: The effect of [CO₂] and nutrient concentration on RGRD (mean \pm SE) of the plants grown in the competition tubs. Positive values indicate *S. molesta* gained relatively more biomass compared to *A. filiculoides* while negative values indicate *A. filiculoides* gained relatively more biomass compared to *S. molesta* in the competition tubs. Error bars

represent ± 1 SE and the asterisks (*) indicate where the RGRD values are significantly different from zero ($P < 0.05$)

Discussion

The aim of this study was to determine how [CO₂] and altered nutrient conditions may alter the competitive interactions between native and invasive exotic freshwater plant species, using *A. filiculoides* (native) and *S. molesta* (exotic) as our study species. We first predicted that an interaction between elevated [CO₂] and high nutrient concentration would result in higher RGR for both species. However, we found that RGR of *A. filiculoides* was increased under both the elevated CO₂ and high nutrient treatments but there was no interactive effect. This finding is in contrast to that of previous studies where the stimulatory effect of elevated [CO₂] has been found to be mediated by other environmental factors such as phosphorus availability, temperature and irradiance (Cheng *et al.*, 2010; Espinar *et al.*, 2015; van Kempen *et al.*, 2016). A possible explanation for this may be that the amount of phosphorus supplied in both nutrient treatments was non-limiting and that *A. filiculoides* was able to alleviate the nitrate limitation in the low nutrient treatment under elevated [CO₂] by fixing its own nitrogen (Wagner, 1997). Although *A. filiculoides* had greater growth in the high nutrient compared to low nutrient treatment, the difference in nutrient availability may not have been sufficient to drive an interactive effect. Previous studies have shown similar levels of responsiveness to nutrient enrichment in this species (Cary & Weerts, 1992).

For *S. molesta* we did find an interactive effect between [CO₂] and nutrient concentration which resulted in faster growth of *S. molesta* under the resource enriched conditions (elevated [CO₂] and high nutrients) compared to low resource conditions.

Interestingly, the largest increase in RGR between the treatments occurred under low resource conditions. This was unexpected because the responsiveness of plant growth to elevated [CO₂] is usually greatest when nutrient supply is also high (Poorter & Navas, 2003). However, previous studies on *S. molesta* found that it grew well in low nutrient conditions (Henry-Silva, Camargo & Pezzato, 2008; Tipping *et al.*, 2009). For example, at nitrate concentrations ranging from 0.02 – 0.9 mg N-NO₃ L⁻¹, *S. molesta* experienced the highest growth rate at a mid-range concentration of 0.1 mg N-NO₃ L⁻¹ (Gufu *et al.*, 2018). It is therefore possible that the low nutrient treatment in our experiment did not limit *S. molesta* growth and that in the high nutrient treatment, *S. molesta* might have already been growing at close to its full capacity under ambient [CO₂] such that an increase in [CO₂] would have had little effect.

In terms of competition effects, we predicted that competition would negatively affect the RGR of both species relative to plants grown in monocultures. We found this not to be the case for *S. molesta* plants which had a neutral effect on their *A. filiculoides* neighbours. This finding is similar to that reported for a congeneric species, *S. minima*, which also had a neutral effect on the growth rate of *Azolla caroliniana* (Dickinson & Miller, 1998). In contrast, *A. filiculoides* had a facilitative effect on *S. molesta*, which has been previously reported for aquatic plant species (Espinar *et al.*, 2002; Boschilia, Oliveira & Thomaz, 2008; Michelan *et al.*, 2010; Wundrow *et al.*, 2012). For example, the long floating stems of *Eichhornia azurea* were found to provide protection against wind and waves for other co-occurring free-floating species (Boschilia, Oliveira & Thomaz, 2008). Facilitation can also occur when one of the species increases nitrogen availability for the other species through its N-fixation process. However, for our study species, facilitation was unlikely to be a result of *S. molesta* having direct access to excess nitrogen fixed by *Anabaena* (*Azolla* nitrogen fixing symbiont) because excess nitrogen is

normally conveyed directly to its *Azolla* host through interconnecting transfer hairs and only becomes available in the water column after mineralisation of the *Azolla-Anabaena* complex (Wagner, 1997). However, *S. molesta* may have indirectly had access to this nitrogen through the decomposition of senescent *Azolla* parts in the water column. It should be noted that other studies have found that *A. filiculoides* suppresses the growth of other free-floating species such as *Lemna minor/gibba* and *L. minuta* by overtopping them (Peeters, Neefjes & Zuidam, 2016; Paolacci, Jansen & Harrison, 2018). In our study, overtopping was not observed and given the relatively larger stature of *S. molesta*, we speculate that even if the experiment had run for longer, *A. filiculoides* might not have been able to overtop *S. molesta*.

