IMPACTS OF NON-NATIVE *HYDROCOTYLE RANUNCULOIDES* ON NATIVE MACROPHYTE COMMUNITIES, AND THE EFFECTS OF MANAGEMENT, NUTRIENTS AND TEMPERATURE.

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Abstract

Hydrocotyle ranunculoides L.f. (Floating Pennywort) is native to South America and is an aggressively competitive aquatic plant species in the family Araliaceae and native to South America. It is invasive and non-native in the United Kingdom, Europe, and many countries worldwide. It has a large capacity for vegetative spread and dominance of waterways causing considerable problems for their ecology and costing over £25 million annually in management costs and recreation losses in the UK and Europe. The study site, Pevensey Levels in southern England, is a globally important wetland and is particularly species rich in aquatic macrophytes but 10% (45km) of the watercourses are infested by *H. ranunculoides*. This research investigates *H. ranunculoides* in relation to its impacts on native macrophyte communities and the effectiveness and sustainability of management methods, the effects of nutrients on its growth, and the potential influence of climate change through increasing temperatures. Macrophyte communities on 60 sample ditches on the Levels, both infested and un-infested, managed, and un-managed, were surveyed and nutrient levels and environmental variables measured in the sediment and water over a period of four years. Greenhouse experiments were run to investigate the influence of nutrients and temperature on growth, and temperature on seed germination and production. Results show that *H. ranunculoides* has a negative impact on native macrophyte species diversity (when cover >50%), may alter rare plant communities ($P=0.001$ for one rare species) and threaten international site designations. Traditional mechanical removal alone is not effective and reduces native macrophyte diversity. *H. ranunculoides* abundance was lower in sites receiving mechanical removal followed by chemical spraying ($P=0.031$) and this management treatment did not impact on native macrophyte diversity. Whilst no specific nutrient seemed to drive the growth of *H. ranunculoides*, it appeared to preferentially use nitrogen from water and phosphate from sediment. There were interactions between *H. ranunculoides* nutrient uptake and pH values ($P=0.002$). There were positive correlations between rising temperature and increased leaf abundance ($P<0.001$), biomass, and seed abundance ($P=0.01$). In addition, *H. ranunculoides* was shown to be capable of reproducing using sexual means in its invaded regions in Europe under current climate conditions. These findings have implications for the conservation of aquatic and riparian communities worldwide, challenge the effectiveness of management methods, and illustrate the potential for the increasing invasive capability of *H. ranunculoides* because of global climate warming.

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Author's Declaration

Declaration:

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree and does not incorporate any material already submitted for a degree.

Signed:

[redacted]

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Chapter 1 - Introduction

1.1. Research context

1.1.1. Rationale

Biological invasions are considered to be one of the most damaging disturbances to native species (Caffrey et al., 2014) and a great deal of funding has been tied up in managing these without understanding the true nature of the impact (Williams et al., 2010). Freshwater macrophyte communities are particularly threatened by invasive non-native species (INNS) (EPPO, 2014). Species richness is commonly used to identify if an invasive non-native plant species is having an impact, but this is just one measure and does not consider changes in the plant communities. *Hydrocotyle ranunculoides* is well established as an invasive non-native aquatic plant species in Europe but despite the many years of invasion history the only evidence of the impact of *H. ranunculoides* on plant communities is on the reduction in species richness (Stiers et al., 2011). In the UK, surveys of macrophytes in the ditches on the Pevensey Levels (Durkota and Friend, 2009) indicate a decline in macrophyte diversity and an increase in *H. ranunculoides* (Royal Haskoning, 2011) but there was insufficient data to make statistical comparisons between those watercourses containing *H. ranunculoides* and uninvaded sites. The reduction in diversity is consistent with Stiers et al.'s (2011) findings in Belgian ponds, where a strong negative relationship between INNS and macrophytes was found.

No work has been undertaken to describe the communities where *H. ranunculoides* is now found outside its native region or the impact it has on native macrophyte communities. The impact of regular disturbance, as annual removal of *H. ranunculoides* biomass, on community composition has not been studied. The data collected and analysed during this research will provide novel evidence about changes to plant diversity, species assemblage and community dynamics because of *H. ranunculoides* invasion. The study will also present evidence of the impact of different management methods on the plant diversity, species assemblage and community dynamics of native macrophyte communities, and show which is the most effective of the methods at reducing abundance of *H. ranunculoides*.

It is important to understand which nutrients play key roles in determining the growth and expansion capability of INNS . Hussner and Lösch (2007) showed that an increase in nitrogen and phosphate combined, resulted in increased growth rate of *H. ranunculoides* but it is not known which of the nutrients, and at what levels, might drive growth and expansion. This research will outline which, if any, of these nutrients could be controlled to limit the growth and expansion capability of *H. ranunculoides*. It will also consider whether pH influences the growth and spread of *H. ranunculoides.*

As the climate changes in its invasive range, understanding the consequences of this on the reproductive capabilities of *H. ranunculoides* is necessary to evaluate whether existing control methods are sustainable and resilient. The initial invasion and rapid local spread of *H. ranunculoides* is due to its exceptional asexual (vegetative ramets) regenerative capacity (Robert et al., 2013) but long term viability and resistance to dynamic regimes and extreme climate events is associated with the ability to reproduce sexually as this facilitates genetic diversity and adaptability (Li, 2014). This research will establish whether *H. ranunculoides* is able to produce viable seed within its invasive regions and whether rising temperatures could facilitate invasive traits. As a result, *H. ranunculoides* could reproduce both vegetatively and sexually, thus increasing its invasive capabilities, adaptability, and long-term resilience.

The results of this research have a practical application and provide evidence to support a more effective management protocol, which could have a positive impact on the economy of countries in which *H. ranunculoides* is now established. The seed germination data will be of considerable importance when considering the management methods. The principles underlying this research on macrophyte communities could be used to inform other pest risk analyses and be transposed to the ecology and management of other freshwater INNS. This information can be used to assess the potential to maintain the quality and resilience of freshwater ecosystems for the future under a changing climate.

1.1.2. Background

H. ranunculoides is a perennial macrophyte (water plant) and is found in more than fifty countries around the world (Centre for Agriculture and Biosciences International, 2020b). It is non-native in most of these countries, having thought to have originated from southern North America or, more likely, South America where the majority of its natural enemies are found (Centre for Agriculture and Biosciences International, 2020b). It is an invasive, non-native plant in five of the seven of the European countries where it has been recorded, as well as in Australia and some African countries (Centre for Agriculture and Biosciences International, 2020b). It is considered so serious an invader in Europe that a pest risk analysis was undertaken by the European and Mediterranean Plant Protection Organization to assess the risk of it spreading further throughout Europe, the current and future impact (EPPO, 2010).

The pathways of introduction for *H. ranunculoides* between continents are principally through the tropical aquarium trade or the horticultural aquatic trade (Centre for Agriculture and Biosciences International, 2020b). Once in a continent or island country, the routes of invasion are more diverse. Deliberate introduction by man, garden escapes or garden waste disposal of vegetative propagules are common, but it is also thought to have established from seed disposed of through sewage treatment works (Newman, 2013) but mostly by hydrochory. Vegetative propagules or seed are spread downstream or across wetlands in flood events, but can also be spread by animals and birds using watercourses or wetlands (Centre for Agriculture and Biosciences International, 2020b).

H. ranunculoides was first recorded in the United Kingdom (UK) in 1990 and by 2010 it was costing the UK economy £25 million, a figure that is likely to have increased given the ineffectiveness of control using conventional methods (Centre for Agriculture and Biosciences International, 2020b). *H. ranunculoides* is listed under Schedule 9 of the Wildlife & Countryside Act 1981 and is a ‘species of concern’ on the European Union (EU) Invasive Alien Species Regulation (IAS) Regulation 1143/2014 which came into force on 03 August 2016 (UK Government, 2020b). It is now banned from sale in the UK (since April 2014) but it is still not an offence to have this plant in a garden pond (Fitzsimons and Wade, 2019). It appeared on the Pevensey Levels study site in southern England in 1995 and by 2008, over 10% of the watercourses, 45 km, had become infested. Despite annual mechanical clearance by the Environment Agency (EA) there has been no reduction in infestation as this method breaks up the plant and creates the asexual vegetative ramets that are so effective at spreading. The site has three statutory designations for its importance for nature conservation: a national Site of Special Scientific Interest (SSSI), a European Special Area of Conservation (SAC) and an international designation, Ramsar.

The Pevensey Levels national SSSI and international Ramsar site designations cite ‘nationally rare…and scarce aquatic plants’ (Natural England, 1990) and ‘an outstanding assemblage of wetland plants’ (Natural England, 1990, Joint Nature Conservation

Committee, 1999). One hundred and ten of the 160 plant species in Great Britain that can be described as aquatic can be found on the Levels (Diston et al., 2007). The current SSSI condition assessment of 'unfavourable recovering' could deteriorate to an 'unfavourable' state, if experimental trials fail to find and implement an adequate control method to reduce the current cover of INNS to <5% (Natural England, 2013a). If *H. ranunculoides* were the cause of the Pevensey Levels not achieving a European or international conservation designation (SAC or Ramsar) then this would fail the Water Framework Directive (WFD). In addition, the EA has to contribute to getting 50% of SSSIs in to favourable condition as required by Outcome 1A of the England Biodiversity Strategy 2020 (Department for Environment Food and Rural Affairs, 2011). Clearly, the macrophyte species richness and community composition for the Pevensey Levels Ramsar and SSSI is critical.

1.2. Research approach

The research combines studies in the field with controlled laboratory experiments. This is a novel approach but practically effective, because the often-variable findings in the field can be supported by evidence from replicable experiments that source material from the field. Multivariate and inferential statistical methods were used to analyse the results. The field site used was the Pevensey Levels because it has a controlled water regime, thus reducing the risk of fluctuating water levels affecting the results, and one of the most diverse aquatic plant communities in the UK.

1.3. Research aims and objectives

The aims of this research are to:

- Identify the impact of *H. ranunculoides* on native macrophyte communities.
- Assess the effectiveness of management treatments being used to control *H. ranunculoides,* and to determine whether these impact on the native macrophyte communities.
- Ascertain the influence of nutrients on its abundance and invasiveness.
- Determine whether climate warming might influence *H. ranunculoides* invasive capacity.

The objectives are to:

- Quantify macrophyte community change in relation to *H. ranunculoides* infestation and its management treatments.
- Evaluate the effectiveness of management treatments on *H. ranunculoides*.

- Examine the influence of key nutrients nitrogen and phosphorus on the growth of *H. ranunculoides.*
- Investigate the effects of a rise in late summer/autumn temperature of 2°C on the reproductive and invasive capacity of *H. ranunculoides*.

1.4. Thesis structure

In addition to this introduction there are seven further chapters. A narrative literature review is presented in chapter two. The practical and analytical general methodology, and a description of the field sites, is described in chapter three. A field study and greenhouse studies were undertaken to meet the aims and objectives of the project. In chapter four, the impact of *H. ranunculoides* invasion, and the management treatments used to control it, on macrophyte communities are examined along with the effectiveness of these treatments. Chapter five investigates *H. ranunculoides* growth in response to key nutrient levels and pH in water, and nutrient levels in sediment. Chapter six explores *H. ranunculoides* growth and reproductive capacity under increased temperatures. Chapter seven critically discusses the results of the field and laboratory studies in relation to the literature. Finally, the conclusions and key implications of the findings from this study are presented in chapter eight.

Chapter 2 - Literature Review

2.1. Introduction

Biological invasions are dependent on the invasiveness of a species and the invasibility of the recipient habitat (Hui et al., 2016). *Hydrocotyle ranunculoides* is a robust, aquatic perennial plant originating from South America (Centre for Agriculture and Biosciences International, 2020b). It has been introduced outside of its native range in many countries worldwide and has since naturalised in numerous of these due to lack of natural enemies and availability of suitable wetland habitat (Centre for Agriculture and Biosciences International, 2020b). Due to a superior vegetative regenerative capacity it has become a serious invader in many of these regions, continuing to spread and unlikely to be contained long-term once established (Millane and Caffrey, 2014). This chapter will explore the existing body of academic research into the ecology and history of *Hydrocotyle ranunculoides* (Floating Pennywort) as an INNS. It will review the management methods and the evidence available to date of the impact *H. ranunculoides* has on native plant communities.

2.2. Invasion ecology

2.2.1. Invasiveness

The Invasive Species Specialist Group (2005) description of invasiveness is: ‘A species that has established and spread – or has the potential to do so – outside of its natural distribution range, and which then threatens ecosystems, habitats and/or other species, potentially causing economic and/or environmental damage, or harm to human health’.

Arim et al. (2006), investigating the spread dynamics of invasive species, discovered that invasion is a regulated process with distinct phases and that it is consistent across diverse taxa; birds, amphibians, fish, invertebrates, and viruses as well as plants. Assuming there a no predators or competitors involved, the invasion pattern is of a linear first-order dynamic with three phases, i. establishment with low spread, ii. expansion with increased spread and ii. saturation where the spread plateaus (Arim et al., 2006). The expansion phase may be limited by biological constraints such as a requirement to grow to a certain size before being able to propagate, the lag phase before being able to produce and disperse propagules and the Allee effect where the more individuals there are, the more likely they are to survive so population numbers need to increase before dispersal occurs

(Arim et al., 2006). Highly successful invaders may not be influenced by these constraints and are able to grow exponentially during the expansion phase until the space is saturated (Arim et al., 2006). However, there will be depletion of resources as sites are invaded and new sites must become available before further invasion can occur, but the spread pattern appears consistent regardless of the taxa, environment, temporal scale or dispersal mechanism (Arim et al., 2006).

To facilitate a successful invasion there are two key requirements, the ecosystem must be susceptible to invasion and the species must possess invasive qualities (Hui et al., 2016). Van Kleunen's (2010) meta-analysis of 125 invasive plant species concluded that performance related traits were key to invasiveness. These key traits were physiological, leaf area and shoot allocation, growth rate, size and invasion fitness (Van Kleunen et al., 2010). Hui et al. (2016) define invasion fitness as the population growth rate where propagule pressure (the number of individuals introduced and the number of events) is very low and the recipient habitat is ecologically balanced. Propagule pressure is consistently found to be a positive part of successful establishment across a wide range of taxa (Lockwood et al., 2005).

2.2.2. Non-native species

The term 'non-native species' is defined as 'a species, subspecies or lower taxon, introduced (i.e. by human action) outside its natural past or present distribution and includes any part, gametes, seeds, eggs, or propagules of such species that might survive and subsequently reproduce' (GB non-native species secretariat, 2019). In Britain there are 1875 established non-native species of which approximately 70% (1377 species) are higher plants (Roy et al., 2012). To become invasive, a non-native species must have the capacity to reproduce, spread and out-compete native species; this process is concisely described by the Convention on Biological Diversity (2019), as 'it must arrive, survive and thrive'.

Biological invasions are not restricted to the UK or Europe, they are a global threat to biodiversity but biodiversity loss due to invasion varies between regions and taxa and is subject to change over time (Bellard et al., 2016). Gallardo and Aldridge's (2015) global meta-analysis of aquatic ecosystems found a strong negative influence of INNS on macrophyte communities and on species diversity, sometimes causing local extinctions.

These invasions are largely driven by global trade networks with live plant imports being responsible for the most recent invasions (Chapman et al., 2017). In Europe, one of the main pathways of spread for aquatic invaders is from the Ponto-Caspian region (S.E. Europe) along the Rhine river to north-western Europe and, subsequently, to the UK from The Netherlands (Gallardo and Aldridge, 2015).

There are approximately 10-12 new non-native species establishing in the UK annually, a pattern that is found across the world, and likely to continue without intervention (GB Non-native Species Secretariat, 2015). Approximately 10-15% of these are likely to become invasive and cause significant environmental, economic, or social harm in their new location (GB Non-native Species Secretariat, 2015). The least damaging way to reduce this impact is by preventing their entry by identifying entry pathways, using horizon scanning and biosecurity techniques (Caffrey et al., 2014). Aquatic non-native species are more likely to become invasive than terrestrial species (GB Non-native Species Secretariat, 2015).

2.2.3. *H. ranunculoides* as an INNS

H. ranunculoides is typical of most aquatic weeds in that it possesses the key invasive species traits of very rapid growth rate, adaptability, and extremely efficient reproduction (Robert et al., 2013). It can produce many vegetative propagules which are viable for more than one year and disturbance in the form of mechanical control benefits its expansion as the propagules are as are very mobile (Centre for Agriculture and Biosciences International, 2020b). In the UK, in 1999, there were 29 sites recorded from a single clone but by 2008 there were at least 156 sites. As no viable seed has been recorded to date this spread is likely to be due to vegetative spread (EPPO, 2010). Hussner and Lösch (2007) studying *H. ranunculoides* in Germany, observed vegetative ramets breaking off from plants and colonising downstream. There is the lack of controlling factors such as host specific pests and diseases, and it can also resist herbivory (Robert et al., 2013). Being aquatic, it can survive overwinter submerged to avoid low temperature stress (Hussner and Lösch, 2007) and the combination of waxy leaf surfaces and the water environment can make chemical control difficult (Robert et al., 2013).

When *H. ranunculoides* is introduced to a new watercourse suitable for its growth, it spreads quickly by vegetative fragmentation (ramets) (Centre for Agriculture and Biosciences International, 2020b). The optimum time of year for this is autumn/winter when the plant structure is less stable due to senescence and there is more water movement and higher velocities (EPPO, 2010). Once established, it has been shown to be capable of doubling its biomass in three days in Australia and between 4-7 days in the UK (Robert et al., 2013). This extremely rapid growth rate, up to 20cm per day, means it can produce thick floating mats that completely dominate waterbodies (Millane and Caffrey, 2014) and out-compete other plant species, particularly submerged vegetation, due to its high leaf area index (Hussner and Lösch, 2007). It also produces biochemicals that affect the growth or survival of algae (called allelopathy) thus maximising light available for photosynthesis (Greca et al, 1992). These biochemicals comprise three types of polyoxygenated oleanane triterpenes (Greca et al, 1992) which have been shown to supress the growth of the green algae *Chlorella vulgaris* and the cyanobacterium *Synechocystis sp*. (Reynolds and Aldridge, 2020).

Hussner and Meyer (2009) found that the biomass of the root systems was not affected by semi-drained or drained conditions so that *H. ranunculoides* can recover well from drought and re-establish faster than native submerged aquatics leading to dominance of the species again when the water level recovers. However, it does not become invasive in this type of marginal or periodically inundated habitat (GB non-native species secretariat, 2019). The rapid growth rate is thought to be linked to nitrate availability (Centre for Agriculture and Biosciences International, 2020b) but it adapts to nutrient availability, with biomass allocated to roots in low nutrient conditions and shoots/leaves where higher nutrient availability predominates (EPPO, 2010).

H. ranunculoides also exhibits phenotypic plasticity (Robert et al., 2013) which is a particularly important method by which plants can cope with disturbance, environmental stress, and climate change (Gratani, 2014). The most common morphological factor that changes, and can be attributed to phenotypic plasticity, is leaf dimension (Ganie, 2014). This is certainly true of *H. ranunculoides* as the size of peak season growth (late summer) leaves can be up to 10-15 cm in diameter compared to small overwintering leaves of 2-4 cm (Centre for Agriculture and Biosciences International, 2020b) making it a very adaptable species.

2.3. Hydrocotyle ranunculoides

2.3.1. Description

Stace (2019) places *Hydrocotyle ranunculoides* L.f., , 1781 (Figure 1), in the Apiaceae, subfamily Hydrocotylaceae and in the genus Hydrocotyle. However, the taxonomy of Araliaceae is under review using molecular phylogenetics and the subfamily Hydrocotyloideae appear to be more closely related to Araliaceae so Nicolas and Plunkett (2009) now include it in the family Araliaceae (Table 1).

Figure 1: Leaves of *H. ranunculoides* (GB Non-native Species Secretariat, 2020a).

Table 1: Scientific classification of *H. ranunculoides (Nicolas and Plunkett, 2009).*

Domain	Eukaryota
Kingdom	Plantae
Phylum	Spermatophyta
Subphylum	Angiospermae
Class	Dicotyledonae
Order	Apiales
Family	Araliaceae
Genus	*Hydrocotyle*
Species	*Hydrocotyle ranunculoides*

H. ranunculoides is an aquatic, herbaceous and perennial plant (Stace, 2019). Table 2 describes the morphological characteristics of *H. ranunculoides.*

Table 2: Morphological description of *H. ranunculoides.*

Root	There are 20-40 filiform roots at each node (Poland, 2009). These hang free in the water column up to 50cm deep or root in the marginal substrate (Centre for Agriculture and Biosciences International, 2020b).
Stem	The slightly fleshy, stoloniferous stems, often floating, have nodes 4-15 cm apart (Centre for Agriculture and Biosciences International, 2020b).
Leaf	The Araliaceae family has simple, alternate, petiolate and stipulate leaves (Stace, 2019). *H. ranunculoides* has dark green, reniform leaves, from 2-4 cm up to 10-15 cm in diameter dependent on nitrate availability (Centre for Agriculture and Biosciences International, 2020b), (Figure 1). There are 3-7 rounded, crenate lobes (Hussner et al., 2012), one division of which extends almost to the base, and are held up to 40cm above the water surface (Millane and Caffrey, 2014). The long fleshy petiole is 5-35 cm long and glabrous, as is the whole plant (EPPO, 2006).
Flower	These are hermaphrodite, small and greenish white with no sepals, 5 petals, 5 stamens and a 2-lobed inferior ovary (Stace, 2019). Approximately 3mm in diameter (Centre for Agriculture and Biosciences International, 2020b). they emerge from the leaf node in umbels of 5-10 at the ends of 1-5 cm long peduncles (Washington State Department of Ecology, 2020). The single umbels averaging 9 flowers each (Millane and Caffrey, 2014) are held erect and on peduncles shorter than the petioles (EPPO, 2006). In Europe *H. ranunculoides* flowers between May and October (Hussner and Lösch, 2007).
Fruit	The fruits are brownish, flattened, sub-orbicular, 2 celled schizocarps, 2-3mm across (Stace, 2019) bending down to the water to disseminate when ripe (Washington State Department of Ecology, 2020). Fruits are produced throughout the summer and into October in the United Kingdom (UK) (personal observation). The 2 mericarps, each with a residual stalk, have 5 ribs which, in their native environment, help with floating dispersion (Centre for Agriculture and Biosciences International, 2020b).

2.3.2. Ecology

The ecological preferences of *H. ranunculoides* regarding light, moisture, pH, nitrogen, and salinity are illustrated in Figure 2 (Hill et al., 2004).

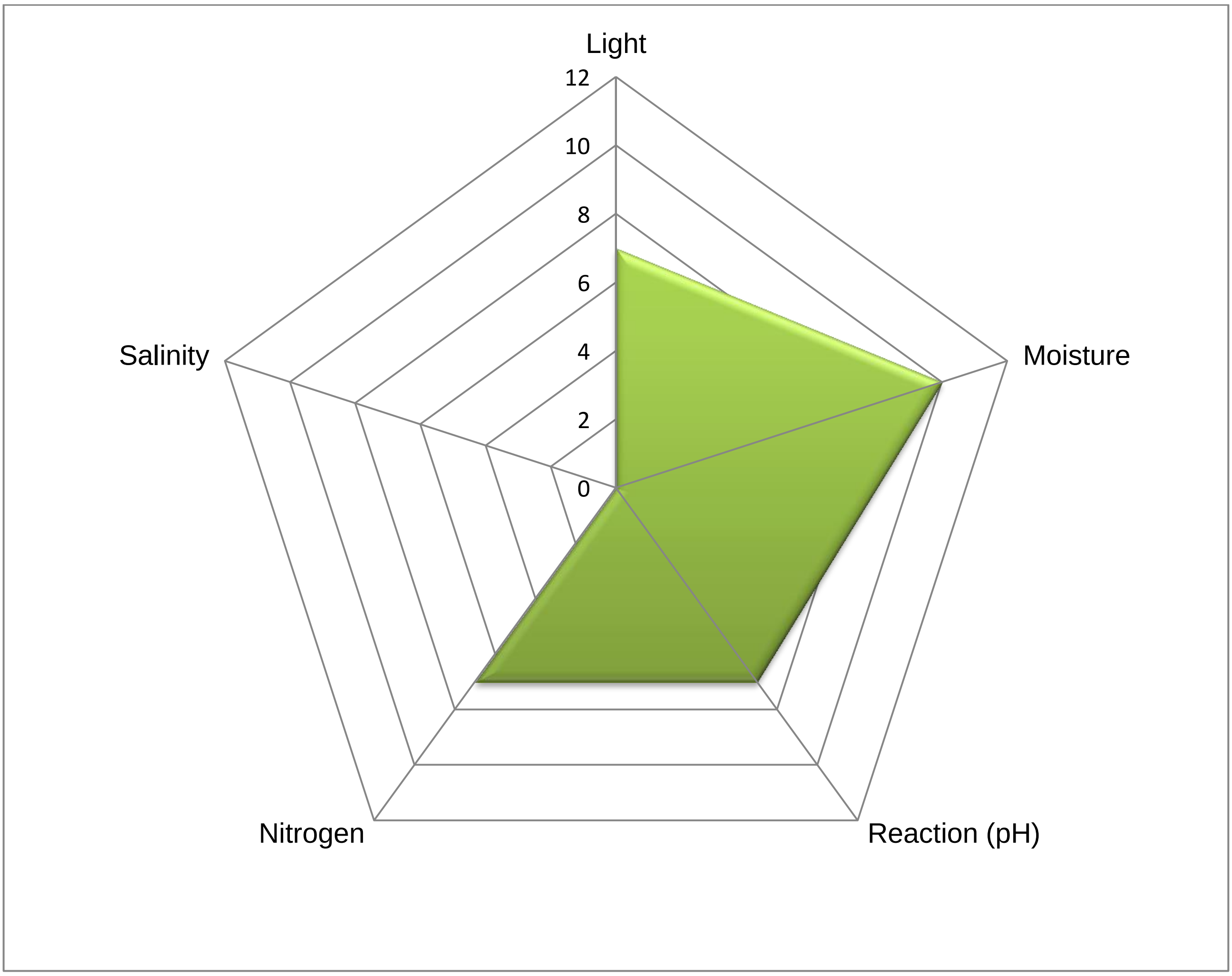


Figure 2: *H. Ranunculoides* Ecological Preferences Using Ellenburg Indicator Values (Hill et al., 2004).

