The effects of elevated atmospheric CO_2 on the biological control of invasive aquatic weeds in South Africa

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Declaration: I have read and adhered to the Rhodes University plagiarism policy

Abstract

There has been a rapid increase in atmospheric CO_2 concentration, from pre-industrial values of 280 ppm to more than 400 ppm currently, and this is expected to more than double by the end of the 21st century. Studies have shown that plants grown above 600 ppm tend to have an increased growth rate and invest more in carbon-based defences. This has important implications for the management of invasive alien plants, especially for the field of biological control which is mostly dependent on herbivorous insects. This is because insects reared on such plants have been shown to have reduced overall fitness.

Nevertheless, most of the studies on potential changes in plant-insect interactions under elevated CO_2 are based on agricultural systems, with only a limited number of these types of studies conducted on alien invasive weeds. However, climate change and invasive species are two of the most prevalent features of global environmental change. Therefore, this also warrants active research and experimental studies to better understand how these systems will be affected by future climates.

Thus, the aim of this study was to investigate the effects of elevated atmospheric CO_2 on the biological control of four invasive aquatic weeds (*Azolla filiculoides, Salvinia molesta, Pistia stratiotes,* and *Myriophyllum aquaticum*). These species are a threat to natural resources in South Africa but are currently under successful control by their biological control agents (*Stenopelmus rufinasus, Cyrtobagous salviniae, Neohydronomus affinis,* and *Lysathia n.* sp.). To achieve this, the selected plant species were grown in a three-factor experimental design in winter (CO_2 X nutrients X herbivory), and another two-factorial design in summer (CO_2 X herbivory). Atmospheric CO_2 concentrations were set at ambient (400 ppm) or elevated (800 ppm), as per the predictions of the IPCC.

As per my hypothesis, the results suggest that these species will become more challenging in future due to increased biomass production, asexual reproduction and a higher C: N ratio which is evident under high CO_2 concentrations. Although the biological control agents were in some instances able to reduce this CO_2 fertilisation effect, their efficacy was significantly reduced compared with the levels of control observed at ambient CO_2. These results suggest that additional biological control agents and other management methods may be needed for continued control of these invasive macrophytes, both in South Africa and further afield where they are problematic.

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Chapter 1: General introduction and literature review

"*Climate change and invasive species are two of the most pervasive aspects of global environmental change*" (Rahel & Olden, 2008).

1.1. Global climate change

The past two centuries have seen a rapid increase in the atmospheric concentration of carbon dioxide [CO_2] from pre-industrial values of 280 ppm to ~400 ppm currently (Ehleringer & Cerling, 2002; O'Brien & Leichenko, 2000; Sage & Coleman, 2001; IPCC, 2007; Cornelissen, 2011). Although climate change is not a new phenomenon to the world, as similar concentrations were recorded some 3.5 million years ago (Jansen et al., 2007; Field et al., 2014), the rate at which it is taking place currently is unprecedented (Ehleringer & Cerling, 2002). For example, CO_2 concentration in the atmosphere has reached a million-year record high at a fast rate of approximately ~1 ppm/yr (Field et al., 2014).

One obvious and useful trend that can be picked up from these past climates is that increased greenhouse concentrations tend to lead to similar increases in temperatures (Idso et al., 1987; Field et al., 2014). Indeed, due to the recent increase in atmospheric CO_2, current global temperatures are warmer than they have ever been during the past eight centuries (IPCC, 2007). The Intergovernmental Panel on Climate Change (IPCC) predicts a mean increase of atmospheric temperatures in the magnitudes of 1.1 to 6.4°C by the year 2100 (IPCC, 2007). If this warming continues unchallenged, the resulting climate change within this century would be extremely unusual even in geological terms (Jansen et al., 2007; Field et al., 2014).

Evidence from ice cores suggests that human activities such as the burning of fossil fuel, agriculture and deforestation are the main drivers of climate change (Sage & Coleman, 2001; Ehleringer & Cerling, 2002; Reuveny, 2007; Moss et al., 2011). Given the current [CO_2] in the atmosphere, it is now evident that climate change is inevitable and this will continue into the future unless drastic mitigation measures are implemented (O'Brien & Leichenko, 2000; IPCC, 2007; Dennis & Dennis, 2012). According to the IPCC report (2007), CO_2 uptake by the terrestrial ecosystem is likely to peak and then weaken or even reverse before 2050, facilitating further climate change. Depending on the scenario used, the IPCC (2007) predicts that atmospheric CO_2 will reach some 800 to 1200 ppm by the year 2100. As such, climate change is a major threat worldwide to both managed and natural ecosystems (Reeves, 2017).

1.2. Plants and elevated CO_2

Atmospheric CO_2 is not only one of the principal greenhouse gasses, but is also the main source of carbon for plants and therefore important for all life on earth (Lawlor et al., 1987; Ziska & George, 2004). Atmospheric CO_2 enrichment will influence plant physiology, with direct consequences for plant production and biochemical composition (Cornelissen, 2011). Given that CO_2 is one of the primary requirements for photosynthesis, the projected increase in CO_2 is likely to translate to increased plant growth and biomass production for a number of species (Cornelissen, 2011). Furthermore, since different plants species are likely to exhibit different optimal ranges for CO_2 concentrations, those that have higher requirements will likely benefit more from elevated CO_2 (Ehleringer & Cerling, 2002). The result will be novel ecosystems with altered plant community composition and increased possibilities for biological invasion (Reeves, 2017).

Although selected crops in some areas might perform better, the overall impact of climate change on agriculture is expected to be negative above a global mean temperature rise of 2 °C, threatening global food security (Dube et al., 2013). Climate change is thus expected to have a negative impact on agricultural activities across Africa (Bryan et al., 2009). The most vulnerable groups are poor people and developing countries (Kiker, 2000;

Reuveny, 2007; Gregory et al., 2009), which rely mainly on agricultural productivity for both economic and food security (O'Brien & Leichenko, 2000; Bryan et al., 2009; Dennis & Dennis, 2012). South Africa, like many other developing countries, is faced with many environmental, social, and economic issues, including climate change (O'Brien & Leichenko, 2000; Dennis & Dennis, 2012; Dube et al., 2013). As such, it has become an urgent matter to develop predictions of how all these issues will interact to affect the resilience of our ecological and agricultural systems.

Although some aspects of climate change may be viewed as advantageous, the rise in atmospheric CO_2 is not selective as it stimulates the growth of both wanted and unwanted plants (Ziska & George, 2004). As such, climate change is expected to alter both food availability and biodiversity through changes in phenology (Cornelissen, 2011), genetic composition, species ranges, species interactions and ecosystem processes (IPCC, 2007). Some authors argue that biological invasions will be favoured under this new atmospheric regime, as exotic species are more adapted to take up and use available resources at a faster rate than native communities (Vilà et al., 2007).

Nevertheless, experimental studies tend to focus mainly on beneficial plants such as food crops and native species. Only a handful of studies have investigated the effects of elevated CO_2 on invasive species, although these also have important economic and ecological implications (Hellmann et al., 2008). Invasive species require different management strategies compared with native species (i.e., control vs. conservation), and according to Hellmann et al (2008), it is important that we investigate the response of invasive species separate to native ones since they differ significantly in terms of reproductive success and abundance.

1.2.1. C3 and C4 photosynthesis under elevated CO_2

Plant response to elevated atmospheric CO_2 will differ significantly between C3, C4 and CAM plants (Ehleringer & Cerling, 2002). Most plants use the more common C3 photosynthetic pathway (Bowes, 1991; Ehleringer & Cerling, 2002). However, a number of monocotyledons such as sugar cane, maize, sorghum and most grasslands, including a selected number of dicotyledonous plants use the more advanced C4 and CAM variations of photosynthesis that have evolved under certain adverse conditions such as drought and low CO_2 (Lawlor et al., 1987; Ehleringer & Cerling, 2002, Sage, 2004).

The performance of C3 plants is limited by photorespiration, especially at high temperatures (Ripley et al, 2007; Ehleringer & Cerling, 2002; Ripley et al., 2013), where photorespiration can use up to 25% of the resources of photosynthesis under current climates (Idso et al., 1987; Bowes, 1991; Sage & Coleman, 2001). C4 species, on the other hand, are already operating under high CO_2 concentrations since they have a specialised photosynthetic pathway which allows them to concentrate CO_2 in the bundle sheath, significantly reducing the oxidative function of the Rubisco enzyme relative to carboxylation (Lawlor et al., 1987; Bowes, 1991; Ehleringer & Cerling, 2002; Sage, 2004).

These physiological differences between C3 and C4 species have important implications for modelling and predicting species response to elevated CO_2. Indeed, a number of studies have shown that C3 plants tend to be more responsive to elevated atmospheric CO_2 compared with their C4 counterparts (Bowes, 1991; Ripley et al., 2013). For example, plants that are grown under increased atmospheric CO_2 conditions have shown an increased rate of photosynthesis (Gregory et al., 2009), reduced photorespiration and improved water use efficiency due to a reduction in stomatal conductance (Woodward, 2002; Ripley et al., 2007; Taylor et al., 2010; Robinson et al., 2012; Ripley et al., 2013). This may manifest itself through increased biomass accumulation and reproduction (Stiling & Cornelissen, 2007; Taylor et al., 2010), especially in cases where photosynthesis is limited by water availability (Ripley et al., 2007; Robinson et al., 2012 Ripley et al., 2013). The rate of this increase is however governed by the accompanying changes in temperature (Idso et al., 1987), and the type of photosynthetic pathway used by the plants (Bowes, 1991). Generally, C3 crops are estimated to increase yield by 10-20%, while estimates for C4 crops lie between 0-10% increases (Gregory et al., 2009). This CO_2 fertilisation effect may saturate above a certain temperature threshold (Kiker, 2000), or even decrease when considering the effect of pests and other factors limiting plant growth (Gregory et al., 2009). According to Spencer and Bowes (1986), many plants do not have the necessary storage organs to store the extra carbohydrates. As a result, they tend to acclimate (exhibit phenotypic plasticity) to high CO_2, meaning that they become less responsive (Bowes, 1991). Indeed, long-term experiments show that photosynthesis can acclimate downwards to increased atmospheric CO_2 in order to reduce photo-inhibition (Woodward, 2002; Ehleringer & Cerling, 2002).

The function of acclimation is to restore the natural balance within a system that has been disturbed by natural environmental change, ensuring improved efficacy of resource use within the plant (Bowes, 1991; Sage & Coleman, 2001). It is important to note however those acclimation responses to increased CO_2 tend to differ, depending on the plants used and the general condition of the environment on which the plants are grown (Woodward, 2002; Ripley et al., 2013). As such, reduced stomatal conductance, root volume, plant age, photoperiod, lack of soil nutrients and source-sink ratio may also be contributing factors which determine the degree to which the acclimation process occurs (Spencer & Bowes, 1986; Bowes, 1991; Sage & Coleman, 2001; Dolezal et al., 2019). For example, Sage and Coleman (2001) showed that juveniles with an extensive rooting system and adequate nutrients tend to show little to no down-regulation of photosynthetic enzymes, while some plants may even be able to increase enzyme activity. With regards to ageing plants, however, nutrient limitations under elevated CO_2 lead to the reduction of key photosynthetic enzymes (Sage & Coleman, 2001).

1.3. Climate change and plant-insect interactions

At present, insects make up almost half of all biodiversity that has been described on earth, and they play a fundamental part in ecosystem structure and functioning (Cornelissen, 2011). They are herbivores, predators and parasitoids of harmful pests, and provide pollination services for both agricultural and natural ecosystems. Climate change will undoubtedly have direct as well as indirect effects on these organisms. Firstly, insects are ectotherms and therefore are directly affected by changes in temperatures (Cornelissen, 2011; Zavala et al., 2013). Increased winter temperatures tend to be beneficial to insects as this reduces overwintering mortality, leading to increased pest populations in the following season (DeLucia et al., 2008; Gregory et al., 2009). Warmer temperatures also reduce development time, while increasing the number of generations (Rosenzweig et al., 2001). However, extremely high temperatures are said to be detrimental to insect populations because this reduces overall fitness (Rosenzweig et al., 2001). Insects also respond to changes in precipitation as wet and humid conditions are said to promote pest infestations. As such, cooler and more humid regions will be more vulnerable to these infestations.

Unlike temperature, elevated CO_2 has no direct effects on insect herbivores (Cornelissen, 2011; Zavala et al., 2013; Reeves, 2017); although changes may sometimes disrupt cues used for identifying favourable oviposition sites or desirable food sources (DeLucia et al., 2008). Observed responses are usually through indirect effects as a result of changes in host plant quality (Figure 1.1) (Scherm et al., 2000; Cornelissen, 2011; Zavala et al., 2013; Reeves, 2017). Herbivory patterns, insect activity, abundance and diversity are some of the aspects that will be affected (Cornelisen, 2011). For example, insects that feed on plants that were grown under elevated CO_2 tend to have reduced fitness as they have to engage in compensatory feeding (DeLucia et al., 2008; Gregory et al., 2009; Zavala et al., 2013; Reeves, 2017), and deal with the additional carbon-based defences (Stiling & Cornelissen, 2007).

Increased CO_2 has been found to change the nutritional value of plants (Cornelissen, 2011), leading to high C: N ratio of up to 26% (Stiling & Cornelissen, 2007), although this may sometimes be countered by the addition of nitrogen fertiliser in managed systems (Robinson et al., 2012). As a result, insects tend to feed more in order to compensate for reduced nitrogen availability from the plants (DeLucia et al., 2008; Gregory et al., 2009). According to Zavala et al. (2013), increased feeding time reduces fitness as it makes insects more vulnerable to predation (Figure 1.1). This is because both temperature and CO_2 also change the production of plant volatile compounds used to attract natural enemies of herbivores (Gregory et al., 2009; Cornelissen, 2011).

Additionally, crops grown under elevated CO_2 tend to have increased carbon-based secondary metabolites such as tannins (>29%) and phenolics (Stiling & Cornelissen, 2007; Cornelissen, 2011), including other structural defences (DeLucia et al., 2008; Zavala et al., 2013). This has negative consequences for insect herbivores as they need to invest more energy and resources in order to break down these chemicals (Zavala et al., 2013). A meta-analysis conducted by Stiling and Cornelissen (2007) verified this statement. They found that insects reared on plants which were grown under elevated CO_2 had reduced relative growth rate (8.3%), food conversion efficiency (19. 9%), and pupal weight (5.0%), while development time and total consumption rates increased by 3.9% and 9.2%, respectively (DeLucia et al., 2008).

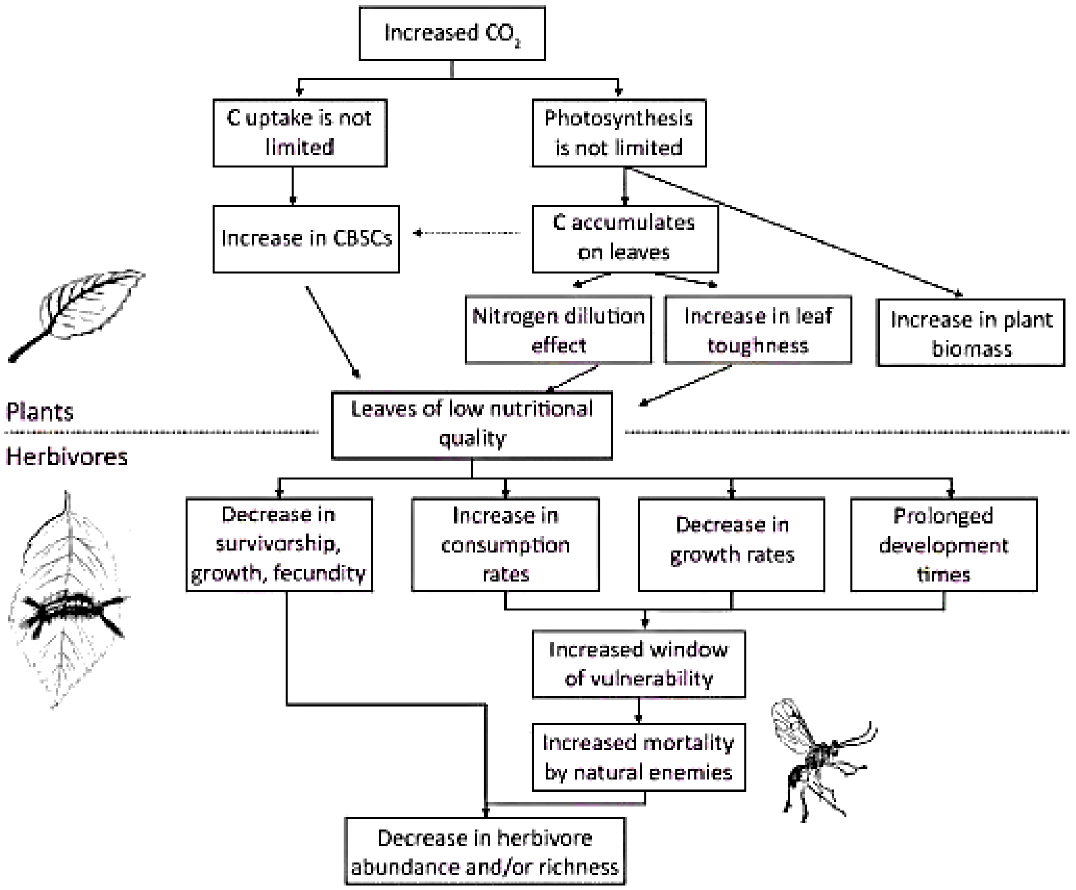


Figure 1. 1: A summary of the predicted effects of elevated CO_2 on plant-insect interactions (adopted from Cornelissen, 2011).

It is important to note that insect responses may differ between the different species, life stages and between different feeding guilds (DeLucia et al., 2008; Robinson et al., 2012; Reeves, 2017). For instance, leaf toughness may be more effective in reducing feeding by folivores, while trichomes may be more detrimental to small, sap-sucking insects such as aphids (Zavala et al., 2013). Additionally, although some folivores and leaf miners may be able to compensate for nutritional changes in the plants at elevated CO_2 by increased feeding, the overall abundance of these insects might be reduced if larval development is delayed, as is the result when nitrogen is limiting (Reeves, 2017).

The general trend, however, is that folivores will be the most negatively affected guild (Stiling & Cornelissen, 2007; Cornelissen, 2011), while sapsuckers are shown to have a moderate response when reared on these plants (Robinson et al., 2012). According to Reeves (2017), phloem-feeding insects are able to maintain or even advance their performance on plants grown under elevated CO_2, leading to decreased development time and increased overall population growth. Some scholars such as Cornelissen (2011) however argue that current studies do not offer a clear response trend when it comes to this feeding guild as there is a lot of variation, and more studies that are not focused solely on aphids are essential.

Atmospheric CO_2 and temperature are co-dependent and both play a vital role in determining plant-insect interactions. However, most studies have tended to investigate the effects of temperature and elevated CO_2 in isolation. The few that have studied these two variables in combination have demonstrated that although CO_2 might promote populations of sap-suckers (Zavala et al., 2013); the end result is usually negative when predictions of increased temperatures are taken into consideration (Gregory et al., 2009). Therefore, it is likely that certain crops may become more vulnerable to insect herbivory than others since defences differ according to the different plant functional groups (Robinson et al., 2012). Additional warming, in conjunction with increased atmospheric CO_2, may also lead to earlier and faster development of crops, causing increased pest damage at the sensitive earlier stages of development (Gregory et al., 2009).

1.4. Biological invasion and control under the new regime

Numerous studies have shown that increased CO_2 levels in the atmosphere have huge impacts on photosynthesis, and therefore plant-insect interactions (Stiling & Cornelissen, 2007). For example, plants grown under elevated CO_2 tend to have increased rates of photosynthesis, reduced photorespiration (Gregory et al., 2007), high C: N ratio, invest more on carbon-based secondary metabolites and decreased overall plant quality (DeLucia et al., 2008; Zavala et al., 2013; Reeves, 2017). This has important implications for the management of invasive alien plants, especially for the field of biological control. Most of the studies on potential changes in plant-insect interactions under elevated CO_2 are based on agricultural systems (Scherm et al., 2000; Vilà et al., 2007). However, according to Rahel and Olden (2008), climate change and invasive species are the most prevalent features of the Anthropocene. It is therefore important that we have more studies focused on understanding how these two facets of global change will interact, and the effects thereof.

4.1.1. The problem of plant biological invasions

The problem of alien species invasion is prevalent in both terrestrial and aquatic ecosystem. In both cases, native biodiversity tends to be under threat as the introduced species are usually adapted to grow and reproduce faster (Vilà et al., 2007). Thus, native species are out- competed, leaving their associated species vulnerable to predation and with no food sources (Sheldon & Creed, 1995). Furthermore, plant invasion also threatens the general development of a country as biological invasions may interact with other existing stressors (Cilliers et al., 2003; Vilà et al., 2007). In addition to changing atmospheric CO_2, climate change will also alter stream flow patterns, increase water temperatures and the frequency of storm events (Rahel & Olden, 2008). Such changes will not only affect the production of ecosystem services in those systems but will also have profound effects on the distribution of both plant and animal species in time and space (Rahel & Olden, 2008).

Invasive plant species are highly problematic because of their fast reproductive and photosynthetic rates which allow them to outcompete indigenous species, resulting in reduced biodiversity (Van Ginkel, 2011; Hill & Coetzee, 2017). Plant invasions, especially within the aquatic environment, have negative implications for both economic and ecological systems (Zimmermann et al., 2004). They produce dense floating mats which block sunlight from penetrating the water, reduce dissolved O_2 concentration in water, resulting in fish die-off. They form ideal habitats (slow-moving/ still waters) for disease vectors like mosquitoes and blackflies. In addition, they block waterways, clog irrigation systems, damage hydroelectric power infrastructures and reduce recreational activities (Van Ginkel, 2011). As a result, a significant amount of funds and research has gone into trying to control alien invasive plants in South Africa (McConnachie et al., 2003; Zimmermann et al., 2004; De Lange & van Wilgen, 2010; Moran & Hoffmann, 2015; McConnachie et al., 2016).

1.4.1. The enemy release hypothesis and biological control

There are many explanations for why alien invasive plants are likely to be more successful outside their native range. One such explanation is offered by the enemy release hypothesis which states that plants automatically become superior competitors outside of their natural range due to the release from stress (herbivory and parasites and diseases) that is evident in the absence of their natural enemies (Williamson & Griffiths, 1996). This is because unlike their native counterparts, they experience reduced regulatory pressure from their natural enemies and therefore are able to spread and reproduce more rapidly (Mitchell & Power, 2003). The underlying assumption of the enemy release hypothesis is that natural enemies are important regulators of plant species populations, and that the pressures from these natural enemies are felt more readily by the native species compared with alien plants, and also that in the absence of such pressures, the exotic plants are able to redirect more resources towards growth and reproduction while also successfully keeping the accumulated biomass (Keane & Crawley, 2002).

This is the premise on which the concept of biological control is founded, the definition of which is the use of introduced natural enemies of the problematic plant for its control. These enemies are referred to as biological control agents, consisting of a variety of macro-invertebrates, mostly insects and some pathogens (Cilliers, 1991a; Hill, 2003). The selection process involves rigorous specificity tests, and as a result, biological control agents are usually highly specialised, in many cases only attacking one plant species (Zachariades et al., 2017). In general, the agents work by reducing plant fitness through damaging plant tissue, killing roots and reproductive parts such as seeds and flowers, and by reducing resource allocation to growth and reproduction by putting pressure on plant defences (Coetzee et al., 2007). As such, biological control is deemed one of the most effective and sustainable ways of controlling invasive alien plants, with minimal to no risk of non-target effects (Reeves, 2017; Zachariades et al., 2017).

This type of invasive plant management strategy has been used successfully in 130 countries, with over 550 biological control agents having been released (Winston et al., 2014; Schwarzländer et al., 2018). In 2013, South Africa celebrated 100 years since the inception of biological control in the country (Zachariades et al., 2017). Until the 1970s, the programmes were focused on terrestrial ecosystems, introducing agents that had already been used successfully elsewhere, with no novel South African projects until the 1960s (Olckers, 2004; Zachariades et al., 2017). At present, 13 biological control agents (11 of which are insects) have been used to target the five most devastating aquatic weeds in the country (see Chapter 2), with four of these under complete control (Coetzee et al., 2011; Hill & Coetzee, 2017). However, some scholars claim that these intimate interactions between control agents and their target plants may be disrupted by changes in climatic factors (Reeves, 2017), thus making this type of control strategy under threat due to changes in atmospheric CO_2 (Hellmann et al., 2008).