Following on from the above hypothesis, we predicted that although RGR of both species would be suppressed by competition, *S. molesta* would be more favoured by high resource conditions relative to *A. filiculoides* and vice versa for low resource conditions. The basis for this prediction was that invasive exotics tend to be more responsive to high resource conditions than native species (Dukes & Mooney, 1999; Manea & Leishman, 2011; Liu *et al.*, 2017). Surprisingly, we found the opposite to be true for our study species, with *S. molesta* favoured relatively more under low resource conditions and *A. filiculoides* favoured relatively more under high resource conditions. A possible explanation for this finding is that the N-fixing ability of *A. filiculoides* means it has a larger carbon and nutrient sink compared to *S. molesta* and is therefore more responsive to enriched resource conditions. Moreover, *A. filiculoides* is a fast-growing native species that is considered highly invasive outside its native range (Espinosa *et al.*, 2015; van Kempen *et al.*, 2016) thus it is not surprising that it responds similarly to high resource conditions as invasive exotic species.

Our final hypothesis predicted that both species would allocate more biomass to their roots under elevated [CO₂] and low nutrient concentration compared to ambient [CO₂] and high nutrient concentration respectively. The basis for this prediction is that plants tend to optimise resource acquisition by allocating more biomass to the organs responsible for uptake of the most limiting resources (Bloom, Chapin III & Mooney, 1985). For nutrient concentration, our findings supported this hypothesis with both species, particularly *S. molesta*, allocating significantly more biomass to their roots in the low nutrient treatment. Contrary to our hypothesis, *A. filiculoides* allocated less biomass to roots under elevated [CO₂] compared to ambient [CO₂] while *S. molesta* did not shift its biomass allocation in response to [CO₂]. While we do not know why *A. filiculoides* allocated more biomass to its roots under ambient CO₂ compared to elevated CO₂, the lack *S. molesta* R:S responsiveness to elevated [CO₂] is consistent with findings for many terrestrial C₃ species (Poorter & Nagel, 2000; Poorter *et al.*, 2012).

We further predicted that elevated [CO₂] and low nutrient concentrations would result in both species producing longer roots, based on the premise that plants tend to maximise their root length in response to elevated [CO₂] and limited nutrient supply (Craine, 2006; Craine & Dybzinski, 2013). As expected, both species produced longer roots under elevated [CO₂]. However, the increase in *A. filiculoides* root length in response to elevated [CO₂] was dependent on nutrient concentration. Contrary to our expectations, it was the plants grown under elevated [CO₂] in the high nutrient treatment that produced longer roots. Although unexpected, this result is not surprising as fast-growing species such as *A. filiculoides* require longer roots for the fast and efficient uptake of nutrients needed to sustain rapid growth rates even under enriched resource conditions (Ryser, 2006). In contrast, *S. molesta* root length was not affected by nutrient concentration. A possible explanation for these contrasting results is that *S. molesta* invested more in

production of fine roots as indicated by our R:S results, rather than increasing its root length in response to low nutrient availability.

In conclusion, our results show that growth of both *A. filiculoides* and *S. molesta* was favoured by high resource conditions. However, *S. molesta* growth was stimulated by an interaction between elevated [CO₂] and nutrient enrichment, while the growth of *A. filiculoides* was stimulated by these factors independent of each other. Nevertheless, *A. filiculoides* was relatively more favoured by high resource conditions than *S. molesta*. In small enclosed water bodies (e.g. ponds and dams) where space and nutrients are limited, these differing degrees of responsiveness may lead to one species displacing the other depending on the environmental conditions present in the water body. For oligotrophic conditions, we would expect *S. molesta* to displace *A. filiculoides* and vice versa for eutrophic conditions. However, for larger more dynamic water bodies (e.g. rivers, creeks) it is likely that this displacement will not occur. This is because in these systems space is usually not limited (depending on currents) and nutrients are constantly replenished (through flow) so there will be little or no competition from neighbours (as we found in this study). Rather, it is the differing growth responses to environmental conditions (e.g. nutrient, CO₂, temperature) between species that will likely determine the species composition in these systems. From a management point of view, we suggest different strategies need to be implemented depending on the water body. For smaller enclosed water bodies where the risk of species displacement is high but the risk of spread is low, eradication of invasive exotic species should be the primary objective. In contrast, for larger dynamic water bodies where the risk of spread is high but the risk of species displacement is low, prevention of entry of invasive exotic species should be the management goal.