2.3.2.1. Light

The Ellenberg Indicator for Light (L) is 7 (Hill et al., 2004) where Light score of 1 would be deep shade and 9 is full sun. *H. ranunculoides* grows best in bright, sunny conditions (high photon flux densities) (Hussner and Lösch, 2007). EPPO (2006) state that it is limited by shade and Hussner & Lösch's (2007) laboratory experiments show an almost linear reduction in gas exchange rate as light is reduced from the optimum of 800 mmol photonsm^{-2}s^{-1} down to zero. There are no studies on shade tolerance, but it seems accepted that this is a limiting factor and Newman and Duenas (2010) suggested this could be an effective environmental control method.

2.3.2.2. Moisture

H. ranunculoides is described by Hill et al. (2004) as having the Ellenberg indicator value for Moisture (F) of 10. A score of Moisture 1 indicates tolerance of extreme dry conditions and 12 would indicate a continuously submerged plant. Although *H. ranunculoides* is described as an emergent aquatic, and grows best in fully aquatic conditions, it can survive periods of low water and drainage, adapting phenologically to drained conditions (Hussner and Meyer, 2009). Hussner & Meyer (2009) showed that *H. ranunculoides* can also grow in marginal zones or wet grassland in Europe. Gantes & Sánchez Caro (2001) studied the distribution of emergent macrophytes, including *H. ranunculoides,* in relation to water velocity in Argentina, where it is indigenous. They found that lower water velocities were preferred but water velocities up to 35cm/s did not affect the distribution of emergents. In Europe, *H. ranunculoides* can survive in water velocities up to 1m/s and depths of several metres (EPPO, 2010) but Hussner and Meyer (2009) note that only small populations were found in waters with high velocities. This is borne out in the UK, where *H. ranunculoides* is found mainly in static or slow-flowing water bodies such as ponds, lakes, ditches, and canals (Newman and Dawson, 1999).

2.3.2.3. pH

The *H. ranunculoides* Ellenberg Indicator for Reaction (pH) was stated by Hill et al. (2004) to be 7, where a score of 1 would be extreme acidity and 9 indicates alkalinity, for example calcareous soils. This indicator value is supported by the EPPO (2010) report which describes the ideal water pH as 7.1 which would be considered slightly basic or alkaline but the full range is broader, 6.7 – 7.5 which covers acidic, neutral, and basic (EPPO, 2010). pH is related to orthophosphate use by plants with the ideal range for uptake being pH 5.5 – 7.0 (Garbey et al., 2004).

2.3.2.4. Nutrients

Nitrogen, phosphorus, and potassium are three of the essential macronutrients required for plant growth (Barko et al., 1991). Nitrate (NO_3^-) is the principal form of nitrogen and orthophosphate (HPO_4^{2-}) the principal form of phosphorus in freshwater taken up by macrophytes (Diston et al., 2007). The most important source of nutrients is likely to be the sediment (Barko et al., 1991). Land use will also affect nutrient loading, run-off from arable crop fertilisers and manure from livestock grazing can input considerable quantities of phosphorus and nitrogen into the water (Pedde et al., 2017). Potassium is key to regulating processes within the plant, and the primary source is likely to be the water (Barko et al., 1991).

Free-floating plants tend to be more limited by nitrogen than phosphorous (Smith, 2014). In addition to this, because sediments are almost anaerobic, they promote denitrification and the assumption that nitrogen fixing can compensate for this is debatable, usually being only 2% of the total budget (Moss et al., 2013). Nitrogen fixing bacteria become more abundant in the summer (Moss et al., 2013) and this would coincide with the maximum growth rates of *H. ranunculoides* (Newman and Duenas, 2010). However, higher nitrate in water (particularly in winter) can lead to a decline in macrophyte species richness as higher nitrate availability increases floating leaved species (such as *H. ranunculoides*) and hence shading out submerged species (Bakker et al., 2013).

H. ranunculoides is generally accepted to prefer eutrophic conditions (EPPO, 2010) and its Ellenberg Indicator for Nitrogen (N) is 7, where a score of 1 would be extremely infertile conditions and 9 would be very rich or polluted situations (Hill et al., 2004). High concentrations of nitrate and phosphate, often because of intensive use of agricultural fertilizers, has therefore made waterbodies in many European countries ideal for the establishment and spread of the species (Millane and Caffrey, 2014).

In water, the mean values for optimum *H. ranunculoides* growth are phosphorus: 21 µM and nitrogen: 116 µM (EPPO, 2010). The EPPO report (2010), describes the following range of nutrient ranges in the field: total phosphate: 0.066-0.82 mg/l, orthophosphate: 0.005-0.21 mg/l and dissolved inorganic nitrogen: 0.018-4.14 mg/l. Hussner & Lösch (2007) found dense stands of *H. ranunculoides* growing under field conditions, with 6.2 - 11.5 mg of nitrate and 2.9 - 61.9 mg of total phosphate per 100 mg/kg of sediment. Using controlled laboratory experiments they then showed that increasing soil nutrient availability promoted increasing relative growth rates of *H. ranunculoides*.

2.3.2.5. Salinity and conductivity

Salinity inhibits the growth of *H. ranunculoides* (EPPO, 2010). Hill et al., (2004) gave *H. ranunculoides* a Salinity (S) Ellenberg indicator value of 0, where Salinity 0 means not found in saline sites and 9 means extremely saline conditions such as lower salt marsh.

Experiments at Rothamsted Research in 2000 showed that leaf numbers declined, and leaf death rate increased when salinity was more than 6.5ppt (EPPO, 2010) which suggests that it may be able tolerate brackish water. The EPPO (2010) report put *H. ranunculoides* water conductivity tolerance between 232-699 µSiemens/cm.

2.3.2.6. Oxygen

H. ranunculoides oxygen requirement is between 6-11 mg/l (EPPO, 2010). Dissolved oxygen (DO) varies considerably with environmental influences, but low DO can indicate pollution and nutrient loading (Diston et al., 2007). Biochemical oxygen demand (BOD) is the amount of oxygen needed to oxidise the organic matter in water to stable inorganic forms and can also be used as a measure of potential pollution (Diston et al., 2007). For example, unpolluted water would have a BOD of less than 2mg/l whereas downstream of a sewage treatment works values of up to 10mg/l might typically be found (Diston et al., 2007). Low DO can slow the nitrification process, which is where ammonia or ammonium is biologically oxidised into nitrite and nitrate which are the forms of nitrogen utilised by plants (Andrews et al., 2013). The impact that dense mats of *H. ranunculoides* has is to decrease the oxygen levels (CABI, 2020b) and it can reduce BOD to below detectable levels (Strosnider et al., 2011).

2.3.2.7. Temperature

The core native distribution range of *H. ranunculoides* is in temperate regions, those without extremes of temperature and precipitation, both maritime and continental (EPPO, 2006). It is found in water temperatures between 0-30^{o}C. Air temperature follows a similar range with the minimum average daily of 1.5 °C and the maximum of 30.8 °C (EPPO, 2010). Poor frost tolerance seems to be a regulating element, particularly of its northern range, although it is hardy to -15^{o}C (EPPO, 2006). Frost acts by causing emergent and floating vegetation to die-back but the submerged leaves, stems and roots survive (Hussner and Lösch, 2007). However, the frost aided die-back would thin the mats out allowing removal of the remaining vegetation with less chance of viable fragments breaking up. Even seedlings seem to be frost hardy, Walsh et al., (2013) found that they can survive even after being completely enclosed in ice. EPPO (2010) state that *H. ranunculoides* can survive 51 consecutive days of frost and still regenerate quickly in the spring. Hardy bankside scrub and marginal species provide shelter from severe frosts (EPPO, 2006) and personal observation (2014).

The average maximum air temperature is stated as 30.8°C (EPPO, 2010) but *H. ranunculoides* can grow well in air temperatures above this as Hussner and Lösch (2007) showed that gas exchange does not drop off until 35°C. This is confirmed by the presence of *H. ranunculoides* in some tropical climates in Africa, America, and Asia. Neither high humidity nor air drought are limiting factors if the root system is established in water or damp ground (Millane and Caffrey, 2014). Hussner & Lösch (2007) suggest that the optimum temperatures for photosynthesis (based on gas exchange experiments) are likely to be between 25°C and 35°C indicating that the upper limit of temperature has not yet been reached in the UK. In addition to this, climate change predictions for higher summer temperatures and lower summer rainfall (Hussner and Meyer, 2009) will increase the growing season and cause wider fluctuation in water levels to which the plant is well adapted (Hussner and Lösch, 2007).

2.3.3. Reproduction

In its native environment, *H. ranunculoides* reproduces both asexually and sexually, by creeping stems and by seeds (Washington State Department of Ecology, 2020).

Asexual reproduction seems to be dominant amongst macrophytes, particularly because of the removal of water stress, with non-specific organs being most common (Li, 2014). *H. ranunculoides* is unusual in macrophyte asexual reproduction in that it only has one type of vegetative propagule; most have more than one non-specific organ (Li, 2014). In the case of *H. ranunculoides*, stem fragments of at least 1 cm and with one node are the main means of dispersal within adjoining water systems (Robert et al., 2013). The expansion process often takes place throughout the growing season as a way of maintaining the population (Li, 2014). Wider dispersal between waterbodies often occurs by waterfowl or human intervention as management or recreational activities (Hussner et al., 2012).

Although asexual reproduction is highly successful, most macrophytes still retain the ability to produce seed as this can be critical for re-population after extreme events (Li, 2014) and to maintain genetic capacity to respond to temporal changes. Seed production can be problematic for macrophytes as flowers usually need to be above water for pollen to survive (Li, 2014) but *H. ranunculoides* is able to overcome this difficulty as the flowers are produced on emergent foliage.

In its native range in Argentina Walsh et al., (2013) observed seed production in summer and found floating seedlings in the autumn. The seeds also float, the majority germinating in the autumn/winter of the same year in the water, not on the soil (Walsh, 2016). Other accounts describe it as germinating in spring on mud banks as light levels increase (EUPHRESCO DeCLAIM, 2011). It has also produced viable seeds in Australia, possibly more than 9,000 m^{-2} (Newman and Dawson, 1999). Little information is available on seed longevity, but it is thought they persist for more than one season (Walsh, 2016). It is not known if there is a seed bank in the soil or if the seeds remain in the water and germinate the following year as a generation of resistance seeds, as some plants do (Walsh, 2016). Although seed is set in many European countries, viable seed has not yet been found in the Western Palearctic (Robert et al., 2013). This may be because the warm autumn temperatures that are needed for seed germination (Millane and Caffrey, 2014) have not been found in the areas in which it is now become established as a non-native species.

Baas & Duistermaat (1999) suggested that *H. ranunculoides* may be more likely to produce seed when conditions for vegetative growth become sub-optimal. This is a reasonable suggestion because sexual propagation is likely to favour long term viability of the species for future persistence, as opposed to asexual propagation which favours rapid establishment and expansion (Li, 2014) and is a trait of invasive species.

2.3.4. Native distribution

2.3.4.1. Native range

The native range of *H. ranunculoides* is traditionally thought to be south-eastern USA and South America (Figure 3) and possibly Central America, Yemen, and Tropical Africa (Hussner et al., 2012). The origin, however, is probably Brazil and Argentina where H*ydrocotyle* species abound (Millane and Caffrey, 2014) and co-evolved insect herbivores are found (Centre for Agriculture and Biosciences International, 2020b). The presence in Central America is likely to be due to introduction (Centre for Agriculture and Biosciences International, 2020b).

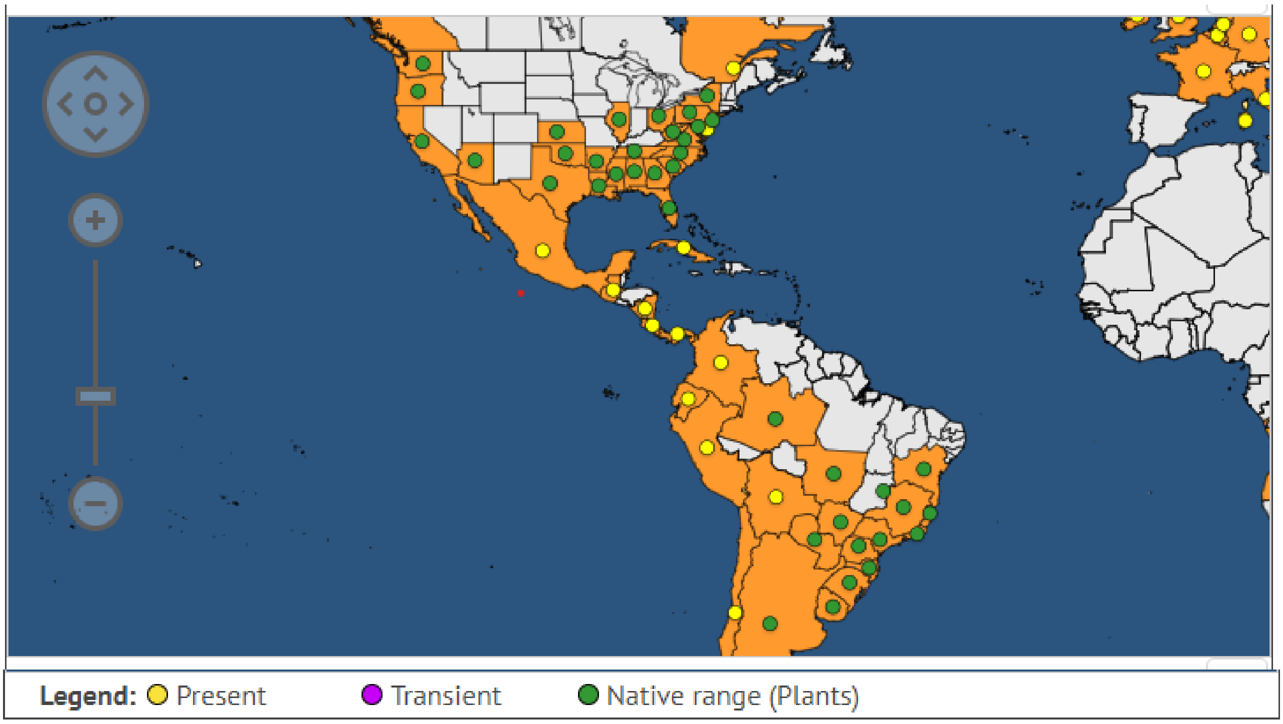


Figure 3: Native distribution *H. ranunculoides* in North and South America (EPPO, 2021).

2.3.5. Alien distribution

It is most likely that *H. ranunculoides* was introduced to its non-native sites through the aquatic plant trade, but this pathway has only been confirmed in Europe (Centre for Agriculture and Biosciences International, 2020b). The earliest record as an INNS was in Australia in 1983 (Centre for Agriculture and Biosciences International, 2020b). *H. ranunculoides* is now found in many countries worldwide (Figure 4); in Asia, Australia, and Europe (Hussner et al., 2012).

Figure 4: *H. ranunculoides* world-wide distribution, both native and invasive (Centre for Agriculture and Biosciences International, 2020b).

H. ranunculoides was present in the UK since the late-1980s in aquatic plant nurseries (Centre for Agriculture and Biosciences International, 2020b) but was first recorded in the wild in the UK in 1990 (GB Non-native Species Secretariat, 2020b). The introduction to the wild in mainland Europe has a similar timeline; it was first recorded in the wild in the Netherlands and Belgium in 1995 as an escape from an aquatic plant nursery (Centre for Agriculture and Biosciences International, 2020b). Since then, it has been recorded in France, Germany, Italy, and Ireland (Centre for Agriculture and Biosciences International, 2020b). The invasive strain found in northern Europe is thought to have originated from North America (Hussner et al., 2012).

Newman and Dawson (1999) noted *H. ranunculoides* was present in 29 sites in the south east of England and South Wales. Currently, there are more than 1,500 confirmed sites in England, Wales & Ireland but none in Scotland (Centre for Agriculture and Biosciences International, 2020b).

The status of *H. ranucuIoides* infestation in the UK at the start of the study (2013) is illustrated in Figure 5.

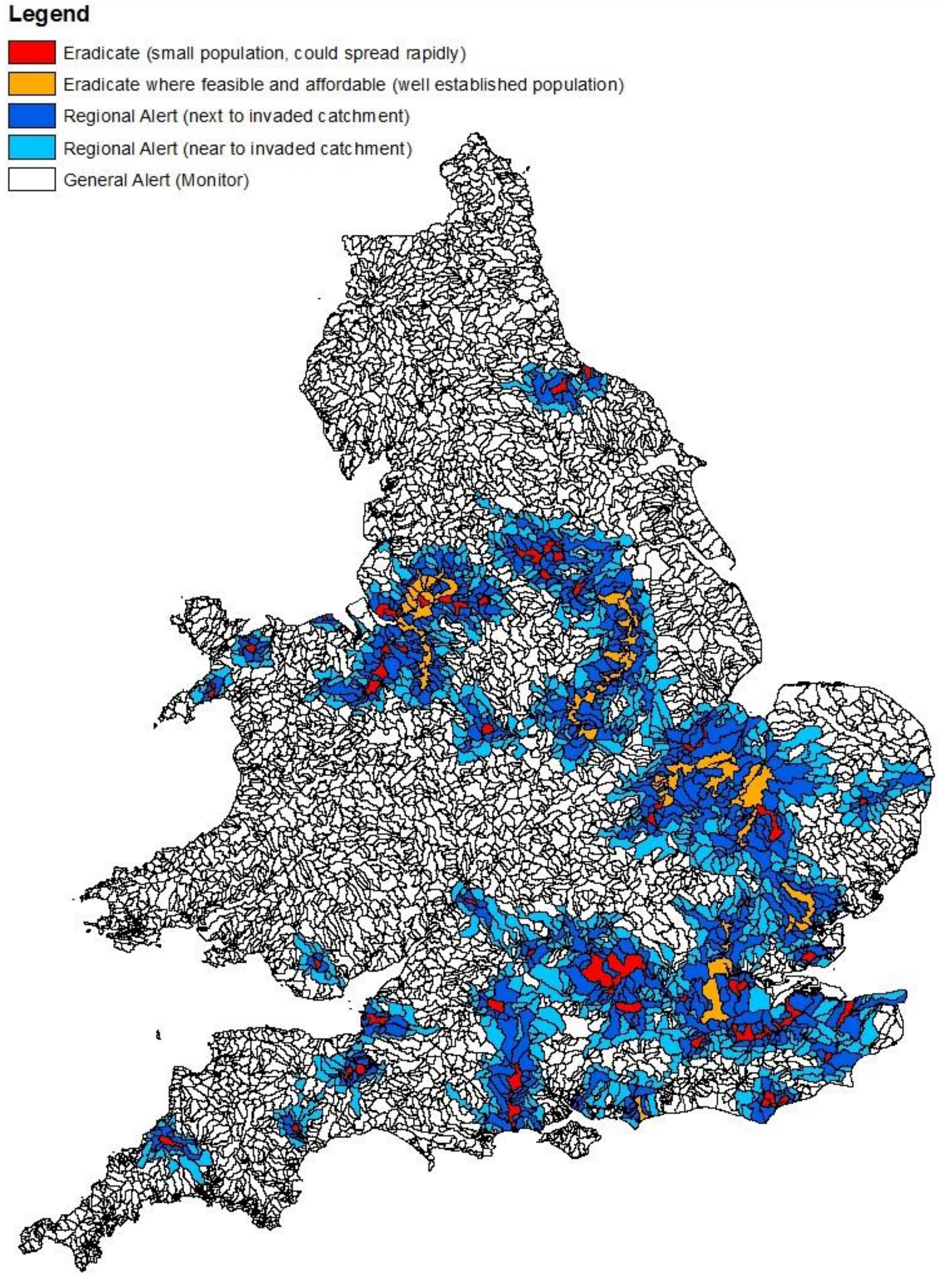


Figure 5: 2013 status of *H. ranunculoides* in the UK and management response by water catchment area (based on known distribution). (GB Non-native Species Secretariat, 2013).

It was first recorded on the Pevensey Levels in 1995 (Figure 6) and by 2008 had spread to 45km of the watercourses, approximately 10% of the site (Royal Haskoning, 2011).

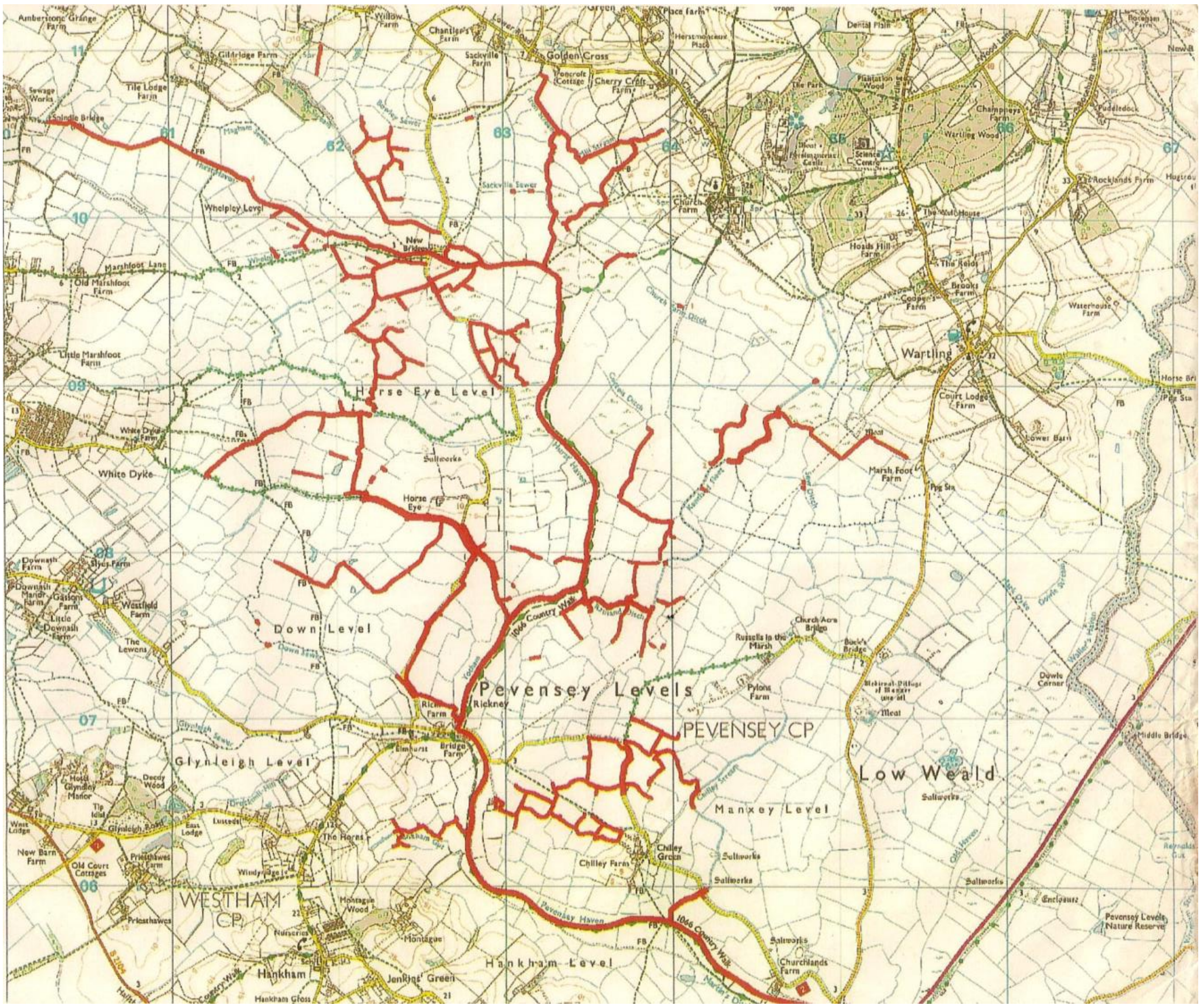


Figure 6: *H. Ranunculoides* distribution on the Pevensey Levels at the start of the study in 2013 (Natural England, 2013b).