Predicting the effects of increased CO_2 has become a hot topic in plant science (Sage & Coleman, 2001). Nevertheless, biological control under the same conditions has not yet received any attention; regardless of the fact that it forms a fundamental part of plant-insect interactions. Ensuring the continued effectiveness of biological control in the face of climate change is of utmost importance as it would be detrimental to the environment and the economy of the country to re-employ previous control measures like the use of chemicals and mechanical removals. This requires extensive research that will allow us to accurately predict the response of invasive plants and their biological control agents to climate change, and hence the interaction between them under these novel conditions (Reeves, 2017). However, it is highly improbable that all invasive species and control agents will respond in a similar manner given the vast range of species-specific responses to increased atmospheric CO_2 and climate change. Nevertheless, there are some general trends that offer some insight into possible response trajectories.

According to Van Ginkel (2011), the USA state of Florida spent an estimated US$ 43 million between 1980 and 1991 on programmes aimed at controlling aquatic weeds. In South Africa, an estimated R12 million is spent annually in the efforts of controlling the most noxious aquatic weed, water hyacinth, *Eichhornia crassipes* (Matr.) (Van Ginkel, 2011). McConnachie et al. (2003) found that the use of biological control agents in the management of *Azolla filiculoides* saves the country some US$589 per ha/yr.

1.4.2. Success and challenges to biological control

Considerable research has been conducted on the management of aquatic weeds in South Africa. Control measures were previously dominated by chemical and mechanical means of removal (Cilliers, 1991a). These were found to be unsuccessful in the long run as they are expensive and effects are felt by non-target species (Olckers, 2004). Biological control has provided the most permanent satisfactory results (Zachariades et al., 2017). This is because the agents are very specific and the process is natural and therefore environmentally friendly (Cilliers, 1991a). Biological control is also the most economically sound way of managing invasive plants compared with chemical control which usually requires extensive research, repeated applications and assistance from manual clearing which is also labour intensive and therefore expensive (Zimmermann et al., 2004; De Lange & van Wilgen, 2010; Hill & Coetzee, 2017; Zachariades et al., 2017). This type of management strategy only requires extensive funds at the inception of a biological control program in order to find the origins of an invasive plant and therefore its natural enemies (McConnachie et al., 2003; Hill & Coetzee, 2017). Considerable savings can be made in cases where biological control has been achieved elsewhere and is being transferred (Zachariades et al., 2017). Nevertheless, biological control is often only considered once other traditional methods have failed (Olckers, 2004).

To date, over 100 species of biological control agents, including insects, mites and plant pathogens have successfully established on more than 60 alien invasive plants in South Africa, offering significant control to approximately 50% of these (Klein, 2011; Winston et al., 2014). Around 24% of these alien invasive plants are controlled solely by biological control agents, with no need for additional interventions (Zachariades et al., 2017). As a result, South Africa has been recognised as one of the leading countries in biological control research (Moran & Hoffmann 2015). The Working for Water programme of the Department of Environmental Affairs (DEA) has been very instrumental in facilitating the control of invasive weeds in South Africa (Olckers, 2004; McConnachie et al., 2016; Zachariades et al., 2017). This includes funding research focused on biological control of these weeds and manual clearing of other terrestrial weeds which are known to reduce the volume of water available in the catchment (Zimmermann et al., 2004; De Lange & van Wilgen, 2010; Hill & Coetzee, 2017).

A notable example of a success case study of biological control is that of Lake Victoria in Uganda, given by Wilson et al. (2007). Water hyacinth was first reported there in 1989. This is the world's second-largest freshwater lake, which provides people with hydropower and other ecosystem services. By the mid-1990s, water hyacinth had covered up to 20 000 ha of the lake. These dense floating mats had huge negative economic and ecological impacts in the surrounding areas as they infested some 80% of the Ugandan shorelines. This weed was a huge hindrance to socio-economic activities such as agriculture, lake transport, hydroelectric power generation, and general water supply. Ultimately, this also affected trade routes in and out of the ports, resulting in GDP losses for Kenya, Tanzania and Uganda which surround the lake. Control measures initiated in the early 1990s had limited success as they were focused on manual removal and conducting public awareness campaigns. Two water hyacinth weevils, *Neochetina bruchi* Hustache, (Coleoptera: Curculionidae) and *Neochetina eichhorniae* Warner (Coleoptera: Curculionidae) were introduced to the area in 1995, and within four years of introduction, they had removed 95% of the water hyacinth population. These agents continue to keep this weed at manageable levels which no longer pose a significant threat to the lake.

In South Africa, one of the most iconic biological control successes is that of water hyacinth in New Year's Dam, in the Eastern Cape Province. This is a 150 ha impoundment which was covered by more than 80% of this weed, until 1990 when 200 adult *N. eichhorniae* were released to control it (Hill & Olckers, 2001). Four years later, these biological control agents had reduced water hyacinth cover to less than 10% of the total surface area of the dam. Due to a number of factors, the remaining weed population grew again to around 80% by 1998.

However, *N. eichhorniae* was able to bring it down again to 10% by 2000 without further introductions.

Despite the above-mentioned successes, biological control in South Africa is varied (Klein, 2011), especially for water hyacinth (Van Ginkel, 2011; Zachariades et al., 2017). Indeed, no other aquatic system has been able to enjoy the same degree of success as the New Year's Dam when it comes to the control of water hyacinth in the country. This is because there are a number of factors that inhibit the success of biological control agents. Hill and Olckers (2001) highlight some of these factors. Firstly, they found that cold temperatures increase the time taken to control the weed. This is mainly observed in winter when insects become dormant and inactive (Coetzee et al., 2011). They also state that highly eutrophic water- bodies enable water hyacinth to overcompensate for herbivory.

Biological control activity may also be interrupted by periodic removal of the weed, natural enemy population fluctuations due to flooding and drought, interference from herbicide applications, and mechanical removal of the weed (Cilliers, 1991a; Coetzee et al., 2011). Furthermore, the biological control agents may require additional disturbance of the mats by wind and wave action in order to increase mortality caused by feeding damage, hence there may be less success in still waters (Hill & Olckers, 2001). In these instances, successful control of aquatic weeds requires an integrated approach that is also sensitive to the needs of the biological control agents (Van Ginkel, 2011; Zachariades et al., 2017).

1.4.3. Biological control and elevated CO_2

Many aspects of climate change play a vital role in modulating plant-insect interactions, and therefore successful prediction on the response of biological control agent populations will be highly dependent in the ability to incorporate these climate components onto research experiments. For example, changes to plant quality as a result of elevated CO_2 may not always lead to increased insect damage, especially if these changes reduce the successful recruitment of the control agents into the population (lower insect performance and survival) (Reeves, 2017). Additionally, compensatory feeding by the control agents, which is also not a uniform response, might not necessarily translate to increased plant damage and control since invasive plants also grow faster at elevated CO_2 (Reeves, 2017). Nevertheless, there may be cases where CO_2 will facilitate biological control, especially in eutrophic water-bodies where good plant quality might promote insect populations.

According to Reeves (2017), there is not enough evidence to suggest which feeding guilds would be more suitable for biological control under this new climate regime. This is because even within each feeding guild, the different life stages may respond differently. Caution should, therefore, be taken not to draw very general conclusions from single factors studies (Vilà et al., 2007), as plant-insect relationships and responses are highly complex. For this reason, it is important that multifactor investigations are conducted for a large variety of species. As shown previously, there are many factors which govern biological control, including nutrient availability, temperature, and plant quality.

1.4.4. Complexities of biological control in a changing world

The continued success of biological control will not only be determined by changes in atmospheric CO_2, but also a variety of other existing stressors that were previously mentioned here. One of the most important challenges to biological control that has existed for decades is eutrophication (Hill & Olckers, 2001). Additional phosphorus from human settlements and nitrogen from agricultural systems are the main cause of this as the phosphorous and nitrogen cycles have been massively altered by these human activities (Oberholster & Ashton, 2008;

Jeppesen et al., 2010; Van Ginkel, 2011). In South Africa, the most eutrophic waterbodies are those in the most developed cities, such as Pretoria and Johannesburg (Figure 1.2). This is because the water reserves are located downstream of these busy urban and metropolitan areas. According to Oberholster and Ashton (2008), the mean phosphorus concentration (as orthophosphate) in the natural water resources of South Africa is around 0.73 mg/liter, despite the adoption of a 0.2mg/liter P standard for the country. Eutrophication is an expensive problem as it threatens human health, water supply and alters biodiversity (Jeppesen et al., 2010; Moss et al., 2011; Van Ginkel, 2011).

For many years, only the symptoms were treated by using algaecides instead of addressing the real issue which is nutrient loading (Moss et al., 2011). However, it has become increasingly clear that eutrophication and climate change are likely to act together in reinforcing the impact that they cause on the aquatic environment (Jeppesen et al., 2010; Vilà et al., 2007). According to Moss et al. (2011), these two stressors may even produce a positive feedback loop, where climate change intensifies eutrophication and vice versa. This is because eutrophication leads to the emission of gases such as methane and nitrous oxides which are greenhouse gases. Additionally, climate change promotes eutrophication through changes in rainfall patterns and more frequent floods that lead to nutrients being washed down into dams and rivers (IPCC, 2007; Jeppesen et al., 2010).

The continued rapid increase in the human population and climate change is likely to lead to more pressure on farmers to produce more food (Oberholster & Ashton, 2008), resulting in increased use of fertilisers and more sewage production, placing our freshwater ecosystems at more risk of eutrophication (Jeppesen et al., 2010). This means that efforts to mitigate the effects of climate change should be made so that they do not exacerbate existing environmental problems. For example, more intensive farming should also be followed by the application of additional nutrient retention measures in order to avoid nutrient runoff into neighboring water bodies.

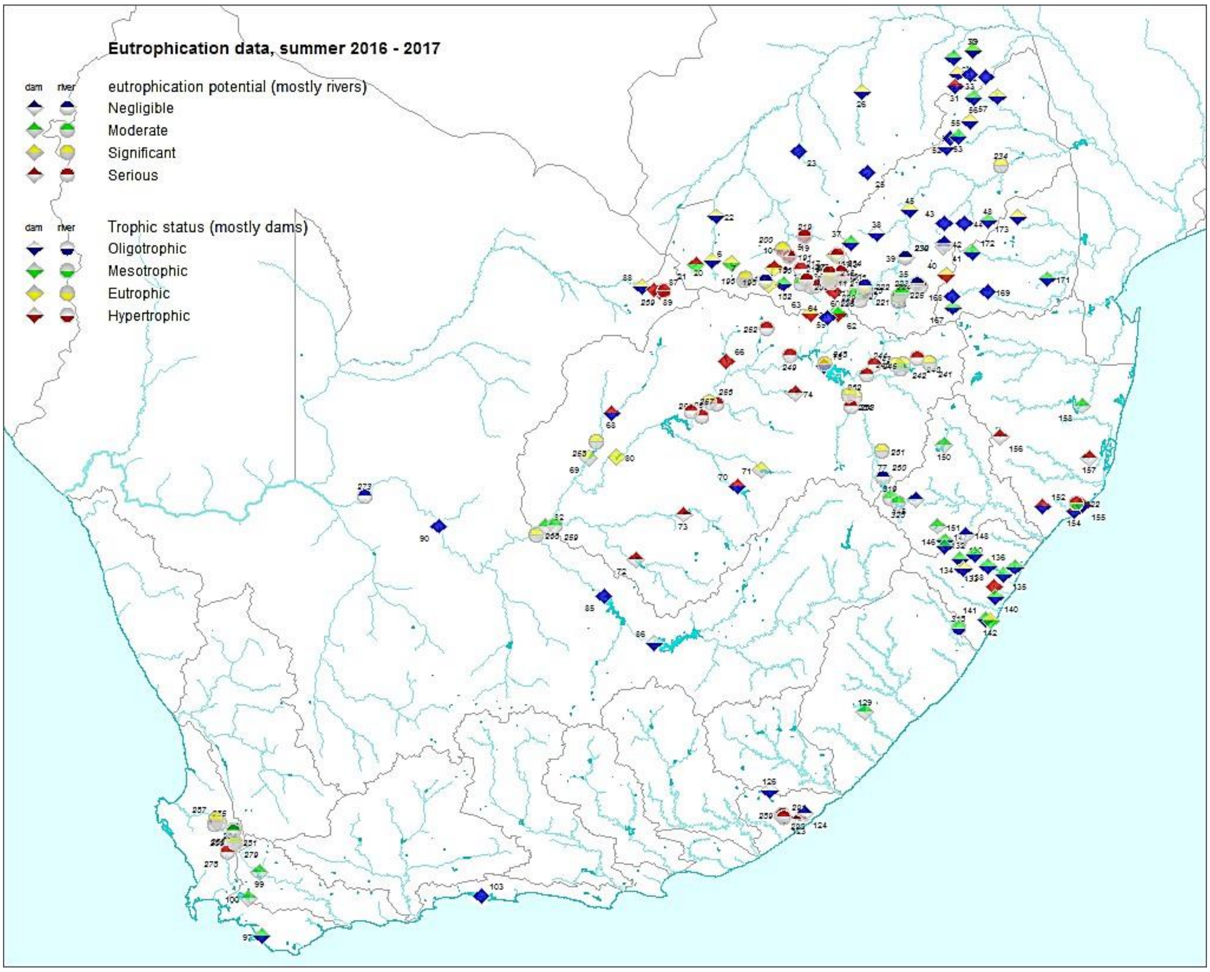


Figure 1. 2: Eutrophication status of selected areas in South Africa during the summer of 2016-2017 (by the National Eutrophication Monitoring Programme, Department of Water Affairs, 2018).

1.4.5. Effects of eutrophication on biological control

Plants respond positively to nutrient addition, especially to phosphorus which is the main limiting factor for many natural ecosystems (Van Ginkel, 2011). Therefore, eutrophic water-bodies tend to be easy targets of invasive weeds (Coetzee et al., 2007). Although eutrophication also promotes the population growth of herbivorous insects, macrophyte infestations in such systems are very difficult to control using biological control due to rapid compensatory growth which negates the effects of increased feeding by the agents (Coetzee et al., 2007). For example, a study conducted by Coetzee et al (2007) on the effect of nutrient status on biological control of water hyacinth by *Eccritotarsus catarinensis* Carvalho (Hemiptera: Miridae) found that the main factor governing the proliferation of water hyacinth was nutrients, especially under eutrophic conditions. The study concluded that the most effective way to reduce infestations would be lowering water nutrient concentrations, as *E. catarinensis* alone would not be able to control this weed under these conditions.

Not only does nutrient runoff promote the growth of invasive plants (Hill & Olckers, 2001), but it also increases the production of defensive compounds such as phenolics (Center & Wright, 1991), similar to the effects of elevated CO_2 (Moss et al., 2011). Due to the relatively high frequency and longevity of droughts in South Africa, there has been an increased shortage of water supply over the past few years, and this is predicted to worsen into the future (Van Ginkel, 2011). Additionally, eutrophication, climate change and biological invasions are likely to further increase the cost associated with using the remaining resources while decreasing the benefits associated with the use of those resources if there are no significant improvements in eutrophication management approaches and treatment technologies (Oberholster & Ashton, 2008). It is of paramount importance therefore that we protect the remaining reserves from eutrophication and invasion by exotic weeds.

1.5. Aims and hypothesis

"Climate change represents a new challenge for resource managers charged with preventing, controlling, and eradicating invasive species" (Rahel & Olden, 2008).

It is clear that atmospheric CO_2 is on the rise, and since this is one of the primary requirements for photosynthesis, elevated CO_2 is likely to impact both individual plants and plant communities. According to Reeves (2017), there is a need to incorporate biological control in studies that are related to climate change. Therefore, the aim of this study was to investigate the effects of elevated CO_2 and nutrient status on four invasive aquatic weeds (*Azolla filiculoides, Salvinia molesta, Pistia stratiotes* and *Myriophyllum aquaticum*) that are a threat to natural resources but are currently being successfully controlled by their respective biological control agents (*Stenopelmus rufinasus, Cyrtobagous salviniae, Neohydronomus affinis* and *Lysathia n.* sp.).

To achieve this, the plants were grown in winter and in summer at different CO_2 and nutrient, concentrations with and without their respective biological control agents, to determine the effects of these combinations on plant growth, and the performance of the control agents under these conditions. Plant quality was analysed and correlated with plant growth and insect performance. The results of this study will make a significant contribution in predicting how increased CO_2 is likely to affect biological control in South Africas' highly eutrophic water bodies.

My hypothesis is that plants grown at elevated CO_2 will have increased fitness, especially with added nutrients, as this allows for more growth, faster reproduction and increased plant defenses. Plants that engage in compensatory growth will especially be more problematic under these conditions since the element of limiting resources for growth will be reduced. This has negative implications for biological control of alien aquatic weeds in South Africa, as most of our waters have high levels of eutrophication.

Chapter 2: Study system

Changes in atmospheric CO_2 will undoubtedly affect the success of invasive species (Dukes & Mooney, 1999; Amare, 2016; Reeves, 2017). This is particularly true for disturbed ecosystems such as eutrophic water bodies (Hill & Olckers, 2001; Vilà et al., 2007; Jeppesen et al., 2010). As such, global climate change will likely have important implications for the field of biological control, a management strategy that has allowed South Africa and many other countries relief from some of the worst aquatic weeds (Winston et al., 2014). The following sections will introduce four of these weeds, their biological control agents, and the methods which were employed in order to experimentally quantify the effects of elevated CO_2 and nutrient enrichment on these plants and their respective agents. It is important to note that all four of the macrophytes that were studied here use the C3 photosynthetic pathway to fix CO_2 (Ray et al., 1979; Longstreth, 1989), and therefore are expected to show a clear response to changes in atmospheric CO_2. Results from this study will allow us to better understand the dynamics of biological control under the conditions predicted by the IPCC (2007).

2.1. *Azolla filiculoides*

The redwater fern *Azolla filiculoides* Lam. (Azollaceae) is a small (10-25 mm) free-floating aquatic macrophyte which originates from South America (Figure 2.1) (Lumpkin & Plucknett, 1982) and has become invasive in many parts of the world, including Asia, Australia, America, Europe (Hussner, 2009) and Africa (Hill, 1999; Cilliers et al., 2003; Cheng et al., 2010). In South Africa, this plant was first recorded in 1948 by Oosthuizen and Walters (1961) in the Oorlogspoort River, Northern Cape. Its mode of introduction is still unknown (Hill, 1998), but was most probably an ornamental fishpond plant that was poured into a river (Guillarmod, 1979). Due to its symbiotic relationship with the nitrogen-fixing cyanobacteria *Anabaena azollae* Strasburger (Nostocaceae), *A. filiculoides* is able to grow successfully and outcompete indigenous plants in areas which have low nitrogen (N) content (van Kempen et al., 2016). Furthermore, although this plant is considered a noxious weed in many natural ecosystems, it has been used as a biological fertiliser in paddy fields to improve N availability for rice in China and Japan due to its close association with the cyanobacteria (Wagner, 1997; Cilliers et al., 2003).

Figure 2. 1: Invasive macrophyte, *Azolla filiculoides*.

Azolla filiculoides reproduces through rapid vegetative growth and can reach a daily growth rate of 15%, meaning it can double its size in 7-10 days under favourable conditions (Lumpkin & Plucknett, 1982). When conditions are not favourable, this plant can switch to sexual reproduction (Cilliers et al., 2003); producing drought-resistant spores that can overwinter and germinate again once conditions become favourable (Lumpkin & Plucknett, 1982; Ashton, 1992). Its growth is limited mainly by phosphorus (van Kempen et al., 2016), although Lumpkin and Plucknett (1982) have shown that *A. azollae* is also able to take-up phosphorus. In South Africa, *A. filiculoides* was initially confined to small dams and rivers in Colesberg, but by 1995, it had spread across the country (Figure 2.2) due to phosphate enrichment of the country's water-bodies (Cilliers et al., 2003). Indeed by 1999 more than

150 sites were infested by this invasive weed, with the majority of these infestations occurring in the Free State Province (Henderson, 1999). Other factors which contributed to the proliferation of this invasive weed include the absence of its natural enemies and dispersal facilitated by man through water collection in the infested sites.

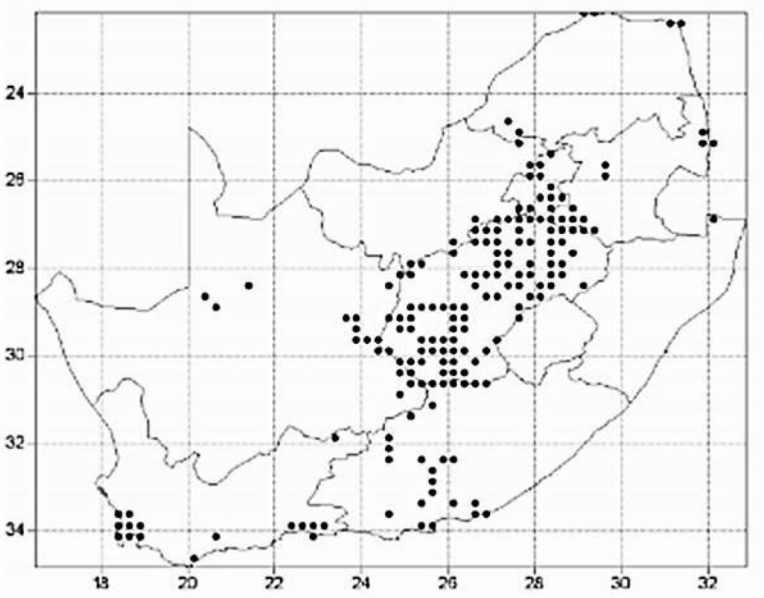


Figure 2. 2: Distribution of *Azolla filiculoides* in South Africa in 1995 (Hill, 2003).

2.1.1. Impacts

Cilliers et al. (2003) and Hill (2003) studied the impacts of the red-water fern. It produces dense floating mats with a thickness of 5-30 cm which can increase the saltation of dams and rivers. As a result, infested waters tend to have increased turbidity, bad colour and odour, reducing the overall quality of the water. These mats also form ideal breeding grounds for disease vectors such as mosquitos and blackflies. They also reduce water surface area available for transport and recreational activities such as swimming, fishing and water-skiing. Infested waters also pose a danger to livestock as the animals are unable to distinguish between green pastures and the mats produced by *A. filiculoides.* The dense mats have also been reported to clog irrigation systems and reduce aquatic biodiversity by blocking sunlight and using up the oxygen available in the water.

2.1.2. Management

Similar to many other invasive macrophytes, control measures suggested for *A. filiculoides* include both chemical and mechanical means of removal. However, due to the labour- intensive nature of mechanical control and the expenses associated with using herbicides such as diquat, glyphosate and paraquat (Ashton, 1992; Cilliers et al., 2003), alternative programmes such as biological control had to be considered. According to Hill (1998), this presented a challenge because *Azolla* species are mainly attacked by generalists, with only a few specialist insects that have evolved with these plants (Lumpkin & Plucknett, 1982). One such species is *Stenopelmus rufinasus* Gyllenhal (Coleoptera: Curculionidae) (Figure 2.3), a biological control agent that has afforded South Africa complete control of *A. filiculiodes*.

Stenopelmus rufinasus is a frond-feeding weevil that was imported from Florida, the United States of America in 1995 (Hill, 1999). It was then released to infested sites around South Africa after laboratory host range tests were concluded in 1997 (Hill, 1998). A summary of the life history strategy of *S. rufinasus* as described by Hill (1998) is presented here. Adults are able to mate as soon as they emerge and oviposition may take place after 1 or 2 days. Each female chews a hole at the tip of the frond and oviposits a single yellow-orange egg and up to 10 eggs may be laid per day. The exposed part of the egg is then covered with a cap of frass. All three larval stages of the weevil feed on the leaves of the redwater fern. Each instar larval stage lasts for 2 to 3 days, with the first instar mining in the upper lobes of the leaves, while the second and third instars feed externally. Before pupating, the larvae select a site, burrowing themselves into the fronds by constructing chambers through chewing. The pupal stage lasts for 4 to 6 days and the whole lifecycle from egg to adult can range between 16 and

23 days. Adults can live for up to 55 days, with each female having produced an average of 325 offspring by the end of its lifetime.

Figure 2. 3: *Stenopelmus rufinasus* a biological control agent of *Azolla filiculoides.* (Image by Grant Martin).