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621 **Supplementary Materials**622 **Supplementary Table 1:** Summary ANOVA table showing the effect of the treatments on RGR and root length of *A. filiculoides* and *S. molesta*.

623 Significant effects are indicated in bold font style

Species	Factor(s)	Dependent variable					
		Relative Growth rate (RGR)			Root length		
		<i>df</i>	F	<i>P</i>	<i>df</i>	F	<i>P</i>
<i>Azolla</i>	CO ₂	1, 71	69.036	<0.001	1, 40	16.375	<0.001
	Nutrient	1, 71	119.139	<0.001	1, 40	4.56	0.039
	Competition	1, 71	0.000	0.985	1, 40	6.048	0.018
	CO ₂ ×Nutrient	1, 71	1.287	0.260	1, 40	8.318	0.006
	CO ₂ × Competition	1, 71	0.806	0.372	1, 40	0.158	0.693
	Nutrient × Competition	1, 71	0.115	0.736	1, 40	1.545	0.221
	CO ₂ × Nutrient × Competition	1, 71	0.246	0.622	1, 40	0.050	0.824
<i>Salvinia</i>	CO ₂	1, 64	32.621	<0.001	1, 64	27.95	<0.001
	Nutrient	1, 64	69.667	<0.001	1, 64	3.616	0.062
	Competition	1, 64	11.28	0.001	1, 64	1.148	0.288

CO ₂ × Nutrient	1, 64	14.418	<0.001	1, 64	0.945	0.335
CO ₂ × Competition	1, 64	0.882	0.351	1, 64	3.229	0.077
Nutrient × Competition	1, 64	3.069	0.085	1, 64	0.336	0.564
CO ₂ × Nutrient × Competition	1, 64	0.339	0.563	1, 64	0.207	0.650

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633 **Supplementary Table 2:** A summary of one-way Kruskal-Wallis test results showing the effect of treatments on the R:S of *A. filiculoides* and *S.*
 634 *molesta*. Significant effects are indicated in bold font style

Species	Factor	df	N	H	P
<i>Azolla</i>	CO ₂	1	79	4.612	0.032
	Nutrient	1	79	14.476	<0.001
	Competition	1	79	0.505	0.477
<i>Salvinia</i>	CO ₂	1	72	1.426	0.232
	Nutrient	1	72	52.604	<0.001
	Competition	1	72	0.427	0.414

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CHAPTER SEVEN

Discussion

1 **General summary**

2 Freshwater ecosystems provide habitat to a disproportionately high number of
3 specialised plant and animal species compared to terrestrial and marine ecosystems
4 (Balian *et al.*, 2008). However, these ecosystems are experiencing severe declines in
5 biodiversity due to a number of interacting human-mediated threats including
6 overexploitation, flow modification, habitat degradation, eutrophication, climate change,
7 and invasive exotic species (Dudgeon *et al.*, 2006). Some of these threats affect the
8 functioning of the biosphere and are thus referred to as global environmental change
9 factors. Despite being extremely species-rich and highly threatened (Abell *et al.*, 2008),
10 studies of freshwater ecosystems with regards to global environmental change are
11 relatively few compared to the terrestrial and marine ecosystems (Brundu, 2015). Through
12 a series of manipulative glasshouse experiments, this thesis explored how global change
13 environmental factors might affect the selected invasive exotic freshwater species in terms
14 of growth, reproduction, and functional traits relative to their native and naturalised
15 counterparts. Specifically, it examined the effects of rising atmospheric CO₂ concentration
16 ([CO₂]) and nutrient enrichment on common co-occurring freshwater plant species in
17 Australia. The aim of this final chapter is to synthesise the main findings of this thesis and
18 put them into a broader context of global change effects on freshwater ecosystems in
19 general.