2.4. Macrophyte communities

2.4.1. Plant communities and *H. ranunculoides*

Plant communities are a description of the combination of frequency and abundance of a particular group of plant species (Rodwell, 1998). Rodwell (1998) said that his community classification was meant to be ‘a working tool for the description, assessment and study of vegetation’. This ability to define different plant communities has many uses, one of the most useful being the ability to observe spatial or temporal environmental changes (Rodwell, 1998). Macrophytes are ‘aquatic photosynthetic organisms, large enough to see with the naked eye, that grow permanently or periodically submerged below, floating on, or growing up through the water surface' (Chambers et al., 2007). Macrophyte communities are, therefore, a description of aquatic plant assemblages (Rodwell, 1998).

2.4.2. Macrophyte communities in native regions

There is no complete phytosociological description of plant communities in *H. ranunculoides* native regions, probably because it is too large and diverse an area. However, there are some descriptions of plant communities which include *H. ranunculoides*. Murphy et al. (2003) identified an *Eichornia azurea – Limnobium laevigatum* sub-community on the River Parana in Brazil where *H. ranunculoides* is native. Milne (2004) then found that *H. ranunculoides* was unique to the group that makes up this sub-community. *H. ranunculoides* is also listed in association with *Eichornia azurea* in southern Brazil (Maltchik et al., 2007). Plants found with *H. ranunculoides* in its native Argentina were listed by Walsh et al., (2013) as: *Alternanthera filoxeroides, Azolla filiculoides, Egeria densa, Enydra anagallis, Ludwigia spp., Myriophyllum aquaticum, Paspalum repens, Pistia stratiotes, Pontederia rotundifolia* and *Salvinia biloba*.

2.4.3. Macrophyte communities in invaded regions

Although *H. ranunculoides* has been managed in its invaded regions across the world little evidence exists to show what impact it has on communities except on species richness. No work has been done to describe the macrophyte communities where *H. ranunculoides* is now found, although it could be assumed that it would be able to invade any community within its environmental parameters. Several of the species with which it co-habits in its native regions are also INNS in European regions, *A. filiculoides, E. densa, L. grandiflora and M. aquaticum* (Roy et al., 2012). The component native macrophytes that make up the rarer communities in *H. ranunculoides* invaded regions are unlikely to be highly competitive species, having adapted to their distinct niches, and thus unlikely to be able to compete with an invader (Grime, 2006).

2.4.4. Succession

All vegetation changes over time with the species composition and prominence of each species altering as it progresses (Grime, 2006). There are two types of succession; primary which is colonisation of a new habitat, and secondary which is re-colonisation of a disturbed habitat (Grime, 2006). Successional processes are essential in maintaining plant diversity and varied structure in aquatic systems and disturbance in the form of weed-cutting or de-silting can trigger this successional process (Natural England, 2013a). As *H. ranunculoides* possesses a very rapid growth rate and a superior reproduction rate (Robert et al., 2013), it is likely to re-colonise faster than native macrophytes and trigger successional shifts where the competitive invasive species dominates (Maskell et al., 2006).

2.4.5. Competition and invasion

Grime (2006) defines competition as '*the tendency of neighbouring plants to utilise the same quantum of light, ion of mineral nutrient, molecule of water, or volume of space'*. He also describes three strategies that plants use to form the structure and composition of vegetation: competitors (C), stress-tolerators (S) and ruderals (R), the 'CSR' theory. However, a competitive species colonising (the increase of a species within its existing range) is distinct from it invading (the establishment of a species outside its former limit) and out-competing neighbouring plants (Grime, 2006). The extent to which plant communities can be invaded is predominately controlled by changes in the availability of resources and competitors have the ability to rapidly take over resources and space by vigorous expansion (Arim et al., 2006). So, vegetation becomes invasible when fluctuating resources create an opportunity niche where the resources, especially nutrients, rise again promoting positive invasion fitness in plants (Hui et al., 2016). *H. ranuculoides* has been shown capable of effectively utilising nutrients from enriched aquatic environments, particularly nitrogen (Basílico et al., 2017).

2.4.6. Changes related to invasion

Invasions can cause habitat degradation, changes in the ecosystem and decline in native flora and fauna with endangered species being particularly susceptible (Arim et al., 2006). The process by which this happens appears to be a regulated one, with a low level of initial infestation, a rapid expansion stage and then saturation (Arim et al., 2006). A report by Maskell et al. (2006) showed that the composition and ecological traits of native species within invaded terrestrial communities can change significantly. However, once invaded, an increase or decrease of the invasive species does not necessarily impact further on the native species (Maskell et al., 2006) suggesting that the communities do not necessarily recover. Bakker et al. (2013) studying restoration of lake macrophytes found that, even when abiotic constraints are removed, diverse macrophyte communities often did not return pointing to plant competition as a possible factor.

Invasion has the potential to affect natural successional processes and community composition in two ways: the dominance of one species and the management of the invasive species. Santos et al. (2011), studying the effects of invasive species on plant communities, found that the dominant invasive species, *Egeria densa,* growth habit did not affect the growth of some native species so they survived but others could not establish. Stiers et al. (2011) found that INNS presence directly reduced species richness and that with more than 50% cover of invasive species certain functional groups of native species were also impacted, particularly submerged species. Both results would indicate that the community composition is likely to change because of invasion.

Management of invasive species is a form of disturbance so, in theory, could promote successional processes but the competitive nature of invasive species would suggest they are better suited to re-colonize and more likely to do so than the native species (Bakker et al., 2013). Santos et al. (2011), noted that invasive species formed a much larger component of aquatic plant communities than native species despite continual management. Santos et al. (2011) suggested that the continual management of the major invasive species, *E. densa*, had contributed to a change in the native species and facilitated the colonisation of other invasive species, which would affect community composition. But, Kovalenko et al. (2010), studying lake communities treated to remove *Myriophyllum spicatum,* found that total macrophyte abundance did not differ from the reference sites, species richness did not change and no macrophyte species disappeared during the study period. In addition, they found that habitat complexity only had minor changes because of treatment as native macrophytes re-colonised.

So, although evidence from studies of invasions points to changes in community composition, the actual changes in communities are not well described. Additionally, little data are available on the recovery of the native communities because of reduction in invasive species cover or because of the control methods used.

2.5. Management of *H. ranunculoides*

It is an offence under Section 14(1) of the Wildlife and Countryside Act 1981 to plant or otherwise cause to grow in the wild *H. ranunculoides*, which is listed in Schedule 9 of the

Act (UK Government, 2020c). Landowners therefore have a duty to prevent the spread of this aquatic plant from their property. Methods currently used for control are manual, mechanical, chemical, environmental, biological, and integrated plant management (Hussner et al., 2017, Centre for Agriculture and Biosciences International, 2020b) (Table 3).

Table 3: Some of the commonly used methods for managing *H. ranunculoides*.

Type	Method	Efficiency	Comments	References
Manual	Hand Pulling	Selective, labour intensive, removed vegetation often remains on-site close to the bank top	Can be good for small, local infestations, needs follow-up	(Clout and Williams, 2009, Hussner et al., 2017)
Mechanical	Excavator or Weed boat	Not species specific, can manage large areas, removed vegetation can be disposed of on-site away from bank top	Causes fragmentation which may exacerbate spread, needs follow-up	(Duenas and Newman, 2010, Hussner et al., 2017)
Chemical	Hand-held or Boat application	Targeted, but not effective on large biomasses, removed vegetation decomposes in-stream	Effective if biomass low and part of a long-term programme	(Duenas and Newman, 2010)
Environmental	De-silting, re-profiling, flow, depth, nutrient limitation, and shading	Not species specific, variable results, rapid re-growth, removed vegetation can	De-silting and re-profiling are unsustainable in the long term, shading is successful	(Duenas and Newman, 2010, Hussner et al., 2017)

Type	Method	Efficiency	Comments	References
		be buried under removed silt	where possible	
Biological	A weevil, *Listronotus elongatus*	Uncertain but promising, affected vegetation would decompose in-stream	Still under test	(Centre for Agriculture and Biosciences International, 2020a)
Integrated	A combination of some of the above techniques with site specific choices	Selective, adaptable to site and circumstances	The most used and successful with some documented eradications	(EPPO, 2014)

2.5.1. Manual control

Hand pulling is the least environmentally damaging method and is the preferred choice for local, small and early stage infestations (Clout and Williams, 2009) or as a follow up to other forms of control, particularly if chemicals are not permitted (Newman and Duenas., 2010). However, it is unlikely to be completely successful because it is difficult to remove all the root systems and plant fragments, even if all sources are identified (Clout and Williams, 2009). At a larger scale, it is impossible to use hand pulling for several reasons: access, health and safety issues and the cost of such a labour-intensive process (Clout and Williams, 2009).

2.5.2. Mechanical control

Weed-cutting using long reach excavators with buckets or weed-boats is effective in reducing the biomass in the short-term and is an important technique for dense infestations that are later to be treated with chemicals (Newman and Duenas, 2010). Often annual, biannual, or more frequent mechanical removal is required to reduce infestations to an acceptable level for the function of the waterbody (Clout and Williams, 2009). Mechanical removal alone is not an effective control method for *H. ranunculoides* as it is impossible to remove all plant material and plant fragments inevitably remain which can worsen the spread if downstream flow is not controlled (Newman and Duenas, 2010). Mechanical control can also lead to re-colonisation by seed germination as removing the dense biomass leads to more light penetration (Clout and Williams, 2009).

2.5.3. Chemical control

Newman and Dawson (1999) recommended the herbicide 2,4-D amine applied at 4.23 kg/ha as the most effective treatment. However, this herbicide has been withdrawn for use in or by water (Royal Haskoning, 2011) so Glyphosate is now the only herbicide available to treat watercourses. Control with Glyphosate as Roundup Pro Biactive using 6L per hectare in a total spray volume of 200L has been successful (EPPO, 2010). The level of success can be improved by using adjuvants (which help 'stick' the chemical to the waxy leaf surface) such as TopFilm™ or Codacide Oil, but it requires several years of treatment (EPPO, 2010). Glyphosate is a non-selective, systemic herbicide which has been passed as safe and effective for use in the UK although this is reviewed regularly, the current expiry date being 2022 (Health and Safety Executive, 2020). However, in Europe, chemicals licensed for use near water have been withdrawn which restricts the possibility of eradication of *H. ranunculoides* (Centre for Agriculture and Biosciences International, 2020b).

2.5.4. Environmental manipulation

De-silting alone would not control *H. ranunculoides* because the plant is an emergent species which roots on the bankside, but it may reduce the level of the nutrients which drive growth (Barko et al., 1991). Re-profiling, by removing the top 6-8cm of soil from the bankside, has been used as a control method as it will remove much of the root system (Royal Haskoning, 2011). However, *H. ranunculoides* fragments easily and only requires one node to root from and re-establish (Robert et al., 2013), so it would not eradicate the species. It is also not a sustainable method as repeated re-profiling would cause over-widening and geomorphological changes to the channel. This, in turn would lead to slower flowing water which favours the establishment of the species (Hussner and Meyer, 2009). Duenas and Newman's (2010) growth dynamics study concluded that environmental control options for *H. ranunculoides* would be limited as it could not be controlled using flow, depth, nutrient limitation, or salinity so the focus should be on shading. Shade was used successfully as an effective environmental control method in 2011, in the Netherlands, by planting trees along the south side of a watercourse (EPPO, 2014).

2.5.5. Biological control

Biological control aims to reduce the infestation of an invasive species to a level at which a balance is achieved between the host plant and a natural enemy whilst not affecting native plants (Clout and Williams, 2009). A weevil (*Listronotus elongatus)* has been identified as having the most potential for controlling *H. ranunculoides* in the UK and a pest risk assessment was submitted to the UK government for evaluation in early 2020 (Centre for Agriculture and Biosciences International, 2020a).

2.5.6. Integrated plant management

Effective, long term management of invasive macrophytes needs an integrated approach using appropriate methods, including reduction in nutrient input (Clout and Williams, 2009). Integrated management uses a combination of options, is the most used in practice, and considered the most promising for *H. ranunculoides* in The Netherlands as mechanical and manual control with shading (EPPO, 2014). There have been several case studies in recent years using a combination of mechanical and chemical control that have virtually eliminated the species or reduced it to low enough levels to maintain it with hand pulling (Sims, 2010).

2.6. Impacts of *H. ranunculoides*

2.6.1. Global

One of the foremost impacts of *H. ranunculoides* infestation is flooding of properties and agricultural land, due to blockage of watercourses and control structures (Centre for Agriculture and Biosciences International, 2020b). The impacts on navigation and other recreational activities like fishing, swimming, and water sports, can also be large (Millane and Caffrey, 2014). There are a number of ways in which *H. ranunculoides* could cause ecological impacts, as a result of the dense monospecific stands, including; flow reduction, increase in sedimentation leading to changes in succession, reduction in oxygen and light and loss of open water (Millane and Caffrey, 2014).

Information on the economic costs of controlling *H. ranunculoides* in its invaded regions around the world is rather fragmented. In Australia, in the 1990s, costs of control in the first year of infestation on the Canning River were estimated at AU $200,000 (EPPO, 2010). In the Netherlands, costs of control were approximately one million Euros in the 1990s (EPPO, 2010). By 2007, as the plant expanded its range in The Netherlands, water boards estimated the control of *H. ranunculoides* was costing them an extra €1.8 million in addition to routine maintenance (EPPO, 2010). In Belgium, similar estimates were given, with 1.5 million Euros required for control per annum in 2009 (Robert et al., 2013). This continues to date, with millions of Euros being spent annually without eradication (Centre for Agriculture and Biosciences International, 2020b)

In Europe, there is also the risk that invasion by *H. ranunculoides* will prevent waterbodies from reaching good ecological status under the WFD by out competing native species (Centre for Agriculture and Biosciences International, 2020b) thus reducing diversity of macrophytes and their associated invertebrate assemblages. This becomes more likely as mechanical means of control alone are ineffective and suitable chemical controls are gradually being withdrawn in the EU (Centre for Agriculture and Biosciences International, 2020b).

2.6.2. UK

The UK experiences similar issues to those found globally but there are additional problems reported anecdotally, such as where heavy infestations cause livestock deaths when they get trapped in the dense *H. ranunculoides* mats and drown (Diston et al., 2007). The blockage of drainage ditches by these dense mats leads to more prolonged flooding of agricultural land impacting on plant communities, grazing pasture and reducing crop productivity (Newman and Dawson, 1999).

The impact of *H. ranunculoides* on the UK economy for management and disposal was estimated at over of £1.9 million in 2008 by Williams et al. (2010) based on an estimate of £1,800 - £2,000 per kilometre (EPPO, 2010). These costs were predicted to rise annually and, following a review in 2011, the costs to the leisure and recreational industry were included bringing the true cost to the UK economy of more than £25 million (Centre for Agriculture and Biosciences International, 2020b). Costs of additional flooding as direct result of *H. ranunculoides* blocking watercourses has not been possible to quantify

(Williams et al., 2010) nor has the reduction in amenity value (Centre for Agriculture and Biosciences International, 2020b).

2.6.3. Pevensey Levels

The study site, Pevensey Levels in East Sussex, has three statutory designations: national - Site of Special Scientific Importance (SSSI) (Natural England, 1990), European – Special Area of Conservation (SAC) (Joint Nature Conservation Committee, 2016b) and international – Ramsar (Joint Nature Conservation Committee, 1999). It is particularly species rich in macrophytes, 68% of the species found in the UK are present here (Natural England, 2013a).

In 2008, the cost of controlling the 45km of infested rivers and ditches on the Pevensey Levels was £35,000 per annum (Williams et al., 2010). The cost of implementing control here is likely to have increased as the mechanical control method alone appears to be ineffective as it has not reduced the scale of the infestation since it was started and is not economically sustainable in the long term. In addition, the impact of the constant control of *H. ranunculoides* on the native macrophyte communities that support the designations of the Pevensey Levels is not known.

There is evidence for the economic damage, but the environmental damage caused by *H. ranunculoides* generally has not been adequately measured (Centre for Agriculture and Biosciences International, 2020b). Diston et al. (2007) suggested that *H. ranunculoides* out-competing native macrophytes would lead to a reduction in their associated invertebrate diversity. In several unpublished studies the EA did not find a reduction in invertebrate diversity but did find a change in the species of invertebrates (Centre for Agriculture and Biosciences International, 2020b). Stiers et al. (2011) also found a change in invertebrate assemblages in invaded ponds with sensitive species disappearing and a reduction in macrophyte species richness. The Centre for Agricultural and Biosciences International (CABI, suggest that reduced native biodiversity because of *H. ranunculoides i*nfestation would lead to habitat alteration and ecosystem changes, eventually damaging ecosystem services. Describing these changes in native macrophyte communities that have been invaded would allow a better understanding of the potential impact of these infestations.

2.6.4. Nature conservation

2.6.4.1. Impact on Succession and Competition

It has been noted that disturbance is an essential part of the successional process (Natural England, 2013a) but repeatedly controlling *H. ranunculoides* by annual mechanical removal means that mid to late successional habitat in the invaded watercourses is unlikely to develop as it is constantly disturbed. Following control methods *H. ranunculoides,* being a highly competitive plant (EUPHRESCO DeCLAIM, 2011), would be likely to re-establish faster and more effectively than native species. Furthermore, it is thought that *H. ranunculoides* may produce allelopathic chemicals that inhibit algae (Bakker et al., 2013). This might give it an additional competitive advantage over native macrophytes as algae forms a part of epiphyton that causes native macrophyte decline over the growing season by reducing available light (Bakker et al., 2013). *H. ranunculoides* has a rapid growth phase during the latter part of the growing season (Newman and Duenas, 2010), so this would allow it to store maximal resources in its overwintering form of submerged plants and stolons (Hussner and Lösch, 2007).

2.6.4.2. Impact on plant diversity

8% of the established non-native plants (108) in Great Britain are considered to have a negative ecological or human impact (Roy et al., 2012). However, the impact of an invasive species on native plant species and the reduction in quality of that wet ecosystem is hard to quantify (Centre for Agriculture and Biosciences International, 2020b). Where work has been done on the impact of *H. ranunculoides*, the focus seems to be on species richness as a simple measure of impact. This has its limitations as macrophyte communities are generally relatively species-poor, often dominated by one or two species (Rodwell, 1998). Submerged native macrophyte species are particularly impacted by dense invasions because light is blocked (Stiers et al., 2011) and *H. ranunculoides* will cover 100% of a watercourse if left unchecked. *H. ranunculoides* has been observed to reduce by more than 50% the number of native macrophyte species and up to 100% of the submerged species, and to reduce the native cover from 50% to 10% (EPPO, 2010).

2.6.4.3. Impact on community composition

Stiers et al. (2011) considered how cumulative abundance of submerged, floating, and emergent native species were impacted by the abundance of invasive species, including

H. ranunculoides. Their results confirm the surmise that the degree of dominance of an invasive species is linked to the level of impact. However, there are no studies of the impact of *H. ranunculoides* on macrophyte communities themselves (Newman, 2013).

2.6.5. Nutrients

Since the 1970s, phosphorus has been thought to be the primary limiting factor for biomass production in freshwaters (Moss et al., 2013). Submerged macrophytes obtain approximately half their phosphorus requirements from water (Barko et al., 1991) and the rest from sediment (Moss et al., 2013). On the Pevensey Levels, *H. ranunculoides* is mainly found in the area receiving a major part of its water supply from two sewage treatment works (Diston et al., 2007) and it has been assumed that the phosphate loading from these discharges was driving the growth of this species. However, Diston et al., (2007) found that that *H. ranunculoides* growth rates seemed to be related to nitrate, not phosphate. Diston et al. (2007) predicted that the introduction of phosphate stripping at these sewage treatment works would not limit the growth of *H. ranunculoides*. This appears to be the case as phosphate levels have dropped considerably in the period from 2003 (Diston et al., 2007) to below the WFD requirements of 0.04mg/l in one waterbody in 2015 (Environment Agency, 2016a), but the coverage and density of the *H. ranunculoides* infestation has not reduced despite annual mechanical clearance (Table 4).

Table 4: Phosphate (Soluble Reactive Phosphate) levels in mg/l of water in two waterbodies in the Pevensey Levels.

Site	Pre-2003 Diston et al. (2007)	2007 Diston et al. (2007)	2009 WFD (Environment Agency, 2016a)	2015 WFD (Environment Agency, 2016a)
Downstream Hailsham North Sewage Treatment Works (Site C), Hurst Haven	1.95 mg/l (1951 µ-P l-10)	0.91 mg/l (91 µ-P l-1)	0.06 mg/l	0.04 mg/l
Downstream Hailsham South Sewage Treatment Works (Site G), Horse Eye Sewer	5.38 mg/l (5380 µ-P l-1)	0.64 mg/l (644 µ-P l-1)	0.56 mg/l	0.52 mg/l

Sediment can provide almost all of submerged macrophytes' nitrogen needs in the short term (Barko et al., 1991) but nitrogen is likely to be lost from sediment faster than phosphorus and so is more likely to limit the growth of submerged macrophytes (Barko et al., 1991).

The EPPO Report of a Pest Risk Analysis (EPPO, 2006) concluded that for Europe and the Mediterranean region there are no upper nutrient limits to growth for *H. ranunculoides*. Hussner and Löschs (2009) growth experiments showed that combined low soil nutrient levels limited the relative growth rate of *H. ranunculoides* but they did not establish the lower point at which these impacted on the growth nor which nutrient was the limiting factor. In the UK, *H. ranunculoides* and native species growth rates were comparable under low nutrient conditions (EPPO, 2010), so this species could potentially cease to be a threat if the lower nutrient levels could be achieved. So, it is particularly important to discover what these lower levels are that limit the growth rate, whether they are co-limiting, or if one nutrient, nitrogen, or phosphorus, is the limiting factor.

2.6.6. Climate change

Biological invasions are linked to global change (Arim et al., 2006), including directly in the aquatic environment through changes in water temperature (Patrick et al., 2012). It has been shown that warmer water increases the growth rate and survival of invasive macrophytes, giving them a more competitive ability (Patrick et al., 2012). Patrick et al. (2012) showed how a rise in water temperature particularly improved the late season growth of another aquatic invasive species, *Myriophyllum spicatum*, and Millane and Caffrey (2014) noted that it is warm autumn temperatures that are required to germinate seed in *H. ranunculoides*.

Initial invasion and rapid local spread of *H. ranunculoides* is due to the exceptional asexual (vegetative ramets) regenerative capacity (Robert et al., 2013) but long term viability and resistance to dynamic regimes and extreme events is associated with the ability to reproduce sexually as this facilitates genetic diversity and adaptability (Li, 2014). Although seeds have a minor influence on community composition or population dynamics, sexual reproduction is likely to lead to a more resilient population, allowing the species to recover from extreme events (Li, 2014), which are an increasing feature of climate change, and adapt to chemical control methods through resistance. Macrophytes are thought to be poor germinators in the field but data to support this is scarce (Bakker et al., 2013), so studies on germination requirements and rates would be a valuable addition to the limited existing information.

The Intergovernmental Panel on Climate Change (2014) reported that global atmospheric temperature, driven primarily by anthropogenic CO_2 emissions, is virtually certain to rise by at least 2°C compared to pre-industrial temperatures over the 21^{st} century, under all emissions scenarios, and by 2019, a 1°C rise had already been experienced (The Chartered Institute of Ecology and Environmental Management, 2019). The impact of a rise in temperature on the sexual reproductive capacity of *H. ranunculoides* in its European invaded regions is not known.

2.7. Synthesis

This review has shown that freshwater macrophyte communities are particularly threatened by biological invasions and *H. ranunculoides* is well established as an invasive species in Europe and the UK costing a considerable amount to control. However, despite the many years of invasion history the only evidence of *H. ranunculoides* impact on plant communities is on the reduction in plant species richness; the impact it has on species assemblage is poorly understood and its impact on native macrophyte communities has not been studied. In addition, there have been many attempts across the world to control the spread but the impact of regular disturbance (as annual removal of *H. ranunculoides* biomass) on plant diversity, species assemblage and community dynamics is not known. Also important is understanding which nutrients play the key role in the determining the growth and expansion capability. Finally, as the climate changes in its invasive range, understanding the consequences of this on its reproductive capabilities are necessary to evaluate whether existing control methods will be effective in the future.

Strategic decisions on the assessment of impact and control measures have, to date, largely been made using anecdotal evidence. This research will build on the existing knowledge by investigating the following three topics:

Native macrophyte communities underpin the designations of many protected wetland sites (Natural England, 1990) and are a component element of the European WFD status of waterbodies (Centre for Agriculture and Biosciences International, 2020b). Understanding the interactions between invasive and native species, and the effectiveness of current control methods, will help assess if it is possible to continue to meet the criteria for protected sites and the WFD, inform non-native species impact assessments and determine if there are sustainable and resilient control measures for the future.