According to Hill (1998), *S. rufinasus* was considered a good biological control candidate for redwater fern as the females are long-lived and produce many offspring which cause extensive damage to the frond. For example, older larvae are able to consume more than one plant in a day. Furthermore, the short development time of the offspring allows for multiple overlaying generations. Indeed, *S. rufinasus* has been highly effective in controlling *A. filiculoides* in South Africa, even leading to local extinction of some of the populations on which it was released (Hill, 2003; McConnachie et al., 2004). In less than five years following its release, this biological control agent was able to significantly reduce the negative effects of *A. filiculoides* infestation and the weed no longer poses any threat to the functionality or use of freshwater systems in the country (Cilliers et al., 2003; McConnachie et al., 2004). As a result, some have even suggested that the plant be removed from the National Environmental Management Biodiversity Act (2004) (NEMBA) list of invasive species in South Africa to permit utilisation (Zachariades et al 2017).

2.2. Salvinia molesta

The free-floating water fern (Figure 2.4), *Salvinia molesta* D.S. Mitchell (Salviniaceae) (salvinia) is an aquatic weed native to South America that has invaded many tropical and subtropical parts of Africa, Australia and Asia (Forno & Harley, 1979; Doeleman, 1989; Cilliers, 1991b; Whiteman & Room, 1991). *Salvinia molesta* is commonly known as the Kariba weed due to its history of invasion in the Zimbabwean Lake Kariba, which was then the largest manmade impoundment (Doeleman, 1989; Cilliers, 1991b). This weed invaded more than 20% of the lake by 1963, causing a problem for the hydropower generation plant in the dam (Cilliers et al., 2003). In South Africa, the weed was first recorded in the early 1900s, both inland and around coastal areas with a subtropical climate (Cilliers, 1991b). It was most problematic in the Provinces of KwaZulu-Natal, Western Cape, Mpumalanga and Eastern Cape (Cilliers, 1991b).

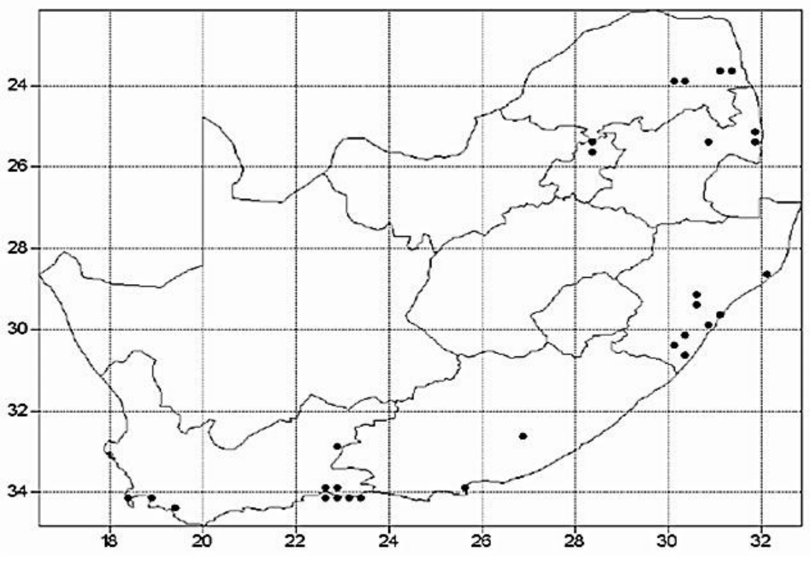


Figure 2. 4: Distribution of *Salvinia molesta* in South Africa in 1995 (Hill, 2003).

2.2.1. Biology and Impacts

Salvinia molesta is a sterile plant that invades stagnant or slow-moving waters, using vegetative reproduction to spread (Figure 2.5) (Forno & Harley, 1979; Doeleman, 1989; Whiteman & Room, 1991; Cilliers et al., 2003). Under ideal conditions, it has the ability to double its biomass every 2-4 days (Cary & Weerts, 1983; Doeleman, 1989), forming dense mats up to 1 m thick held together by horizontal branching rhizomes (Julien & Bourne, 1986; Room, 1990; Whiteman & Room, 1991). The ramets which form these colonies each consist of a node, three leaves, of which one has been modified to act as a root, an apical bud and lateral buds (Sands & Scholtz, 1984; Forno & Bourne, 1985; Julien & Bourne, 1986; Julien et al., 1987; Room, 1988). It is these buds which are responsible for the growth of new ramets (Julien & Bourne, 1986; Room, 1988). Once the old ramets senesce, their rhizome segments break off from the main colony to form a new daughter colony (Room & Thomas, 1985).

Figure 2. 5: Kariba weed, *Salvinia molesta*.

Impacts of *S. molesta* are similar to those of other invasive macrophytes, including the destruction of the invaded habitat, exclusion of native fauna and flora, hindering the utilisation of impoundments and rivers, and promoting waterborne diseases (Cilliers, 1991b).

In the 1980s, this weed was considered the second most problematic weed after water hyacinth in South Africa and elsewhere in the world (Doeleman, 1989; Cilliers et al., 2003; Coetzee et al., 2011). Its destructive nature led to the classification of this weed as a category 1b invader in South Africa, which needs to be controlled according to NEMBA regulations of 2004.

2.2.2. Management

For a while, the control of *S. molesta* in South Africa was primarily dependent on chemical control (Cilliers et al., 2003). Terbutryn, glyphosate, paraquat and diquat based herbicides are some of the chemicals that have been used to control *S. molesta* (Room & Thomas, 1985; Cilliers et al., 2003). However, this type of management only allowed a brief relief from the problem, before surviving buds of the plant regenerated again (Doeleman, 1989). Manual removal has also been used (Room & Thomas, 1985), but with less success (Room, 1990; Cillers et al., 2003).

The unreliability of the above methods due to various environmental and economic implications led to the consideration of the use of biological control (Room & Thomas, 1985; Room, 1990; Zimmermann et al, 2004). A suite of biological control agents, including moths, weevils and grasshoppers have been used to control the weed in Africa (Room, 1990; Cilliers et al., 2003). Due to misidentification of *S. molesta* as *S. auriculata* Aublet (Forno & Harley, 1979; Room, 1990), the first biological control agent that was released on the weed was *Cyrtobagous singularis* Hustache (Coleoptera: Curculionidae) in two African countries, Botswana and Zambia (McFadyen, 1998). Evidently, this weevil and the other biological control agents struggled to control *S. molesta* in the wild (MacFadyen, 1998).

It was not until 1972 that it was reclassified as *S. molesta* based on the male sporocarps which hang from the rhizome (Doeleman, 1989). This was a very important discovery as it allowed for specific surveys of control agent which feed on *S. molesta* (Forno & Harley, 1979; Room, 1990). The discovery of an effective biological control agent, *Cyrtobagous salviniae* Calder and Sands (Coleoptera: Curculionidae), for control of *S. molesta* in Australia was the start of successful control of the weed all around the world (Sands and Schotz, 1984; Doeleman, 1989; Whiteman & Room, 1991; Cilliers et al., 2003; Coetzee et al., 2011). In 1985, *C. salviniae* was imported into South Africa from a population in Namibia which originated from Brazil (Cilliers, 1991b). Host specificity tests were not conducted as the host range of this agent had already been established before its release in Australia (Cilliers, 1991b). As such, *C. salviniae* was introduced directly into the field.

2.2.3. Biological control

The weevil (Figure 2.6), *C. salviniae* is a small (2 mm in length), black, semi-aquatic beetle (Cilliers, 1991b; Pieterse et al., 2003). Following is the summary of its biology as reported by Cilliers (1991b). The females construct oviposition sites by chewing into the stem of *S. molesta* and laying their eggs singly in those cavities. They may also suspend the eggs singly in the roots below the rhizome. Each female is able to lay 2-5 eggs per day over a period of 60 days. After 10 days, the eggs hatch and the newly emerged larvae feed extensively on the young terminal leaf buds for 3-14 days, after which they burrow into the rhizome to moult through three larval instars. Pupation occurs 23 days later in the root mass. The entire lifecycle of *C. salviniae* takes approximately 46 days from egg to adult, and adults can live for up to 60 days (Room & Thomas, 1985).

Figure 2. 6: Adult *Cyrtobagous salviniae*, a biological control agent of *S. molesta*. (Image by Grant Martin).

The control of *S. molesta* is established through the damage caused by both adults and larvae, where the adults feed on the leaf buds, and the larvae feed mainly inside the rhizomes (Forno & Bourne, 1986; Julien et al., 1987). Herbivory pressure from both the larvae and the adults leads to the darkening of the leaves, loss of buoyancy, and subsequent premature separation and sinking of the ramets (Whiteman & Room, 1991).

Nitrogen content and temperature are an important determinant of the development and therefore the effectiveness of *C. salviniae* as a biological control agent (Room, 1990; Cilliers, 1991b). At low temperatures, the reproductive output of the weevil is compromised, as the females are unable to lay eggs below 21 ^{o}C, while the eggs also fail to hatch below 19 ^{o}C (Cilliers, 1991b). Furthermore, this agent is unable to establish on *S. molesta* if the nitrogen content of the dry weight is less than 0.8% (Cilliers, 1991b). However, in instances where the insects have been able to establish, their feeding damage can increase nitrogen concentration in the plant to levels where it becomes a self-sustaining system that is able to support the increasing weevil population (Julien & Bourne, 1987; Room, 1990).

Although high nitrogen content in *S. molesta* supports a large number of control agents, this also allows the plant to engage in ‘traumatic reiteration’, where the destruction of buds by the insects lead to compensatory activation of dormant buds (Julien & Bourne, 1986; Room, 1990). However, there is no compensation for the destruction of vegetative material such as leaves and rhizome segments (Julien & Bourne, 1986; Room, 1990). Despite the increased growth rate of *S. molesta* under high nutrient conditions (Cary & Weerts, 1983), the overall effect is negative as the high numbers of insects it supports results in more damage to the plant than it is able to compensate for. In cases of high densities, the results are not as straight forward as crowding reduces the growth rate of *S. molesta* and the production of new leaf buds. Thus, biological control becomes more challenging under these conditions as the plants become less suitable for the weevils (Schooler et al., 2011).

According to Room (1990), the shape of leaves, the branching system and the size of ramets produced by *S. molesta* may also differ depending on physical damage, temperature, nutrients and space availability. Upon initial colonisation, the plant may take on a slender form with small leaves (15 mm wide) and if conditions are favourable, it may develop into a robust plant with leaves up to 60 mm wide (Pieterse et al., 2003; Schooler et al., 2011). High nutrient concentration in the absence of biological control agents usually exacerbates the growth of problematic weeds such as *S. molesta* (Cary & Weerts, 1983; Room, 1990; Coetzee et al., 2011).

2.3. Pistia stratiotes

Water lettuce, *Pistia stratiotes* Linnaeus (Araceae), is a free-floating macrophyte which is believed to have originated from South America (Figure 2.7) (Holm et al., 1977). It has become invasive in a number of countries, including the USA, Australia India, part of Africa and Asia (Holm et al., 1977). Harley et al. (1990) believe that its use as fodder and medicinal plant is what perpetuated the widespread distribution of *P. stratiotes* (Figure 2.8). According to Holm et al. (1977), this plant is one of the most widespread invasive macrophytes. In South Africa, *P. stratiotes* was first recorded in the early 1950s in the then Transvaal (Cilliers, 1987).

Figure 2. 7: Water lettuce, *Pistia stratiotes*.

The common name water lettuce is derived from the plant’s appearance which is likened to a floating head of lettuce (Figure 2.7). It has rosettes of leaves covered with velvety hairs that arise sequentially from a central meristem, with a tuft of long feathery roots (Wheeler & Center, 1998). The flowers are pale and simple and may appear all year round, but are more common in the middle of summer. Water lettuce is able to reproduce using both sexual and asexual reproduction (Harley et al., 1990), although the latter is more dominant (Coetzee et al., 2011). Under ideal conditions, *P. stratiotes* produces daughter plants through vegetative growth, while seed production ensures persistence of the weed by remaining viable in the soil and only germinating when weed densities are reduced (Cilliers, 1987; Coetzee et al., 2011).

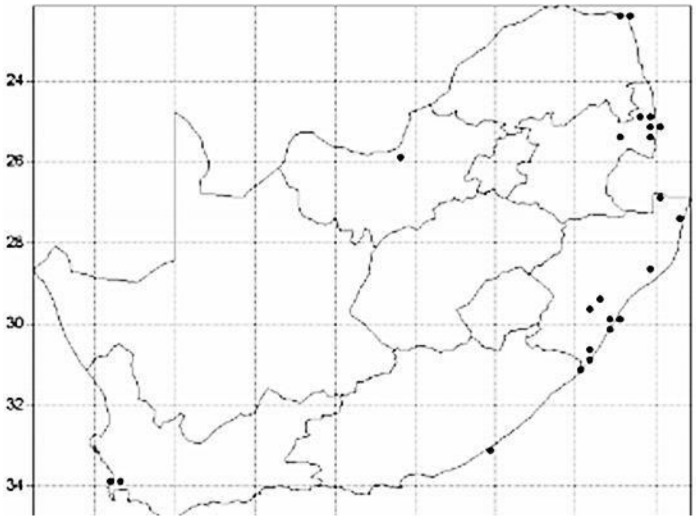


Figure 2. 8: Distribution of *Pistia stratiotes* in South Africa in 1995 (Hill, 2003).

2.3.1. Impact and management

Unlike salvinia and water hyacinth, water lettuce was not as problematic in the 1980s in South Africa (Coetzee et al., 2011), although severe infestations presented similar impacts on water use and ecosystems as the other floating weeds, such as impeding water use for irrigation and recreation (Thompson & Habeck, 1989; Cilliers & Strydom, 1996). Additionally, the roots of *P. stratiotes* are an ideal habitat for mosquito larvae and pupae (Holm et al., 1977; Thompson & Habeck, 1989; Harley et al., 1990). Therefore, control measures were put in place as a preventative measure to stop further spread of this weed in the country. Firstly, legal provisions were made to ensure that no sale or distribution of *P. stratiotes* was permitted under the Conservation of Agricultural Resources Act 43 of 1983 (Cilliers, 1987). Secondly, chemicals such as terbutryn and glyphosate were used with some degree of success, provided that they were administered as soon as the seedlings reappeared after the dry season (Cilliers & Strydom, 1996; Cilliers et al., 2003). Manual removal was not favoured due to the fast growth rate of *P. stratiotes*.

Biological control was considered as it is usually the most effective and environmentally friendly management strategy, and had already been implemented successfully against *P. stratiotes* in Australia (Thompson & Habeck, 1989); a country with relatively similar climatic conditions and vegetation as South Africa. The weevil *Neohydronomus affinis* Hustache (Coleoptera: Curculionidae) is a natural enemy of *P. stratiotes*, which successfully controlled the weed in Australia in the early 1980s (Thompson & Habeck, 1989; Neuenschwander et al., 2009). This success was transferred to South Africa in 1985 through the release of *N. affinis* from the Australian population directly into the field (Cilliers, 1987; Coetzee et al., 2011). No host specificity tests were conducted before this release as *N. affinis* had already been shown to feed only on the target plant in Australia (Cilliers, 1987; Thompson & Habeck, 1989; Harley et al., 1990; Coetzee et al., 2011). Thus far, more than 15 countries depend on this biological control agent for managing *P. stratiotes* infestations (Winston et al., 2014).

2.3.1.1. *Neohydronomus affinis*

Following is the summary of the biology of *N. affinis* as described by Thompson and Habeck (1998) and Harley et al. (1990). The colours of the adult weevils vary noticeably, ranging from brown to bluish-grey with checkerboard patterns or circles of light and dark on the abdomen (Figure 2.9). Feeding activity by the weevils is characterised by round holes on the leaves. The insects are able to penetrate through the leaves when feeding on the more delicate tissue near the apex, but only form punctures which do not penetrate the thicker tissue at the base of the leaves. The punctures on the outer third of the leaves are used for oviposition. The eggs take 2-3 day to hatch and the larvae start to feed through the mesophyll down to the basal area of the leaf. They moult through three instars in about 20 days, after which they pupate within the leaves. Adults emerge 4-5 days later, and the complete lifecycle from oviposition to adult emergence requires 25-30 days. According to DeLoach et al. (1976), *N.*

affinis can produce up to three generations per year, with females laying 1 egg per day. However, Thompson and Habeck (1989) reported 6-8 generations per year, with each adult surviving for up to 3 months in laboratory conditions.

The weevil is more effective at controlling *P. stratiotes* in slow-moving waters in small dams rather than fast-flowing rivers (Coetzee et al., 2011). For example, in South Africa, *N. affinis* showed positive results in less than 10 months of introduction on seasonal pools, while rivers required up to three years for control to be evident (Cilliers, 1987; Cilliers et al., 1991b; Cilliers & Strydom, 1996). Furthermore, a study by Moore and Hill (2012) found a 99% decrease in cover of *P. stratiotes* at a 1.5 ha pond in the Cape Recife Nature Reserve in Port Elizabeth, Eastern Cape, within three months of the weevils' introduction in 2003. Complete control of this weed is dependent on the depletion of the seed bank (Coetzee et al., 2011). In South Africa and many other African countries, the agent has achieved this level of control (Neuenschwander et al., 2009). Thus, no further interventions are needed besides assisted dispersal of the weevil to newly infested sites (Coetzee et al., 2011).

Figure 2. 9: Adult *Neohydronomus affinis*, a natural enemy of *Pistia stratiotes*.
(Image by Grant Martin).

2.4. *Myriophyllum aquaticum*

Parrot's feather, *Myriophyllum aquaticum* (Velloso) Verde (Haloragaceae) is a rooted emergent macrophyte which originates from South America (Figure 2.10) (Godfrey & Wooten, 1981). It is an invasive weed that colonises stagnant and slow-moving waters in many parts of the world, including New Zealand, Central Europe, Japan, North America and some parts of Africa (Moreira et al., 1999; Mafokoane et al., 2007; Hussner et al., 2009). It was introduced to South Africa before the 1920s (Mafokoane et al., 2007). Its main populations are found in the Eastern and Western Cape (Mafokoane et al., 2007), while smaller populations also occur in Mpumalanga, KwaZulu Natal and the North West Province (Cilliers, 1999a). The main mechanism of introduction of *M. aquaticum* to the many invaded areas of the world is through the aquarium trade (Guillarmod, 1979). Parrot's feather is set apart from the rest of the genus *Myriophyllum* by the fact that it is the only emergent species in this genus (Godfrey & Wooten, 1981).

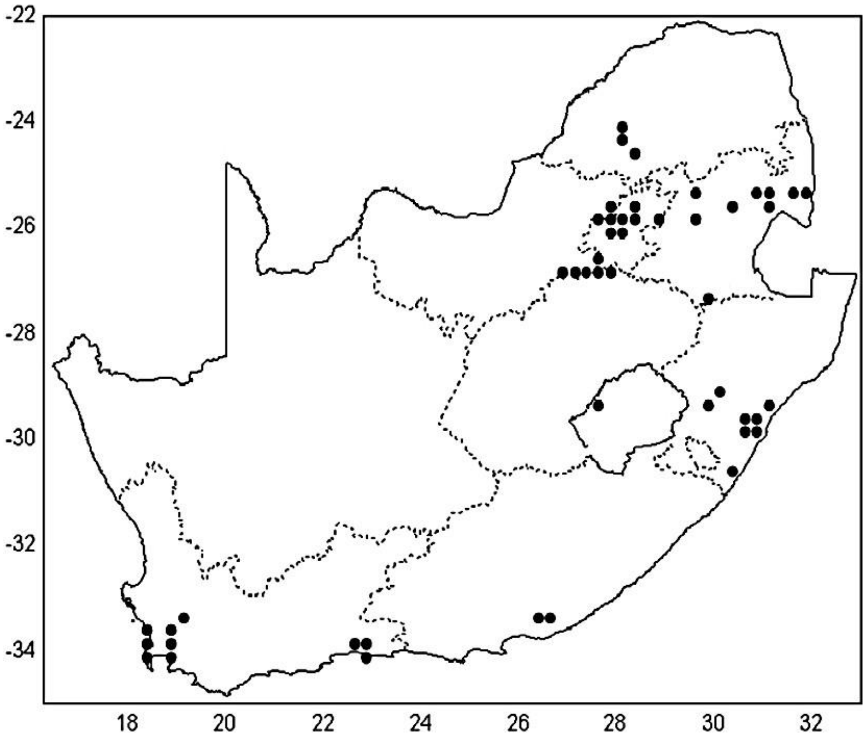


Figure 2. 10: Distribution of *Myriophyllum aquaticum* in South Africa in 2006 (Mafokoane et al., 2007).

2.4.1. Description and biology

Parrot's feather is heterophyllous, meaning that it has distinct submerged and emergent leaf forms (Wersal & Madsen, 2011; Yin et al., 2017). The emergent part of this plant has spiral, stiff, pale-green leaves with 20 or more filiform divisions, resulting in a feathery like appearance (Godfrey & Wooten, 1981). This part of the plant never exceeds 25% of the total plant biomass, although it contains close to 80% of available phosphorus (Sytsma & Anderson, 1993).

The whorls of the submerged shoots are made up of four to six filamentous, red or orange leaves. The stems can grow up to 1.5 m (sometimes as long as 3 m) (Mafokoane et al., 2007; Hussner et al., 2009), while the emergent shoots can be 20 to 50 cm above the water surface (Cilliers, 1999; Hussner et al., 2009). Both the roots and shoots arise from the nodes above and below the water. Once the submerged shoots emerge, they become denser and have more structural tissue (Wersal & Madsen, 2011; You et al., 2013). The growth form also changes from vertical to horizontal stolons, enabling the plant to cover the surface of the water more swiftly (Hussner et al., 2009; Wersal & Madsen, 2011; You et al., 2013).

Myriophyllum aquaticum (Figure 2.11) grows best in areas where the water depth is less than 100 cm, although in some instances it is also able to grow as a submerged plant in deeper waters, up to 2 m (Moreira et al., 1999; Wersal & Madsen, 2007; Hussner et al., 2009; You et al., 2013). The plant is dioecious, but outside of its native range, *M. aquaticum* does not produce seeds and is only able to reproduce asexually as there are no male counterparts in those regions (Cilliers, 1999; Henderson, 2001). Therefore, survival and spread of the plant is dependent solely on vegetative growth of stem fragments (You et al., 2013).

Figure 2. 11: Parrot’s feather, *Myriophyllum aquaticum.*

The impacts of *M. aquaticum* on freshwater systems are similar to those caused by other invasive macrophytes such as *E. crassipes* and *S. molesta*. These include displacing native vegetation, facilitating mosquito population growth, hindering recreational activities, blocking waterways and irrigation systems (Cilliers et al., 1999). In one case, *M. aquaticum* caused up to 50% revenue loss to tobacco farmers by changing the colour of the tobacco to red where field were irrigated with infested water (Cilliers et al., 1999). However, unlike other invasive aquatic plants, *M. aquaticum* has not become problematic in other African countries, except for Lesotho and Zimbabwe (Chikwenhere & Phiri, 1999; Cilliers et al., 2003). In the 1960s, parrot’s feather was considered more damaging than *E. crassipes* in South Africa because of its creeping growth form (Cilliers et al., 2003). Although the plant is classified as a category 1b invader under the NEMBA (2014) legislation (Henderson, 2001); it is still present in nurseries and gardens, facilitating further spread (Mafokoane et al., 2007).

2.4.2. Management

There are no registered herbicides against *M. aquaticum* in South Africa because the chemicals are unable to travel through the water column and reach the stems that are rooted in the soil (Mafokoane et al., 2007). Mechanical control is also relatively difficult, especially when the plant has formed thick intertwined mats (Mafokoane., 2007), and this strategy is not desired as *M. aquaticum* can propagate from remaining fragments as small as 5 mm if they have a node (Cilliers et al., 2003). In an attempt to control this weed, a biological control programme was initiated in 1991 (Mafokoane et al., 2007), resulting in the release of *Lysathia n.* sp. (Coleoptera: Chrysomelidae) as a biological control agent of parrot's feather (Cilliers, 1999). This is a small (3.8 mm in length) metallic green flea beetle which feeds on the leaves of parrot's feather (Cilliers, 1999). According to Cilliers (1999), this species can be distinguished from the two other known species *L. flavipes* (Boheman) and *L. ludoviciana* (Fall) by its characteristic light-yellow legs.