20 As a starting point for this thesis, it was necessary to establish the current status of
21 exotic freshwater plant species in Australia. A comprehensive inventory of the entire
22 naturalised flora in Australia (~2700 species) has been established (Randall, 2007) and has
23 been used for predicting future plant invasions (Duursma *et al.*, 2013), analysing drivers
24 responsible for patterns of naturalisation (Dodd *et al.*, 2015), and identifying areas with

high naturalised species richness (Dodd *et al.*, 2016). However, these outcomes are broad and generalise across many different ecosystems, necessitating ecosystem-specific analyses to better direct management and monitoring practices. *Chapter 2* of this thesis utilised a systematic descriptive approach using literature and database searches to establish the current status of exotic freshwater plant species in Australia. Sixty-three exotic freshwater plant species were identified as occurring in Australia, with the ornamental plant trade recognised as the most important introduction pathway. Therefore, it is unsurprising that these species are concentrated around areas with the highest human population density (NSW, QLD and VIC). At the national level, these species represent about 2% and <0.3% of the exotic and total Australian flora respectively. Despite forming a very small proportion of the Australian flora, six of the 32 Weeds of National Significance are exotic freshwater plant species (<http://www.environment.gov.au/biodiversity/invasive/weeds/weeds/lists/wons.html>), indicating their significant impacts. Moreover, ~40% of the naturalised freshwater plant species are currently considered invasive either at federal or state and territory levels, meaning freshwater ecosystems contribute a disproportionately high number of species to the invasive exotic species pool in Australia. Therefore, an important question to consider is whether ongoing global environmental change will increase the dominance of these exotic species in vegetation assemblages (Leishman & Thomson, 2005; Leishman & Gallagher, 2015). To provide some insight into this question, this thesis empirically tested the effects of rising atmospheric [CO₂] and nutrient enrichment on a range of native, naturalised and invasive exotic freshwater plant species in *Chapters 3-6*.

The overarching hypothesis of this thesis was that all species would have increased growth rates in response to both elevated [CO₂] and nutrient enrichment with the highest growth rates expected to occur when both resources were enriched. More specifically, it

was hypothesised that the invasive exotic species would have the strongest responses to elevated [CO₂] and nutrient enrichment. However, the results indicate that plant growth responses to the treatments were largely species-specific (Table 1).

Table 1: A summary of the growth responses of each study species under the different experimental conditions across the data chapters, showing the percentage difference in final dry mass of plants grown under enriched resource conditions relative to low resource conditions. The numbers in brackets show the relevant thesis chapters. The arrow symbols indicate a significant treatment effect (RGR increased with increasing resource supply (↑), increased up to a threshold (↗)), zero (0) indicates no treatment effect and a dash (-) indicates the treatment effect was not measured. Where there was a significant interaction effect the main effect cells are left blank (e.g. *Zantedeschia aethiopica*).

Species	Status	Nutrient enrichment	CO ₂ enrichment	Competition	Nutrient × CO ₂
<i>Azolla filiculoides</i>	Native	↑416% (6)	↑80% (4) ↑194% (6)	0 (6)	0 (6)
<i>Vallisneria spiralis</i>	Native	↑30% (3)	0 (4)	-	-
<i>Ludwigia palustris</i>	Naturalised	0 (5)	↑60% (5)	-	0 (5)
<i>Mentha aquatica</i>	Naturalised	0 (5)	0 (5)	-	0 (5)
<i>Rorippa nasturtium-aquaticum</i>	Naturalised	↑33% (5)	0 (5)	-	0 (5)
<i>Egeria densa</i>	Invasive	↑192% (3)	-	-	-
<i>Mentha pulegium</i>	Invasive	↑39% (5)	0 (5)	-	0 (5)
<i>Salvinia molesta</i>	Invasive	↗113% (3)	0 (4)	↑29% (6)	↑288% (6)
<i>Zantedeschia aethiopica</i>	Invasive			-	↑109% (5)