Control methods should consider the two most important factors influencing the growth; namely high nutrient levels and environmental manipulation (shading and flow). Unfortunately, on many sites the manipulation of key environmental factors is constrained or not feasible. Therefore, the emphasis needs to be on determining the nutrient levels and if these are proportional to the growth and spread of the species so that future work can evaluate whether changes in land management practices might help limit invasion potential.

If rising temperatures facilitate the production of viable seeds, control methods using de-silting and re-profiling methods would not work if the sediment were not removed, stockpiled, or buried to prevent germination (EPPO, 2014). In addition, mechanical and chemical control methods may facilitate re-colonisation by seed germination by providing light and a bare substrate (Clout and Williams, 2009), so alternative methods of control may need to be developed.

The data collected and analysed during this research will provide evidence about changes to the community composition because of biological invasion. It will not only provide the scientific knowledge to support management decisions but help inform policy on INNS in the long term.

Chapter 3 - Methodology

3.1. Introduction

Chapter 2 reviewed the existing literature and introduced *H. ranuculoides* as an INNS, describing its ecology, growth, reproduction, and distribution. The European macrophyte communities which it might invade were described and the changes that might occur due to disturbance and competition resulting from invasion, were considered. The potential impacts of these invasions both globally and locally were outlined. Climate in its native and invaded regions were illustrated and the potential for expansion of *H. ranuculoides* invasion because of climate change predictions was considered.

This research approach combines studies in the field with controlled greenhouse and laboratory experiments. This is a novel approach, but practically effective, because the often-variable findings in the field can be supported by evidence from replicable experiments which source material from the same field sites.

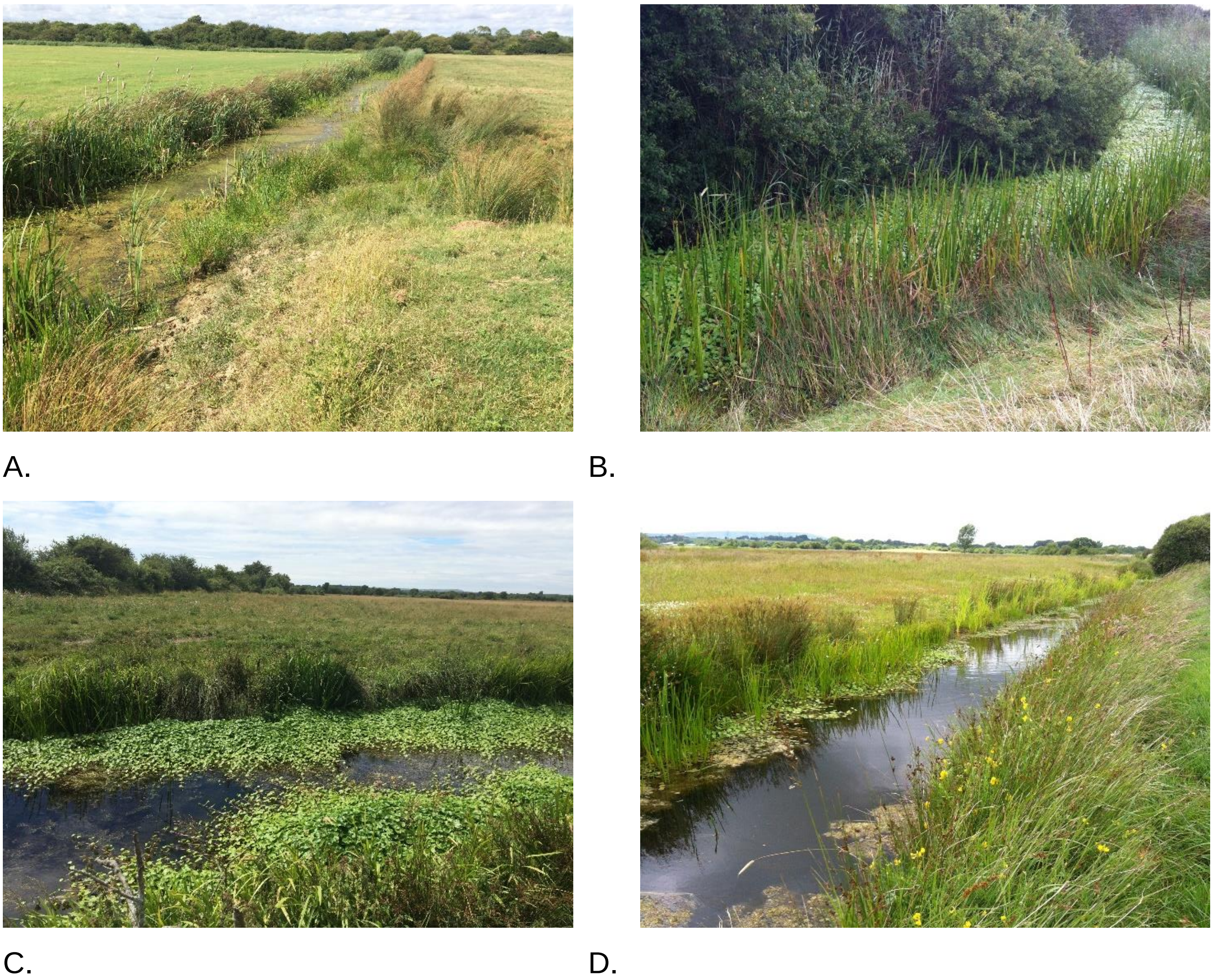
The field site used was the Pevensey Levels in the UK because it has one of the most extensive infestations of the studied INNS, *H. ranunculoides* (Figure 7), in combination with some of the most diverse aquatic plant communities in the UK (Centre for Agriculture and Biosciences International, 2020b). Its water regime is controlled by an EA water level management plan (WLMP) (Atkins Ltd, 2015) which minimises seasonal rainfall effects.

The greenhouse approach was used to examine the effects of nutrient availability on the growth of *H. ranunculoides* and the influence of temperature on growth, seed production, germination, and invasive potential of *H. ranunculoides.*

Figure 7: Field ditch, Rickney, Pevensey (site 18HR), July 2016 to illustrate the level of *H. ranunculoides* infestation that can occur in the field sites before weed-cutting (Photograph Birch, J.).

3.1.1. Selection of field sites

The study location used was the Pevensey Levels, a wetland in Sussex, UK, intersected by a network of watercourses with an exceptional assemblage of macrophytes (Joint Nature Conservation Committee, 1999). Approximately 10% of the watercourses, 45km, are infested by an INNS, *H. ranunculoides* (Royal Haskoning, 2011). The survey sites used in the field studies are heavily modified watercourses or man-made drainage ditches all of which have artificially managed water levels and controlled low flows for most of the year (Atkins Ltd, 2015). Figure 8 illustrates four of the monthly survey sites which are representative of the 60 total sites. The sites are described in terms of location, type of watercourse, biodiversity, land use and water management.



A. B. C. D.

Figure 8: Four typical survey sites photographed (by J. Birch) June/July 2014. A. un-infested site, B. infested site, C. weed-cut site, D. weed-cut and sprayed site.

The Pevensey Levels, central grid reference TQ 628 085, was chosen as it falls wholly within the Environment Agency 2006 WLMP (2006) and routine maintenance programme so there is historic data available, and a degree of consistency in some of the environmental variables such as water level, watercourse and vegetation management. In addition, the EA routine maintenance programme, 2013 – 2017, implemented a previously trialled *H. ranunculoides* management method (Royal Haskoning, 2011). This provided an ideal opportunity to monitor the effects of this new management strategy on *H. ranunculoides* and the native macrophyte communities over a period of four years.

The field surveys were undertaken on 60 sites and included macrophyte surveys of a 100-metre length at each site over four years. Environmental data were collected as observational variables and measured using a handheld water quality YSI meter (Xylem

Inc., 2019). Field measurements of nutrients in sediment and water were taken, sediment was sampled using a core sampler and water was sampled by collecting directly from the watercourse into a new 1L sample bottle. However, the effects of differing levels of nutrients on the growth of *H. ranunculoides* required controlled, replicable conditions. Although seed can be collected from the wild, temperature regimes, viability and germination rate also required controlled replicable conditions. Experimental studies were carried under controlled conditions in a horticultural greenhouse.

3.1.2. Pevensey Levels

3.1.2.1. Location and description

Pevensey Levels (Figure 9) was originally a saltmarsh that was reclaimed for agricultural use and has been pump drained since the 1960s (Toogood, 2005). It is a low-lying area in East Sussex between the South Downs, and the town of Eastbourne, in the west and the town of Bexhill in the East (Atkins Ltd, 2015) (Figure 9). Much of the site is below the five-metre contour line, 2m above ordnance datum (OD), and most lies below the high tide level which can reach 3.9m above OD (Toogood, 2005). The site has a complex network of three types of watercourse: main rivers, pump and feed drains and field ditches. The soils are poorly drained alluvial clays with low hydraulic conductivity of about 0.05 m/day (Atkins Ltd, 2015). With above average daily sunshine hours for the area due to the rain-shadow effect from the South Downs, high mean annual temperatures and the lack of shelter from the westerly winds, evapotranspiration is one of the most important influences (Atkins Ltd, 2015).

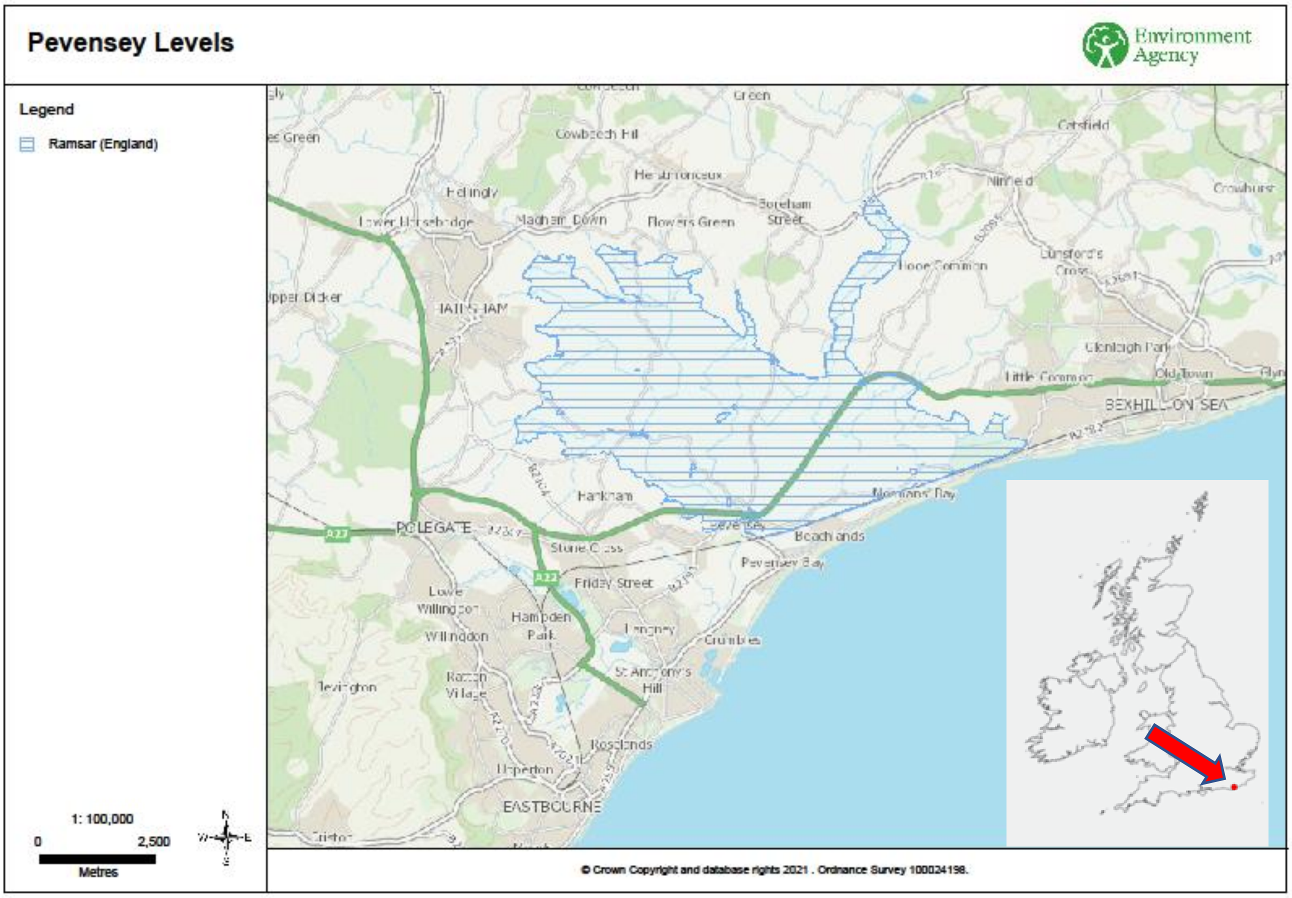


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Figure 9: Map of the study site, Pevensey Levels, East Sussex, UK, with the protected site highlighted in blue.

3.1.2.2. Designations and biodiversity

Pevensey Levels comprises 4,300 hectares (ha) of the habitat of principal importance ‘Coastal and Floodplain Grazing Marsh’ and contains the most diverse ditch community in the UK (Atkins Ltd, 2015). The site has three statutory designations: Site of Special Scientific Interest (SSSI), Ramsar and Special Area of Conservation (SAC) (Natural England, 1990, Joint Nature Conservation Committee, 1999, Joint Nature Conservation Committee, 2016b), which cover 3,500 ha of the Pevensey Levels. The purpose of the designations is to conserve the diversity of wetland and aquatic plant, animals, and birds. Both the Ramsar and SSSI site designations cite ‘an outstanding assemblage’ of macrophytes (Joint Nature Conservation Committee, 1999, Natural England, 1990). Macrophyte communities are usually species-poor and often dominated by one or two species (Rodwell, 1998) but 110 of the 160 plants in Great Britain that can be described as macrophytes can be found on the Pevensey Levels (Diston et al., 2007). Macrophytes are those which grow, or are basally rooted, in permanent water (Preston and Croft, 2001). Of note are the macrophytes listed in Table 5.

Table 5: Macrophytes of conservation interest on the Pevensey Levels (Preston and Croft, 2001, International Union for Conservation of Nature, 2020).

Species	Common Name	Conservation Status
Hydrocharis morsus-ranae	Frogbit	Nationally Scarce
Potamogeton actuifolius	Sharp-leaved Pondweed	Threatened, RDB Vulnerable
Stratiotes aloides	Water-soldier	Nationally Scarce
Potamogeton friesii	Flat-stalked Pondweed	RDB Near Threatened
Sium latifolium	Greater Water-parsnip	Nationally Scarce, RDB Endangered
Wolffia arrhiza	Rootless Duckweed	Nationally Scarce

3.1.2.3. Historical and current land use

There are records of Pevensey Levels since Roman times when it was still saltmarsh and inundated at high tide except for small islands (Pearson, 1999). At the time of the Domesday Book in 1086 it still supported 21 salt pans around the perimeter but by the time of the 1287 flood almost all had been reclaimed for fertile arable land (Salzmann, 1910). It remained principally a freshwater marsh with periodic saltwater floods until the 1500's when the sea wall failed, and it returned to saltmarsh for approximately 100 years. In 1698, when commissioners were appointed to survey the coast, the seawater had been excluded and the marsh looked much as it does today (Salzmann, 1910). Pump drainage was installed between 1950 and 1980 and in the 1990s English Nature (now Natural England) introduced an agri-environment payment scheme to encourage conservation of the freshwater communities of plants and animals and restore the traditional management practices (Atkins Ltd, 2015). WLMPs were produced for the Pevensey Levels in 1998, updated in 2006 and reviewed in 2014 (Atkins Ltd, 2015). These were implemented using a pump and sluice system to provide ditch water levels that provide a compromise between the conservation objectives and farming requirements (Atkins Ltd, 2015). This was reviewed in 2006 dividing the SSSI into eight hydrological units; Manxey, Gravity, Horse Eye & Down, Whelpley, Star Inn, Waterlot and Glynleigh and Barnhorn. (EA, 2006). The Levels are predominantly coastal and floodplain grazing marsh habitat, grazed by cattle and sheep with some of the surrounding land under arable crops (Atkins Ltd, 2015).

The ditches are rotationally managed for use as ‘wet fencing’ and for livestock drinking (Atkins Ltd, 2015). The livestock farming has the potential to increase the nutrients despite the phosphorus stripping.

3.1.2.4. Type of watercourse

The main rivers are generally wide, >10m, canalised and embanked high level channels used not only to take run-off from the upper catchment in times of excess but, also as water storage in winter when the system is tide-locked and to feed into the Levels in the summer (Atkins Ltd, 2015). The non-main rivers are pumped drains and feed watercourses which are also widened, 5-10m, and canalised for much of their length but with greater geomorphological diversity (Atkins Ltd, 2015). These are used to pump water from the Levels to the embanked channels or to transfer water back from the channels to the Levels. The privately owned field ditches are smaller, being <5m, but predominantly heavily modified and usually straightened, being used both to maintain field water levels and as ‘wet fencing’ and drinking water for livestock. The whole system is too low lying to convey water to the sea naturally so the water is pumped and discharged from the two main rivers, the Pevensey Haven and Wallers Haven, into outfalls with tidal flap sluices (Atkins Ltd, 2015).

3.1.2.5. Water management

For Pevensey, the WLMP describes how water levels for the SSSI can be managed to maintain or achieve favourable condition (Atkins Ltd, 2015). In practice this means maintaining water levels 0.3m below mean field level throughout the year in all types of watercourse in all the eight hydrological units (Atkins Ltd, 2015). Ditch management is linked to water management which has historically been for grazing and includes keeping water levels higher in summer (Atkins Ltd, 2015).

The approximate annual mean rainfall between 1968 to 1998 was 763mm, which is less than half the requirement to maintain the soil water deficit during the summer (Atkins Ltd, 2015). The water held in the high level embanked channels, plus the output from the sewage treatment works, is used to supplement the levels in the field ditches in summer to achieve the required level to maintain the ecological designations (Atkins Ltd, 2015).

3.1.3. Field survey sites

3.1.3.1. Geographical location

Sixty, 100m long, survey sites were chosen in accordance with the EAs macrophyte monitoring protocol for the WFD (Environment Agency, 2016c) and a review of methods by Gunn et al. (2010) which found that wading 100m transects was the most practical without reducing efficacy. The sites were chosen using a stratified sampling technique, with representation from all three of the watercourse types; main river, pump and feed drains and field ditches and each of the management treatments; weed-cut, weed-cut and spray, weed-cut and de-silt, weed-cut, spray, and de-silt, (Table 6).

Table 6: Management treatments applied to the survey sites (n=60).

Management treatment	With *H. ranunculoides*	Without *H. ranunculoides*,
Reference, un-cut	8	12
Weed-cut	14	0
Weed-cut and sprayed	8	0
Weed-cut and de-silted	8	0
Weed-cut, de-silted, sprayed	10	0

Weed-cutting was done using a long-arm excavator tracking along the watercourse bank-top (Figure 10). The cut material was placed one excavator arm-length back on the bank-top. This was done in accordance with the EA’s maintenance plan (Environment Agency, 2006), not more than once in five years in line with the SSSI requirements, but annually on *H. ranunculoides* infested sites (Natural England, 2017). Herbicide spraying, using the current appropriate and approved Glyphosate formula and an appropriate adjuvant, Topfilm™ in summer and Codacide Oil for autumn, was applied when *H. ranunculoides* re-growth appeared approximately two weeks later. A three-year programme of de-silting was undertaken on selected watercourses in accordance with the SSSI requirements, each watercourse only being de-silted once (Environment Agency, 2006). The same type of excavator used for the weed-cutting was used but with a different bucket attachment. The sediment was placed one excavator arm-length back on the bank-top, left to dry out for several months, then harrowed back into the field margin. Weed boats are another means of mechanical removal used on the Pevensey Levels, particularly for the larger waterbodies but were not used in this study.

Figure 10: Example of excavator used for mechanical removal and de-silting, weed-cutting *H. ranuculoides* on the Pevensey Levels. (Photograph, Birch J).

Sixty sites (Figure 11) were required for a minimum sample size of six replicates of each management treatment plus baseline sites with and without *H. ranunculoides* and to allow for unforeseen circumstances. Six replicates were considered sufficient because macrophyte communities are relatively species poor and the individual samples were large (100m lengths) compared to the 2x2m botanical survey quadrats used for wet grassland communities (Toogood and Joyce, 2009).

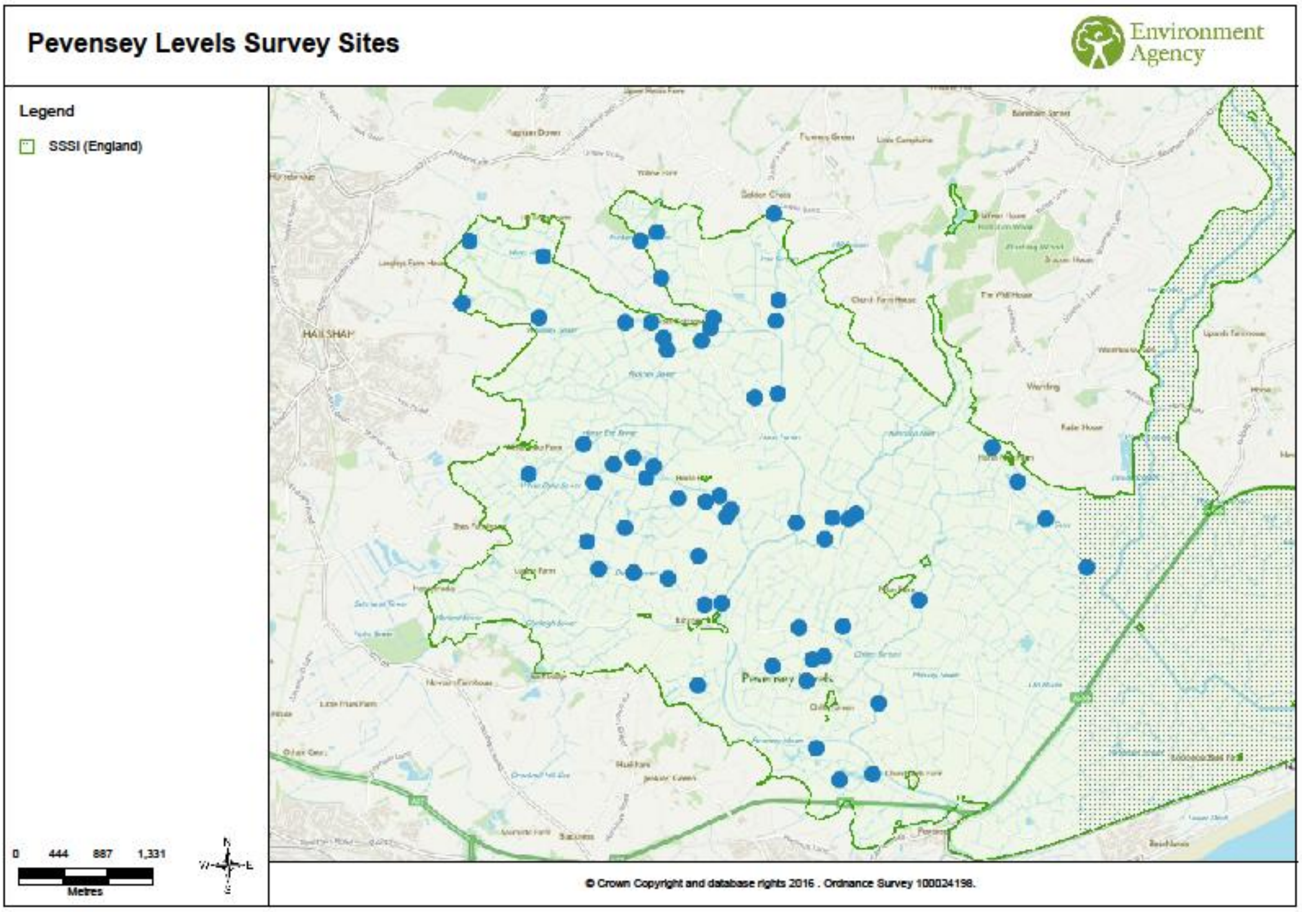


Figure 11: Locations of the 60 survey sites used for the study on the Pevensey Levels.

Once the watercourse types and management treatment areas had been identified, the survey sites could not reasonably be randomly selected due to the small sample size. So, these were systematically selected to represent upper, mid, and lower stretches, where possible (main river and pump and feed drains), and field ditches that were easily accessible to ensure the surveys could all be undertaken during the appropriate season.

The sites were coded using two letters to represent the name and location of the site and a number in order of selection, for example 01HS identifies the first site selected, the Horse Eye Sewer located at ‘Spaghetti Junction’ (a confluence of pump drains). These shortened name codes have no analytical value but were used to identify sites within years, for monthly analyses, and between years, for annual analyses.