Following is the summary of the biology of *Lysathia* sp. (Figure 2.12) in laboratory conditions as described by Cilliers (1999). The adults lay eggs (0.7 mm long) singly or in masses in the growth tips between or within the leaflets. Initially, the eggs are covered with a fine white waxy powder when laid and turn yellow after a day or two. There are four larval instars (measuring 1-8 mm long), and this stage takes 14-18 days, while the pupal stage lasts between three to six days. The complete lifecycle from egg to adult takes 24 to 36 days. Adults may take four to six days before oviposition, and the eggs hatch in three to six days.

This agent is frost tolerant and can produce up to nine generations in summer (Cilliers et al., 2003). Both adults and the larvae cause extensive damage to the vegetative structures of the plant (Cilliers, 1999), after which they disperse in search of undamaged populations. The absence of the control agents allows the rooted stems time to regenerate before the insects are back again to attack them, resulting in a cycle of defoliation followed by recovery. The longer recovery time and fast population growth result in an overall reduction of the infestation after a few years (Cilliers, 1999).

Figure 2. 12: Adult *Lysathia* sp., a biological control agent of *Myriophyllum aquaticum*. (Image by Grant Martin).

The cyclic abundance of the plant led to a consideration of an additional agent, *Listronotus marginicollis* (Hustache) (Coleoptera: Curculionidae), which is a stem-boring weevil that feeds on *M. aquaticum* in its native range (Cordo & DeLoach, 1982). The first tests were conducted by Cordo & DeLoach (1982) in Argentina for release in the USA. Following on the suggestions made by Cordo & DeLoach (1982) for further investigation, Mafokoane et al. (2007) undertook laboratory host range tests on the same insect to assess its suitability for release in South Africa. They found that *L. marginicollis* is a promising candidate for biological control of *M. aquaticum* in the country as it does not feed on any of the native plants.

Cilliers et al. (2003) and Mafokoane et al. (2007) believe that the nature in which *L. marginicollis* feeds (burrowing through the stem) would supplement the damage caused by *Lysathia* sp., helping to bring about the desired control faster (Mafokoane et al., 2007; Coetzee et al., 2011). However, the effectiveness of *Lysathia* sp. as a control agent led to the discontinuation of the *L. marginicollis* programme as additional control was deemed unnecessary (Coetzee et al., 2011). Rather, Coetzee et al. (2011) suggested that supplemental release of *Lysathia n.* sp. from mass-rearing facilities in early summer could accelerate population build-up, thereby putting more strain on the plant early on in the season.

Chapter 3: Materials and Methods

3.1. Growth chambers

To determine the effects of elevated CO_2 (eCO_2) and nutrient (N) concentration on the biological control of four invasive macrophytes (*Azolla filiculoides*, *Salvinia molesta, Pistia stratiotes* and *Myriophyllum aquaticum*) by their respective control agents (*Stenopelmus rufinasus, Cyrtobagous salviniae*, *Neohydronomus affinis* and *Lysathia* sp.), two seasonal experiments were conducted at the Rhodes University Elevated CO_2 Facility (Figure 3.1), in Grahamstown, South Africa. The facility has decagonal Open-Top Chambers (OTCs), 3 m in diameter and 2.8 m tall, that allow CO_2 and temperature manipulation. The chambers are made up of F-Clean plastic panels that transmit 94% visible light. Each chamber was fitted with a 3-phase fan that introduces air into the chamber via a circular diffuser, 34 cm in diameter and perforated with 300, 1 cm holes for equal distribution. The diffuser was positioned 1 m above the ground and airspeed entering the chamber was regulated via the fan speed.

Airspeed was controlled to keep the difference in temperature between ambient and the interior of the chamber within 4^{o} C. Temperatures were monitored via CS215 temperature/humidity sensors (Campbell Scientific) and data were processed via a CR6 data logger (Campbell Scientific) and Loggernet software (Campbell Scientific), using proportional–integral–derivative (PID) procedures, to alter fans speeds according to temperature differentials. CO_2 concentrations within the chambers were measured with open- path CO_2 analysers (GMP343, Visala, Finland) via the same procedure as controlled fan speed, but using different PID equations that altered the CO_2 injection into the chamber ventilation system.

Target CO_2 concentrations were set at 400 ppm and 800 ppm (two OTCs at each concentration). The OTCs are constructed on a flat earth platform in two rows with alternate spacing to ensure maximum light interception in each chamber. Average (±SD) chamber day/night temperatures were 24.4 ± 5.1 and 20.6 ± 3.5˚C, respectively. Average day/night chamber relative humidity was 65.3 ± 21.8 and 88.3 ± 35.1%. The plants received 11 hr of daylight at an average daytime PPFD (Photosynthetic Photon Flux Density) of 1300.9 ± 863.5 µmol m^{-2} s^{-1} and daily maximum values in excess of 2100 µmol m^{-2} s^{-1}.

Figure 3. 1: Rhodes University Elevated CO_2 Facility (Image by Brad Ripley).

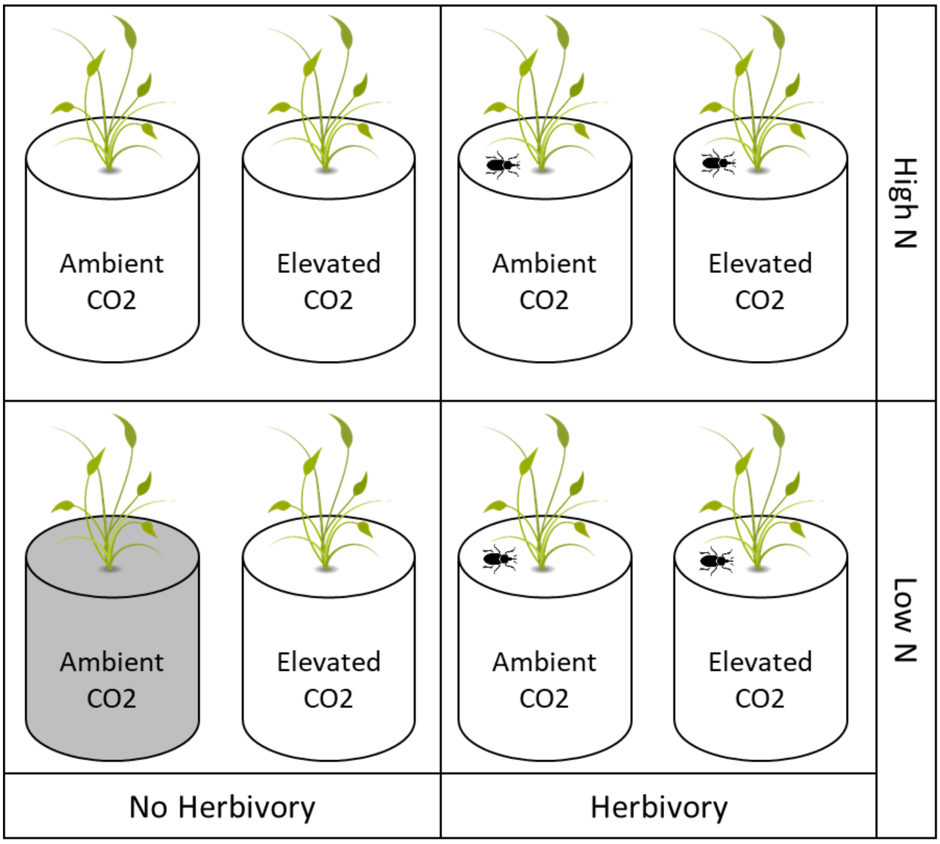
3.2. Winter experiment

The winter experiment was run for 10 weeks, from 6th July to 14th September 2018. The first two weeks were for acclimatisation, and the remaining eight weeks were dedicated to data collection. All plants were sourced from insect-free cultures maintained in the Waainek mass-rearing facility at Rhodes University. Biological control agents of the focal plants were also sourced from the same facilities. The plants were grown under different combinations of nutrients (high N and low N), CO_2 (ambient= aCO_2 and elevated= eCO_2) and biological control (present or absent) following a crossed design layout (Figure 3.2.1). The control treatments for each species were plants that were grown at ambient CO_2 concentration, with no added nutrients and no biological control agents (Figure 3.2.1). Before the commencement of the experiment, the wet biomass of all the plants was measured and plants selected to be approximately of equal mass.

The number of biological control agents used for the herbivory treatment differed between the different plant species as their efficacy is also variable. Starting densities for *A. filiculoides* were approximately 10 g in 5 L ice-cream tubs with two mating pairs of *S. rufinasus* per tub (1:1 sex ratio). For *S. molesta*, round 5 L containers were used to cultivate the four youngest ramets of the plant, with a starting wet weight of approximately 50 g and eight *C. salviniae* agents per tub. For *P. stratiotes,* two plants with 6-8 leaves per plant, each weighing approximately 90 g and two pairs of *N. affinis* insects per plant were grown in round 8 L tubs. All tubs were filled with tap water. Lastly, 10 shoots of *M. aquaticum* (approximately 15 cm x 10 g) were cultivated in 20 L buckets half-filled with sediments and flooded with 10 L tap water. Each shoot was exposed to two pairs of *Lysathia* sp. control agents.

The high nutrient treatments were supplied with 10 mg of nitrogen per litre (eutrophic) using Osmocote fertiliser with an NPK of 19:6:12, while low nutrient treatments were at ~0.1 mg of nitrogen per litre (oligotrophic) obtained solely from tap water. For the CO_2 treatment, there were two OTCs for ambient concentrations set at 400 ppm, while the two other OTCs were for eCO_2 set at 800 ppm. Each CO_2 by nutrient treatment was replicated six times for each plant-insect combination, with each OTC housing half the replicates. For the herbivory treatments, the plants were allowed two weeks to acclimatise to the new growing conditions before being inoculated with their respective control agents. All containers were covered with a transparent mesh sleeve (diameter of 0.5 mm) to avoid cross-contamination between treatments. Plant growth and insect activity were monitored and measured once every two weeks for the duration of the 8-week long experiment (excluding acclimation period). Water and nutrients were replaced during this time and the plants were repositioned to avoid any spatial effects in the OTCs.

Figure 3.2 1: Schematic layout of the winter experiment for each of the four species. Each treatment was replicated six times. Ambient CO_2 (aCO_2) = 400 ppm, Elevated CO_2 (eCO_2) = 800 ppm, Nutrients [(High N) = 10 mg L^{-1}, (Low N) = 1 mg L^{-1}] and Herbivory (HERB) = No/Yes. Dark shaded pot or column represent the control treatment.



3.2.1. Data collection

Fresh weight (FW) (g/tub) was determined by letting the plants drip off the excess water before weighing. Relative growth rates (RGR) (g tub-^{1} day^{-1}) were calculated for total biomass increase in each treatment using the following equation: RGR = (lnW$_{2}$-W$_{1}$)/(t2- t1) where W$_{1}$ and W$_{2}$ are dry weights at times t$_{1}$ and t$_{2}$ (at the beginning and at the end of the experiment). Doubling times (days) were obtained by dividing ln(2) by RGR. Chlorophyll content was measured for three randomly chosen leaves per tub using a chlorophyll meter (Model CCM 200, Apogee instruments).

Destructive measurements to determine changes in plant tissue chemistry (C: N ratio and ash percentage) were conducted at the end of the experimental period after all the visible insects were removed from the plants. No plant chemical analyses were done at the beginning of the experiment as the control treatments would be used as the baseline. In preparing for these measurements, plant samples were oven-dried at 60 ^{0}C for 72 hours and then powdered using a pestle and mortar which were cleaned in methanol and dried with a paper towel before grinding each sample to avoid contamination. These were then weighed to ±2 g and sent to BemLab (Strand, PO Box 684, Somerset Mall, 7137) for C: N analysis.

Some of the remaining ground plant material was used to determine the percentage ash content. This was done by putting the plant powder (~1 g) into crucibles, weighing them to four decimal places and burning them using a muffle furnace for an hour at 250 ^{0}C. The heat was then increased to 550 ^{0}C and left for another 2 hours. At the end of the 3-hour burning session, the samples were removed from the furnace and left to cool down for 30 min, after which they were measured again, and the difference in mass between before and after burning was used to calculate ash percentage.

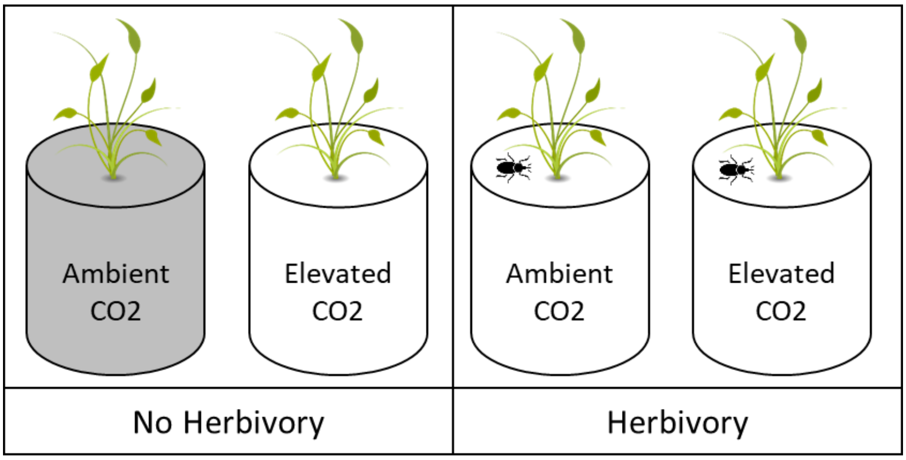
In addition to the above general measurements, there were also some specific data measurements for the different plants in each tub. Additional parameters for *S. molesta* included the number of new ramets and the total number of leaves produced. Similar to *S. molesta*, the total number of leaves and the number of daughter plants were also recorded for *P. stratiotes*, in addition to leaf thickness for leaf position 4 which was measured with electronic calipers. For *M. aquaticum*, the number of daughter plant was counted for each branch, as well as the number of mother branches (referred to as number of leaves) and the total length (cm) of the longest stolon/branch in each tub was measured

3.3. Summer experiment

A separate but similar experiment was conducted to investigate how biological control of our four selected plant species will be affected by CO_2 during the summer season (4th October to 6th December 2018). There were slight changes to the design and setup of this experiment compared with the winter season in order to simplify it and because of lessons learnt previously.

The most important change was that there was no nutrient treatment for the summer experiment as all plants were given equal concentrations of nutrients (10 mg N/L), allowing focus solely on the CO_2 effects in the presence and absence of biological control agents (Figure 3.3.1). Minor changes included the use of similar 20L containers for all the plants to allow for equal distribution of light and using equal starting densities (90 g of plant material).

Figure 3.3 1: Layout of the summer experiment for each of the four species, replicated six times. Ambient CO_2 (aCO_2) = 400 ppm, Elevated CO_2 (eCO_2) = 800 ppm and Herbivory (HERB) = No/Yes. Dark shaded pot or column represent the control treatment.



3.3.1. Data collection

Similarly to the changes made in setting up the summer experiment, there was also a change in the way data collection was conducted for this experiment. Some of the measurements done during the winter season were omitted or changed in the summer experiment and there were also some new additions. Chlorophyll content was not measured in this experiment, while new additions include the measurement of photosynthetic rate, dead plant mass and insect feeding activity.

The photosynthetic rate was measured using a LI-6400 Portable Photosynthesis System (LI-COR, Lincoln, Nebraska, USA) and was conducted only on clear days between 10: 00 and 15:00 h local time at week 10 of the experiment period, between the 10th and the 13th of September 2018. These measurements were only taken for *P. stratiotes* and *M. aquaticum* as the leaves on the other two plants were not suitable for inclusion in the LI-6400. It was also not possible to quantify dead mass for these two plants as their control agents did not leave any damaged plant material. Thus, this was only measured for *A. filiculoides* and *S. molesta*.

To quantify insect feeding activity on *S. molesta*, the number of leaves showing the characteristic shot-hole feeding scars were counted on 10 randomly selected leaves and presented as a percentage of the 10 selected leaves. The percentage feeding activity of the control agents on *P. stratiotes* was determined using a criterion analogous to that set out by Diop et al. (2010). Percentage scores were allocated using the following observed criteria: 0-20= No adult feeding scars and larval mines, 21-40= Evidence of feeding scars, larval mines and healthy bud leaves, 41-60=Bud leaves start decomposing, larval mines on 25–50% of the leaves, 61-80=Older leaves start decomposing, and 25% of the leaves affected, 81-90=Decomposing of leaves, 25–50% of the leaves affected, 91-100=All plants are dead. A similar method was also used to determine insect feeding activity for *A. filiculoides* and *M. aquaticum*. The plants were then harvested and separated into leaves, daughter plants and roots where possible, and their biomass (g/tub) was determined before and after drying at 60 ^{o} C for 72 h.

3.4. Data analyses

Generalised Linear Models (Gaussian, Gamma and Quasipoisson regression) were used to analyse the effects of CO_2 (ambient and elevated), nutrient status (low N and high N) and herbivory (no and yes) on plant growth parameters and plant chemical constituents for all four species. GLM was chosen over Mixed models because upon inspection, the amount of variance explained by chamber (random effect with four categories=OTCs) was not distinguishable from zero for all the response variables. Statistical comparisons were only made within species, and not between species, although responses and trends are compared in the results. The summer experiments did not include a nutrient treatment, thus, the models for this period only had the two factors (CO_2 and herbivory). Multiple *post hoc* comparisons of the factors (CO_2, herbivory and nutrients where applicable) on the dependent variables (plant parameters and chemical constituents) were conducted after the significance of at least the main effects was established with a previously fitted GLM. These comparisons were made using the lsmeans function (least-squares means) within the lme4 package, and the family- wise error rate was corrected using the Bonferroni adjustment. All analyses were conducted in R Studio v 3.4.3 (R Core Team 2017) at an alpha level of 0.05 for all statistical tests. The data were graphed using the ggplot2 package also available in R.

Chapter 4: Results

The effects of eCO_2 on the biological control of four invasive macrophytes (*Azolla filiculoides, Salvinia molesta*, *Pistia stratiotes*, and *Myriophyllum aquaticum*) by their respective control agents (*Stenopelmus rufinasus*, *Cyrtobagous salviniae*, *Neohydronomus affinis* and *Lysathia* sp.) were investigated using a three-level factorial design in winter (CO_2 X nutrients X herbivory) and two factor experimental design in summer (CO_2 X herbivory). Although this was a repeated measure study, only data obtained at the end of the experiment period (week 8) for both growing seasons are presented here as this is the point where the plants would be showing a response to the treatment. Results for plant responses to the treatments are presented separately for each plant species and include measures of growth (wet or dry biomass and number of leaves), reproduction (number of side shoots), and insect activity (number of insects, percentage plant damage and dead plant weight). Measured plant parameters that did not respond to any treatment for any of the species of interest such as ash%, RGR and doubling time were not analysed any further in this study.

4.1. *Azolla filiculoides*

4.1.1. Winter

Biomass production by *A. filiculoides* had a similar positive response to eCO_2 regardless of herbivory or nutrient status, although differences in biomass were more pronounced at low nutrients, but not significantly. Increasing the CO_2 concentration increased the biomass produced. However, the biomass of the plants with insects was noticeably higher than that of the plants not subject to herbivory, and this was so at both growth CO_2 concentrations (Figure 4.1.1a, Table 4.1.1). Unlike wet biomass, the C: N ratio of *A. filiculoides* was neither affected by CO_2 nor herbivory under low nutrients. However, when supplied with nutrients, there was a significant difference between aCO_2 and eCO_2 treated plants, but only in the absence of herbivory. In this case, eCO_2 resulted in a significantly lower C: N ratio (Table 4.1.1, Figure 4.1.1b).

Table 4 1.1: Summary of GLM results for the effects of CO_2, (CO_2; Ambient, Elevated), Nutrients (N; low N, high N), and Herbivory (HERB; No, Yes) on plant parameters of *Azolla filiculoides* at the end of the winter season experiment. Figures in bold indicate significant results.

Response	Factor	Estimate	Std. Error	*t/z value*	*P-value*
Wet biomass/tub (g)	(Intercept)	135.5	5.393	25.124	**< 2e-16**
	N	-86.5	7.627	-11.341	**4.54E-14**
	CO_2	25.333	7.627	3.322	**0.00192**
	HERB	5.833	7.627	0.765	0.44887
	N*CO_2	53	10.786	4.914	**1.56E-05**
	N*HERB	25	10.786	2.318	**0.02566**
	CO_2*HERB	5.833	10.786	0.541	0.59164
	N*CO_2*HERB	12.333	15.254	0.809	0.42357
Chlorophyll	(Intercept)	1.8219	0.1642	11.097	**< 2e-16**
	N	-0.3907	0.2584	-1.512	0.13056
	CO_2	-0.2886	0.2508	-1.15	0.24997
	HERB	0.8511	0.1961	4.34	1.42E-05
	N*CO_2	1.5154	0.3383	4.479	7.48E-06
	N*HERB	-0.6896	0.335	-2.059	**0.03951**
	CO_2*HERB	-0.4338	0.3133	-1.385	0.16615
	N*CO_2*HERB	-1.4562	0.4993	-2.916	**0.00354**
C: N ratio	(Intercept)	3.26391	0.07983	40.885	**< 2e-16**
	N	0.04687	0.1116	0.42	0.674473
	CO_2	-0.44571	0.12777	-3.488	**0.000486**
	HERB	-0.36112	0.12457	-2.899	**0.003745**
	N*CO_2	0.22354	0.17321	1.291	0.196839
	N*HERB	0.16754	0.17023	0.984	0.325008
	CO_2*HERB	0.43394	0.18634	2.329	**0.019874**
	N*CO_2*HERB	-0.30062	0.25267	-1.19	0.234143

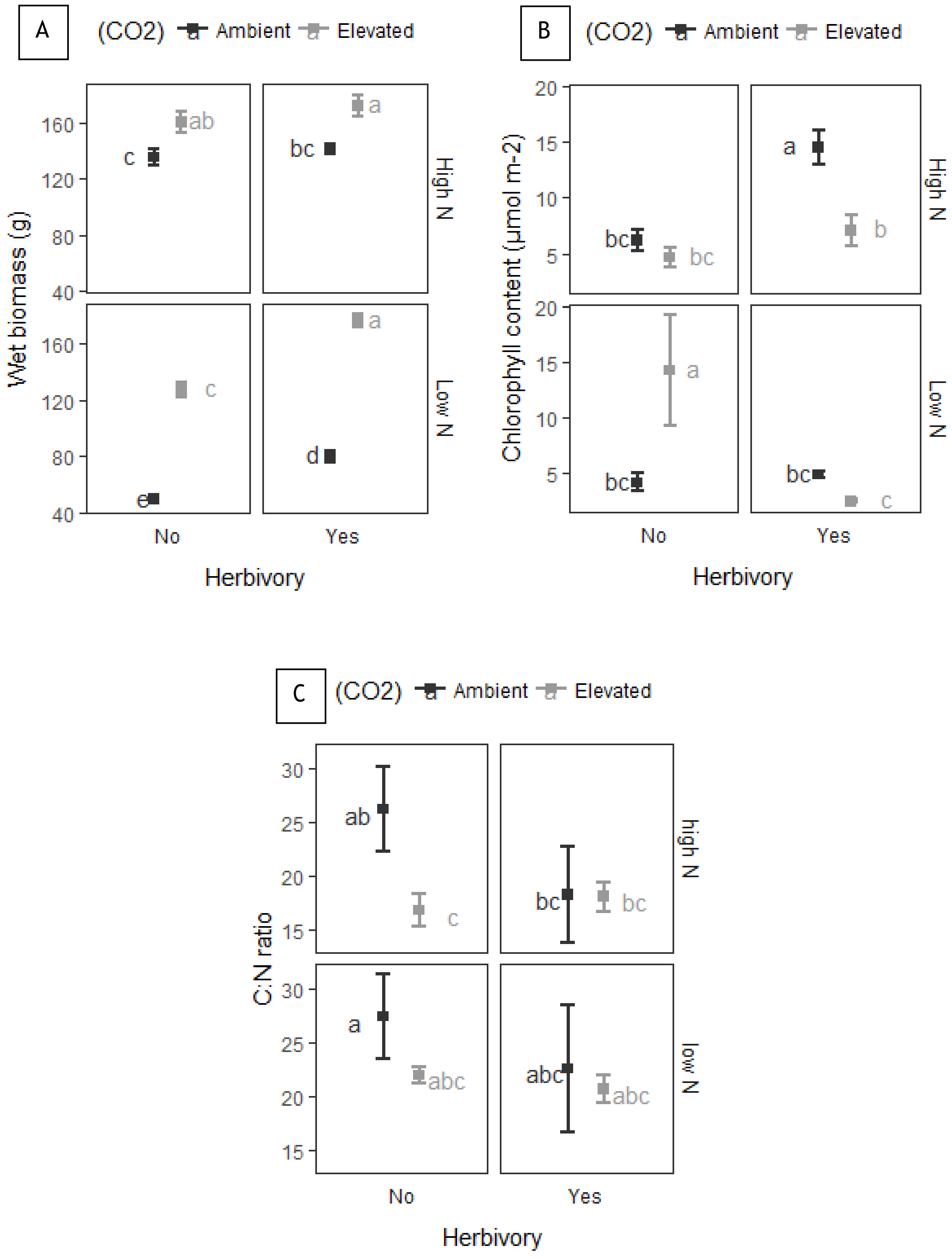


Figure 4.1. 1: Mean wet biomass produced by *Azolla filiculoides* per tub (A) and mean C: N ratio (B) as affected by CO$_2$ (Ambient and Elevated), Nutrients (high N and low N) and Herbivory (No and Yes), in winter. Error bars= SE, different letters indicate significant differences (P<0.05).