In the experiments where the nutrient (*Chapter 3*) and CO₂ (*Chapter 4*) effects were tested separately, it was predicted that elevated [CO₂] and nutrient enrichment would each enhance plant growth rates. In *Chapter 3*, the growth of the invasive *E. densa* and native *V. spiralis* increased with increasing nutrient concentration as expected while the invasive *S. molesta* growth did not differ at nitrate concentrations above 0.1 mg N-NO₃ L⁻¹. The findings for *E. densa* and *V. spiralis* are similar to those of other studies of members of Hydrocharitaceae (Ozimek, van Donk & Gulati, 1993; Barker *et al.*, 2008; Li, Zhang & Jeppesen, 2008). In *Chapter 4*, elevated [CO₂] did not affect growth of the native *V. spiralis* and invasive *S. molesta* but enhanced that of the native *A. filiculoides*. As a species with CAM metabolic pathway (Helder & Van Harmelen, 1982) that uses both free CO₂ and HCO₃⁻ (Hussner *et al.*, 2016), *V. spiralis* is less carbon limited and was therefore not expected to be responsive to elevated [CO₂]. Moreover, studies have shown that when DIC was not limiting, growth of other submerged species of the same family (Hydrocharitaceae) did not differ across a [CO₂] gradient of 400-800 ppm (Hussner *et al.*, 2019). However, it was surprising that *S. molesta*, a C₃ species, was not responsive to elevated [CO₂] given that nutrient was not limiting (see *Chapter 3*). Other studies (Cary & Weerts, 1984; Madsen & Wersal, 2008) and the results of *Chapter 3* showed that *S. molesta* reaches its growth threshold at relatively low nutrient concentrations. In addition, the results of *Chapter 6* showed that when either CO₂ or nutrient availability was increased, *S. molesta* growth was only marginally enhanced further by enrichment of the other resource. Considering these observations, it is unsurprising that *S. molesta* growth did not respond to elevated [CO₂] in *Chapter 4* since the non-limited nutrient supply would have enabled the plants to reach their growth threshold under ambient [CO₂].

In the experiments where the effects of both CO₂ and nutrient concentration were tested simultaneously, it was predicted that while each factor could enhance plant growth

independently, the greatest growth response would occur under enrichment of both resources. Of the seven species grown across these experiments (*Chapter 5* and *6*), only the growth of the invasive species *S. molesta* and *Z. aethiopica* was affected by an interaction between [CO₂] and nutrient enrichment. More specifically, both species had significantly lower growth rates under ambient [CO₂] and low nutrient concentration compared to all other treatment combinations. However, their growth rate was not affected when both resources were enriched relative to when only one was enriched. For *S. molesta*, this unexpected result may be attributed to it having reached its growth threshold as discussed above. For *Z. aethiopica*, this unexpected growth response to resource enrichment (both CO₂ and nutrients) may be due to its growth being constrained by the size of the tub, which is an important determinant in the growth of potted plants (Poorter *et al.*, 2012). Alternatively, the growth of both species may have been limited by other environmental factors such as micronutrients, space and intraspecific competition which meant they could not utilise any other additional resources such as CO₂ and nutrients.

Growth of the remaining species studied in *Chapter 5* and *6* (except *M. aquatica*) was stimulated by either one of the factors (CO₂ - *L. palustris*; nutrients - *M. pulegium* and *R. nasturtium-aquaticum*) or both factors independently of each other (*A. filiculoides*). Although an interactive effect between elevated [CO₂] and nutrient enrichment was predicted, studies have shown that elevated [CO₂] (Cao & Ruan, 2015; Hussner *et al.*, 2016; van Kempen *et al.*, 2016) and nutrient enrichment (Fernandez-Going, Even & Simpson, 2013) often enhance freshwater plant growth rates independently of each other. Surprisingly, *M. aquatica* was unaffected by either elevated [CO₂] or nutrient enrichment. While unexpected, this was not surprising as a previous study has reported similar response in the same species under varied levels of sediment fertility (Pedersen & Sand-Jensen, 1997).

Generally, doubling [CO₂] has been shown to result in an average of 41% increase in biomass production among the terrestrial C₃ species, although species-specific differences have been observed with N-fixing species (as also shown by *A. filiculoides* in *Chapter 4* and *6* of this thesis) being the most responsive (Poorter, 1993; Poorter & Navas, 2003; Wang *et al.*, 2012). However, contrary to findings of other studies, the majority of the species studied in this thesis did not respond to elevated [CO₂]. This disparity could be explained by the relatively small difference between ambient and elevated [CO₂] used in this thesis (200 ppm) compared to similar studies of freshwater plant species where the elevated [CO₂] treatment ranged between 3- to 10-times the ambient concentration (Xie *et al.*, 2004; Yan, Yu & Li, 2006; Cao & Ruan, 2015; Titus & Wendlberger, 2016; van Kempen *et al.*, 2016). Furthermore, since photosynthetic CO₂ assimilation rates of C₃ species tend to increase only marginally as [CO₂] rises above 400 ppm (Caemmerer & Edmondson, 1986; Bazzaz, 1990; Ainsworth & Rogers, 2007), it is not surprising that plant growth differences between ambient (400 ppm) and elevated (600 ppm) [CO₂] in this thesis were only marginal.