3.1.3.2. Baseline conditions

All 60 sites were surveyed in 2013, prior to any de-silting or spraying management treatment, weed-cutting being the only management method used up until 2014. Table 7 shows this information which includes the site code, watercourse size, watercourse depth, silt depth, land use and community type.

Table 7: Baseline condition of the sites, 2013, including watercourse size, depth, silt depth, land use and community type. Size of watercourse: S – Small <5m, M – Medium 5-10m and L – Large > 10m. Land use codes: 1 - Unimproved, 2 - Semi-improved, 3 - Rush Pasture, 4 - Rough Pasture, 5 - Other (trackway, road or modified). The British National Vegetation Classification (NVC) codes are explained in Table 8.

Site Code	Size Width	Depth % 1m+	Silt depth mm	Land use N/E	Land use S/W	NVC
01HS	M	100	750	1	5	A11
02CH	S	80	500	3	3	S4
03WS	M	100	450	5	1	A3
04RR	M	90	850	2	5	A11a
05KR	L	90	750	3	3	A5b
06HB	M	80	500	1	1	A11a
07WH	M	90	750	2	2	A3
08CR	S	100	500	2	5	A3
09HG	M	90	750	1	1	A3
10RU	S	80	450	1	3	A3
11HG	S	90	500	5	1	S14
12CK	M	80	300	1	1	A3
13PN	M	90	1200	2	2	A3
14IU	M	70	900	3	3	S14
15DU	M	100	500	4	3	A3
16DM	M	90	450	5	4	A3
17DL	M	90	400	4	5	A5b
18HR	S	80	600	1	5	S12d
19WF	M	50	750	3	3	A3
20HU	M	90	600	2	2	A3
21CU	M	80	750	4	1	A3
22CM	S	80	650	3	1	A3
23MB	S	70	350	3	3	A4
24MD	M	80	450	3	3	A3
25MN	S	60	600	4	4	S8b
26CF	S	80	300	3	2	A3
27HP	L	100	550	3	2	A3

Site Code	Size Width	Depth % 1m+	Silt depth mm	Land use N/E	Land use S/W	NVC
28HM	L	100	650	3	4	A11a
29HN	L	100	750	2	4	A3
30HR	L	100	600	3	3	S14
31ML	M	90	600	2	3	A3
32MM	M	90	600	2	3	A3
33MU	M	90	500	2	3	A3
34HL	M	90	900	5	5	A3
35WE	S	80	750	3	3	A3
36WW	S	80	500	3	3	S8b
37HS	S	80	450	3	3	S14
38CG	S	50	300	2	2	A3
39MW	S	80	450	3	3	A3
40DN	S	80	450	4	4	S14
41WU	S	90	950	5	4	A4
42WM	S	90	300	1	3	A4
43WL	S	100	300	2	5	A3
44RL	M	90	600	4	2	A3
45PM	S	100	950	4	3	A11a
46PU	S	100	550	3	3	S14
47IM	M	90	600	2	2	A5b
48IL	M	90	850	2	2	S14d
49CM	M	90	500	2	4	A3
50CB	L	100	300	4	4	A11a
51VS	S	30	600	2	2	A4
52CC	M	80	300	2	2	A3
53HF	S	80	450	1	1	S12c
54HW	S	80	300	1	1	A4
55KM	L	100	500	3	3	S5b
56HK	S	70	600	3	3	S14
57VN	S	50	300	4	4	A3
58HD	S	90	300	1	1	A3
59KF	M	90	600	2	2	S14
60HH	S	50	600	4	5	A4

Table 8 lists the NVC communities found in the survey sites based on analysis using the Modular Analysis of Vegetation Information System (MAVIS) plot analyser (Centre for Ecology and Hydrology, 2016). This provides a percentage probability of the community types according to the species found and their abundance. Each analysis returns the 10 top communities but only the community with the highest percentage probability was used to describe each site in Table 8. A minimum of five quadrats are recommended to determine an NVC but only one 100m survey length was available, in each year, for each site. These were sampled by wading the stretch, recording percentage cover of observed species and, if the water was turbid, grapnel samples were taken every 10m to ensure submerged species were not missed. However, the 100m survey lengths used for this study are far larger than the 1-2m quadrats commonly used for terrestrial habitats so would encompass considerably more than five 2m squares at each individual site. An accepted workaround for individual quadrats or survey length is to convert cover values to the constancy values that would be obtained for each species appearing in the five quadrats (Smart et al., 2016). Converted percentage cover to constancy values were as follows: <2% = I, 2-5% = II, 5-10% = III, 10-20% = IV, >20% = V (Smart et al., 2016).

Table 8: NVC community types found in the 60 survey sites, where A are aquatic communities and S are swamp and tall herb fen communities (Rodwell, 1998).

Code	**Community**
A1	*Lemna gibba* community *Lemnetum gibbae* Miyawaki & J. Tx. 1960
A2	*Lemna minor* community *Lemnetum minoris* Soó 1947
A3	*Spirodela polyrhiza - Hydrocharis morsus-ranae* community
A5	*Ceratophyllum demersum* community *Ceratophylletum demersi* Hild 1956
A11	*Potamogeton pectinatus - Myriophyllum spicatum* community
A15	*Elodea canadensis* community
S4	*Phragmites australis* swamp and reed-beds *Phragmitetum australis* (Gams 1927) Schmale 1939
S5	*Glyceria maxima* swamp *Glycerietum maximae* (Nowinski 1928) Hueck 1931 emend. Krausch 1965
S8	*Scirpus lacustris ssp. lacustris* swamp *Scirpetum lacustris* (Allorge 1922) Chouard 1924
S12	*Typha latifolia* swamp *Typhetum latifoliae* Soó 1927
S13	*Typha angustifolia* swamp *Typhetum angustifoliae* Soó 1927
S14	*Sparganium erectum* swamp *Sparganietum erecti* Roll 1938
S16	*Sagittaria sagittifolia* swamp

Table 9 describes the communities found on the Pevensey Levels in the surveyed sites.

Table 9: NVC community descriptions based on Rodwell (1998).

Community Name	**Description**	**Notes**
A1 *Lemna gibba* community	Characterised by floating mats of duckweeds with emergent marginal vegetation. This community is typical of standing eutrophic and base rich waters, especially in S.E. England.	Often found in situations too disturbed to support other communities and those where it can make a quick recovery from weed-cutting and de-silting. It also tends to dominate after herbicide treatment.
A2 *Lemna minor* community	This community consists almost entirely of floating mats of *Lemna minor* and can be found widely throughout lowland Britain. There is a *L. trisulca* sub-community which prefers clearer water.	Found in standing or slow-moving mesotrophic to eutrophic waters and less base-rich demanding than the A1 community.
A3 *Spirodela polyrhizza – Hydrocharis morsus-ranae* community.	Characterised by floating mats of duckweeds with associated submerged species and a few shorter emergent that do not provide shade. It requires unpolluted and unshaded mesotrophic to eutrophic standing waters, particularly those that are calcareous or with a saline influence.	It tolerates disturbance well; in fact, disturbance is necessary to prevent succession to taller swamp communities. Herbicide treatment combined with disturbance or pollution can result in simpler duckweed communities
A5 *Ceratophyllum demersum* community *Ceratophylletum demersi* Hild 1956.	Characterised by dense submerged mats of *Ceratophyllum demersum* often with *Elodea spp*.	Typical of slow flowing or standing, eutrophic watercourses and prefers higher nutrient levels both of phosphorus and inorganic nitrogen
A11 *Potamogeton pectinatus - Myriophyllum*	This community can contain several other *Potamogeton* species but apart from	Prefers clear, eutrophic, base rich and sometimes slightly saline waters. Although it is

spicatum community.	*Myriophyllum spicatum* not many other plants are abundant due to the density of these submerged species. It has several sub-communities one of which is particularly associated with *Chara* spp.	found in standing and slow-moving waters it can tolerate quite fast-moving water but not those that are polluted or turbid
A15 *Elodea canadensis* community	A species poor community dominated by this North American pondweed preferring still to sluggish moving and nutrient rich waters.	It has spread widely since its introduction approximately 150 years ago. After the initial invasion it declines after 7-15 years. It is found in the lowlands of Britain where there is more nutrient enrichment and warmer summers
S4 *Phragmites australis* swamp and reed-beds *Phragmitetum australis* (Gams 1927) Schmale 1939.	This community is dominated by *Phragmites australis,* which is the only constant species, and it is usually very species-poor. However, it is variable, both morphologically and composition-wise; there are four sub-communities.	Prefers eutrophic, undisturbed conditions and, although tolerant of moderately saline conditions, salinity limits growth. Low nutrients, both nitrogen and phosphorus, have been shown to limit growth
S5 *Glyceria maxima* swamp *Glycerietum maximae* (Nowinski 1928) Hueck 1931 *emend.* Krausch 1965.	This community is dominated by *Glyceria maxima* and is typically found in eutrophic, often base rich, conditions.	Prefers slow-moving water or marginal conditions and is usually species poor.
S8 *Scirpus lacustris ssp. lacustris* swamp *Scirpetum lacustris* (Allorge 1922) Chouard 1924.	This is a community of deep, eutrophic, standing or slow-moving water and it often overlaps with *Phragmites* communities	(Now *Schoenoplectus lacustris*).

S12 *Typha latifolia* swamp *Typhetum latifoliae* Soó 1927	*Typha latifolia* is always dominant in this community and no other plant species are frequent.	Found in neutral to basic, slow-moving mesotrophic to eutrophic waters and can tolerate some salinity.
S13 *Typha angustifolia* swamp.	This is dominated by *T. angustifolia* and rarely species rich. Found in standing or slow-moving neutral to base-rich lowland waters.	It can grade to rich-fen vegetation but is declining due to disturbance and eutrophication.
S14 *Sparganium erectum* swamp *Sparganietum erecti* Roll 1938.	*Sparganium erectum* is the only dominant species but it can be quite a species rich community.	This community is common and found in shallow, mesotrophic to eutrophic waters, preferring slow flowing conditions.
S16 *Sagittaria sagittifolia* swamp.	This is characteristic of moderately deep eutrophic waters such as canals and wide dykes. Typical of standing or slow-moving waters, it can tolerate turbulence as it is submerged, and strap-shaped early emergent leaves are streamlined.	It is found mostly in central and southern England but declining due to its susceptibility to pollution. Generally, species-poor, although may co-exist as a marginal habitat with other floating and submerged communities

3.1.3.3. Water nutrient parameters

Diston et al. (2007) suggested that nitrate is the main type of nitrogen driving the growth of *H. ranunculoides* on the Pevensey Levels. Diston et al. (2007) found a median value of nitrate in water across their study sites of 2.49 mg/l which is very low on the EA GQA grading. Soluble Reactive Phosphorus (SRP) is the primary form of phosphorus available to plants and has historically been elevated in the water on the Pevensey Levels due to the discharge from two sewage treatment works (Diston et al., 2007). SRP in the Diston et al. study (2007), post phosphate stripping at the sewage treatment works was between 91-498 µg-P l^{-1}.

3.1.3.4. Sediment nutrient parameters

Toogood (2005) sampled soils from wet grassland on the Pevensey Levels for the major nutrients and found levels of 5-7.5 mg/l of nitrogen in swamp communities (which are the closest community type to the macrophyte communities) but virtually no phosphate. However, four of the field grassland sites were adjacent to the ditch sites in this study and had high levels of both nitrogen 10-45 mg/l and phosphate 10-70 mg/l in the soils. The phosphate in the sediment of the watercourses may have accumulated from the high levels present in the water downstream of sewage treatment works (Diston et al., 2007).

3.1.4. Climate

3.1.4.1. During study

The climate during the field study is shown in Figures 12-14, as: average maximum and minimum monthly temperature, monthly rainfall, and monthly sunshine. This was taken from the nearest Met Office weather station to the study site, Eastbourne, Latitude 50.762, Longitude 0.285. Sunshine data were taken using a Campbell Stokes recorder (Met Office, 2020d).

2014 exhibited higher maximum summer temperatures than the other three years, with four summer growing months, June, July, August, and September above 20^{o}C, but had a colder winter than 2013 or 2015 (Figure 12). 2015 had higher rainfall during the late summer growing months of July, August, and September than the other three years except August 2014, which was a wet month (Figure 13). 2013 had the highest summer sunshine hours of the study period, with July and August being above 250 hours for each month but September 2013 had the lowest sunshine hours compared to the other three years (Figure 14). 2014 was the warmest year since 1910 (Met Office, 2020a) and this, combined with the most consistent summer sunshine hours, is likely to have improved growing conditions. The colder 2014 winter may have suppressed the growth of *H. ranunculoides* which might have influenced the lower abundance levels in 2015. The rainfall was the most variable across the field study period but, as the site has managed water levels (Environment Agency, 2006), this is less likely to have affected the results.

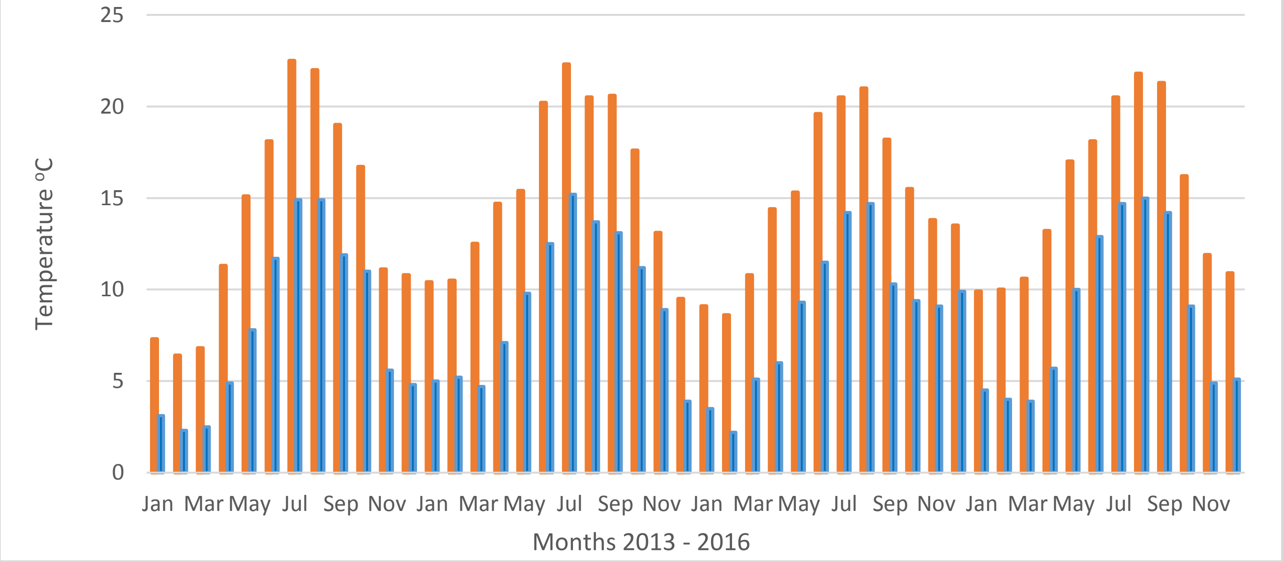


Figure 12: Average maximum (red) and minimum (blue) monthly temperature, in ^{o}C, for Eastbourne during the field study period, January 2013 to December 2016 (Met Office, 2020d).

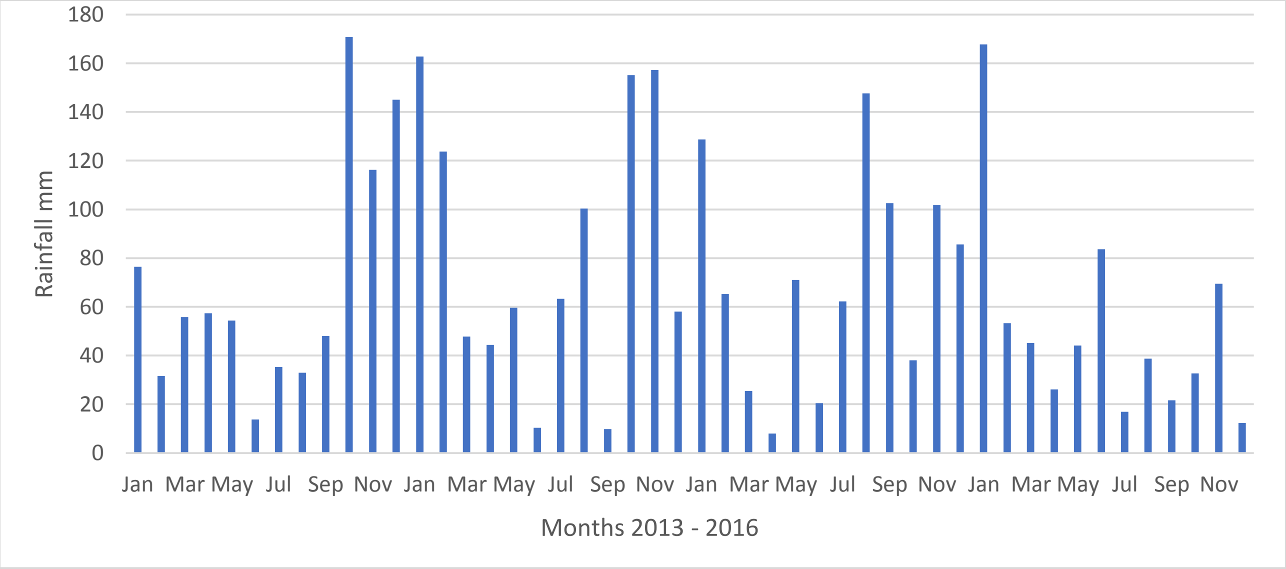


Figure 13: Monthly rainfall for Eastbourne during the field study period, January 2013 to December 2016 (Met Office, 2020d).

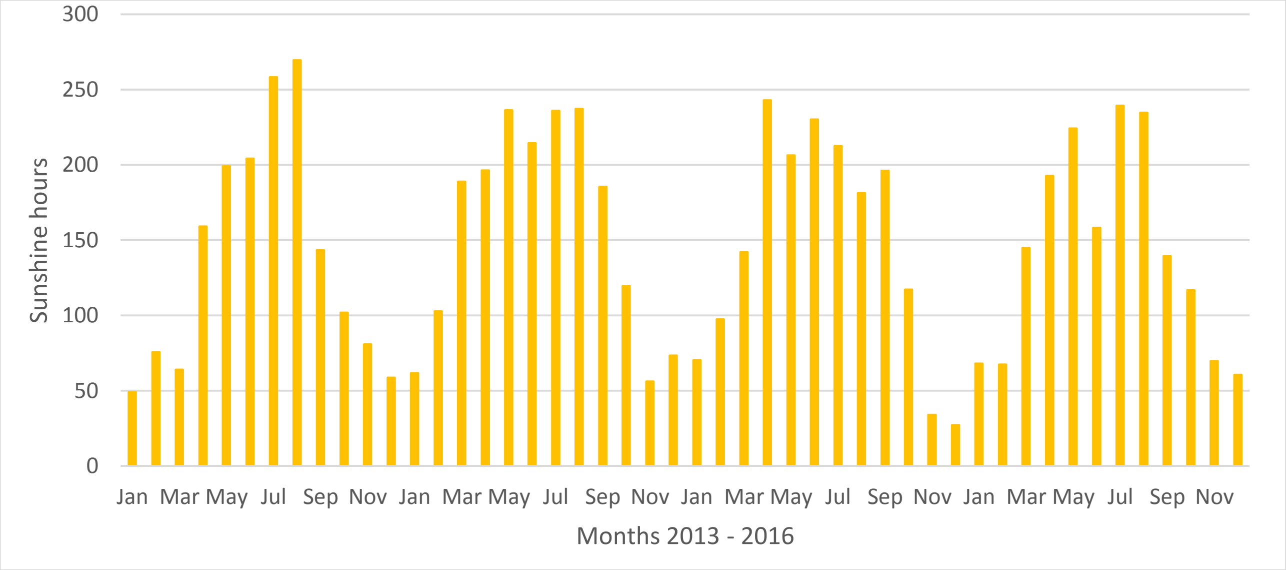


Figure 14: Monthly sunshine hours for Eastbourne during the field study period, January 2013 to December 2016 (Met Office, 2020d).

3.1.4.2. Climate context

During the field study period, 2013-2016, mean summer and autumn temperatures in England were generally above the mean average compared to the period 1981-2010, particularly 2014, where all months were warmer than average except August (Met Office, 2020c). 2014 being the third warmest in a series since 1910, with the highest above average temperature reaching 2.0°C in the UK, in April (Met Office, 2020c).

3.2. Introduction to data collection

Observational field data were collected to quantify any macrophyte community change because of the presence of the INNS *H. ranunculoides* and to define the macrophyte community composition of watercourses treated to remove the species. Experimental data were collected from two greenhouse studies to describe the regeneration capacity as growth at different nutrient availabilities and to determine the impact of a rise in mean summer / autumn minimum temperature of 2°C.

3.2.1. Field surveys

3.2.1.1. Aims and objectives

The aims of the field research were to identify the impact of *H. ranunculoides* on native macrophyte communities, the effectiveness and impact of management treatments and the influence of nutrients on its abundance. The field study objectives were to quantify macrophyte community change in relation to *H. ranunculoides* infestation and its management treatments, evaluate the effectiveness of management treatments in reducing abundance of *H. ranunculoides* and examine the influence of key nutrients on the growth of H. ranunculoides.

To achieve these objectives, field surveys were undertaken, in the period June to September, on sites that had *H. ranunculoides* present and those that were *H. ranunculoides* free, over a period of four years, 2013 to 2016. The EA's macrophyte monitoring protocol for the WFD, Operational Instruction (OI) 131_07 (Environment Agency, 2016c) was used. A copy of the recording form can be found in Appendix 1. Using the same survey protocol, the community composition of untreated *H. ranunculoides* sites was compared with those where management treatments were undertaken. Abundance of *H. ranunculoides*, as percentage cover, obtained from these surveys was used to evaluate the effectiveness of the management treatments. Water and sediment samples were taken from the same sites during the surveys, and these were used to investigate the influence of nutrient on the abundance of *H. ranunculoides.* Water samples were taken directly from the watercourse using a clean, new 1L sample bottle. Sediment samples were taken manually using a core sampler and a clean, new 1L tub filled with an appropriate portion avoiding base clay layers or anoxic surface layers. These were stored in a cool box and placed in a refrigerator at the end of each survey day for transport by overnight courier to the EA's accredited laboratory for analysis (Environment Agency, 2016b).

3.2.1.2. Macrophyte survey techniques

At each of the 60 sites, a 100m survey length was measured out using a tape measure and the grid references of the start and finish recorded. Where possible, for practical field reference, clear visible markers such as a fence post or adjacent field ditch confluence were used to mark the beginning or end of the 100m survey length. Navigation of the survey length was undertaken by wading in a dry suit for at least 80% of the length and any inaccessible areas then surveyed from the bank. If the bed of the channel was not clearly visible, a grapnel was also used every 10m along the length to sample submerged species. The channel was waded in an upstream direction so disturbed sediment did not obscure the visibility and traversed every 10 metres at each site. All macrophytes that grow and flower under conditions of nearly permanent saturation or submergence of their basal parts were recorded using an EA SD01 survey form modified to reflect the taxa present in the geographical region (Environment Agency, 2016c). For the purposes of this study, and in accordance with the EA survey form, a macrophyte includes any higher plant, bryophyte or filamentous algae that can be observed with the naked eye. Samples were taken of taxa that require specialist identification skills and sent to a recognised expert aquatic botanist for verification. Macrophyte nomenclature used was in accordance with Stace (2019). Once all macrophyte taxa were recorded the length was then assessed for percentage cover of each species. Given that there are submerged, emergent and floating species, in some cases, there could, theoretically, be more than 100% cover due to layers of vegetation being present. However, due to the statistical methods used for analysis, in practice, a maximum value of 100% was used in the field. Finally, a measure of confidence was assigned to each survey to decide if any factors may have distorted the survey results. Factors affecting the survey could include de-silting, weed-cutting, herbicides, livestock grazing, recent extreme flooding events, or excessive turbidity. The measures of confidence were as follows: A – less than 25% of the survey length affected, B – 25-50% affected and C – more than 50% affected.

Practically, due the requirement of double-manning because of entry to water, reliance on volunteers, studying part-time and the length of time required to access the sites, the surveys were undertaken over a period of several months. To assess potential seasonal effects, eight sites were also surveyed monthly for the three years of the management treatment, from May to October. The monthly sites were chosen to be geographically as close as possible without direct connectivity, to be of similar width, and for ease of access due to frequency of sampling and time restraints. One site for each treatment was selected with two reference sites without *H. ranunculoides* in case infestation occurred during the survey period and a further 'spare' in case management changed during the survey period. These sites remained the same for all three years. Within each year the timing of the annual surveys was randomised. In addition, within each year, results from a randomised stratified sample (one from each management treatment) of five sites surveyed in June, compared to those surveyed in September, were subject to a Student's t-test ($p<0.05$) to determine any seasonal effects.