4.1.2. Summer

The mean total dry weight and mean dead weight per tub of *A. filiculoides* was not affected by changes in CO_2 for the no herbivory treatment (Figure 4.1.2, Table 4.1.2). However, the presence of herbivory stimulated the growth of *A. filiculoides* at eCO_2 compared with aCO_2 (Figure 4.1.2). In contrast, the C: N ratio of *A. filiculoides* was higher at eCO_2 for the control treatment. Upon addition of herbivory, there was not enough plant material left for C: N analysis for the aCO_2 treatment (as the insects had eaten it all), but the relationship is assumed to be similar to that of the control treatment (Figure 4.1.2c, Table 4.1.2).

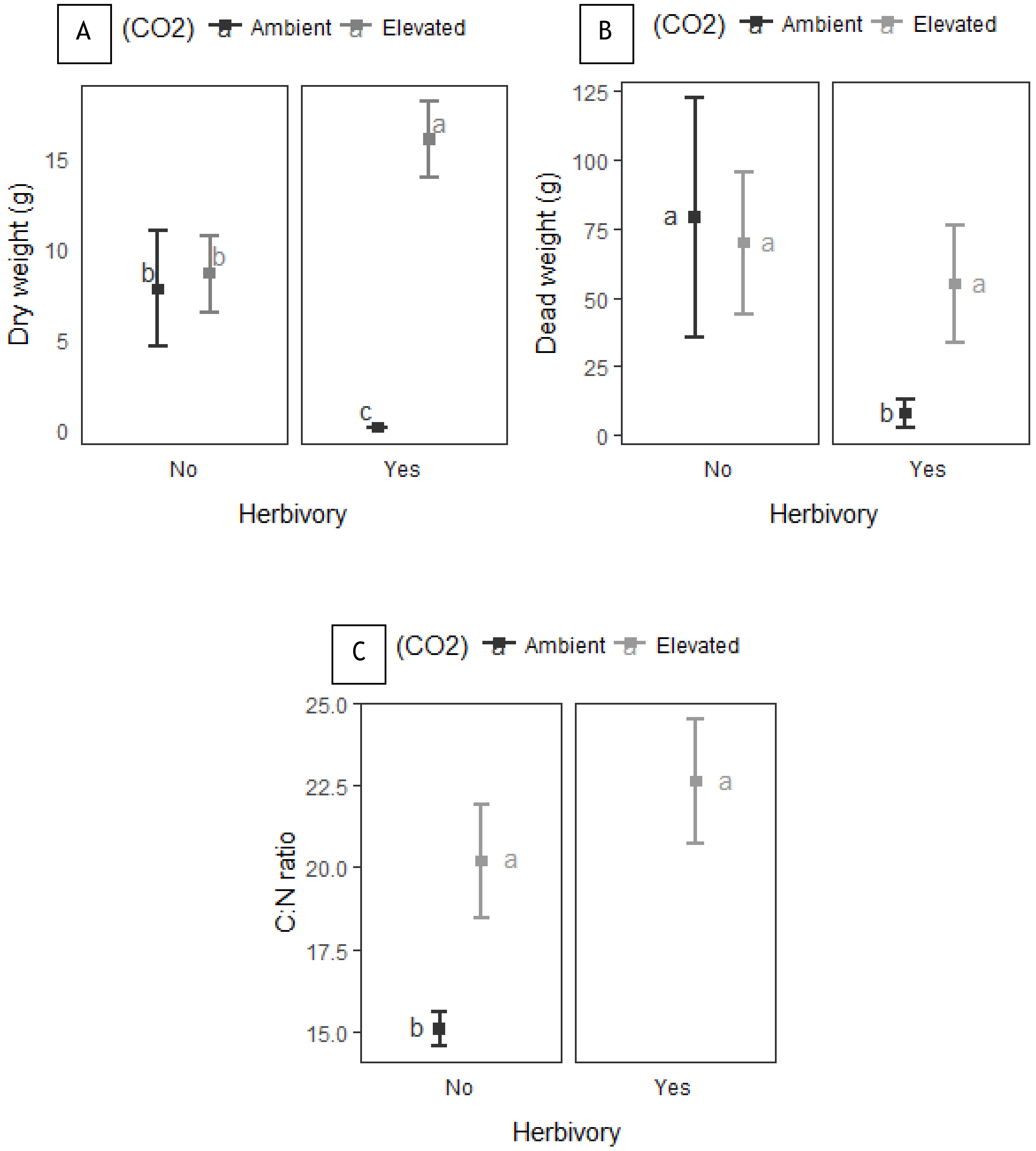


Figure 4.1. 2: Plant parameters of *Azolla filiculoides* as affected by CO_2 (Ambient and Elevated), and Herbivory (No and Yes) in summer. (A) Dry weight, (B) Dead weight and (C) C: N ratio. Values are means per tub ± SE, different letters indicate significant differences ($P<0.05$).

The mean number of live *S. rufinasus* recovered from *A. filiculoides* (Figure 4.1.3a) and the damage thereof (Figure 4.1.3b), was significantly reduced under eCO_2 (t value $_{(1,\ 10)}$ = -3.616, *P*=0.0047 and t value $_{(1,\ 11)}$ = -5.912, *P*=0.0001).

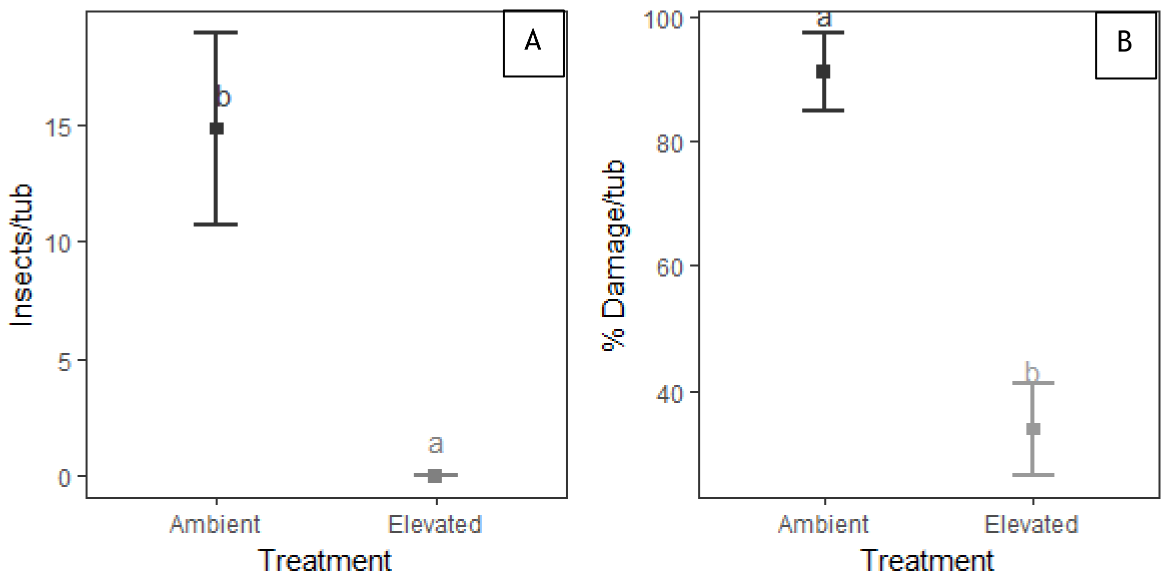


Figure 4.1. 3: (A) Mean number of insects per tub and (B) Mean damage caused by *Stenopelmus rufinasus* on *Azolla filiculoides* in summer as affected by changes in CO_2 concentration. Error bars= SE, different letters indicate significant differences (*P*<0.05).

Table 4.1. 2: Summary of GLM results for the effects of CO_2, (CO_2; Ambient, Elevated) and Herbivory (HERB; No, Yes) on plant parameters of *Azolla filiculoides*, at the end of the summer season experiment. Figures in bold indicate significant results.

Response	Factor	Estimate Std	Error	t value	p-value
Dry biomass/tub (g)	(Intercept)	2.052	0.2599	7.896	**1.42E-07**
	CO_2	0.1056	0.3582	0.295	0.7712
	HERB	-4.3546	2.3073	-1.887	0.0737
	CO_2*HERB	4.9727	2.3275	2.137	**0.0452**
Dead weight/tub (g)	(Intercept)	4.3716	0.5266	8.302	**< 2e-16**
	CO_2	-0.1254	0.7447	-0.168	0.86623
	HERB	-2.3047	0.7447	-3.095	**0.00197**
	CO_2*HERB	2.0659	1.0532	1.962	**0.04981**
C: N ratio	(Intercept)	0.066475	0.004436	14.986	**1.40E-09**
	CO_2	-0.01697	0.005725	-2.963	**0.011**
	HERB	-0.00327	0.004952	-0.661	0.52
	CO_2*HERB	NA	NA	NA	NA

4.1.3. Winter vs summer

As evident from Figure 4.1.1a, biomass production by *A. filiculoides* was very responsive to eCO_2 for both herbivory treatments in the winter season. However, in summer, a positive response was only triggered by the presence of herbivory (Figure 4.1.2a). The C: N ratio of *A. filiculoides* was higher at aCO_2 for the control plants during the winter season (Figure 4.1.1b). This relationship was reversed in summer for the same treatment (Figure 4.1.2c). Addition of herbivory reduced the measured C: N ratio, but this was not different from the eCO_2 treatment for the winter months. There was not enough plant material left in the aCO_2 treatment of the summer month to make the same comparisons, but herbivory did not reduce the C: N ratio at eCO_2 compared with the no herbivory treatment with the same CO_2 concentration.

4.2. *Salvinia molesta*

4.2.1. Winter

Biomass production by *S. molesta* only had a positive response to eCO_2 at high nutrients for the control treatment (Figure 4.2.1a). The asexual reproductive output of *S. molesta*, on the other hand, had a more varied response. More specifically, there were more side shoots in the control treatment when nutrients were added at eCO_2, while in the presence of herbivory it was only those plants grown at aCO_2 that benefited from nutrient addition (Figure 4.2.1b, Table 4.2.1). Lastly, the C: N ratio in the leaves of *S. molesta* was not significantly affected by any treatment combinations (Figure 4.2.1c).

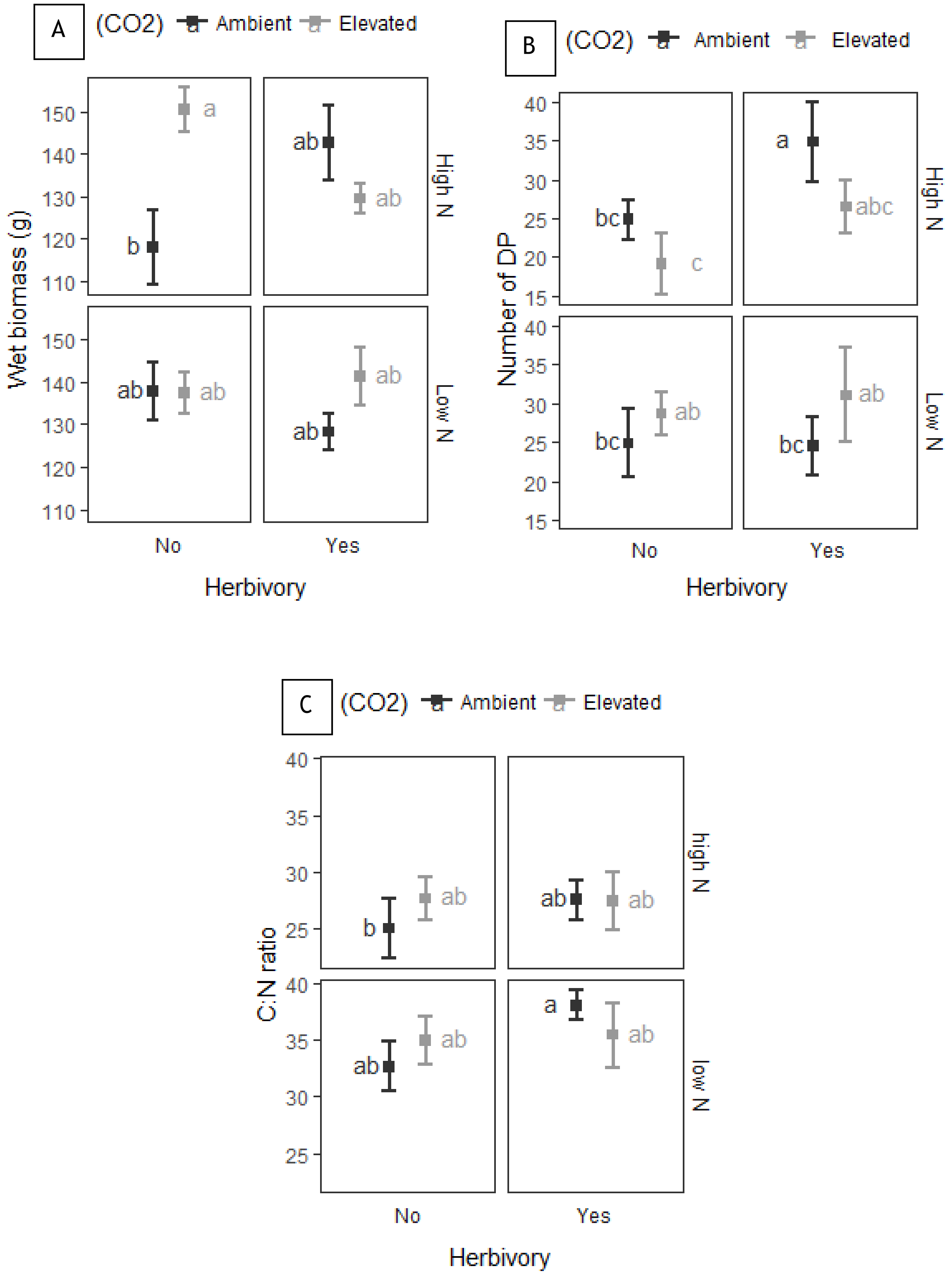


Figure 4.2. 1: Plant parameters of *Salvinia molesta* as affected by CO$_2$ (Ambient and Elevated), Nutrients (high N and low N) and Herbivory (No and Yes) in winter. (A) Wet biomass, (B) Number of side shoots and (C) C: N ratio. Values are means per tub ± SE, different letters indicate significant differences (P<0.05).

Table 4.2. 1: Summary of GLM results for the effects of CO_2, (CO_2; Ambient, Elevated), Nutrients (N; low N, high N), and Herbivory (HERB; No, Yes) on wet biomass production of *Salvinia molesta* at the end of the winter season experiment. Figures in bold indicate significant results.

Response	Factor	Estimate	Std. Error	t value	P-value
Mean wet biomass/tub (g)	(Intercept)	78	6.444	12.105	**5.94E-15**
	N	19.833	9.113	2.176	**0.03548**
	CO_2	32.333	9.113	3.548	**0.00101**
	HERB	64.5	9.113	7.078	**1.46E-08**
	N*CO_2	-32.667	12.887	-2.535	**0.01527**
	N*HERB	-34.167	12.887	-2.651	**0.01144**
	CO_2*HERB	-45.333	12.887	-3.518	**0.0011**
	N*CO_2*HERB	58.833	18.225	3.228	**0.00249**
Mean number of side shoots/tub	(Intercept)	3.21E+00	8.19E-02	39.21	**<2e-16**
	N	7.54E-12	1.16E-01	0	1
	CO_2	-2.59E-01	1.24E-01	-2.087	**0.0369**
	HERB	3.38E-01	1.07E-01	3.156	**0.0016**
	N*CO_2	4.03E-01	1.67E-01	2.409	**0.016**
	N*HERB	-3.52E-01	1.58E-01	-2.225	**0.0261**
	CO_2*HERB	-1.44E-02	1.63E-01	-0.089	0.9294
	N*CO_2*HERB	1.06E-01	2.26E-01	0.469	0.6388
C: N ratio	(Intercept)	25.045	2.223	11.267	**7.84E-14**
	N	7.583	3.143	2.412	**0.0207**
	CO_2	2.658	3.143	0.846	0.4029
	HERB	2.528	3.143	0.804	0.4261
	N*CO_2	-0.405	4.446	-0.091	0.9279
	N*HERB	2.839	4.555	0.623	0.5367
	CO_2*HERB	-2.803	4.446	-0.631	0.532
	N*CO_2*HERB	-2.039	6.365	-0.32	0.7504

4.2.2. Summer

There was an increase in total dry weight at eCO_2 compared with aCO_2 for the control treatment (Figure 4.2.2a, Table 4.2.2). Dead weight did not show any significant response to this treatment (Figure 4.2.2b). In the presence of herbivory however, both measured plant parameters were not affected significantly by changes in CO_2 (Figure 4.2.2, Table 4.2.2). Furthermore, dry weight was reduced by the addition of herbivory, but only at eCO_2 (Figure

4.2.2a). The opposite relationship was observed for dead weight at eCO_2, while at aCO_2, deadweight had no significant response to herbivory (Figure 4.2.2b). Lastly, the C: N ratio of *S. molesta* was not significantly affected by CO_2 manipulation in the control treatment. However, upon addition of herbivory, there was an increase in C: N ratio at eCO_2 compared with aCO_2 (Figure 4.2.2c, Table 4.2.2).

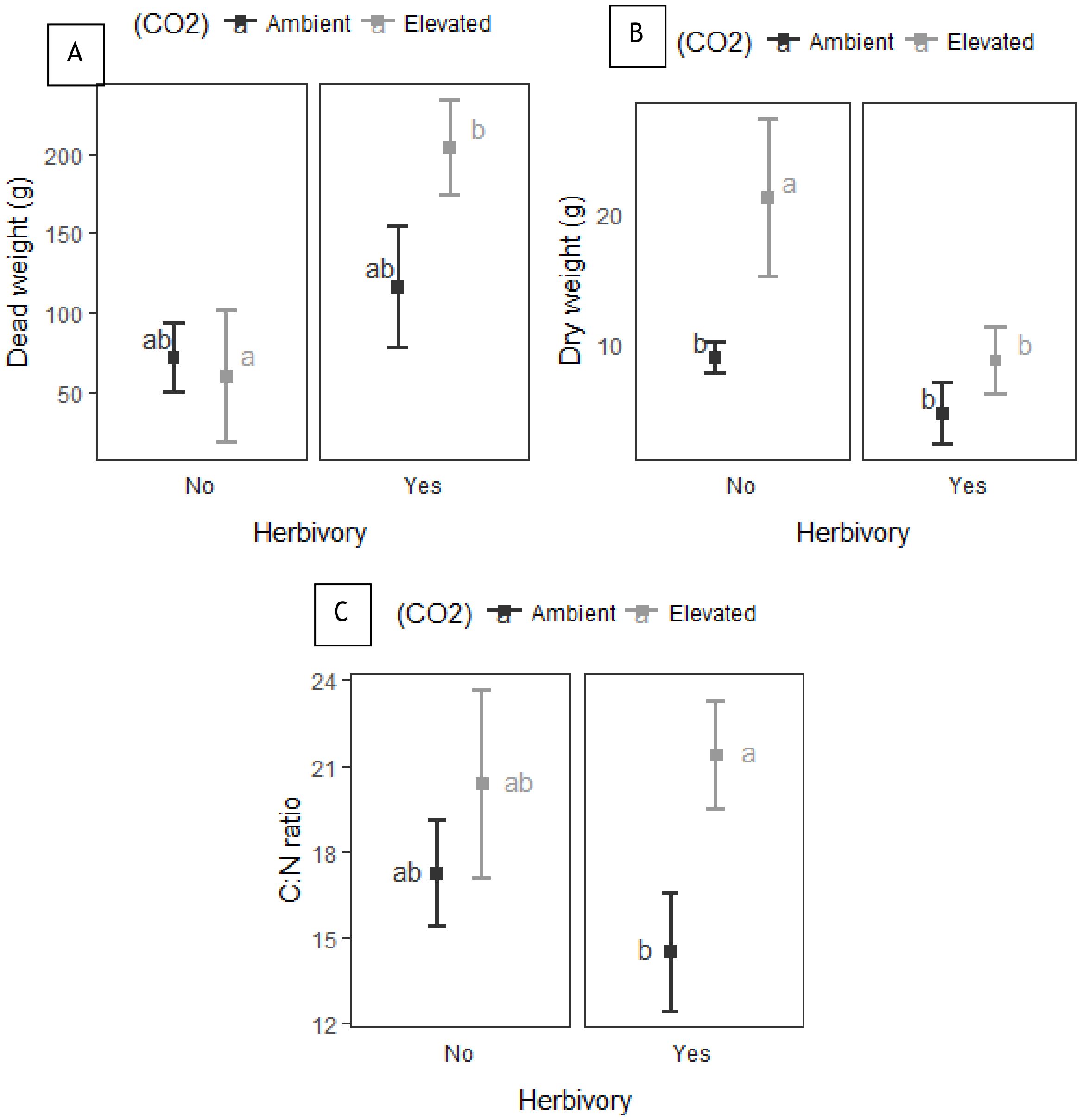


Figure 4.2. 2: Plant parameters for *Salvinia molesta* in summer as affected by CO_2 (Ambient and Elevated), and Herbivory (No and Yes). (A) Dry weight, (B) Dead weight and (C) C: N ratio. Values are means per tub ± SE, different letters indicate significant differences ($P<0.05$).

Mean percent damage caused by *C. salviniae* on *S. molesta* was not significantly affected by CO_2 manipulation due to high variability (t value $_{(1,\,12)}$ = -0.768, *P*=0.4169) (Figure 4.2.3).

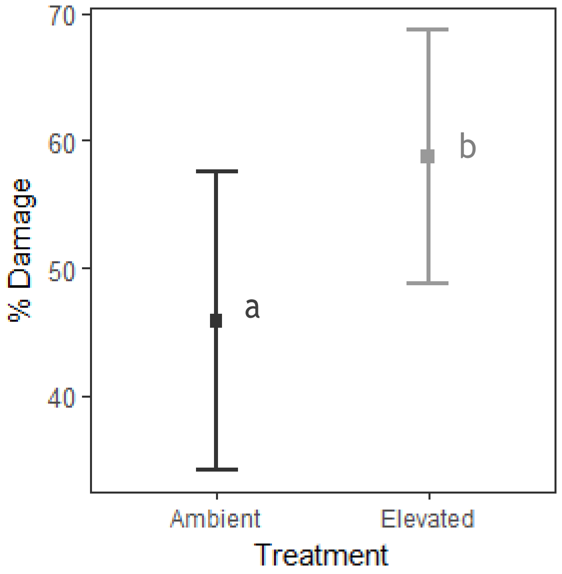


Figure 4.2. 3: Mean percent damage caused by *Cyrtobagous salviniae* on *Salvinia molesta* in summer as affected by changes in CO_2 concentration. Error bars= SE, different letters indicate significant differences (*P*<0.05).

Table 4.2. 2: Summary of GLM results for the effects of CO_2, (CO_2; Ambient, Elevated) and Herbivory (HERB; No, Yes) on biomass production by *Salvinia molesta* at the end of the summer season experiment. Figures in bold indicate significant results.