Overall, the results across the data chapters of this thesis did not provide evidence for a consistent interaction effect between [CO₂] and nutrient concentration on growth differences among the invasive, native and naturalised study species in response to these factors. Nevertheless, within species growth differences between treatment levels tended to be more dramatic for the invasive species, except *M. pulegium*, compared to the native and naturalised species, except *A. filiculoides*. However, these dramatic growth responses of the invasive species did not always translate into them having greater growth rates compared to their native counterparts. For example, in *Chapter 5* the invasive species *Z. aethiopica* had significantly lower RGR than three naturalised study species irrespective of the treatment.

Based on the findings of this thesis, it is suggested that nutrient enrichment is likely to be a more important determinant of community structure and function of freshwater systems than rising atmospheric [CO₂]. Furthermore, these results indicate that species responses to global change environmental factors may be better predicted based on functional groups rather than invasive status. For example, the free-floating native *A. filiculoides* (N-fixing) consistently showed enhanced growth responses under elevated [CO₂] and nutrient enrichment where it had greater RGR compared to even the free-floating invasive exotic *S. molesta* across the chapters. This is consistent with studies of N-fixing terrestrial plants (Hungate *et al.*, 1999; Rogers, Ainsworth & Leakey, 2009; Li *et al.*, 2017) and aquatic free-living cyanobacteria (Levitan *et al.*, 2007; Czerny, Barcelos e Ramos & Riebesell, 2009) that have shown that elevated [CO₂] increases the N-fixation capacity of the species and hence their growth rates. Considering these findings, N-fixing plants will likely be the most responsive to resource-enriched conditions in freshwater systems in the future. If this occurs, the natural senescence and mineralization of the N-fixers could exacerbate the eutrophication of freshwater bodies, thus favouring exotic species invasions with cascading detrimental impacts on submerged plant communities as well as at higher trophic levels (e.g. herbivores). This is based on the fact that facilitation among aquatic plant species is quite common (Espinar *et al.*, 2002; Boschilia, Oliveira & Thomaz, 2008; Michelan *et al.*, 2010; Wundrow *et al.*, 2012) and is supported to some extent by the finding that *A. filiculoides* had a facilitative effect on *S. molesta*, which points to a potential shift towards free-floating species dominated communities in the future.

Experimental caveats

Although the glasshouse experiments in this thesis tried to simulate field conditions as realistically as possible, it is important to acknowledge aspects of the experiments that need to be considered when applying the results to a field context.

Firstly, in the experiments that had submerged study species (*Chapters 3 and 4*), air was continuously bubbled into the water column to simulate flowing waters. The purpose of doing this was to stimulate turbulence which tends to reduce the boundary layer thickness, thereby enhancing the supply rates of dissolved inorganic carbon (DIC) and nutrients to the plants (Madsen & Søndergaard, 1983; Bornette & Puijalon, 2011). Although the pH of the water column was measured as a proxy for DIC availability in both experiments it would have been ideal to measure DIC directly to ascertain whether bubbling had the intended effect.

Secondly, due to glasshouse space constraints, it was necessary in *Chapter 3* that multiple plants were grown in each tub. To account for this, each individual tub was considered to be one experimental unit and its random effect was included in the statistical models. It is also important to acknowledge that due constraints (mainly species unavailability), it was not always possible to control for all the differences (e.g. phylogeny, growth forms, and physiology) that might confound the treatment effects. However, these differences were always highlighted (e.g. *V. spiralis* – CAM, *A. filiculoides* – N-fixing, *Z. aethiopica* – rhizomatous) and their possible influence discussed.

Finally, significant algal growth was a commonly encountered problem in all of the experiments. Algae was considered to be detrimental to the growth of the study species because if uncontrolled it competes for nutrients with all species regardless of growth form

and reduces light availability to the submerged species. A number of different techniques were utilised to reduce the algal effect on plant growth including regularly circulating the nutrient medium through an ultraviolet sterilisation unit (*Chapter 3*) as well as replacing the nutrient media and thoroughly cleaning the tubs on a regular basis (*Chapter 4* and *6*). Although these techniques did not prevent algal growth completely, they were relatively successful in reducing its impact on plant growth. However, some algal influence cannot be ruled out.