3.2.1.3. Environmental data collection

Environmental data were collected over a four-year period from 2013 to 2016 at the 60 selected sites in the period June to September. Environmental data were collected from each site using the following: observed physical variables, a hand held water quality and monitoring YSI, Professional Plus, meter at the time of macrophyte survey (Xylem Inc., 2019). Annual 1L water sample and pre and 1kg sediment samples taken post desilting management treatment. Observed physical variables recorded were width, depth, water clarity, shading, substrate, bed stability, water habitat and land use. YSI variables recorded were: date, time, temperature, dissolved oxygen, conductivity, and pH (Xylem Inc., 2019). The water analysis used was a standard EA WFD basic rivers suite which includes biological oxygen demand, ammoniacal nitrogen, chloride, nitrogen, orthophosphate, conductivity, turbidity, and pH (Environment Agency, 2016b). The sediment analysis used was an EA basic rivers suite which includes: ammoniacal nitrogen, nitrate, nitrite, total nitrogen, orthophosphate, phosphorus, and dry solids. Salinity can be determined, if necessary, from conductivity (Environment Agency, 2016b).

3.2.1.4. Sediment sampling protocol

Sediment samples were collected between October 2013 and July 2014 before sites were de-silted and between June and September 2016 after de-silting. Samples were taken at the start of each 100m reach. A direct manual sample collection technique was employed using a core sampler. This was a 1m long, clear, heavy duty plastic tube fitted with an open-close suction valve at the top. The samples were taken to the depth of the sediment at each site. The core sampler was then cleaned in water from the current site after use and rinsed in water from the next site before re-using. An appropriate portion of the sediment, avoiding anoxic surface layers or lower clay layers, was immediately used to fill a clean, new, 1 litre (L) EA sediment sampling container. All sample containers were labelled with prescheduled laboratory labels stating site name and number, and sample type and date. At the end of each sampling day, these containers, with pre-analysis data sheets, were refrigerated and sent by courier overnight for analysis at the EAs UKAS accredited Starcross laboratory, Exeter (Environment Agency, 2016b). The analyses are detailed in Chapter 5., 5.2.2.2 Field Methods, sediment analysis.

3.2.1.5. Water sampling protocol

Water samples were collected, annually, one for each site per year, over a four-year period from 2013 to 2016. These were undertaken in the period June to September. Samples were taken at the start of each 100m reach. A clean, new, 1L EA water sample bottle was filled directly from the watercourse at each site. All sample bottles were labelled with prescheduled greenhouse labels stating site name and number, sample type and date. These were kept in a cool box during transit prior to refrigeration at the end of each sampling day. They were then sent by overnight courier for analysis within 24 hours at the EAs UKAS accredited Starcross laboratory. The analyses are detailed in Chapter 5., 5.2.2.4 Field Methods, water analysis.

3.2.1.6. Biosecurity and compliance with legislation

H. ranunculoides and *Crassula helmsii* (present at some sites) are both listed in Schedule 9 (UK Government, 2020c). Reference sites, without *H. ranunculoides,* were always scheduled for surveying or sampling first before moving to sites known to have INNS present. All personal protective equipment (PPE) and collection equipment was rinsed off and checked for the presence of plant fragments, seeds and invertebrates when leaving each site. At the end of the survey day the ‘Check, Clean, Dry’ protocol was used for all equipment.

H. ranunculoides is listed on the EU Invasive Alien Species Regulation (IAS) Regulation 1143/2014 which came into force on 03 August 2016 (UK Government, 2020b). A derogation, under Article 8, for growing and propagating (seed germination) *H. ranunculoides* for research purposes was obtained.

3.2.2. Greenhouse experiments

3.2.2.1. Aims and objectives

The aims of the greenhouse experiments were to identify the influence of nutrients on *H. ranunculoides* abundance and invasiveness and to explore if it can be sustainably managed considering climate change. The greenhouse experiment objectives were to examine the influence of key nutrients on the growth of *H. ranunculoides* and investigate the effects of a rise in temperature of 2°C on the reproductive and invasive capacity of *H. ranunculoides*.

To achieve these objectives three greenhouse experiments were undertaken. To test the influence of nutrients on *H. ranunculoides*, an experiment using the nutrients nitrogen, as nitrate and ammoniacal nitrogen, and phosphorus, as orthophosphate, was used to obtain relative growth rate, biomass, and root to shoot ratio at different levels of each nutrient. To test the gemination capacity of *H. ranunculoides,* seeds from plants from the UK, Europe, its native range and from a seed bank, were germinated under different temperature regimes. Once germination from UK plant material was confirmed, *H. ranunculoides* cuttings from its invaded UK location, were grown under different temperature regimes to test the effect of rising temperatures on time to flowering, flowering abundance and seed production.

3.2.2.2. Greenhouse design

The experiments took place in a brick based, 2.4 x 3.0m Hartley Botanic horticultural greenhouse, 3km from the centre of the field study site, grid reference TQ 62188 05451. Figure 33d (Chapter 5) illustrates the interior of the greenhouse. The experimental design for the nutrient experiment is described in chapter 5, section 5.2.3.2.

For the germination and growth and seed production experiments, Geopod 37w horticultural propagators with controlled lighting and digitally controlled heating thermostats were used (Harrod Horticultural Ltd, 2019). These were capable of regulating heating between 5–30°C and with lid vents to prevent excessive condensation. Two ThermoPro, TP50 maximum / minimum thermometers per propagator were used to measure temperature. The lighting for each propagator was 2 x 6400k grow lamps producing 2,232 initial lumens in the photosynthetically active radiation range, housed in a nanotech enhanced reflector which increases the lumen availability by 300% to 6,696 (Harrod Horticultural Ltd, 2019). Detailed experimental design for each experiment is described in chapter 6, the germination experiment in section 6.2.5.2 and the growth and seed production experiment in section 6.2.5.3,

3.3. Data analysis

The analyses comprised: MAVIS to describe the macrophyte communities (Smart et al., 2016), multivariate statistical analysis using the Canoco 5 programme (ter Braak and Šmilauer, 2012) to summarise the variation in community composition, effects of environmental conditions and management methods, and classical statistics using Minitab

19 (2019). Microsoft® Excel™ 2007 was also used to calculate the Simpson's and Shannon's diversity indices and produce randomisation designs. Figures and Tables were produced using either the Canoco 5 or Excel™ programmes. Cluster analysis, such as TWINSPAN, was not used because this forms end-groups of sites with least variation in species composition. It is likely that TWINSPAN would separate out those sites with and without *H. ranunculoides* which is already known. The aim was to discover the direction and magnitude of impact of *H. ranunculoides* which was done using the multivariate analyses (ter Braak and Šmilauer, 2012). The MAVIS analysis is described in chapter 4, section 4.2.3.2. The Simpson's and Shannon's diversity indices and their equations are described in chapter 4, section 4.2.3.5.

3.3.1. Field data analysis

3.3.1.1. Multivariate analysis

Multivariate statistical analyses were used to investigate several of the aims and objectives: the impact of *H. ranunculoides* on community composition, the impact of the management methods on community composition, the impact of the management methods on *H. ranunculoides* abundance and nutrients in water and sediment.

Multivariate statistical methods investigate the complicated ecological interactions in large, species-rich, but sparse data sets typical of ecological community surveys (Šmilauer and Lepš, 2014). These techniques are appropriate because ecological data often does not conform to standard statistical methods with normal distributions. The data were tested for normality using the Anderson-Darling test in Minitab 19. This test was chosen in preference to the other two tests available, Ryan-Joiner and Kolmogorov-Smirnov, because it is more effective in detecting departures from normality at the tails of the distribution (Minitab, 2019).

The first step in investigating the relationships between species composition and environmental factors, is to ordinate the data to explore the factors with the most important influence. Canoco 5 is one of the most widely used statistical programmes that analyses ecological data using ordination methods (Šmilauer and Lepš, 2014). The Canoco 5 programme can be used to arc sine transform non-normal data if required during the analysis process.

Ordination is done to discover connections between species distributions and environmental variables (Šmilauer and Lepš, 2014). It orders sets of objects (here species assemblages) in dimensions to approximate a pattern of response and where each site is a point (Šmilauer and Lepš, 2014). This will highlight those factors likely to be of overriding importance in determining the occurrence of a species or type of vegetation. Then a concept or hypothesis can be formed about the relationship between species composition and the underlying factors (Smilauer and Leps, 2014).

The initial exploration of the data was done using a Principal Components Analysis (PCA). This is an unconstrained ordination method (indirect gradient analysis) which displays the variation in data without assuming any structure of the samples themselves (Šmilauer and Lepš, 2014). It is used for linear distributions and overall trends in abundance where all species are combined. It includes any variable that best explains the species composition. The data points are deconstructed into Eigenvectors; a direction on a graph and Eigenvalues; how much variance is in the data in that direction and they explain the importance of the axis (Šmilauer and Lepš, 2014). It is particularly good for dimension reduction by reducing the number of components and indicating the most important. Interpretation of this is subjective and uses the experience and knowledge of the user but essentially it shows which component(s) are largest in magnitude and furthest away from zero on a graph so that these can then be targeted for further investigation (ter Braak and Šmilauer, 2012). This was used in preference to Detrended Correspondence Analysis (DCA) because the response data were compositional and had a gradient 2.8 Standard Deviation (SD) units long, so a linear method was recommended (ter Braak and Šmilauer, 2012). Site points close together will have similar species assemblages and those further apart are more dissimilar. Species at the edge of a PCA diagram are the most important for indicating site differences. Strong negative correlation is implied by points diagonally opposite, although they may be responding to the same variable but in opposite ways (ter Braak and Šmilauer, 2012).

Once PCA was completed, a constrained method was used to test the principal components. This explicitly includes two or more different sets of data (here species, sites, and environmental variables) into a single analysis and directly examines the relationships between these sets of variables (Šmilauer and Lepš, 2014). Then a Redundancy Analysis (RDA) was performed in preference to a Canonical Correspondence Analysis (CCA) because the response data were compositional and had a gradient 2.8 SD units long (ter Braak and Šmilauer, 2012). This method tests all axes, using Monte Carlo permutation tests, and gives a P value and a pseudo-F ratio statistic to ensure that the reported P-value is accurate (ter Braak and Šmilauer, 2012).

3.3.1.2. Classical analysis

Classic statistical methods were performed using Minitab 19. Percentage data were tested for normality in Minitab and transformed in Excel™ using the formula =ASIN(SQRT(cell/100)) if non-normal. All significant differences were set at $P<0.05$ for both the field and greenhouse data analyses. Spearman rank correlation was used to describe the relationship between variables. A non-parametric test, the Spearman rank correlation, was used in preference to Pearson’s because Spearman rank does not assume normal data and not all data were normal. Indirect gradient analysis is carried out by correlation of variables with their positions on the ordination axes. Indirect gradient analysis considers the ordination axes as hypothetical environmental variables.

Once the key factors were identified, classical statistical analysis were used to test the hypotheses using Minitab 19. These included parametric tests, Analysis of Variation (ANOVA) to test the difference between means of groups and the Student’s two-sample t-test to determine if the means of two populations significantly differ. These are explained relevant to each set of results under the appropriate section in each results chapter.

3.3.2. Greenhouse data analysis

3.3.2.1. Classical analysis

The greenhouse experiments incorporated classical statistics following tests for normality. These included ANOVA with Tukeys tests where ANOVA indicated significant differences, and Spearman rank correlations to examine relationships between variables.

The impact of the nutrients nitrogen, ammoniacal nitrogen and phosphorus on the dry weight (DW), relative growth rate (RGR) and root to shoot ratio (R:S) of *H. ranunculoides* using classical statistics, ANOVA and Tukey tests, if significant. The equations used to calculate DW, RGR and R:S are described in chapter 5, section 5.2.4.3.

Final biomass (DW) data for each treatment were tested for normality and arcsine transformed to provide normal data. A one-way ANOVA was used with *H. ranuculoides* DW being the dependent variable and the nutrients being the independent variables.

3.4. Summary of methods and survey sites

This chapter has introduced the field survey sites, the baseline conditions and rationale for selection. It also describes the suitability of the sites and experimental methods to answer the aims of the study. The baseline condition of the sites, including watercourse size, water depth, silt depth, land use, community type, water nutrients and weather during the study were described. To investigate which of the nutrients, nitrogen, or phosphorus, might limit the growth of *H. ranunculoides*, growth experiments were carried out under controlled conditions. Germination experiments were carried out under controlled conditions to investigate the impact of raised temperatures in the germination and growing periods. The results of these field, greenhouse and studies are presented in the next three chapters.

Chapter 4 - The impact of *H. ranunculoides* and the effects of management on native plant communities.

4.1. Introduction

Chapter 2 explored the existing body of academic research into the ecology and history of *Hydrocotyle ranunculoides*, as an INNS, the effects of the plant itself and its control methods on native plant communities. Using this research, chapter 3 described the methods used in this study to provide novel evidence about changes to plant diversity, species assemblage and community dynamics as a result of *H. ranunculoides* invasion and the management treatments used to control it, under field conditions. In this chapter the results of macrophyte surveys on 60 sites over four years, the impact of *H. ranunculoides* on native plant communities and the effects of the management methods used to control it are presented.

4.1.1. Invasion ecology

Invasive species are organisms that have been introduced to an environment outside their native region and cause damage to the new environment or harm to human health (The Invasive Species Specialist Group, 2005). A non-native species is one introduced outside its natural past or present distribution (GB non-native species secretariat, 2019) and to become invasive it must reproduce, spread and out-compete the native species of its new location (Convention on Biological Diversity, 2019). With regard to plants, Grime (2006) notes that an invasive species should be a ‘competitor’ capable of rapidly taking over resources and space from resident plants.

Invasions are a major component of global change, threatening many endangered species, causing economic impact, and disrupting ecosystem services (Arim et al., 2006). Species invasions were considered idiosyncratic but there do seem to be general patterns in invasion dynamics, including vegetation (Arim et al., 2006). Vegetation becomes invasible when depleted resources, particularly nutrients, rise again (Grime, 2006) or disturbance occurs (Bakker et al., 2013). In aquatic ecosystems, invasive species are more likely to colonise disturbed sites leaving a low likelihood of colonisation by rare or endangered macrophyte species (Bakker et al., 2013). Stiers et al. (2011) found that aquatic INNS reduce diversity and that the degree of dominance of an invasive species is linked to the level of impact.

As macrophytes have a fundamental part to play in the structure and functioning of aquatic ecosystem services (O'Hare et al., 2018) aquatic INNS may be particularly damaging.

4.1.2. *H. ranunculoides* and invasiveness

H. ranunculoides is a contender for the most harmful aquatic INNS in the UK and has spread across other European countries (Centre for Agriculture and Biosciences International, 2020a). It possesses the key invasive traits of very fast growth, adaptability and very efficient propagation (Robert et al., 2013). Outside its native regions there is a lack of controlling factors such as host specific pests, diseases and herbivory (Robert et al., 2013).

H. ranunculoides rapidly dominates watercourses, spreading quickly by vegetative fragmentation (Centre for Agriculture and Biosciences International, 2020a). The rapid growth rate of up to 20cm a day means it can double its biomass in a week (Robert et al., 2013) in Europe and form thick floating mats. Its capacity for phenotypic plasticity (Robert et al., 2013) means it can cope very well with disturbance, environmental stress and climate change. The morphological factor most evident is leaf size, the Centre for Agriculture and Biosciences International, (2020b) have recorded peak season leaves up to 10-15cm in diameter with overwintering leaves only 2-4 cm. In addition, root biomass is not affected by semi-drained or drained conditions, so it is capable of recovering well from drought and re-establishes faster than native macrophytes (aquatic plants) (Hussner and Meyer, 2009).

4.1.3. Aims and objectives

The aim of these macrophyte surveys, on 60 sites over four years in the field, was to investigate the impact of *H. ranunculoides* on native macrophyte communities, assess the effectiveness of management treatments being used to control *H. ranunculoides,* and to determine whether these impact on the native macrophyte communities. The objectives were to:

- Quantify macrophyte community change related to *H. ranunculoides* infestation.
- Establish the effectiveness of the management treatments in controlling *H. ranunculoides.*

- Evaluate the macrophyte community composition of watercourses treated to remove *H. ranunculoides*.

4.2. Methods

4.2.1. Site selection and description

The unusually high diversity of aquatic macrophytes (Joint Nature Conservation Committee, 1999) provided an excellent opportunity to study the impact of the INNS, *H. ranunculoides,* on native macrophyte communities. The EA was beginning a five-year programme of works in the invaded sector, and the implementation of a previously trialled *H. ranunculoides* control programme. There were two reference groups, those sites with no *H. ranunculoides* present and un-cut (CO), and sites with *H. ranunculoides* present but un-cut (PW). The remaining sites, with *H. ranunculoides* present, were subject to the following management treatments; an annual weed-cut (WC), annual weed-cut and sprayed (WS), annual weed-cut and de-silted (WD) and annual weed-cut, de-silted and sprayed (DS). Landowner permission for access was granted as the infestation of *H. ranunculoides* has caused them considerable problems over the past 20 years with no effective control measure yet identified.

4.2.2. Field methods

Field surveys were undertaken, annually for each site, over a four-year period from 2013 to 2016 at the 60 selected sites. A site comprised a 100m length of watercourse with a minimum of six replicates for each management treatment. The sites were chosen to represent the three types and sizes of watercourse on the Pevensey Levels, small field ditches <5m, medium sized pump and feed drains 5-10m and larger main rivers >10m. Infested and non-infested watercourses and the proposed management method was taken in to account in all three sizes. Accessibility and time constraints for surveying also influenced the choice of watercourse. The 100m length chosen represented the conditions and flora in the water body and is in in accordance with the EAs macrophyte monitoring for the WFD OI 131_07, over a 100-metre length (Environment Agency, 2016c). The macrophyte surveys were undertaken in the period June to September, also in line with OI 131_07 (Environment Agency, 2016c). This protocol is in accordance with the WFD UKTAG (2014) guidance for surveying freshwater rivers, streams, and ditches.

To assess potential seasonal effects, eight sites were surveyed monthly for the three years of the management treatments. These were done from May to October which includes one month at each end of the season outside the usual recording period. Of these eight sites, six were selected for statistical analysis, one for each management treatment. One (17DL) was rejected as it was one of two reference sites without *H. ranunculoides* that became infested during the study period and a second (54CC) as it duplicated the management treatment weed-cut and spray.

The annual survey months were randomised across sites over the four years of the study. However, the average survey month did differ over the four years. Timing of surveys were subject to practical restrictions and weather. For the 2013 (baseline) and 2014 the average survey month was early August. For 2015 this was early July and 2016 mid-July. In addition to macrophytes, environmental data were collected as observational variables and measured using a handheld water quality measuring YSI Pro Plus Multiparameter Instrument (Xylem Inc., 2019).

The impact of nutrients on the growth of *H. ranunculoides* is discussed in Chapter 5 but the environmental factors must be considered to rule out any effect, either alone or in combination with the management treatment, on the macrophyte community composition and the potential impact of *H. ranunculoides* on those communities. Timing of sampling and surveys were subject to practical restrictions and weather. To visually assess any potential physical changes, each site was photographed on every visit and compared over the four years.

4.2.3. Statistical methods

4.2.3.1. Introduction

The complex interactions between plant communities and environmental influences, and large datasets with small sample numbers, influenced the choice of statistical analyses. These require multivariate statistical analyses and the ability of the statistical programme to allow for the data not to follow a normal distribution (Šmilauer and Lepš, 2014). Canoco 5 was selected and is default set to log transform but can be changed to alternative methods. Selected data can then be tested by classical methods if required, here Minitab19 was used. All plant community and environmental data collected were tested for normality in Minitab 19, using the Anderson-Darling test, and transformed if non-normal, using the arcsine transformation in Excel™, prior to analyses.

4.2.3.2. The Modular Analysis of Vegetation Information System (MAVIS)

The NVC community for each of the sites, prior to treatments taking place in 2013 and post-treatment in 2016, was investigated using the MAVIS program (Centre for Ecology and Hydrology, 2016). MAVIS analyses vegetation data using different types of classification systems but for this purpose it was used to describe the NVC communities of the study sites (Smart et al., 2016). Lists of plant species recorded within fixed areas (usually quadrats but in this case 100m stretches of watercourse) make up the basic information required to investigate spatial and temporal variation in plant species composition. To provide a descriptive framework for understanding these different compositions the data are placed within various classifications. MAVIS links botanical data with several commonly used plant species and vegetation classifications (Smart et al., 2016). This gives a standard description of various types of plant community allowing comparisons between sites, regions, and even biogeographic zones (Smart et al., 2016). The NVC is a core phytosociological standard used to understand the character and value of vegetation and is accepted by UK conservation bodies, agencies, universities and many other organisations (Rodwell, 1998).

The NVC underpins the selection of biological sites for SSSI designation and may be used to inform the SSSI threats and remedies database of any changes due to *H. ranunculoides* infestation or management (Natural England, 2013a). Here it was used to identify which plant communities are most likely to be impacted by the presence of *H. ranunculoides.*

The MAVIS (Centre for Ecology and Hydrology, 2016) NVC output provides a percentage probability of the community types according to the species found and their constancy. Each analysis returns the10 top coefficients but only the community with the highest percentage probability was used to describe each site.

4.2.3.3. Annual macrophyte community dynamics

Six of the monthly surveyed sites, one for each management treatment, were subject to a PCA of community composition for the three years of the management treatment. All species abundance data, for all three years, were combined and grouped by month to show the changes in species abundance associated with the season, and whether *H. ranunculoides* abundance varied through the growing season. The PCA plant species vectors indicate the steepest increase of the values and the length of the vector acts as a measure of fit for the plant species (ter Braak and Šmilauer, 2012).

4.2.3.4. Plant community change and *H. ranunculoides* infestation

The annual sites were subject to further analysis using PCA, which included all species recorded, for all four years of the study. From this analysis a diagram was used to illustrate the variation of plant species diversity in the plots across the ordination space. The distance between the symbols (individual plots) is a measure of their dissimilarity using Euclidean distance. The value (as number) and size of the plot reflects the size of the attribute, so the larger the number and size, the greater the diversity (ter Braak and Šmilauer, 2012).

To investigate the strength of the negative association between *H. ranunculoides* and the key native species identified by the PCA, classical statistics was used. A Spearman Rank correlation, a non-parametric measure of statistical dependence between the ranking of two variables, was used. Spearman Rank was chosen rather than Pearson product-moment correlation because the latter benchmarks linear relationships whereas Spearman benchmarks monotonic relationships and where data may not fit a normal distribution (Minitab, 2019).

4.2.3.5. Impact of H. ranunculoides on plant communities

Species richness was used as a measure of plant diversity but there are other more detailed indices, the commonest being the Shannon-Wiener and Simpson’s D (Toogood, 2005). These index values were used to quantify the biodiversity and are based on the proportional abundance of species (Morris et al., 2014). The Shannon-Wiener diversity index (Equation 1) combines both species richness and the proportion of abundance. It is sensitive to changes in less abundant species and it increases as both species richness and evenness increase (Morris et al., 2014). The value ranges between 0 (a mono-culture) and 4 (highly diverse community).

$$H = -\sum_{j=1}^{s} p_i \ln p_i$$

Equation 1: Shannon-Wiener diversity index where *s* is species richness and p_i are the proportion of abundance.

Simpson's D index (Equation 2) gives more weight to evenness and common species, by taking into account the number of species present and the abundance and is therefore more sensitive to changes in more dominant species (Morris et al., 2014). As dominance increases, diversity decreases; with 0 representing infinite diversity and 1 a mono-culture. However, as this is counter-intuitive and for ease of comparison with the Shannon-Wiener diversity index, the Simpson's Index of Diversity was used, (1-D), so that the higher the number the more diverse in both cases (Morris et al., 2014).

$$\mathrm{D} = 1 - \sum_{i=1}^{s} (pi_i)^2$$

Equation 2: Simpson's diversity index where *s* is species richness and p_i are the proportion of abundance.

Due to the high diversity levels found at Pevensey Levels the Shannon-Wiener diversity index would ensure variations in rarer species were captured. However, as the study needed to capture the influence of *H. ranunculoides*, which was dominant on many sites, the Simpson's index of diversity was also relevant. Morris et al. (2014) show there is no ideal diversity measure and suggest parallel use of two indices, which improves the output information of the dataset, so both indices were used. For ease of comparison with the Shannon-Wiener diversity index, D was subtracted from 1 to give Simpson's index of diversity 1 – D, so that both index values rise as diversity increases. These tests were run using Excel™. An ANOVA was then run in Minitab 19 to test the significance of these indices using abundance of *H. ranunculoides* over all four years. The index values were then Arcsine transformed to provide normal data and a one-way ANOVA was run to test the significance of impact of *H. ranunculoides* on community composition and the effect of management treatment on community.

4.2.3.6. Impact of management methods on macrophyte communities

To investigate the effect of management over the period of four years, an analysis of repeated observations of the species composition of the 60 sites was undertaken. Using baseline data from 2013 and three more samples over the following three years, 2014, 2015 and 2016, the interaction between time and management treatment investigated the dynamics of the managed and reference sites (Šmilauer and Lepš, 2014). The response of the macrophyte communities to the management treatments was tested as temporal dynamics. The data are in the form of repeated measurements and for species composition the RDA was used because the species composition is relatively similar (Šmilauer and Lepš, 2014). The analysis subtracts the average of each site and so only the changes within the site are analysed.