Response	Factor	Estimate Std	Error	t value	p-value
Dry biomass per tub (g)	(Intercept)	0.11278	0.01546	7.294	**3.01E-13**
	CO_2	-0.06572	0.01601	-4.104	**4.06E-05**
	HERB	0.10781	0.04503	2.394	**0.0167**
	CO_2*HERB	-0.03948	0.04797	-0.823	0.4105
Dead weight per tub (g)	(Intercept)	2.1823	0.3312	6.59	**2.03E-06**
	CO_2	0.8741	0.3942	2.217	**0.0384**
	HERB	-0.6708	0.5693	-1.178	0.2525
	CO_2*HERB	-0.226	0.6943	-0.326	0.7482
C:N ratio	(Intercept)	2.8477	0.0983	28.97	**<2e-16**
	CO_2	0.1654	0.1336	1.238	0.216
	HERB	-0.1749	0.1455	-1.202	0.229
	CO_2*HERB	0.2239	0.1928	1.162	0.245

4.2.3. Winter vs. Summer

Mean wet biomass produced by *S. molesta* was increased by eCO_2 for the control treatment in the winter season (Figure 4.2.1a). However, in the presence of herbivory, there was no significant response to CO_2 (Figure 4.2.1a). For the summer experiment, none of the treatments showed a significant response to CO_2 manipulation for this plant species (Figure 4.2.2b), although there was a herbivory effect at aCO_2. Furthermore, the C: N ratio of *S. molesta* was not affected by changes in CO_2 for the control plants during both seasons (Figure 4.2.2a). However, the addition of herbivory increased the measured C: N ratio under eCO_2 in the winter season, while no response was observed in summer.

4.3. *Pistia stratiotes*

4.3.1. Winter

Biomass production by *P. stratiotes* was increased by the addition of nutrients to plants grown at eCO_2 compared with aCO_2 when control agents were absent. Within the herbivory treatment, only the low nutrient treatment triggered a positive response to eCO_2 compared with aCO_2 (Figure 4.3.1a, Table 4.3.1). Leaf production, on the other hand, showed a reduction at eCO_2 compared with aCO_2 under high nutrients for the control treatment (Figure 4.3.1b, Table 4.3.1). There was also a positive response to nutrient addition at eCO_2 for the herbivory treatment. When comparing between treatments, leaf production had a positive response to the addition of herbivory at eCO_2 provided that nutrients were not limiting (Figure 4.3.1b). Nevertheless, the mean number of side shoots produced by *P. stratiotes* was not significantly affected by any of the treatment combinations (Figure 4.3.1c, Table 4.3.1). Lastly, the C: N ratio of this species was higher at eCO_2 and high nutrients for the control treatment (Figure 4.3.1d). The introduction of herbivory to this treatment resulted in a reduction of this plant parameter. There were no significant responses measured in the other treatments (Figure 4.3.1d, Table 4.3.1).

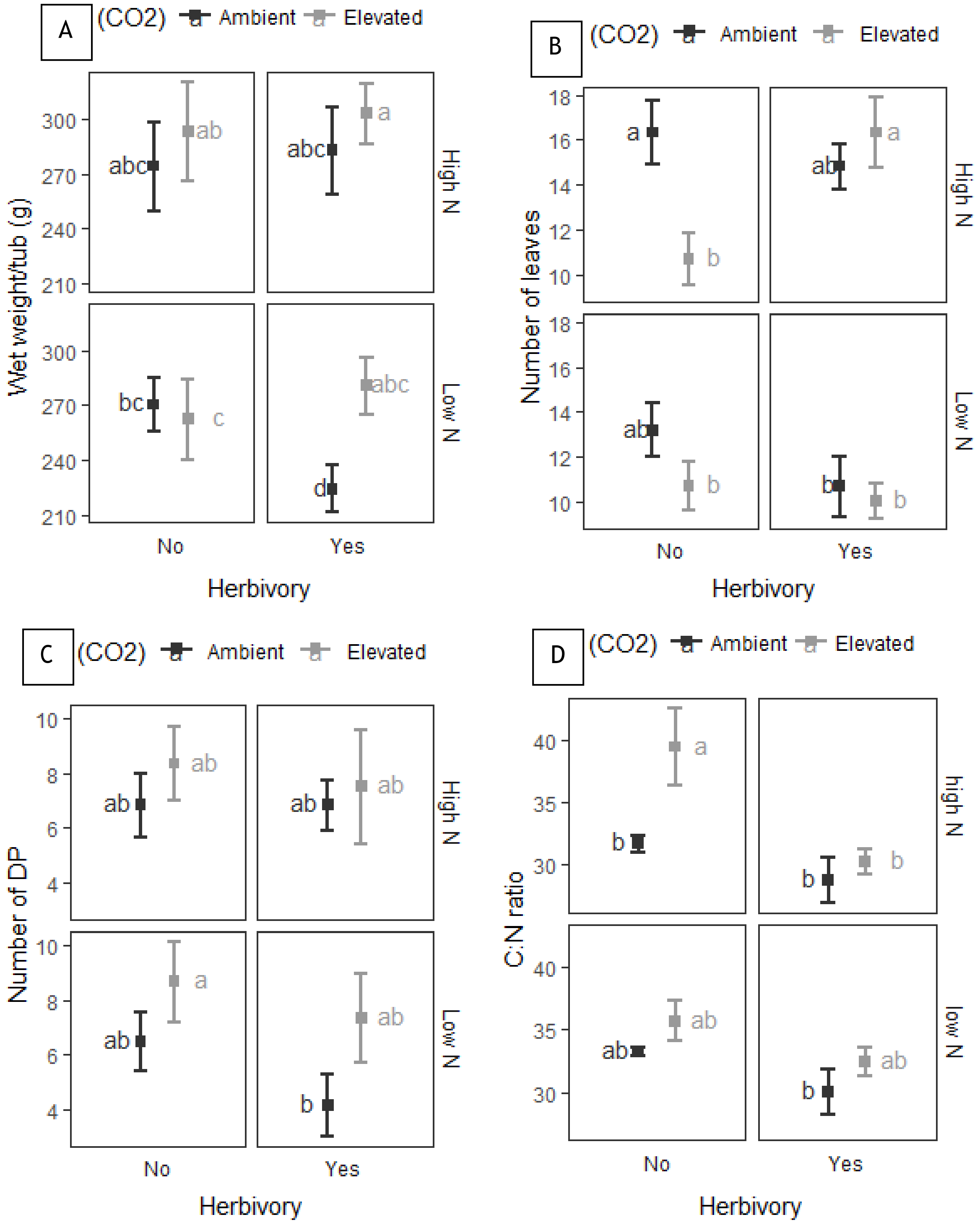


Figure 4.3. 1: Plant parameters of *Pistia stratiotes* as affected by CO_2 (Ambient and Elevated), Nutrients (high N and low N) and Herbivory (No and Yes) in winter. (A) Wet biomass, (B) Number of leaves, (C) Number of side shoots (DP) and (D) C: N ratio. Values are means per tub ± SE, different letters indicate significant differences ($P<0.05$).

Table 4.3. 1: Summary of GLM results for the effects of CO_2, (CO_2; Ambient, Elevated), Nutrients (N; low N, high N), and Herbivory (HERB; No, Yes) on plant parameters of *Pistia stratiotes* at the end of the winter season experiment. Figures in bold indicate significant results.

Response	Factor	Estimate	Std. Error	t value	p-value
Wet weight per tub (g)	(Intercept)	274.167	20.248	13.541	**<2e-16**
	N	-3.667	28.634	-0.128	0.899
	CO_2	18.667	28.634	0.652	0.518
	HERB	8.5	28.634	0.297	0.768
	N*CO_2	-26.833	40.495	-0.663	0.511
	N*HERB	-54.667	40.495	-1.35	0.185
	CO_2*HERB	1.5	40.495	0.037	0.971
	N*CO_2*HERB	63	57.269	1.1	0.278
Number of leaves per tub	(Intercept)	2.79321	0.08404	33.236	**< 2e-16**
	N	-0.21552	0.1258	-1.713	0.09441
	CO_2	-0.42608	0.13371	-3.187	**0.00279**
	HERB	-0.09633	0.12182	-0.791	0.43374
	N*CO_2	0.21552	0.19353	1.114	0.27209
	N*HERB	-0.11423	0.18552	-0.616	0.54154
	CO_2*HERB	0.52242	0.18088	2.888	**0.00623**
	N*CO_2*HERB	-0.37639	0.27321	-1.378	0.17598
Number of side shoots per tub	(Intercept)	1.92E+00	1.56E-01	12.306	**<2e-16**
	N	-5.00E-02	2.24E-01	-0.224	0.823
	CO_2	1.99E-01	2.11E-01	0.942	0.346
	HERB	-1.40E-16	2.21E-01	0	1
	N*CO_2	8.92E-02	2.99E-01	0.299	0.765
	N*HERB	-4.45E-01	3.38E-01	-1.315	0.189
	CO_2*HERB	-1.05E-01	3.02E-01	-0.349	0.727
	N*CO_2*HERB	3.83E-01	4.46E-01	0.859	0.39
C: N ratio	(Intercept)	3.456369	0.049846	69.34	**< 2e-16**
	N	0.047034	0.069679	0.675	0.50356
	CO_2	0.217439	0.066961	3.247	**0.00236**
	HERB	-0.09814	0.072288	-1.358	0.1822
	N*CO_2	-0.14635	0.095195	-1.537	0.13209
	N*HERB	-0.00396	0.101101	-0.039	0.96897
	CO_2*HERB	-0.16606	0.099137	-1.675	0.10172
	N*CO_2*HERB	0.172232	0.139506	1.235	0.22419

4.3.2 Summer

The photosynthetic rate of *P. stratiotes* was reduced by eCO_2 for both herbivory treatments (Figure 4.3.2a, Table 4.3.2). Furthermore, the addition of herbivory increased the photosynthetic activity of *P. stratiotes* for both CO_2 treatments relative to plants that did not experience herbivory. There was an increase in mean total dry weight per tub when CO_2 was increased for the control plants (Figure 4.3.2b, Table 4.3.2). The same increase was observed in the presence of herbivory in this treatment (Figure 4.3.2). On the other hand, the mean mass of side shoots per tub was not significantly affected by CO_2 manipulation for both herbivory treatments (Figure 4.3.2d). Herbivory did play an important role in reducing total weight, and the mass of side shoots per tub as it reduced overall plant mass growth regardless of CO_2 concentration (Figure 4.3.2, Table 4.3.2). Lastly, the C: N ratio in the leaves of *P. stratiotes* was higher at eCO_2 for both herbivory treatments, but was only significant in the presence of herbivory (Figure 4.3.2e, Table 4.3.2). In terms of feeding, the damage caused by *N. affinis* on *P. stratiotes* was significantly reduced by eCO_2 (t value $_{(1, 10)}$ = -2.674, P=0.0233) (Figure 4.3.3).

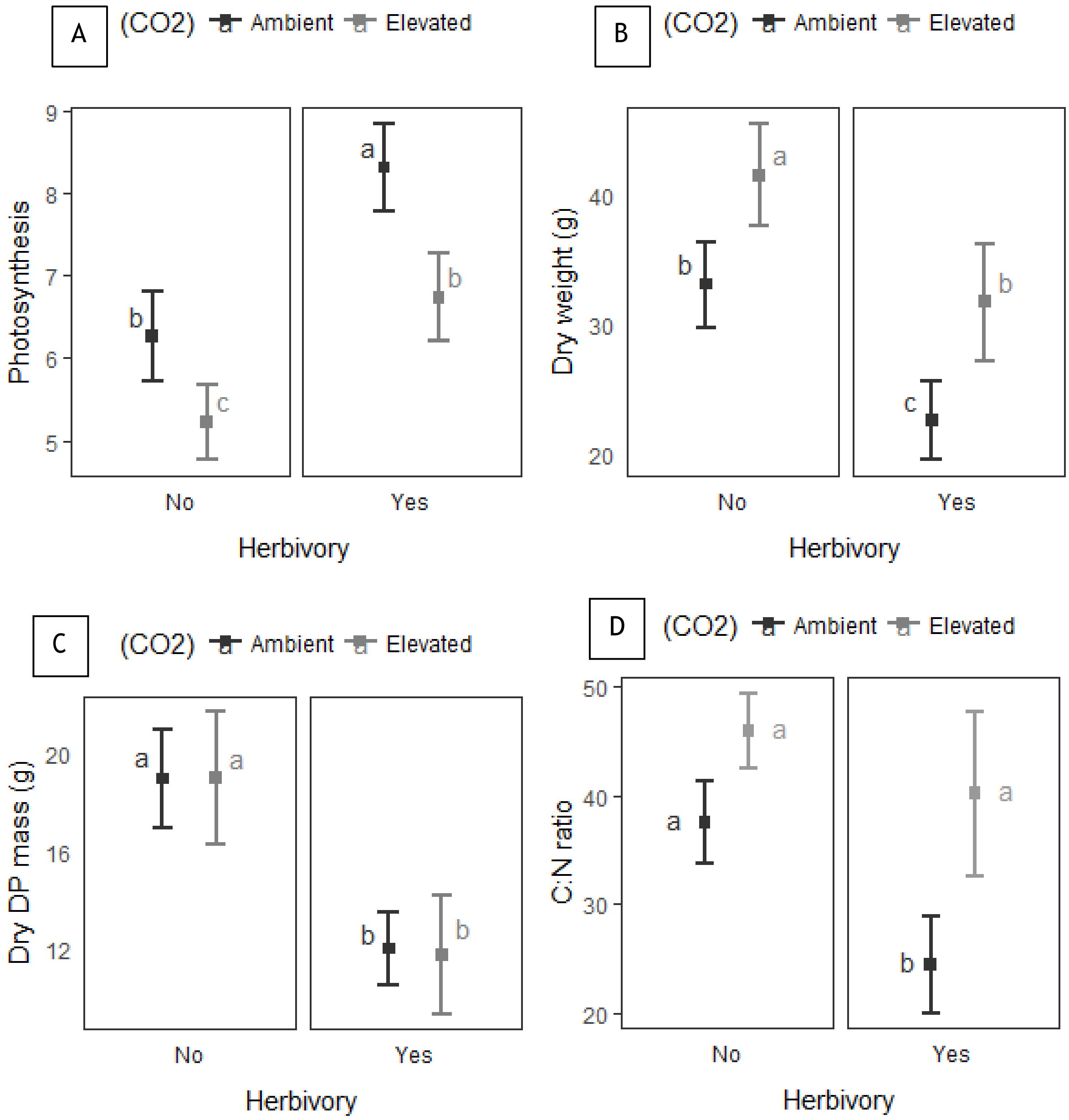


Figure 4.3. 2: Plant parameters for *Pistia stratiotes* in summer as affected by CO_2 (Ambient and Elevated), and Herbivory (No and Yes). (A) Rate of photosynthesis, (B) Dry weight, (C) Dry mass of side shoots and (D) C: N ratio. Values are means per tub ± SE, different letters indicate significant differences (P<0.05).

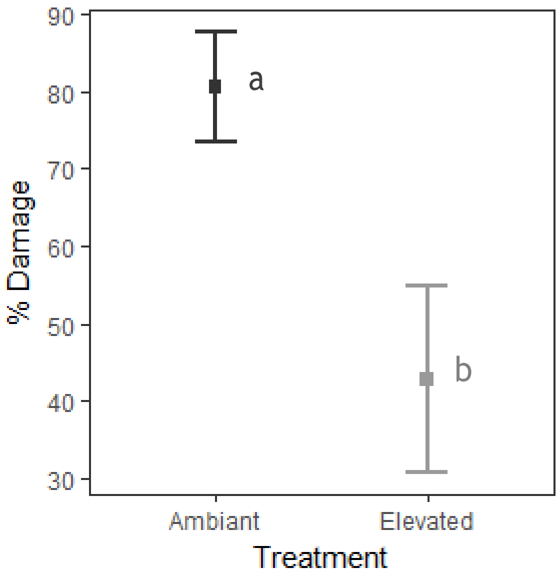


Figure 4.3. 3: Mean percent damage caused by *Neohydronomus affinis* on *Pistia stratiotes* in summer as affected by changes in CO_2 concentration. Error bars= SE, different letters indicate significant differences (*P*<0.05).

Table 4.3. 2: Summary of GLM results for the effects of CO_2, (CO_2; Ambient, Elevated) and Herbivory (HERB; No, Yes) on biomass production by *Pistia stratiotes* at the end of the summer season experiment. Figures in bold indicate significant results.

Species	Factor	Estimate Std	Error	t value	p-value
Photosynthesis	(Intercept)	1.83485	0.07858	23.349	**< 2e-16**
	CO_2	-0.1807	0.11868	-1.523	0.13057
	HERB	0.28297	0.10481	2.7	**0.00797**
	CO_2*HERB	-0.02955	0.15757	-0.188	0.85155
Dry weight per tub (g)	(Intercept)	3.4995	0.1158	30.224	**<2e-16**
	CO_2	0.2282	0.1552	1.471	0.1569
	HERB	-0.3837	0.1819	-2.11	**0.0477**
	CO_2*HERB	0.1134	0.2403	0.472	0.642
Dry side shoot mass per tub (g)	(Intercept)	2.941804	0.129553	22.707	**9.44E-16**
	CO_2	0.001757	0.183135	0.01	0.992
	HERB	-0.45412	0.207882	-2.185	**0.041**
	CO_2*HERB	-0.02275	0.294891	-0.077	0.939
C: N ratio	(Intercept)	3.6247	0.06665	54.38	**< 2e-16**
	CO_2	0.20126	0.08987	2.24	**0.0251**
	HERB	-0.42412	0.10598	-4.002	**6.29E-05**
	CO_2*HERB	0.29089	0.14088	2.065	**0.0389**

4.3.3. Winter vs Summer

Mean biomass production for both growing seasons was not significantly affected by CO_2 manipulation both in the presence and absence of herbivory, although there was an effect of herbivory at aCO_2 for the summer experiment (Figure 4.3.2b). The C: N ratio of *P. stratiotes* was higher at eCO_2 for the control plants during the winter season (Figure 4.3.1a). The addition of herbivory reduced the measured C: N ratio at eCO_2, but this was not different from the aCO_2 treatment for the winter months. Although there were indications of increased C: N ratio at eCO_2 for both herbivory treatments, no significant responses were measured in summer (Figure 4.3.2b).

4.4. *Myriophyllum aquaticum*

4.4.1. Winter

The mean number of leaves produced by *M. aquaticum* was increased by the addition of nutrients to the growing medium for the herbivory treatment at aCO_2 (Figure 4.4.1a, Table 4.4.1). All the other treatment combinations had no significant effects on the number of leaves produced by *M. aquaticum*. Side shoot production, on the other hand, was only affected by herbivory at high nutrients and under aCO_2 (Figure 4.4.1b). Lastly, the C: N ratio present in the foliage of *M. aquaticum* was increased significantly by eCO2, but only for the high nutrient treatment and in the presence of herbivory (Figure 4.4.1c, Table 4.4.1).

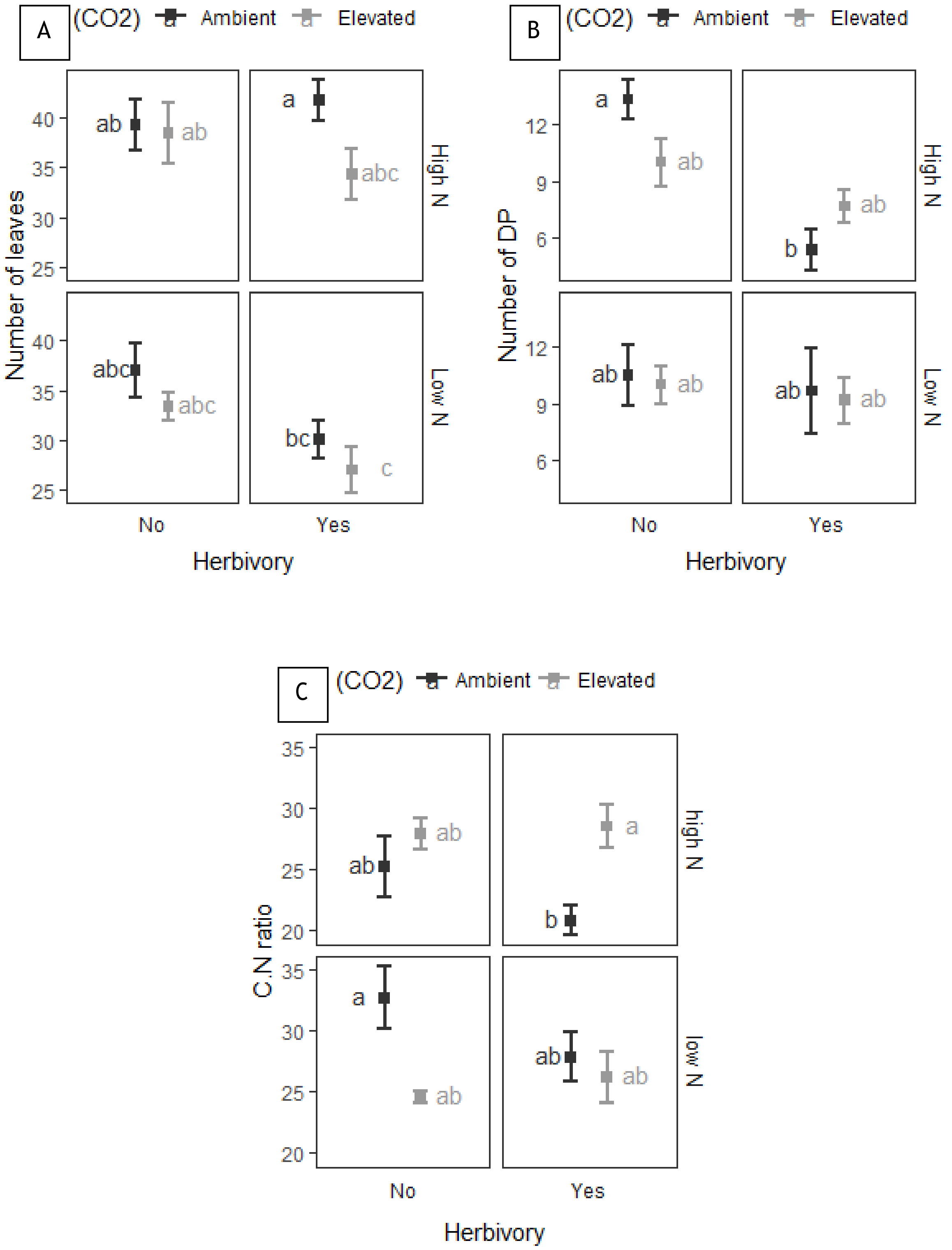


Figure 4.4. 1: Plant parameters of *Myriophyllum aquaticum* as affected CO_2 (Ambient and Elevated), Nutrients (high N and low N) and Herbivory (No and Yes) in winter. (A) Number of leaves, (B) Number of side shoots and (C) C: N ratio. Values are means per tub ± SE, different letters indicate significant differences (P<0.05).

Table 4.4. 1: Summary of GLM results for the effects of CO_2, (CO_2; Ambient, Elevated), Nutrients (N; low N, high N), and Herbivory (HERB; No, Yes) on plant parameters of *Myriophyllum aquaticum* at the end of the winter season experiment. Figures in bold indicate significant results.

Response	Factor	Estimate	Std. Error	t value	p-value
Number of leaves	(Intercept)	3.37872	0.07538	44.824	**< 2e-16**
	N	-0.08289	0.10888	-0.761	0.446489
	CO_2	-0.02882	0.10738	-0.268	0.788388
	HERB	0.35497	0.09832	3.611	**0.000306**
	N*CO_2	-0.11713	0.15762	-0.743	0.457411
	N*HERB	-0.24961	0.14627	-1.707	0.087912
	CO_2*HERB	-0.16876	0.14272	-1.182	0.237026
	N*CO_2*HERB	0.20935	0.2131	0.982	0.325906
Number of side shoots	(Intercept)	2.5903	0.1118	23.168	**< 2e-16**
	N	-0.2389	0.1684	-1.418	0.15612
	CO_2	-0.2877	0.1708	-1.684	0.09209
	HERB	-0.9163	0.2092	-4.381	**1.18E-05**
	N*CO_2	0.2389	0.2484	0.962	0.3362
	N*HERB	0.8336	0.2772	3.007	**0.00264**
	CO_2*HERB	0.6506	0.2866	2.27	**0.02322**
	N*CO_2*HERB	-0.6549	0.3874	-1.69	0.09097
C: N ratio	(Intercept)	0.03973	0.00274	14.501	**< 2e-16**
	N	-0.00913	0.003458	-2.641	**0.01175**
	CO_2	-0.00383	0.003693	-1.036	0.30649
	HERB	0.008478	0.004308	1.968	0.05602
	N*CO_2	0.014016	0.005099	2.749	**0.00894**
	N*HERB	-0.00313	0.0054	-0.579	0.56578
	CO_2*HERB	-0.00929	0.005527	-1.68	0.10077
	N*CO_2*HERB	0.001408	0.007485	0.188	0.85177

4.4.2. Summer

The photosynthetic activity of *M. aquaticum* was increased at eCO_2 compared with aCO_2, regardless of herbivory (Figure 4.4.2a, Table 4.4.2). Furthermore, the addition of herbivory reduced the photosynthetic activity of *M. aquaticum* at aCO_2 relative to plants that did not experience herbivory, while at eCO_2 there was no significant response of photosynthesis to this treatment. There was an increase in total dry weight (Figure 4.4.2b), mean mass of side shoots (Figure 4.4.2d) and the C: N ratio (Figure 4.4.2e) of *M. aquaticum* when CO_2 was increased for the control plants (Table 4.4.2). The same increase was observed in the presence of herbivory for dry weight and side shoot mass, while C: N ratio showed no significant response to this treatment (Figure 4.4.2). Herbivory played an important role in reducing total weight, the mass of side shoots, and the C: N ratio as it reduced overall plant mass regardless of CO_2 concentration, except for the aCO_2 treatment where herbivory did not change C: N ratio of *M. aquaticum*. The mean number of *Lysathia* sp. recovered from *M. aquaticum* was higher at eCO_2 ($z=4.202$, $P<0.0001$) (Figure 4.4.3a), while the opposite relationship was observed for the damage caused by these agents ($z=-8.066$, $P<0.0001$) (Figure 4.4.3b).