Future management and research directions

Despite many statutory and regulatory measures being in place at the local-, state- and federal-levels to control the trade of invasive exotic species in Australia, a few continue to be traded. Therefore, a stricter enforcement of these controls is necessary by regularly assessing nursery compliance through the monitoring of the online aquarium market. For naturalised species, it is important that weed risk assessments are regularly undertaken in light of ongoing global environmental change. Furthermore, it is also vital that wild populations of these species are monitored regularly to determine if they are spreading or not. Finally, accessing information on the ecology and management of exotic freshwater plants at the local- and state-level is difficult as data is contained within disparate sites. Therefore, a centralised system of collating and storing data would be desirable for better knowledge sharing.

The species-specific growth responses of the tested species to the experimental conditions underscore the importance of phylogeny. When designing experiments to understand mechanisms that drive species responses to environmental change (e.g. origin and physiology), it is advised that where possible, closely related species with the same

growth form be used. However, if the objective is to evaluate how the invasive species might alter freshwater ecosystems under changed environmental conditions, then using species of different growth forms, origin, and physiological pathways may be more informative. In addition, not much insight would be gained when comparing the native vs invasive species responses, if the native species are also strong invaders elsewhere as is the case with *Azolla filiculoides*. It would therefore be more insightful to think of species functional groups when making comparisons rather than just origin.

At a species-level, this thesis provides evidence that *S. molesta*, the focal study species of this thesis and one of the worst freshwater invaders globally (Courchamp, 2013), warrants continued management attention. Despite the use of the biological control *Salvinia* weevil *Cyrtobagous salviniae* (Curculionidae) being declared successful in the 1980s, *S. molesta* continues to persist in many water bodies in eastern Australia (Schooler *et al.*, 2011). Because of its superior competitive ability in enclosed spaces where the risk of native species displacement is high, future management of this species should target eradication in small enclosed oligotrophic water bodies (e.g. ponds and dams). In contrast, for larger dynamic water bodies where the risk of spread is high but the risk of species displacement is low, prevention of entry of invasive exotic species should be the management goal.

As stated above, this research field is in its infancy and is ripe for further research efforts. To date, the majority of experiments on this topic including the ones in this thesis have investigated the responses of a few species to one or two global change environmental factors over a short period of time. If we consider what we have learnt from similar studies in the terrestrial realm it is clear that multiple global change environmental factors can interact with one another to alter plant performance (Ainsworth & Rogers,

2007; Wang *et al.*, 2012). Furthermore, these changes in performance are not necessarily evident in the short term. Therefore, future research efforts should put an emphasis on testing multiple environmental factors simultaneously over a long term period. These studies should also incorporate factors known to affect plant performance but have received less research attention including pH, turbidity, altered thermal and hydrological regimes and biotic factors such as herbivory.

As alluded to earlier in the ‘experimental caveats’ section, the majority of experiments on freshwater plant species responses to global change environmental factors have been conducted in controlled glasshouse conditions. As glasshouse and field conditions can vary greatly (Poorter *et al.*, 2016), it is important to verify the findings of controlled glasshouse experiments in order to be confident that they can be applied to natural environments. The best way to do this is to conduct large scale field experiments, as have been carried out in terrestrial ecosystems for decades (e.g. FACE experiments, open top chambers). However, the feasibility of these experiments in an aquatic context is questionable due to the large costs and logistic difficulties often associated with them (Norby *et al.*, 2016). Nevertheless, if conducted, they would provide the necessary link between glasshouse experiments and the natural environment that would ensure that the correct management decisions have been and continue to be made for freshwater ecosystems.

Conclusion

The ornamental plant trade has led to the introduction of many plant species into novel ecosystems. A small proportion of these species have naturalised in their introduced range while an even smaller proportion has spread and become invasive. Usually, species

with wide environmental tolerances are selected for importation and are likely to benefit from ongoing global environmental change. However, there is insufficient evidence that global change affects exotic species differently than native species in freshwater systems. The overarching aim of this thesis was to address this question in terms of freshwater plant responses to rising atmospheric [CO₂] and nutrient enrichment. Overall, the effect of elevated [CO₂] and nutrient enrichment on freshwater plant species was found to be largely species-specific with no clear trend emerging, in terms of invasive status, from the experiments. Nevertheless, this thesis has contributed a meaningful body of work that provides an important insight into the poorly understood research field of freshwater plant ecology and global change.

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