To investigate the effect of management treatment on species diversity as number of species, an ANOVA was used on the normalised data. The management treatments were grouped, and the means plotted by year to investigate the species diversity over the three years of management treatment, 2014, 2015 and 2016. The null hypothesis was that all means of species richness were equal for untreated sites without *H. ranunculoides* and for treated sites with *H. ranunculoides*.

To investigate the impact of the presence of *H. ranunculoides* and the management treatments on species diversity Simpson's 1-D and Shannon-Wiener diversity index Indices were used. The management treatments were grouped, and the means plotted by year to investigate the species diversity as index values in 2016 after three years of management treatment, using data from 2013, 2014 and 2015. The data were Arcsine transformed to produce normal data, and an ANOVA was run using management treatments. The null hypothesis was that all means of diversity index values were equal for untreated sites without *H. ranunculoides* and for treated sites with *H. ranunculoides*.

To investigate the effect of the management treatments on *H. ranunculoides,* the management treatments were grouped, and the means plotted by years. Then an ANOVA was run using Minitab 19 with management treatment (the independent variable), and percentage cover of *H. ranunculoides* (the dependent variable), followed by a Tukey Test.

To investigate the community change related to the four management treatments, the average species richness for each year was calculated. Then an ANOVA was run using Minitab 19 with management treatment (the independent variable), and species richness (the dependent variable), including a Tukey test and if significant.

4.2.3.7. Community change over time

In order to assess the relationship between time and management treatment effects the development of the communities was studied over time (Šmilauer and Lepš, 2014). To investigate these temporal changes, an RDA was used to search for the best explanatory variable to explain species composition for each of the four years. The results of each of the four years RDAs were illustrated as diagrams mapping the environmental variables and management method symbols and comparing between the years. The distance between the environmental symbols approximates the dissimilarity of plant species composition between the plots based on their Euclidean distance (ter Braak and Šmilauer, 2012).

4.3. Results of impact of *H. ranunculoides* on community composition

Over the four years of the study, 60 x 100m stretches of watercourse were surveyed annually. 76 macrophyte species were observed in total, over the course of the four years, plus three species of macro-algae and three species of stonewort. The maximum number of species observed in any one ditch, at any time, was 26 species.

4.3.1. Classification of macrophyte communities

The analysis of survey data using the MAVIS plot analyser classified the macrophyte communities into two broad groups Swamp (S) and Aquatic (A) (Table 10), with the majority of the sites placed in traditional swamp (S) communities, and very few as true aquatic (A) communities. More specifically, across all sites a total of fifty-four swamp sub communities were identified, and a further six aquatic sub communities identified in the 2013 surveys These transitioned to fifty-two swamp and eight aquatic sub communities in the 2016 surveys.

4.3.1.1. MAVIS results

Table 10: MAVIS plot analyser results as NVC macrophyte communities of pre (2013) and post (2016) management treatments. The sites, n=60, are coded with a two digit and two letter code. The ✓ denotes presence of *H. ranunculoides*.

Site Code	*H.ranunculoides*	Treatment	NVC 2013	NVC 2016
01HS	✓	De-silt, spray	A11a	S4
02CH	✓	Cut only	S4a	S4a
03WS	✓	Cut only	S4a	A5a
04RR	✓	Cut only	A11a	S4
05KR	✓	De-silt, spray	S5	S5
06HB	✓	Cut, de-silt	S5	S5
07WH	✕	Un-cut	A1	A5
08CR	✓	Cut, spray	S12	S4
09HG	✓	De-silt, spray	S5	S5
10RU	✓	Cut, spray	S4	S14
11HG	✓	Cut only	S14	S4
12CK	✕	Un-cut	S14	S5
13PN	✓	Cut, de-silt	S14	S5b
14IU	✓	De-silt, spray	S5	S5
15DU	✓	Un-cut	A11a	A2b
16DM	✓	Cut only	S8	A5
17DL	✓	Cut only	S8	S5
18HR	✓	Cut, de-silt	S5	S5
19WF	✕	Un-cut	A1	A3
20HU	✓	Cut, spray	S4a	A11a
21CU	✓	Cut only	S8	S8
22CM	✓	Cut only	S8b	A15
23MB	✓	Un-cut	S8	S5
24MD	✓	Cut, spray	S5	S14
25MN	✓	Cut only	S14	S14
26CF	✓	Cut, spray	S14	S4
27HP	✓	Cut, de-silt	S5	S4a
28HM	✓	De-silt, spray	S8	S16
29HN	✓	De-silt, spray	S16	S4a
30HR	✓	De-silt, spray	S4	S5

31ML	✓	Un-cut	S14	S4
32MM	✗	Un-cut	S14	S4
33MU	✗	Un-cut	S4	S4
34HL	✓	Cut only	S14	A11a
35WE	✓	Cut only	S14	S14a
36WW	✓	Cut only	S8b	A11a
37HS	✓	Un-cut	S4	S14
38CG	✓	Cut, spray	S4a	S4
39MW	✓	Cut, spray	S5	S13
40DN	✓	Un-cut	S5b	S14
41WU	✗	Un-cut	S14	S4a
42WM	✓	Un-cut	S5b	S4a
43WL	✓	Cut only	S4	S16
44RL	✓	Cut only	S8	S4
45PM	✓	De-silt, spray	A11a	S4
46PU	✓	De-silt, spray	S14	S4
47IM	✓	Cut, de-silt	S8	S4
48IL	✓	De-silt, spray	S4	S4
49CM	✗	Un-cut	S4	S5
50CB	✗	Un-cut	S13	S13
51VS	✓	Cut, de-silt	S4	S4
52CC	✓	Cut, spray	S12	S14
53HF	✗	Un-cut	S4	S14
54HW	✗	Un-cut	S8	S14
55KM	✗	Un-cut	S5	S5
56HK	✓	Un-cut	S5	S5
57VN	✓	Un-cut	S4	S4a
58HD	✗	Un-cut	S14	S14
59KF	✓	Cut, de-silt	S14	S4
60HH	✓	Cut, de-silt	S4	S16

70% (42 of 60) of the sites' NVC communities changed during the survey period, 2013 to 2016, including six of the eight reference sites without *H. ranunculoides* (CO). Figure 15 shows this breakdown by management treatment. This suggests that some community change occurs whether management is undertaken or not and whether the site is infested or not. This shift in NVC community was the same for untreated and un-infested sites (CO) and untreated infested sites (PW), with six changing and four remaining unchanged (75%). Fewer sites changed in de-silted sites, sprayed (DS) (60%) or unsprayed (WD) (58%). The greatest change was observed in the weed-cut and sprayed sites (87%).

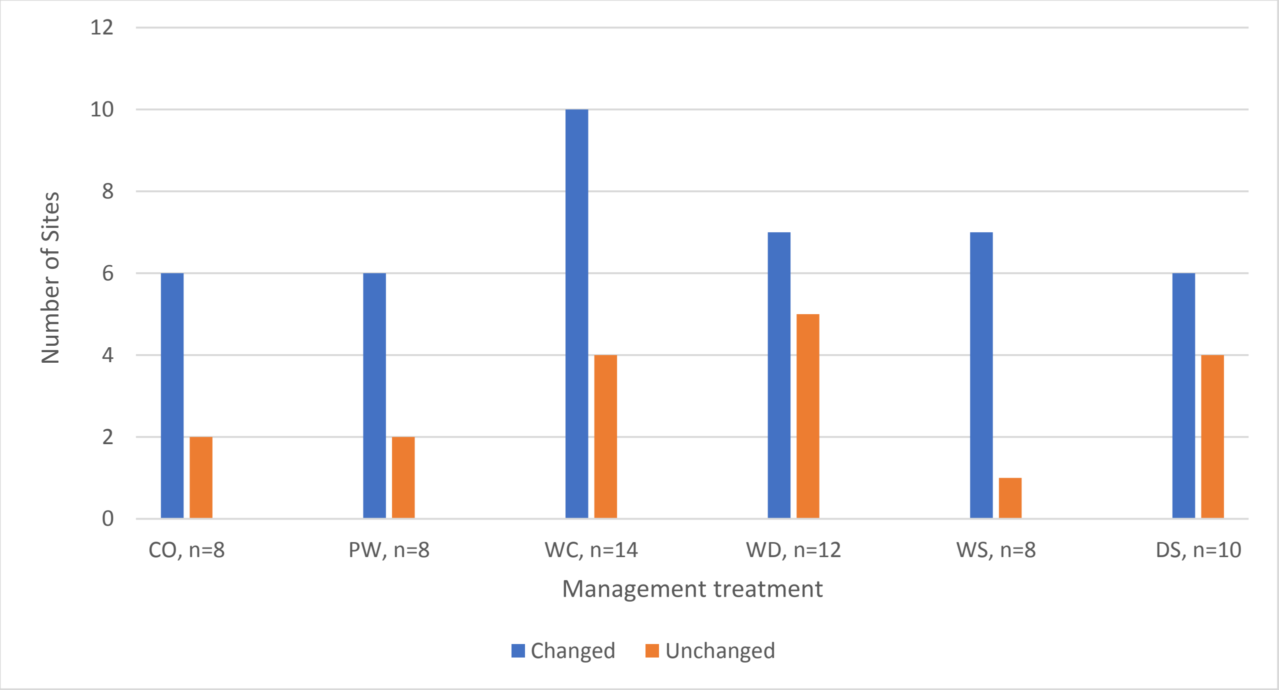


Figure 15: The 60 survey sites showing the number of sites where the NVC community changed or remained unchanged between the baseline year 2013 and the end of the survey period in 2016. Management treatments were: un-cut without *H. ranunculoides* (CO), un-cut with *H. ranunculoides* (PW), weed-cut (WC), weed-cut and de-silted (WD), weed-cut and sprayed (WS), and weed-cut, de-silted and sprayed (DS).

4.3.2. Annual macrophyte community dynamics

Assessment of the seasonal effects on community composition were completed for the five infested sites that were surveyed monthly, for the three years of the management measures. Apart from obvious physical intervention such as de-silting and weed-cutting, which affected abundance for that season, the data showed that community composition, as type of species and species richness, remained consistent over the year at each site. However, the months did show variation with certain plant species being associated with certain months and *H. ranunculoides* increasing in abundance in the latter part of the season, September to October. The plant name abbreviations used in the diagrams are shown in Table 11. The results of an unconstrained, linear PCA for the infested monthly sites for the three years of the study (2014, 2015 and 2016) and grouped based on sampling month, are shown in Table 12 and Figure 16.

Table 11: Plant names as latin binomials, common names and the abbreviations used in diagrams.

Latin name	**Common Name**	**Abbreviation**
Azolla filiculoides INNS	Water Fern	AzoFil
Alisma lanceolatum	Narrow-leaved Water-plantain	AliLan
Alisma plantago-aquatica	Water Plantain	AliPla
Blanketweed agg.	Blanket Weed	BlaAgg
Berula erecta	Lesser Water-parsnip	BerEre
Bolboschoenus maritimus	Sea Club-rush	BolbMar
Callitriche spp.	Water-starwort	CalSpp
Carex otrubae	False Fox Sedge	CarOtr
Carex pseudocyperus	Cyperus Sedge	CarPse
Ceratophyllum demersum	Rigid Hornwort	CerDem
Cladophora spp.	Green algae	ClaSpp
Elodea canadensis	Canadian Waterweed	EloCan
Elodea nuttallii	Nuttall’s Waterweed	EloNut
Enteromorpha intestinalis	Gut Weed	EntInt
Glyceria maxima	Reed Sweet Grass	GlyMax
Hydrocharis morsus-ranae VU	Frogbit	HydMor
Hydrocotyle ranunculoides INNS	Floating Pennywort	HydRan
Hydrocotyle vulgaris	Marsh Pennywort	HydVul
Juncus articulus	Jointed Rush	JunArt
Lemna gibba	Fat Duckweed	LemGib
Lemna trisulca	Ivy-leaved Duckweed	LemTri
Mentha aquatica	Water Mint	MenAqu
Myriophyllum spicatum	Spiked Water-milfoil	MyrSpi
Nitella mucronata	Pointed Stonewort	NitMuc
Nymphaea alba	White Water-lily	NymAlb
Oenanthe fistulosa VU	Tubular Water-dropwort	OenFis
Persicaria amphibia	Amphibious Bistort	PerAmp
Phalaris arundinacea	Reed Canary-grass	PhaAru
Phragmites australis	Common Reed	PhrAus
Potamogeton actuifolius CR	Sharp-leaved Pondweed	PotAcu
Potamogeton crispus	Curled Pondweed	PotCri
Potamogetum lucens	Shining Pondweed	PotLuc

Potamogeton natans	Broad-leaved Pondweed	PotNat
Potamogeton pectinatus	Fennel Pondweed	PotPec
Ranunculus spp.	Water-crowfoot species	RanSpp
Rumex hydrolapathum	Water Dock	RumHyd
Schoenoplectus lacustris	Common Club-rush	SchLac
Sparganium erectum	Branched Bur-reed	SpaEre
Stratiotes aloides	Water-soldier	StrAlo
Typha angustifolia	Lesser Bulrush	TypAng
Typha latifolia	Bulrush, Reedmace	TypLat
Red Data Book Categories:	Invasive Species:	
VU = Vulnerable	INNS	
CR = Critically Endangered		

The PCA highlighted the differences in plant species composition over the months that were assessed (Figure 16). The 20 plant species with the best fit (vector length) were selected for display on the diagram (Šmilauer and Lepš, 2014). Axis 1 accounted for 39.32% of the variation in the data (Table 12) and showed a clear separation between spring and summer surveys.

Table 12: Summary table of PCA results of all species for all three years for the infested monthly survey sites.

Statistic	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	0.3932	0.2393	0.1755	0.1120
Explained variation (cumulative)	39.32	63.25	80.79	91.99

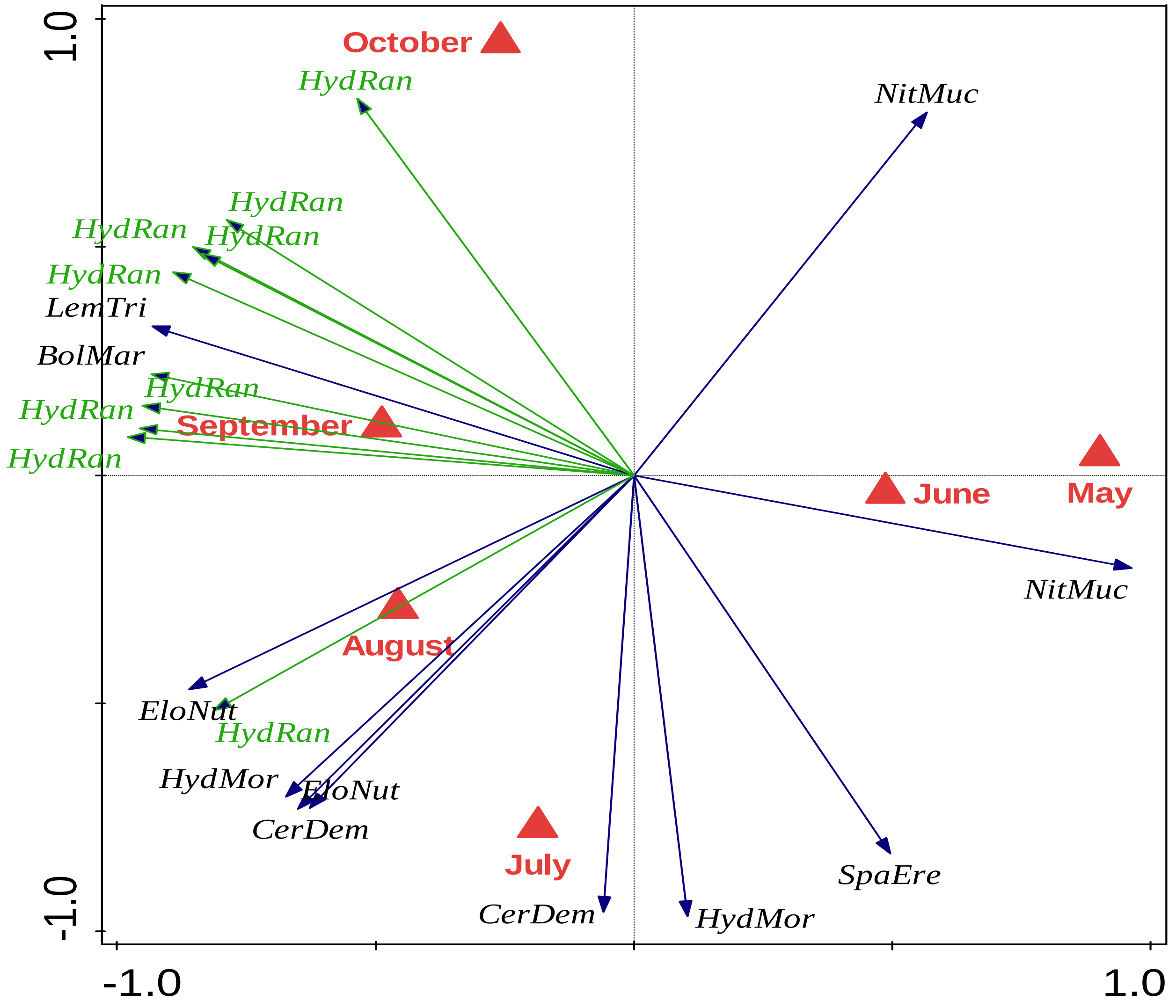


Figure 16: PCA biplot diagram for the *H. ranunculoides* infested five monthly survey sites, for 2014, 2015 and 2016. Triangles represent the months, and the abbreviations are based on the plant latin binomials (Table 11). The first two axes are represented.

The placement of the months was based on species composition and differences between the months. The only species strongly associated with Spring (May/June) was the charophyte *Nitella mucronata*. In summer (July/August) two submerged species *Elodea nutalli* and *Ceratophyllum demersum* were represented, along with the floating species *Hydrocharis morsus-ranae*. Further variation was also evident along Axis 2, which accounted for 23.93% of the variation (Table 11) and separated the autumn surveys from the early season surveys. Most noteworthy, axis 2 showed the stronger association with *H. ranunculoides* vectors clustered around September to October in particular. Moreover, when both axis 1 and axis 2 were considered together (cumulative variation = 63.25%) they evidenced the seasonal differences in plant species composition and the growth of individual species on a monthly/seasonal basis.

Spearman rank correlations indicated a strong positive linear relationship with abundance and month (Table 13). In conjunction with the PCA biplot this demonstrates that abundance of *H. ranunculoides* increases steadily through the growing season, peaking around September.

Table 13: Spearman Rank Correlations with *H. ranunculoides* average abundance and month, n=6, for all infested monthly sites n=30 per year, for the three years of the monthly surveys, May to October 2014 - 2016.

Year	2014	2015	2016
Months and % cover *H. ranunculoides*	Rho 0.943 **P = 0.005**	Rho 1.000 **P = <0.001**	Rho 1.000 **P = <0.001**

The timing of surveys was randomised but to determine any seasonal effects, a Student's t-test of mean number of species of five sites surveyed in June were compared to those surveyed in September for three years, 2014, 2015 and 2016. There were no significant differences, P=0.550.

4.3.3. Plant community change related to *H. ranunculoides* infestation

An unconstrained, linear PCA was completed on all species for all four years of the study. There were 292 plant species records included with the 20 species with the best fit to the shown ordination axes plotted. The first (horizontal) axis explains 14.44% of the total variability in species composition, which is a poor explanation of the variation, due to the number of species in the data set. The second (vertical) axis explains a further 9.6% of the variation (Table 14).

Table 14: Summary of PCA results of all species on all 60 sites for all four years.

Statistic	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	0.1444	0.0959	0.0932	0.0662
Explained variation (cumulative)	14.44	24.04	33.35	39.97

Figure 17 shows *H. ranunculoides* species vectors have low values along both horizontal and vertical axes and are separated from the other species in all cases equal to or more than 90^o.

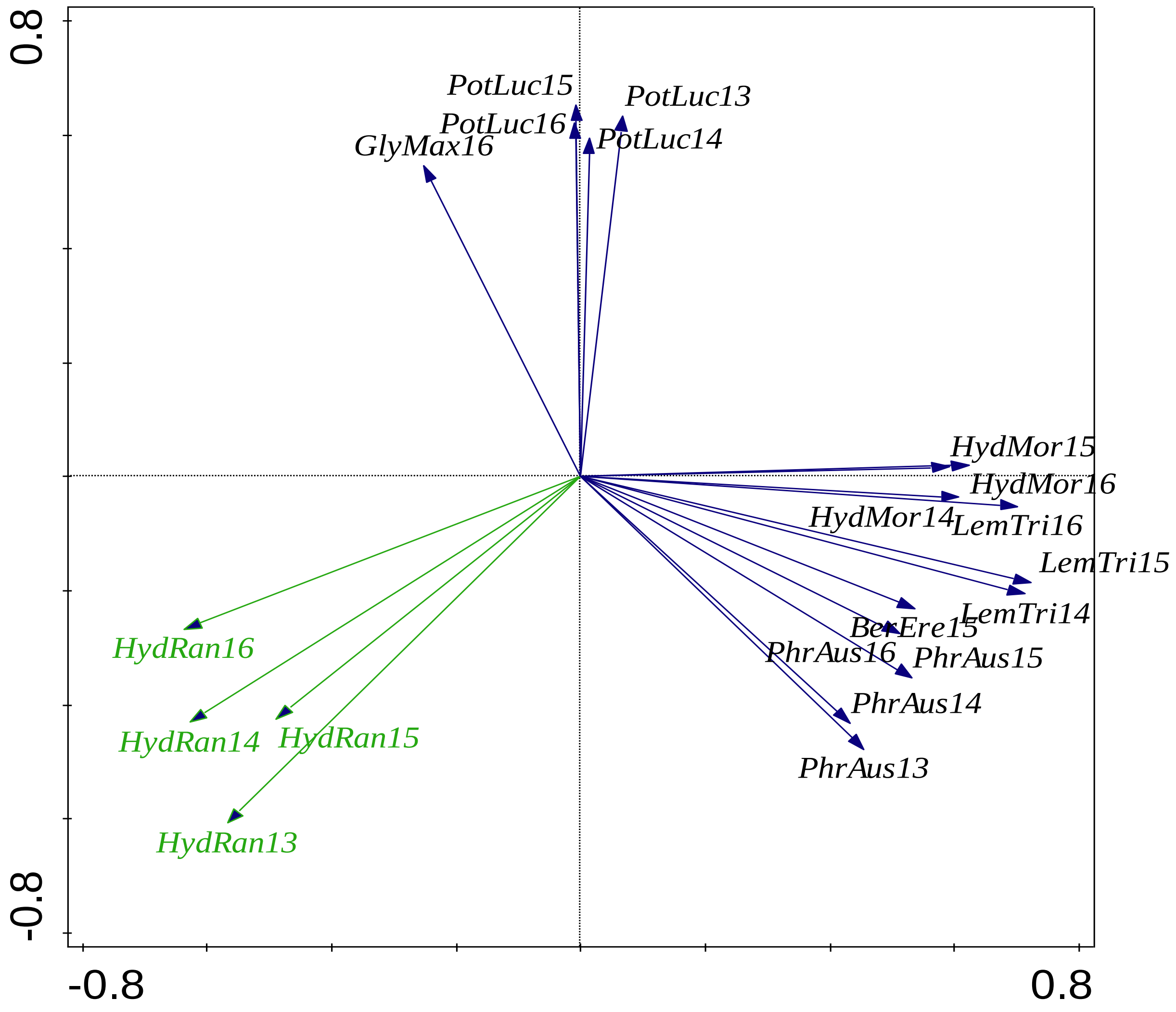


Figure 17: PCA plant species diagram for all species, on all sites for all four years 2013, 2014, 2015 and 2016 with the 20 species with best fit to the ordination axes selected. Plant names are shortened versions of the plant latin binomials with the year suffixed (Table 11). The first two axes are plotted.

It can be inferred from this that community composition could be impacted by *H. ranunculoides* presence and the most likely species to investigate are *Hydrocharis morsus-ranae* and *Lemna trisulca* as these are present three times (out of four years). Their vectors are arranged along the horizontal axis with higher values furthest away from *H. ranunculoides* on the horizontal axis. Included in this group is *Berula erecta*, appearing once, and *Phragmites australis*, present in all four years, however, the latter is more closely aligned with *H. ranunculoides* along the vertical axis. In a different direction, there is a separate group of vectors with high values along the vertical axis with *Potamogeton lucens*, and one occurrence of *Glyceria maxima*, again placed distinctly away from *H. ranunculoides*.

In the plot symbol diagram in Figure 18., the plots with the largest symbols have the higher count of plant species. If this diagram is compared with the diagram in Figure 17., the plots furthest away (vertical axis and upper right quadrant) from the *H. ranunculoides* arrows (vertical axis and bottom left quadrat) have higher species counts and those in the quadrant with the *H. ranunculoides* vectors have lower species counts.