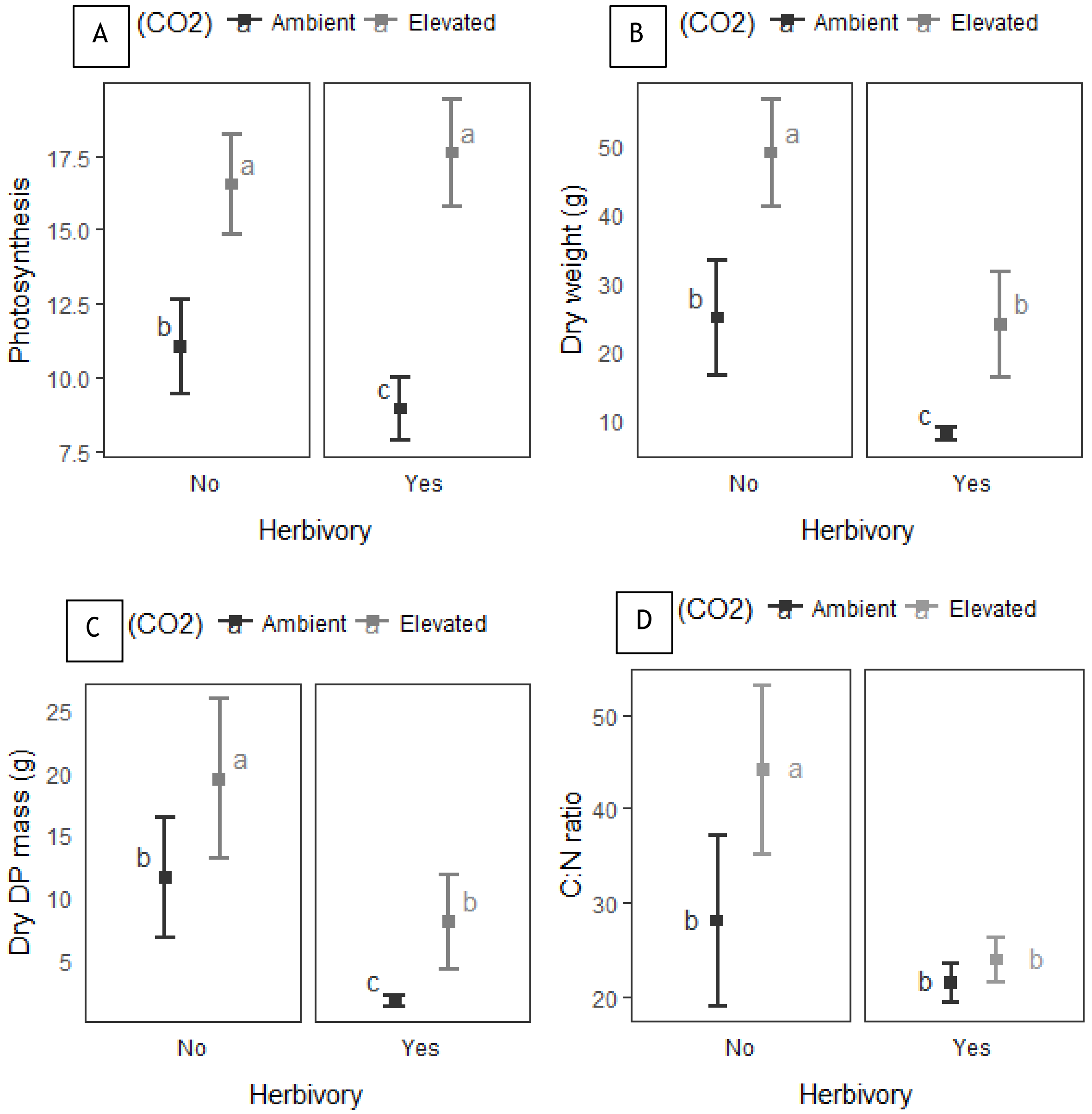


Figure 4.4. 2: Plant parameters of *Myriophyllum aquaticum* in summer as affected by CO$_2$ (Ambient and Elevated), and Herbivory (No and Yes). (A) Rate of photosynthesis, (B) Dry weight, (D) Dry mass of side shoots, (E) C: N ratio. Values are means per tub ± SE, different letters indicate significant differences (P<0.05).

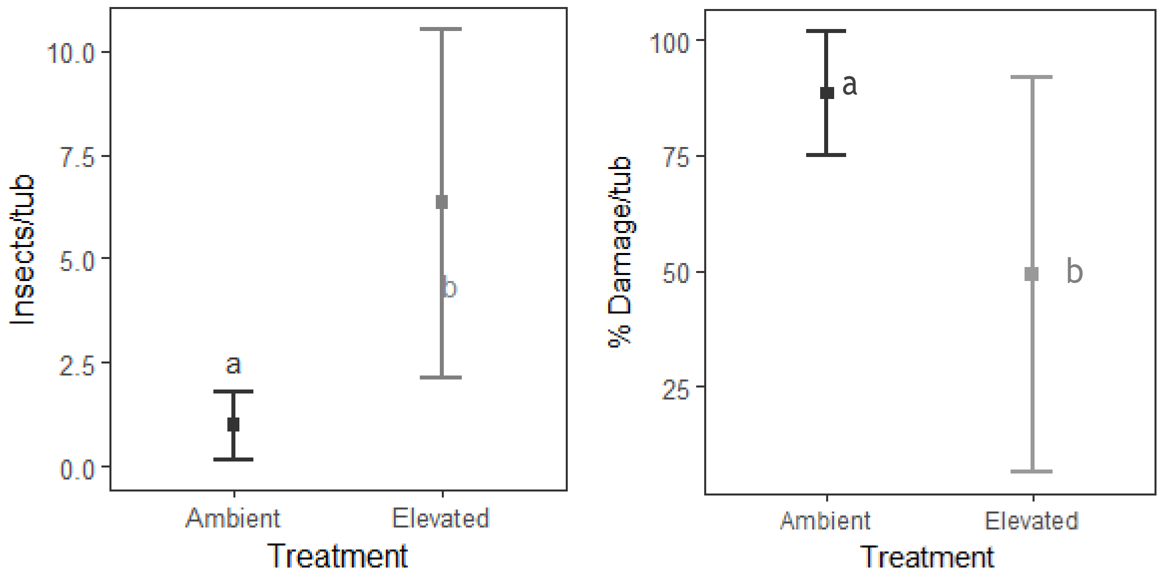


Figure 4.4. 3: (A) Mean number of insects per tub and (B) Mean damage caused by *Lysathia* sp. on *Myriophyllum aquaticum* in summer as affected by changes in CO_2 concentration. Error bars= SE, different letters indicate significant differences (*P*<0.05).

Table 4.4. 2: Summary of GLM results for the effects of CO_2, (CO_2; Ambient, Elevated), Nutrients (N; low N, high N), and Herbivory (HERB; No, Yes) on biomass production by *Myriophyllum aquaticum* at the end of the summer season experiment. Figures in bold indicate significant results.

Species	Factor	Estimate Std	Error	t value	p-value
Photosynthesis	(Intercept)	2.4036	0.1248	19.262	**<2e-16**
	CO2	0.4018	0.1676	2.398	**0.0182**
	HERB	-0.2112	0.1883	-1.121	0.2645
	CO2*HERB	0.2731	0.2403	1.136	0.2583
Dry weight per tub (g)	(Intercept)	3.2162	0.2562	12.554	**6.10E-11**
	CO_2	0.6708	0.3149	2.13	**0.0458**
	HERB	-1.1347	0.5194	-2.185	**0.041**
	CO_2*HERB	0.4236	0.6096	0.695	0.4951
Dry side shoot mass per tub (g)	(Intercept)	2.4496	0.3578	6.846	**1.18E-06**
	CO_2	0.5174	0.452	1.145	0.2659
	HERB	-1.89	0.9876	-1.914	0.0701
	CO_2*HERB	1.0066	1.1119	0.905	0.3761
C: N ratio	(Intercept)	3.33405	0.07708	43.254	**< 2e-16**
	CO_2	0.45392	0.09856	4.605	**4.12E-06**
	HERB	-0.27183	0.11721	-2.319	**0.0204**
	CO_2*HERB	-0.34296	0.15649	-2.192	**0.0284**

4.4.3 Winter vs. Summer

For the winter experiment, none of the treatments showed a response to CO_2 manipulation, although there was a herbivory effect (Figure 4.4.1). Mean side shoot biomass production was however increased by eCO_2 for both herbivory treatments in the summer season (Figure 4.4.2). The C: N ratio of *M. aquaticum* was higher at eCO_2 for the control plants during both seasons, although not significant for the winter experiment. The addition of herbivory reduced the measured C: N ratio at eCO_2 for the summer months. But this was only different from the aCO_2 treatment for the winter experiment (Figure 4.4.1).

4.5. Summary interspecific comparisons: Winter

Mean biomass production in the winter season was increased by eCO_2 for *A. filiculoides* and *S. molesta* in the control treatment (Figure 4.1.1a & 4.2.1a). However, *P. stratiotes* (Figure 4.3.1a) and *M. aquaticum* (Figure 4.4.1a) showed no significant response to CO_2 within this treatment. The relationship did not change for *A. filiculoides* upon the addition of herbivory, while all the other species were not significantly affected by CO_2 manipulation (Table 4.5.1). The C: N ratio measured in the winter experiment had a varied response to eCO_2 and herbivory across the different species. In the absence of herbivory, *A. filiculoides* had a higher C: N ratio at aCO_2 (Figure 4.1.1), while for *P. stratiotes*, an increased C: N was observed at eCO_2 (Figure 4.3.1). On the other hand, *S. molesta* and *M. aquaticum* had no significant responses to CO_2 manipulation (Figure 4.2.1). The addition of herbivory led to a reduction in C: N at eCO_2 only for *P. stratiotes*. When comparing aCO_2 and eCO_2 within the herbivory treatment, only *M. aquaticum* had a higher C: N ratio at eCO_2, all the other species had no significant response to this treatment.

Table 4.5. 1: A summary table showing overall plant species and biological control agent responses to changes in CO_2 and nutrient concentration during winter for the herbivory treatment (with special reference to plant biomass).

Species	**Ambient CO_2**				**Elevated CO_2**			
	Low N		**High N**		**Low N**		**High N**	
	Plant growth	Agent response	Plant growth	Agent response	Plant growth	Agent response	Plant growth	Agent response
Azolla filiculoides	↓	↓	↓	↓	↑	↓	↑	↓
Salvinia molesta	→	↓	→	↓	→	↓	→	↓
Pistia stratiotes	↓	↓	→	↓	↑	↓	→	↓
Myriophyllum aquaticum	→	↓	→	↓	→	↓	→	↓

4.6. Summary interspecific comparisons: Summer

Mean biomass production in the summer experiment showed a varied response to changes in CO_2 concentration for the different species. Specifically, *A. filiculoides* (Figure 4.1.2a) was only responsive to CO_2 manipulation when biological control agents were present, while the reverse was true for *S. molesta* (Figure 4.2.2a) and *P. stratiotes* (Figure 4.3.2a) (Table 4.6.1). It was only *M. aquaticum* that showed a positive response to eCO_2 regardless of whether herbivory was present or absent (Figure 4.4.2b) (Table 4.6.1). The C: N ratio measured in the summer experiment was generally higher at eCO_2 across the different species. More specifically, for the control treatment, *A. filiculoides* (Figure 4.2.2b) and *M. aquaticum*

(Figure 4.4.2e) had a higher C: N ratio at eCO_2. The addition of herbivory did not change this relationship, although it was only significant for *S. molesta* (Figure 4.2.2c). Lastly, much like the C: N ratio, insect responses in summer followed a similar trend across species, where eCO_2 had a negative impact on the damage that the control agents were able to inflict on the plants. Only *C. salviniae*, the biological control agent of *S. molesta*, was not affected by eCO_2 as it caused the same extent of damage to the plants regardless of the CO_2 concentration (Figure 4.2.3).

Table 4.6. 1: A summary table showing overall plant species and biological control agent response to CO_2 manipulation during summer for the herbivory treatment (with special reference to plant biomass and insect damage).

Species	**Ambient CO_2**		**Elevated CO_2**	
	Plant growth	**Agent response**	**Plant growth**	**Agent response**
Azolla filiculoides	⬇	⬆	⬆	⬇
Salvinia molesta	➡	➡	➡	➡
Pistia stratiotes	⬇	⬆	⬆	⬇
Myriophyllum aquaticum	⬇	⬆	⬆	⬇

Chapter 5: Discussion and conclusions

Scholars across different fields agree that climate change is happening (Ehleringer & Cerling, 2002; O'Brien & Leichenko, 2000; Sage & Coleman, 2001; IPCC, 2007), with major consequences already being felt by the most vulnerable of communities (Kiker, 2000; Reuveny, 2007; Bryan et al., 2009; Gregory et al., 2009; Dube et al., 2013; Dennis & Dennis, 2012). Furthermore, results from Malheiro et al. (2013) and Gufu et al. (2019), indicate that aquatic macrophytes have the capacity to respond to increased atmospheric CO_2 concentration in much the same way that terrestrial plants do.

Thus, the aim of this thesis was to investigate the response of four aquatic invasive weeds; *A. filiculoides, S. molesta, P. stratiotes* and *M. aquaticum* and their respective biological control agents (*S. rufinasus, C. salviniae, N. affinis* and *Lysathia* sp.) to high CO_2 concentrations. This was prompted by the need to predict whether the current suite of control agents will still be suitable under future climates predicted by the IPCC climate models in the next 100 years.

The initial hypothesis was that due to their C3 photosynthetic pathway (Ray et al., 1979; Longstreth, 1989), these macrophytes would have increased growth and reproduction, and also be more defended against herbivory by their respective control agents when grown under increased atmospheric CO_2 concentrations, making it more challenging for the insects to control these plants in future climates. There were noticeable differences as well as similar trends in responses between species and between seasons within the same species. These results are discussed below, as well as their implications on biological control programmes, in the context of both the regional and the international sphere.

5.1. *Azolla filiculoides*

Redwater fern, *A. filiculoides*, has received considerable research focus in terms of how elevated CO_2 affects its growth (Allen et al., 1988; Cheng et al., 2010; van Kempen et al., 2016; Gufu et al., 2019) compared with other floating invasive macrophytes such as *P. stratiotes* and *S. molesta*. This is mainly because of its agricultural significance as a natural fertiliser in rice fields, and not as an invasive weed. The current study will contribute to rectifying this, in order to ensure that the invasive aspect of this weed is not neglected in that we have a better understanding of how the projected changes in atmospheric CO_2 will influence the relationship between this plant and its current biological control agent.

Given the fast-reproductive growth rate and nitrogen-fixing capacity of *A. filiculoides* (Lumpkin & Plucknett, 1982), it is important that we investigate how biological control of this invasive aquatic weed will be affected by elevated CO_2. Therefore, one of my objectives for this project was to investigate how carbon assimilation and nitrogen accumulation in *A. filiculoides* was affected by increased atmospheric CO_2 in combination with added nutrients, and how this affected the efficacy of *S. rufinasus* as a biological control agent of this weed. For many plant communities, both nitrogen and CO_2 availability limit photosynthesis (Cheng et al., 2010). The carbon limitation is, however, expected to be reduced under elevated CO_2. Nitrogen-fixing plant species like *A. filiculoides* will likely benefit much more fully from elevated CO_2, especially if other nutrients such as phosphorus are not limiting (Cheng et al., 2010).

Indeed, similar to findings by Cheng et al. (2010) and Gufu et al. (2019), the growth of *A. filiculoides* was significantly stimulated by increased CO_2 concentrations during both experimental seasons. In winter, this plant was responsive to CO_2 manipulations at both nutrient concentrations. However, the difference between eCO_2 and aCO_2 was unconventionally more pronounced for the low nutrient treatment. This is possibly because, under these conditions, the plants experienced more stress and therefore depended on, and responded more, to the supplementary CO_2 with a biomass increase of up to 45 %. Certainly, *A. filiculoides* would be able to show this type of response as it has a symbiotic relationship with a group of highly effective nitrogen-fixing bacteria that is also able to supplement *A. filiculoides* with phosphorus (Lumpkin & Plucknett, 1982).

This idea of an increased plant response to CO_2 under low nutrients has not yet been explored much in existing literature on aquatic plants. However, increased nutrient uptake as a result of eCO_2 has been shown in terrestrial plants, for both nitrogen and phosphorus (Hasegawa et al., 2016). Even in nitrogen-limited environments, *A. filiculoides* exhibits a strong response to eCO_2. Similar results were also reported by Gufu et al. (2019) where using the same species, they found that even at low nutrients, *A. filiculoides* had a large quantity of nitrogen supply due to its symbiotic relationship with the nitrogen-fixing *Anabaena azollae.* Hence this allowed the plant to be able to make use of the added CO_2, even at low nutrient availability.

However, in summer, where all plants were grown under eutrophic conditions, *A. filiculoides* was only responsive to changes in CO_2 concentration in the presence of herbivory, with both dry weight and dead weight following a similar trend. In this case there were two extremes; on the one hand, there was a population boom of the biological control agent at aCO_2, which led to a complete collapse of the plant biomass, while on the other hand, the fertilization effect of eCO_2 led to the flourishing of *A. filiculoides* at the expense of the control agents. A meta-analysis conducted by Stiling and Cornelissen (2007) on how high CO_2 affects plant- herbivore interactions found that eCO_2 reduced herbivore abundance on the treated plants. This was an expected result as this is a nitrogen fixer and is, therefore, able to engage more successfully in compensatory growth under these growth conditions while also increasing its qualitative defense mechanisms.

Chlorophyll content was 29% higher at eCO_2 compared with aCO_2 for the low nutrient treatment without herbivory. This could be an indication of increased photosynthesis, which was expected under high CO_2 (Woodward, 2002; Gregory et al., 2009; Robison et al., 2012). However, at high nutrients and in the presence of biological control, this trend was reversed to reveal a higher chlorophyll content at aCO_2. Although unconventional, this can be explained by the increased availability of nitrogen and phosphorus, which in turn allowed for more enzyme activation for the production of chlorophyll within the leaf (Gufu et al., 2019).

The response of the biological control agent, *S. rufinasus* was not easy to quantify during the winter experiment as it was not as active during this time. Even so, there was stimulated growth of *A. filiculoides* when the agent was added to low nutrient plants regardless of the CO_2 concentrations. Thus, feeding by *S. rufinasus* triggered a compensatory growth response within this nutrient treatment (Coetzee et al., 2011). The C: N ratio was highly variable during winter, especially for the aCO_2 treatments, hence no reliable patterns were observed in this regard. However as expected, in summer eCO_2 increased the C: N ratio of *A. filiculoides* by 25% for the no herbivory treatment compared with aCO_2, due to the increased availability of carbon which resulted in dilution of nitrogen. An equivalent response has also been shown by Stiling and Cornelissen (2007).

A similar pattern was expected in the presence of herbivory but could not be measured as *S. rufinasus* is highly aggressive under ideal conditions (Hill, 2003; McConnachie et al., 2004); it ate all available plant biomass in the aCO_2 treatment. A simple way to sum up the response of *A. filiculoides* and its biological control, *S. rufinasus* to future CO_2 concentrations would be to say that the plant is likely to become more problematic, both in nutrient-limited environments, but more so in eutrophic aquatic systems (Hill & Olckers, 2001; van Kempen et al., 2016), which make up more than 60% of all large standing water-bodies in South

Africa (Matthews & Bernard., 2015). In other words, this is where the currently effective biological control agent might struggle to control this alien invasive weed in future.

Additional biological control agents might need to be introduced in order to ensure continued control of *A. filiculoides* in South Africa. One potential agent is the flea beetle, *Pseudolampsis darwini* Scherer (Coleoptera: Chrysomelidae). This insect was first considered for biological control of *A. filiculoides* in the country in 1995. However, the current biological control agent *S. rufinasus* performed so well in quarantine that it was decided that after its release, its impact needed to be fully evaluated before further research could be carried out on other insects (Cilliers et al., 2003).

Indeed, *S. rufinasus* was able to bring complete control of the weed in many parts of Africa, at least for the last 20 years (Hill & Coetzee 2017). Cilliers et al. (2003) believed that *A. filiculoides* would no longer be a problem for African water-bodies, and no additional agents would be required as the performance of *S. rufinasus* had been outstanding. Thus far, the biological control programme against *A. filiculoides* is still regarded as the most successful programme in controlling alien invasive species in the country (Coetzee et al., 2011). However, besides introducing new biological control agents, under elevated CO_2, the most effective way to control *A. filiculoides* might be to reduce phosphorus-enrichment in our freshwater ecosystems (Hill & Olckers, 2001). This could be implemented by putting in place regulatory clauses on the use and disposal of any phosphorus-containing products.

5.2. *Salvinia molesta*

From inception, biological control of *S. molesta* by *C. salviniae* in South Africa, and throughout the world, has mostly been excellent, except for a few specific areas where cooler temperatures increased the time taken to control the weed (Julien & Bourne, 1986; Room, 1990; Cilliers, 1991b; Cilliers et al., 2003; Sullivan & Postle, 2010). For example, one site was cleared in just over a year after the release of *C. salviniae* in South Africa, while two other sites at which the agent was introduced took less than two years to be cleared of *S. molesta* (Cilliers, 1987). This weed is now considered to be under complete control in South Africa (Martin et al., 2018), meaning that there is no need for additional control measures besides occasional redistribution of *C. salviniae* to newly discovered infested sites (Cilliers, 1991b; Coetzee et al., 2011).

The degree of control obtained through the use of *C. salviniae* is regarded as a prime example of successful biological control (Room & Thomas, 1985). However, continued effectiveness of programmes such as these will depend on our abilities to predict, and wherever possible protect our freshwater ecosystems from unnatural disturbances, such as eutrophication and further invasions. Thus, the objective of this section was to investigate how elevated CO_2 in conjunction with eutrophication, will affect the level of biological control achieved by *C. salvinia* on *S. molesta*.

The response of *S. molesta* to changes in CO_2 concentration was highly variable in winter. However, as expected, the fertilization effect of CO_2 was evident in the absence of herbivory for the high nutrient treatment, where biomass production by *S. molesta* was 21 % higher at eCO_2 compared with aCO_2. These findings correspond with results from a variety of similar studies, which show that plant response to elevated CO_2 is determined by nutrient availability (Ziska & George, 2004; Gregory et al., 2009; Cornelissen, 2011; Zavala et al., 2013).

The reproductive output (number of shoots) of *S. molesta* was also higher at eCO_2, but surprisingly, this was only observed in the low nutrient treatment with no herbivory and not on plants growth with high nutrients. This implies that the plant was able to successfully use the added CO_2 for asexual reproduction even in environments that were nutrient-limited (Sage & Coleman, 2001). Although it is an enigma that a similar response was not realized in the high nutrient treatment, but may possibly be explained by the way this plant grows.

Salvinia molesta has two different growth forms, the expression of which is governed by temperature, physical damage and nutrient availability (Room, 1990; Pieterse et al., 2003; Schooler et al., 2011). The first phase in the growth of this plant is rapid colonization (production of new ramets), with a slender form and small leaves (Gufu, et al., 2019). Thus, the plants supplied only with limited nutrients may have been stuck in this phase. However, it was those plants grown at aCO_2 with added nutrients and herbivory present that had a higher number of shoots compared with the low nutrient treatment, which could be an indication of compensatory growth (Coetzee et al., 2007). This suggests that CO_2 was only important for reproduction when herbivory was absent, and it was nutrient availability that was more important when control agents were present (Coetzee et al., 2007).

There was no difference in chlorophyll content between the treatments, implying that photosynthesis was not affected by any of the treatments. This was similar to results found by Gufu et al. (2019), which showed that even though *S. molesta* has a C3 photosynthetic system; it did not respond to changes in CO_2 concentration as much as other C3 species do. They suggested that this was because the plant was already operating close to or at its optimal nutrient and CO_2 concentration, meaning that any increase in nutrient or CO_2 availability would have only negligible effects on the plant. Indeed, due to its ability to use both its leaves and roots for nutrient acquisition, *S. molesta* has an increased surface area compared with most plants (Julien & Bourne, 1986), thus only requiring 0.1 mg $N\text{-}NO_3\ L^{-1}$ to reach it highest growth rate (Gufu et al., 2019), which was the low nutrient concentration used in the current study.

According to Ehleringer & Cerling (2002), different plant species are likely to differ in their requirements of CO_2 concentration for maximum growth, even for those that use the C3 photosynthetic pathway. An alternative explanation for the lack of chlorophyll response could be that the high density of trichomes on the surface of *S. molesta* lessened the effect of CO_2 by reducing the amount of photosynthetic active radiation reaching the plant's photosystem (Barthlott et al., 2009; Gufu et al., 2019). It is also possible that a direct measurement of photosynthesis was required in order to observe a CO_2 response, as growth parameters (biomass and reproduction) did respond to CO_2 manipulation in some instances. Again, some of the patterns may not be reliable due to the natural reduction in plant growth in winter (Sage et al., 1990; Dolezal et al., 2019).

The insects did not survive the full duration of the winter experiment and this is an expected outcome as this plant and its biological control agents do not overwinter well in cooler regions, typical of Grahamstown's climate, where the experiment took place (Room, 1990; Cilliers, 1991b). However, unlike results obtained by Gufu et al. (2019), in summer there was a clearer response to CO_2, where biomass accumulation of *S. molesta* was significantly stimulated by eCO_2 in the absence of biological control agents. Herbivory, on the other hand, reduced the CO_2 fertilization by up to 56% (Gregory et al., 2009); although the results show that the control agents inflicted highly variable but similar amounts of damage at both CO_2 concentrations. These results are consistent with similar studies conducted on other species (Sage & Coleman, 2001; Stiling & Cornelissen, 2007; Cornelissen, 2011).

When considering the effect of CO_2 on the nitrogen availability of *S. molesta*, it was clear that herbivory was an important determinant of the C: N ratio (Ziska & George, 2004). The plant was less responsive to CO_2 in the absence of biological control agents, but when control agents were added, *S. molesta* was able to use the extra CO_2 to increase its C: N ratio by up to 32%, which could be an indication of increased carbon-based defenses (Stiling & Cornelissen, 2007). Currently, there is no simple way to summarize the response of *S. molesta* and its biological control agent, *C. salviniae* as the results did not conclusively support the prediction that the plant will be more successful under elevated CO_2, and that the efficacy of the biological control agent will be reduced. Although previous studies have investigated the response of *S. molesta* to eCO_2 (Gufu et al., 2019), the current study is the only one that has incorporated herbivory, and therefore a biological control component. I recommend re-evaluating this species response in the context of biological control, with more frequent measurements and a longer time frame.

5.3. *Pistia stratiotes*

In the field, thick mats of *P. stratiotes* are able to support heavily damaged plants and keep them afloat, unless the whole structure is weakened (Thompson & Habeck, 1989). Furthermore, eutrophication allows the plant to tolerate herbivory (Moore, 2005). Consequently, it is important for resource managers to understand how elevated CO_2 will further perpetuate this tolerance and what this means for biological control of this weed. Thus, this section will discuss how elevated CO_2, in combination with eutrophication, will influence the plant-insect relationship between the invasive weed *P. stratiotes* and its biological control agent, *N. affinis* and the implications thereof.

For the winter experiment, *P. stratiotes* did not offer any clear-cut trends with regards to its response to the different treatments. But as expected, biomass production was significantly stimulated by eCO_2 for the no herbivory treatment when grown in eutrophic conditions compared with plants that were not given any extra nutrients (Lawlor et al., 1987; Ziska & George, 2004). The added nutrients allowed *P. stratiotes* to use the available CO_2 more efficiently for biomass accumulation of up to 11% more than the low nutrient treatment, without the limitation of biological control (Cornelissen, 2011). The same trend was observed in the presence of herbivory, but surprisingly this time, the difference in biomass accumulation was only evident in the aCO_2 treatment across the two nutrient concentrations.

High CO_2 also resulted in an unexpected decrease in leaf production in the high nutrient treatment compared with aCO_2 in the absence of herbivory. But as expected, the combination of high nutrients and eCO_2 increased leaf production by up to 38% more than the low nutrient treatment in the presence of herbivory (Gregory et al., 2009). Furthermore, the addition of *N. affinis* also increased the number of leaves produced by *P. stratiotes* for the high nutrient treatment, but only at eCO_2. This might mean that herbivory did affect the response of *P. stratiotes* to CO_2 changes, although the direction of this impact was not clear due to limited insect activity in the winter experiment.

Alternatively, biomass accumulation might have been more dependent on CO_2 availability in the absence of herbivores, while nutrient concentrations were more important in the presence of herbivory (Coetzee et al., 2007). Furthermore, feeding by the control agents within the low nutrient treatment successfully reduced biomass production by 18%, provided there was no supplementary CO_2. This means that, as predicted in the introduction, *N. affinis* was more effective at controlling *P. stratiotes* within this treatment due to its vulnerability as a result of limited nutrients. According to Coetzee et al. (2007) nutrients are an important determinant of the efficacy of biological control, hence; this could be an indication that the plant was using the available CO_2 for growth and defenses in the added CO_2 treatment, making it less affected by herbivory.

Indeed, in eutrophic conditions, *P. stratiotes* was not affected by herbivory, regardless of CO_2 concentration because this allows for more growth and defenses within the plant (Cornelissen, 2011; Zavala et al., 2013). However, leaf production was still vulnerable to the effects of herbivory, but eCO_2 reduces this vulnerability (Scherm et al., 2000; DeLucia et al., 2008; Reeves, 2017), especially at high nutrients (Gregory et al., 2009). The reproductive output of *P. stratiotes* had no response to any treatment during the winter experiment. There have been a number of studies that have shown that there is a trade-off between growth and reproduction in plants, and if conditions are not ideal, as was possibly the case here, plants will use their resources for growth instead (Messina et al., 2002). Certainly, in order to minimize imbalances and optimize resources acquisition, plants tend to differentially allocate biomass to their structures as required (Martorell et al., 2006).

Lastly, the C: N ratio within the leaves of *P. stratiotes* was for the most part not responsive to the treatments in winter; except that there was a 15% higher C: N ratio in the absence of herbivory within the high nutrient plants at eCO_2. These results were contrary to the expectation that high nutrients reduce the C: N ratio of the plant, but may be explained by the response of *P. stratiotes* to the increased CO_2 concentration within this treatment (Stiling & Cornelissen, 2007). The 25% reduction in C: N ratio of *P. stratiotes* when *N. affinis* was added within the high nutrient treatment might be explained by the fact that the plant was using the available nutrients for defenses, as nitrogen-based defenses have been shown to be more effective (Cornelissen, 2011). Given that the C: N ratio can be used as an indication of plant defenses (DeLucia et al., 2008; Zavala et al., 2013), it makes sense, therefore, to think that plants exposed to other treatment combinations might not show a response for this variable as there were not enough abundant resources to invest in both growth and defense (Messina et al., 2002; Martorell et al., 2006).

Unlike the winter experiment, the response of *P. stratiotes* was more predictable in summer with results that were more traditional in nature. Firstly, there was a down-regulation of photosynthesis at eCO_2 for both herbivory regimes, although higher rates were still recorded in the presence of herbivory, which makes sense because feeding by the agents acted as a sink for the extra carbohydrates produced within this treatment (Spencer & Bowes, 1986; Dolezal et al., 2019). According to Sage and Coleman (2001), ageing plants are more likely to express a down-regulatory response to elevated CO_2, which could have been the case here as photosynthesis was only measured on mature leaves after 10 weeks. However, these results are still unexpected because this type of response is usually observed in low nutrient environments. There could be other elements that affected the observed response as all plants were grown in eutrophic conditions in the summer experiment.

The fertilization effect of eCO_2 on the dry weight of *P. stratiotes* was clear both in the presence and absence of herbivory with an increase of 20% and 28% respectively. Nevertheless, herbivory by *N. affinis* was able to significantly reduce this effect by up to 23%. On the other hand, the main determinant of the asexual reproductive potential of *P. stratiotes* was herbivory and not CO_2, as plants did not show a response to CO_2 manipulation. Hence, in the presence of herbivory, the mass of side shoots was significantly lowered compared with the no herbivory treatment regardless of CO_2 concentration (Martorell et al., 2006).

Similar to results by Cornelissen (2011), the C: N ratio was increased by eCO_2, and significantly more so in the presence of herbivory (42%), possibly as a means of increasing carbon-based defenses (Cornelissen, 2011). Indeed, the damage caused by *N. affinis* on *P. stratiotes* was significantly reduced to 46% less at eCO_2 compared with aCO_2 (DeLucia et al., 2008). In summary, there were no obvious trends in winter, but in summer, the plants performed significantly better at a high concentration of CO_2 within the growth atmosphere, especially when nutrients were not limiting, while the biological control agents became less effective under these conditions as a result. As such, it would be recommendable to consider *Spodoptera pectinicornis* (Hampson) as a possible supplementary biological control agent against *P. stratiotes* in South Africa. This agent has already been used and tested in Thailand, and the United States (Wheeler & Center, 1998). Together with *N. affinis*, these agents may be able to reduce the fertilisation effect of CO_2 to levels where the plant does not pose a risk to natural resources.

5.4. *Myriophyllum aquaticum*

Despite the obvious importance of continued control of *M. aquaticum*, after 1999, infestations of this plant in South Africa no longer received the same attention and research as *S. molesta* and *P. stratiotes* (Coetzee et al., 2011). Furthermore, there is relatively small information on how this plant will respond to levels of CO_2 increase predicted by the IPCC (2007) by the end of this century. Thus, this section explored how elevated CO_2, in conjunction with eutrophication, will affect the level of biological control achieved by *Lysathia* sp. on *M. aquaticum*.

According to Hussner (2009), there is a significant difference between the chlorophyll content of the submerged and emergent shoots of *M. aquaticum*, therefore most of its photosynthetic activity occurs in the emergent part of the plant (Wersal & Madsen, 2011; Yin et al., 2017). This allows *M. aquaticum* to escape CO_2 limitations from the water column (Yin et al., 2017). According to Hussner (2009), C3 plants such as this one are said to be more responsive to increased atmospheric CO_2 compared with their C4 counterparts or completely submerged macrophytes. Hence, due to its carbon and nutrient limitation (Sytsma

& Anderson, 1993), this plant species was expected to benefit from the projected climate change.

On the contrary, *M. aquaticum* was not very responsive to changes in atmospheric CO_2 during the winter experiment, although the addition of nutrients at aCO_2 significantly increased leaf production in the presence of herbivory. It has been shown that plants are able to increase growth in eutrophic conditions to make up for the effects of herbivory (Coetzee et al., 2007). It is however surprising that a similar response was not evident at eCO_2, although one might argue that *M. aquaticum* was unable to show its full response to eCO_2 due to the overall limited growth that plants usually experience in winter.

Nevertheless, high CO_2 concentrations led to a 27% increase in the C: N ratio of this plant for the high nutrient treatment and in the presence of herbivory compared with the normal CO_2 concentrations. Thus, as shown elsewhere (Stiling & Cornelissen, 2007), it is possible that the plant was using the extra CO_2 for quantitative defenses instead of the more costly nitrogen-based defenses which is a normal response for plants grown under eCO_2 (Stiling & Cornelissen, 2007). However, the biological control agent could not survive the full duration of the winter experiment, and therefore the results are not conclusive in this regard.

The results of the summer experiment were more convincing for *M. aquaticum*, showing typical C3 responses to eCO_2 that have been reported by Malheiro et al. (2013) on the same species, and elsewhere (Stiling & Cornelissen, 2007; DeLucia et al., 2008; Cornelissen, 2011; Zavala et al., 2013). Firstly, there was an up-regulation of photosynthesis by up to 48% measured for plants grown at eCO_2 compared with aCO_2 regardless of herbivory (Gregory et al., 2009). This may be because *M. aquaticum* reproduces asexually through the production of 'daughter plants' or side shoots and therefore does not need to down-regulate its photosynthetic activity as this serves as a sink for the additional carbohydrates assimilated at

eCO_2 (Sage & Coleman, 2001; Stiling & Cornelissen, 2007; Taylor et al., 2010). Vu et al. (2006) showed that the up-regulation of photosynthetic activity in sugarcane under high CO_2 conditions was an indication of an acclimation response, which led to increased biomass accumulation for the plant. Certainly, biomass production and reproduction followed a similar trend to what was observed for photosynthesis, where the fertilization effect of eCO_2 was clearly visible especially so in the absence of herbivory, where there was 47% more biomass produced for this treatment.

As anticipated, the presence of herbivory significantly reduced the positive effects of eCO_2 on *M. aquaticum* by up to 50%. Additionally, although highly variable, the C: N was increased by eCO_2 for the no herbivory treatment (Malheiro et al., 2013), whereas CO_2 concentration surprisingly had no effect on C: N ratio of *M. aquaticum* in the presence of herbivory. But when comparing across herbivory treatments, there was a 45% reduction in C: N ratio upon addition of biological control agents within the eCO_2 treatment, a possible indication of induced nitrogen defenses, which, is not seen in the absence of herbivory, hence the higher C: N ratio for the said treatment (DeLucia et al., 2008; Gregory et al., 2009; Zavala et al., 2013; Reeves, 2017). These results also make sense because there was no nutrient limitation in the summer experiment which might have allowed the plant the luxury to use the more effective and more costly means of defense for this treatment (Coetzee et al., 2007), and not needing to do the same thing in the of absence of herbivory.

The number of live *Lysathia* sp. insects recovered at the end of the summer experiment was significantly higher at eCO_2. This was contrary to findings by Stiling & Cornelissen, (2007) from their meta-analysis which showed a decrease in phytophagous insects at eCO_2. A possible explanation for this could be that the higher plant biomass available within this treatment was able to support a larger population of insects, although the damage inflicted by these biological control agents on *M. aquaticum* was still lower for this treatment, possibly due to the high ratio of plants per insect. Alternatively, the reduced efficacy of the biological control agents at eCO_2 even when occurring in larger numbers could be linked to increased plant defenses, reduced palatability and reduced nutritional quality within this treatment, which has been shown in similar studies (Julien & Bourne, 1987).

Thus, we can expect that this species will not respond significantly to changes in CO_2 during the winter months, while in summer there might be more growth of this plant species (Malheiro et al., 2013), which may be a challenge for its biological control agents. If this is the case, dense monospecific stands of this species are likely to cause further problems for domestic water use and loss of biodiversity in infested waters (Hussner, 2009). Furthermore, the eutrophic systems in which *M. aquaticum* is found in South Africa might enable the plant to benefit more from the CO_2 fertilisation effect by increasing its growth through increased vegetative output, as shown in this study. Thus, once the submerged shoots of *M. aquaticum* break through the water surface, their nuisance nature is quickly revealed, especially so in nutrient-enriched waters (Hussner, 2009). With the projected increase in atmospheric CO_2, and given the way *M. aquaticum* grows and responds to herbivory by *Lysathia* sp., it might be necessary to re-visit the stem boring beetle, *Listronotus marginicollis* as a potential addition to the biological control programme of this weed as this might complement the damage caused by *Lysathia* sp. since these types of feeders and leaf miners are less likely to be as negatively affected as folivores (Zavala et al., 2013; Reeves, 2017).

5.5. Conclusions

In general, all the study species showed a typical C3 response to eCO_2 concentration (Cornelissen, 2011; Gufu et al., 2019), with increased biomass production and reduced insect damage, however, these responses were more distinct in summer. This means that there is increased possibility of further spread of these plant species in the near future due to reduced efficacy of biological control agents, and increased plant success in growth, defences and reproduction (Reeves, 2017). Responses were less noticeable in winter possibly owing to limited plant physiology at lower temperatures (Idso et al., 1987; Sage et al., 1990; Dolezal et al., 2019). Furthermore, there were a lot of challenges for the winter experiment, one of the biggest ones being the death of biological control agents and the general reduction in plant growth during this time.

According to Parker et al. (1999), the total impact of an invasive species is determined by its spatial extent, abundance and per capita impact on that new environment. However, the significance of that impact is dependent on the size or scarcity of the impacted resources (Parker et al., 1999; Hellmann et al., 2008). Climate modelling by Hoveka et al. (2016) based on a number of bioclimatic parameters investigated the spatial distribution of the top five most problematic aquatic weeds in South Africa, which comprised four of the study species here, and *E. crassipes*. They showed that there is a high probability for range expansion of the most challenging aquatic weed, *E. crassipes* and the two floating ferns, *A. filiculoides* and *S. molesta* under future climates, while *P. stratiotes* and *M. aquaticum* are expected to contract (Figure 5.1). Their models, however, did not account for increased CO_2 concentrations, and consequently, they might have underestimated the capability of these plants to colonise new habitats. Therefore, the results of the current study suggest that the total impact of *A. filiculoides, S. molesta, P. stratiotes* and *M. aquaticum* on the environment is likely to increase, with more negative implications for water resources and aquatic ecosystem functioning in general.

The perceived impacts might be more severe in future as we are currently experiencing heavy drought all over South Africa (Parker et al., 1999; Denis & Denis, 2012; Dube et al., 2013; Vogel & Olivier, 2019). Additionally, as shown here, climate change might also affect the effectiveness of insect herbivores (Hellmann et al., 2008), with some agents such as chewers being at a disadvantage, while other feeding guilds possibly continue to thrive (Robinson et al., 2012; Reeves, 2017). This is because, besides the increased production of tannins and phenolics under high CO_2 concentrations, plants also tend to increase their structural defences such as leaf toughness, a direct hindrance to chewing insects (Zavala et al., 2013). Therefore, it is possible that invasive weeds like *A. filiculoides*, *S. molesta, P. stratiotes* and *M. aquaticum* that are currently under the control of some biological control agents may become problematic again. Alternatively, although not shown here, climate change may facilitate the control of some weeds, especially in cases where the control agent and the target species are not currently in synchrony.

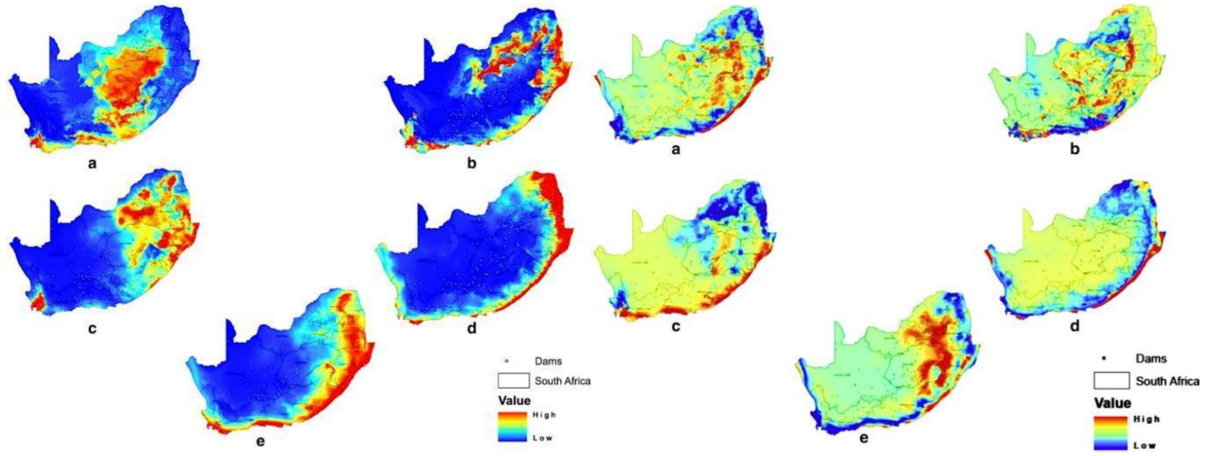


Figure 5. 1: Current (A) and predicted (B) future distribution of the “big five” aquatic invasive plants in South Africa based on climate models: a-*Azolla filiculoides*, b- *Eichhornia crassipes*, c- *Myriophyllum aquaticum*, d- *Pistia stratiotes*, e- *Salvinia molesta*, (from Hoveka et al., 2016).

5.6. Recommendations for future research

The results from this study show that interacting effects of elevated CO_2 and eutrophication alter the relationship between invasive macrophytes and their specific herbivores; therefore, although biological control agents in some instances reduced the CO_2 fertilization effect, current levels of control might still be compromised under future climates as there was reduced efficacy of agents at eCO_2. Further analysis and experimental research is needed in order to investigate and confirm the emerging trends from the above results. Future experiments investigating how plants grown at different CO_2 concentrations affect the life histories of their biological control agents should focus on various aspects of agent population parameters, including, food consumption, mean generation time, growth rate, development time, mortality, population density, reproduction rate, and larval and pupal weight. These data would be highly valuable in making further predictions on how biological control of invasive plants will be affected by climate change.

Moreover, there is a necessity to also incorporate the effects of elevated temperature with these kinds of studies, as increased CO_2 concentrations are synonymous with higher temperatures (Idso et al., 1987). According to a meta-analysis by Ziska and George (2004), alien invasive weeds benefit more than their native counterparts from increased CO_2 availability. This also requires more experimental studies which will look at how competition between invasive and non-exotic species and the resilience of ecosystems will be altered by future elevated concentrations of CO_2. Hence, in order to be able to make generalised statements as to how the vigor of invasive plants and therefore biological control is likely to change in future, more studies such as the current one are needed as responses are usually species-specific.

According to Hellmann et al. (2008), there is a high possibility that projected changes in global climate will affect the management of invasive species, as shown in this study and further supported by Ziska and George (2004). Therefore, this will require coordinated large scale programmes and approaches, innovative research, and more extensive monitoring. In this regard, I would suggest the development of a central database where scholars can upload and access data on invasive species. This could include distribution records, success stories and limitation of biological control programmes that are currently underway, general management plans and eradication efforts. This would help identify changing management effectiveness and disseminate novel techniques (Hellmann et al., 2008).

In conclusion, although this is not a universal response, the general trend from these results illustrates that the efficacy of chewing biological control agents which were investigated here might be reduced under elevated CO_2, while the noxious invasive weeds will become more problematic. This study was the first of its kind, but results could be taken as an early warning to the possible impacts climate change will have on biological control programs across the board.

As shown by Winston et al. (2014) and Schwarzländer et al. (2018), many countries around the world rely on the use of biological control agents to manage alien invasive weeds, both in terrestrial and aquatic environments. Thus, successful programmes such as the one against water hyacinth in Lake Victoria in Uganda (Wilson et al., 2007) could possibly revert to a problematic state. Thus, these results, at the very least should trigger questions about how such systems will be affected by future climates and what are the possible ways in which resilience can be built so as to allow adaptability and continued efficacy of biological control programs.

Biological control programmes could still continue to be regarded as the most sustainable, and cost-effective way to protect natural biodiversity from uncontrollable infestation by alien invasive weeds because there are endless opportunities for these programmes to be adjusted in order to adapt to the changing environmental conditions. These opportunities are due to the availability of a large pool of potential biological control agents, and their capacity to adapt to future climates. In the words of McFadyen (1998), “We owe it to the environment to promote biological control for what it is - the only safe, cost-effective, and sustainable method of weed control, with a success rate of at least 80% overall”. Nevertheless, prevention is better than cure, and in the case of aquatic alien weeds management, long term success will depend on the reduction of nutrient loads that enter the aquatic ecosystems and our ability to prevent future infestations by monitoring species entering South Africa (Hill, 2003).

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