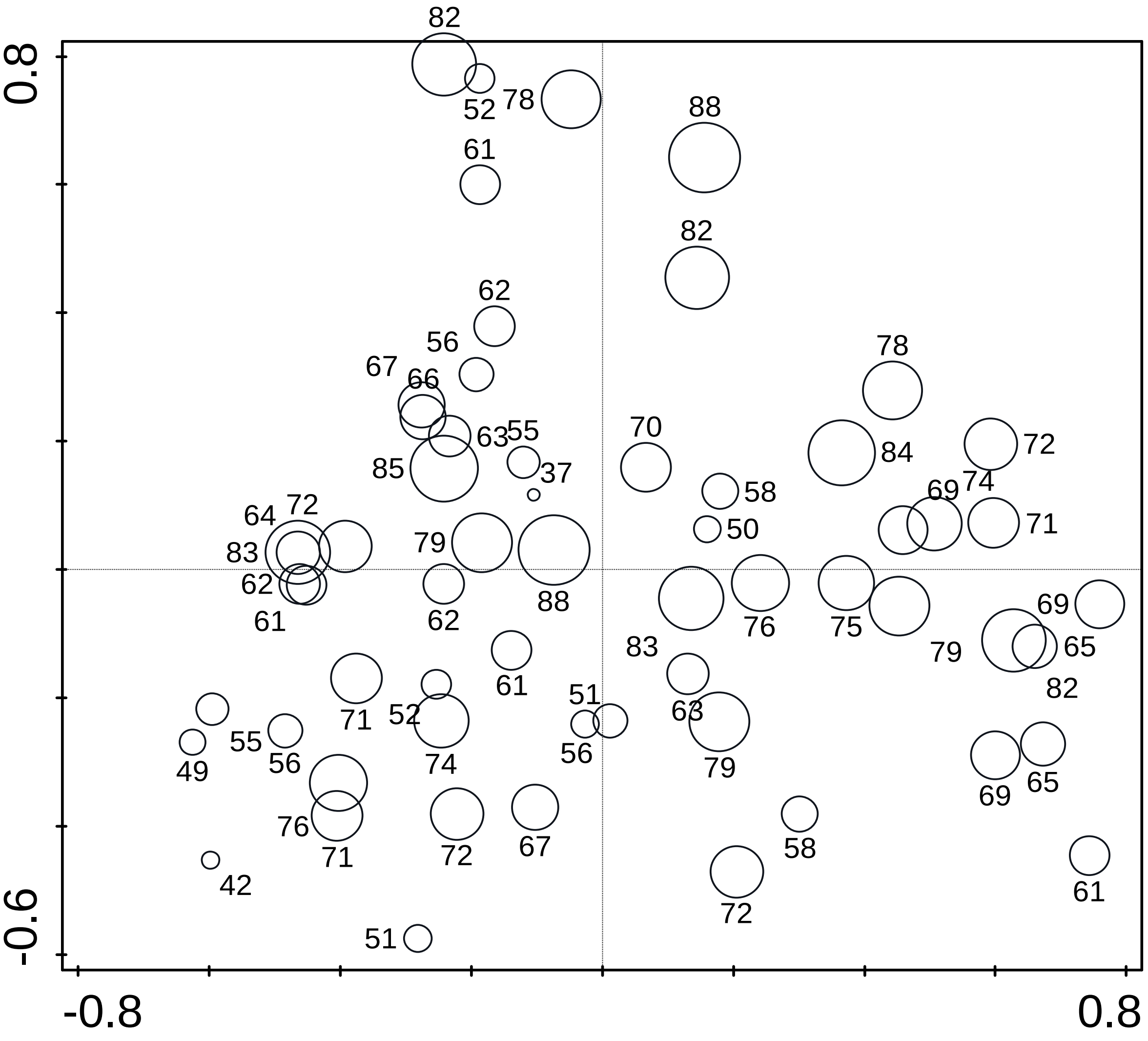


Figure 18: Plot Symbol Diagram for all species on all 60 sites, for all four years 2013 - 2016. Symbol size reflects the site value with the number showing the count of plant species within the site.

If the 10 species with the best fit to the first two ordination axes for all four years are then analysed (40 plant species over the four years) a similar pattern is observed to Figure 17, but the cumulative explained variation is much higher (Table 15). In Table 15. the first (horizontal) axis explains 24.86% of the total variability in species composition, with the second (vertical) axis adding a further 16.32%.

Table 15: Summary of PCA of the10 species with the best fit to the ordination axes, for all 60 sites, for all four years 2013 – 2016.

Statistic	**Axis 1**	**Axis 2**	**Axis 3**	**Axis 4**
Eigenvalues	0.2486	0.1632	0.1306	0.0762
Explained variation (cumulative)	24.86	41.18	54.24	61.87

The plant species diagram (Figure 19) shows the *H. ranunculoides* vectors, in the bottom left of the diagram, clearly separated from the native plant species vectors. The emergent and marginal *P. australis* is distinct in the bottom right of the diagram. The submerged and floating native aquatic species are clustered in the top right of the diagram separated from both the *H. ranunculoides* and *P. australis* groupings.

Based on the results of the 10 species PCA, these nine native species were then subject to a Spearman Rank Correlation with *H. ranunculoides*. Only six of these nine selected species showed a significant relationship, over the four years of study. These six species all had a negative and significant correlation with *H. ranunculoides* are listed in Table 16.; *H. morsus-ranae, P. lucens, S. erectum, L. trisulca, B. erecta* and *P. australis*.

Table 16: Significant Spearman Rank Correlations, of species highlighted by the PCA in Figure 19, with *H. ranunculoides* for all 60 sites for the four years of the study 2013 - 2016.

Species	**2013**	**2014**	**2015**	**2016**
Hydrocharis morsus-ranae	Rho -0.273 **P = 0.030**	Rho -0.367 **P = 0.004**	----	Rho -0.397 **P = 0.002**
Potamogeton lucens	Rho -0.477 **P = <0.001**	----	----	----
Sparganium erectum	Rho -0.281 **P = 0.030**	Rho -0.269 **P = 0.038**	----	----
Lemna trisulca	----	Rho -0.441 **P = <0.001**	----	----
Berula erecta	----	----	----	Rho -0.255 **P = 0.049**
Phragmites australis	----	----	----	Rho -0.340 **P = 0.008**

The nearer the rank correlation to 1 or -1 the stronger the positive or negative correlation, so none are strongly correlated but all are negative and significant. *P. lucens* and *L. trisulca* both had the highest rank correlation values and the most significant relationships with *H. ranunculoides* but only in one year each. *H. morsus-ranae* stands out as there was a significant relationship with *H. ranunculoides* in three of the four years. There were no significant correlations in 2015, although all species had negative correlations with *H. ranunculoides.*

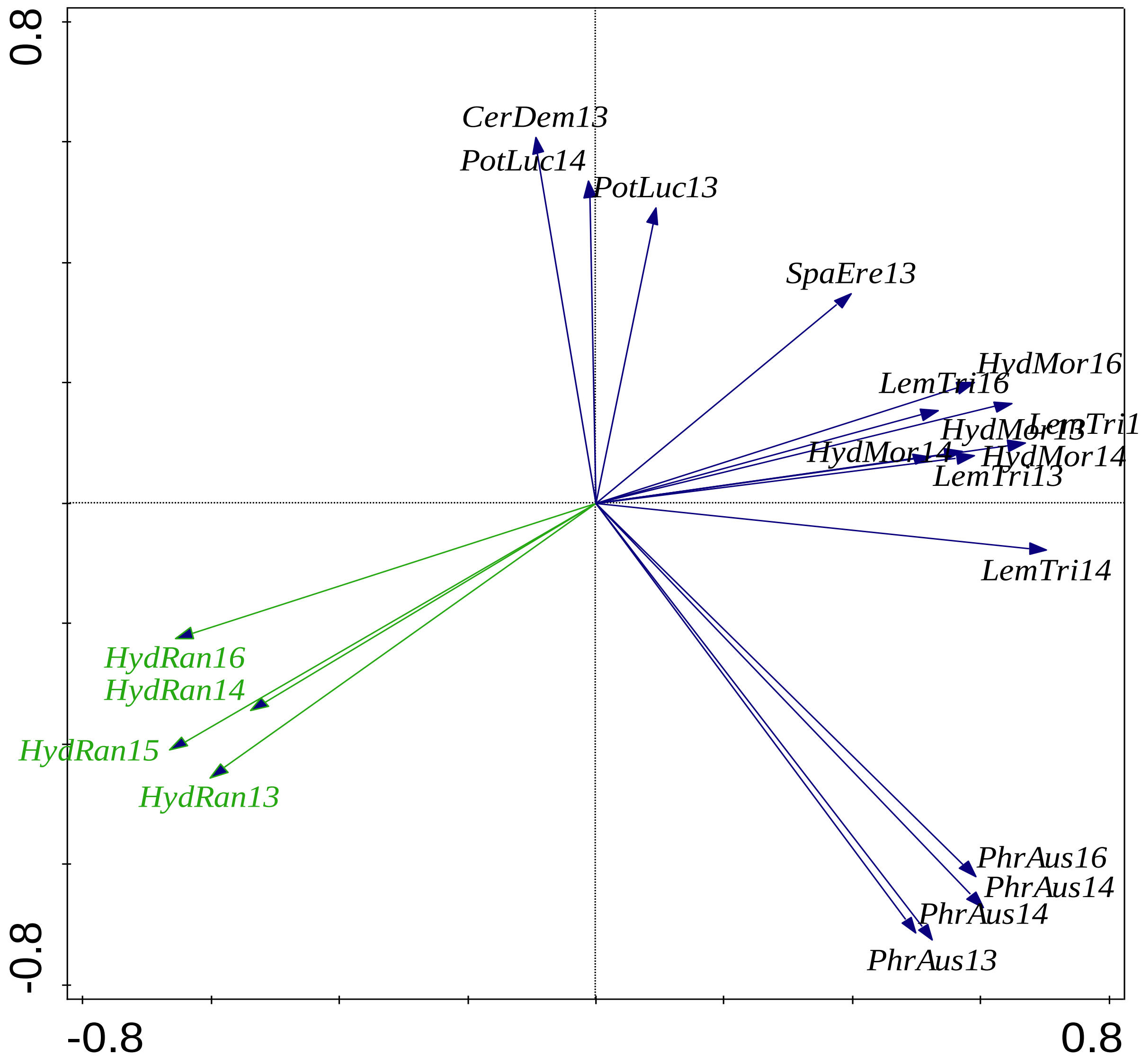


Figure 19: PCA plant species diagram for all four years using the10 species with the best fit to the ordination axes for all 60 sites for all four years 2013, 2014, 2015 and 2016. Plant names are shortened versions of the plant latin binomials with the year suffixed. The first two axes are plotted.

4.3.4. Relationships between *H. ranunculoides* and plant communities

4.3.4.1. Species richness

An analysis of the mean number of species present in sites with *H. ranunculoides* (at any percentage cover), n=48, and without *H. ranunculoides,* n=11, in all years showed that the number of species present varied very little but was consistently slightly lower in all years at the infested sites (Figure 20). One site without *H. ranunculoides* was omitted as an outlier, as it had very low diversity values over the four years, so an outlier test was run using Minitab 19.

The Student's two-sample t-test, for all years combined, was used to determine if there was a significant difference between the sites with and without *H. ranunculoides*. Following a normality test, the data were found to be not normal so were Arcsine transformed to provide normal data. There was no significant difference in species richness between sites with and without *H. ranunculoides* over the four years (df = 4, t = 2.52, p = 0.065).

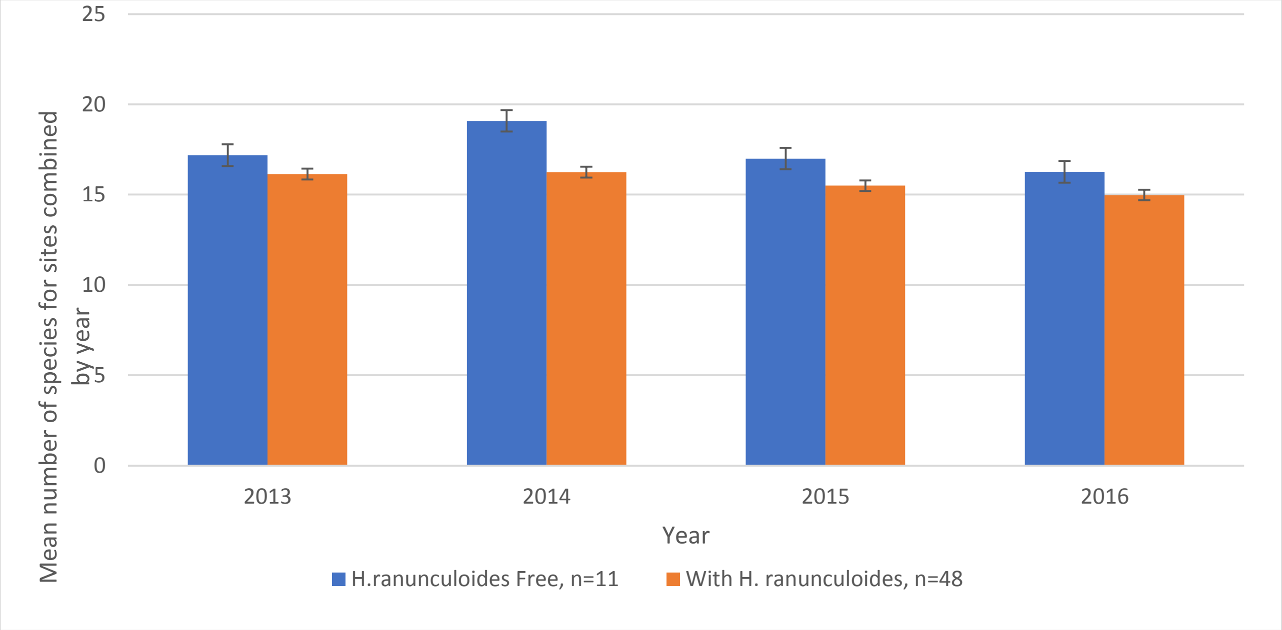


Figure 20: Mean species richness, as mean number of species, for sites with and without *H. ranunculoides*, by year, with standard error bars. There were no significant differences (t-test, P=0.065).

4.3.4.2. Shannon-Wiener diversity index

The diversity index values for all 60 sites (overall) for all four years, for sites without *H. ranunculoides* and those with <25% cover of *H. ranunculoides* do not significantly differ.

However, the index values decrease as the abundance of *H. ranunculoides* increases over a level of 25% (Figure 21). Even though sites with >25% cover of *H. ranunculoides* did not significantly differ from the sites with <25% cover or no *H. ranunculoides*, Figure 21 shows a distinct drop in diversity once this level is reached.

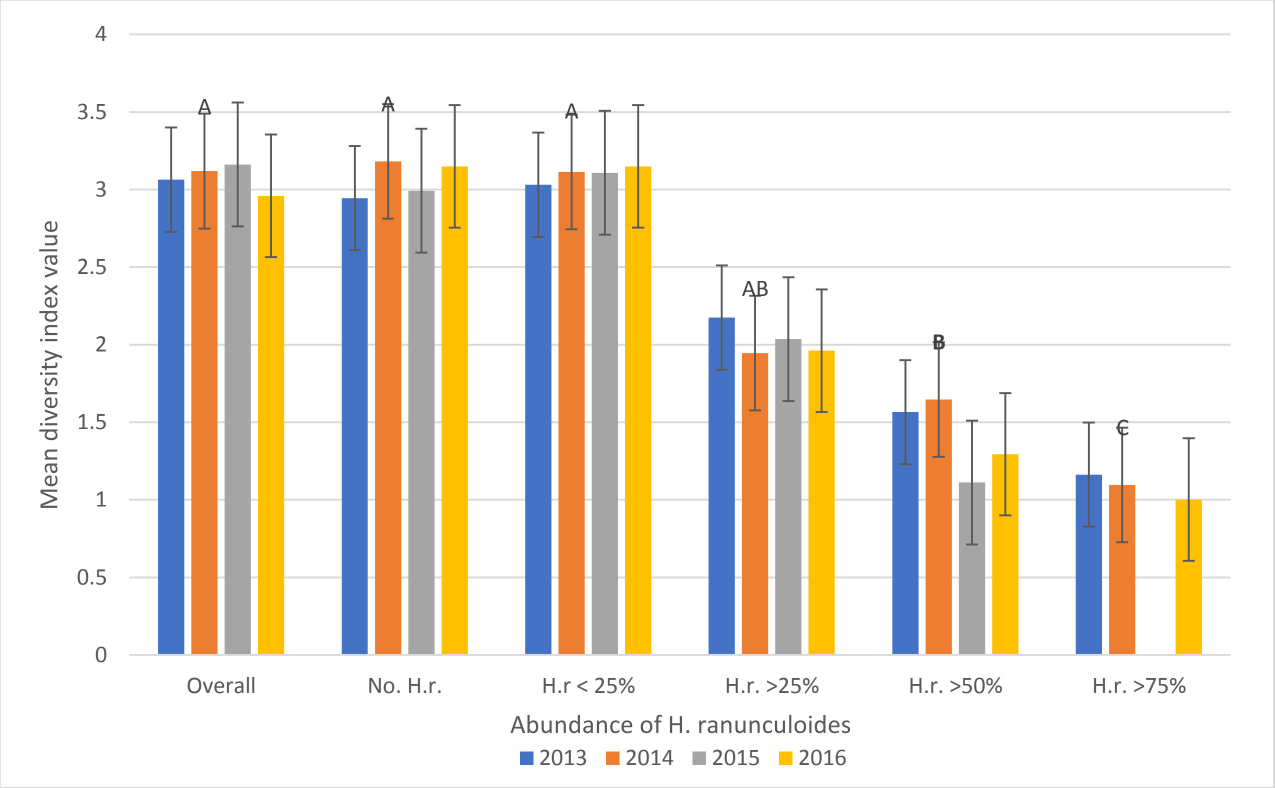


Figure 21: Mean Shannon-Wiener diversity index values for all surveyed sites, for all four years of the study, with abundance of *H. ranunculoides.* There were no sites with more than 75% cover of *H. ranunculoides* in 2015. Significant differences between treatments (ANOVA, P=0.00, n=60) are represented by letters A, B and C following a Tukey pairwise comparison and means that do not share a letter are significant (P<0.05).

At levels of >50% cover of *H. ranunculoides* there is a significant difference from those sites with >25% or less cover. Sites with >75% cover are significantly different to sites with >50% cover and those with >25% cover or less. So, there is a clear trend showing a decrease in diversity as Shannon-Wiener diversity index once a threshold of >25% cover of *H. ranunculoides* is reached but this does not become significant until >50% cover is reached.

2015 did not have any sites with 75%+ *H. ranunculoides.* The most likely reason for this, given the monthly community dynamics analyses presented earlier, is that there were more surveys undertaken earlier in the year, twenty-five surveys in June compared to none during June in 2013 or 2014 and only four in June in 2016. The surveys timings were randomised across the four years using accepted WFD period of June to September, but it was not known at that point that there was an association between the abundance of *H. ranunculoides* and time of year.

4.3.4.3. Simpson's 1-D diversity index

The Simpson's 1-D diversity Index scores have a very similar pattern to the Shannon-Wiener diversity index, with a very clear decline in the Simpson's diversity in the heavily infested 2016 year. Figure 22 shows a clear downward trend in the Simpson's 1-D diversity index as abundance of *H. ranunculoides* increases.

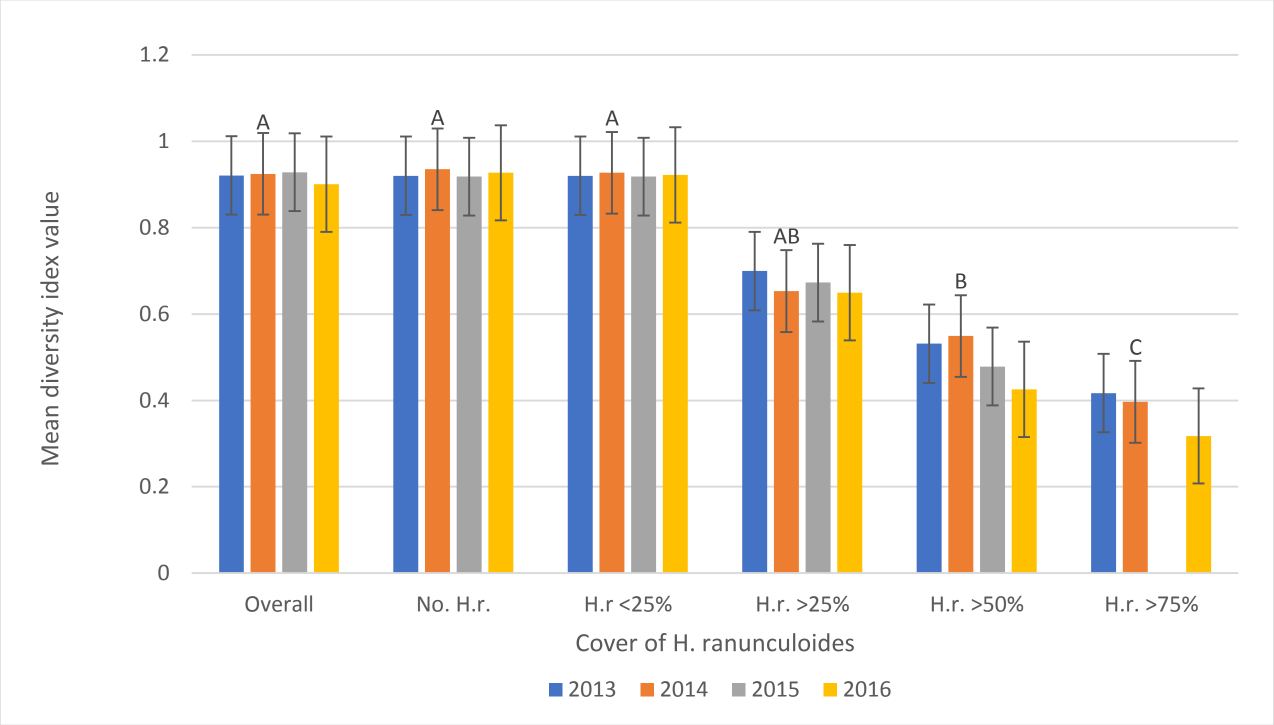


Figure 22: Mean Simpson's 1-D index of diversity values for all 60 surveyed sites, for all years, with different levels of infestation of *H. ranunculoides,* with standard error bars. There were no sites with more than 75% cover of *H. ranunculoides* in 2015. Significant differences between treatments (ANOVA, P=0.000, n=60) are represented by letters A, B and C following a Tukey pairwise comparison and means that do not share a letter are significant (P<0.05).

As with the Shannon-Wiener diversity index, the data were Arcsine transformed and a one-way ANOVA was run to test the significance of these results. The results were consistent with the Shannon-Wiener diversity index, with a decrease in diversity becoming apparent when abundance of *H. ranunculoides* exceeds 25%. There is no significant difference in diversity between all sites (overall), sites with no *H. ranunculoides* or <25%

cover. Sites with >50% cover are significantly different to those with no *H. ranunculoides* or <25% cover. Sites with more than >75% cover are significantly different to those with >50% cover or less.

4.4. Results of impact of management treatments on community composition

4.4.1. Impact of management treatments on macrophyte communities

60 sites were selected for monitoring. Of these 60 sites twelve remained free from *H. ranunculoides* for the three years of management treatments (Table 17). Unfortunately, four of these sites were unexpectedly de-silted in 2016. Of the remaining sites with *H. ranunculoides* eight remained un-cut, fourteen had an annual weed-cut, eight were weed-cut and sprayed annually,10 had a de-silt in one year and were weed-cut and sprayed in the other years and eight sites were de-silted in one year and weed-cut in the other years.

Table 17: The 60 sites and their management with and without *H. ranunculoides* for 2013 – 2016 and their codes used in the analyses described hereafter. Four reference, un-cut sites without *H. ranunculoides*, de-silted in 2016 have been removed from the analysis.

Management treatment	With *H. ranunculoides*	Without *H. ranunculoides*	Treatment Codes
Reference un-cut	0	8 (plus 4 de-silted, 2016)	CO
Reference un-cut	8	0	PW
Weed-cut	14	0	WC
Weed-cut and Sprayed	8	0	WS
Weed-cut, De-silted	8	0	WD
Weed-cut, De-silted, Sprayed	10	0	DS

4.4.1.1. Effect of management treatment on species richness

There were two reference groups, those with no *H. ranunculoides* present and sites with *H. ranunculoides* present but un-cut. The management treatments on the remaining sites with *H. ranunculoides* present were weed-cut annually, weed-cut and sprayed, weed-cut and de-silted and weed-cut, de-silted and sprayed (Table 17). The one-way ANOVA of the management treatments undertaken gave a P-Value of 0.004 indicating a significant result. Mean species number was highest in ditches that were weed-cut and sprayed

(WS) and lowest in ditches that were weed-cut and de-silted (WD) and those weed-cut, de-silted and sprayed (DS) (Figure 23).

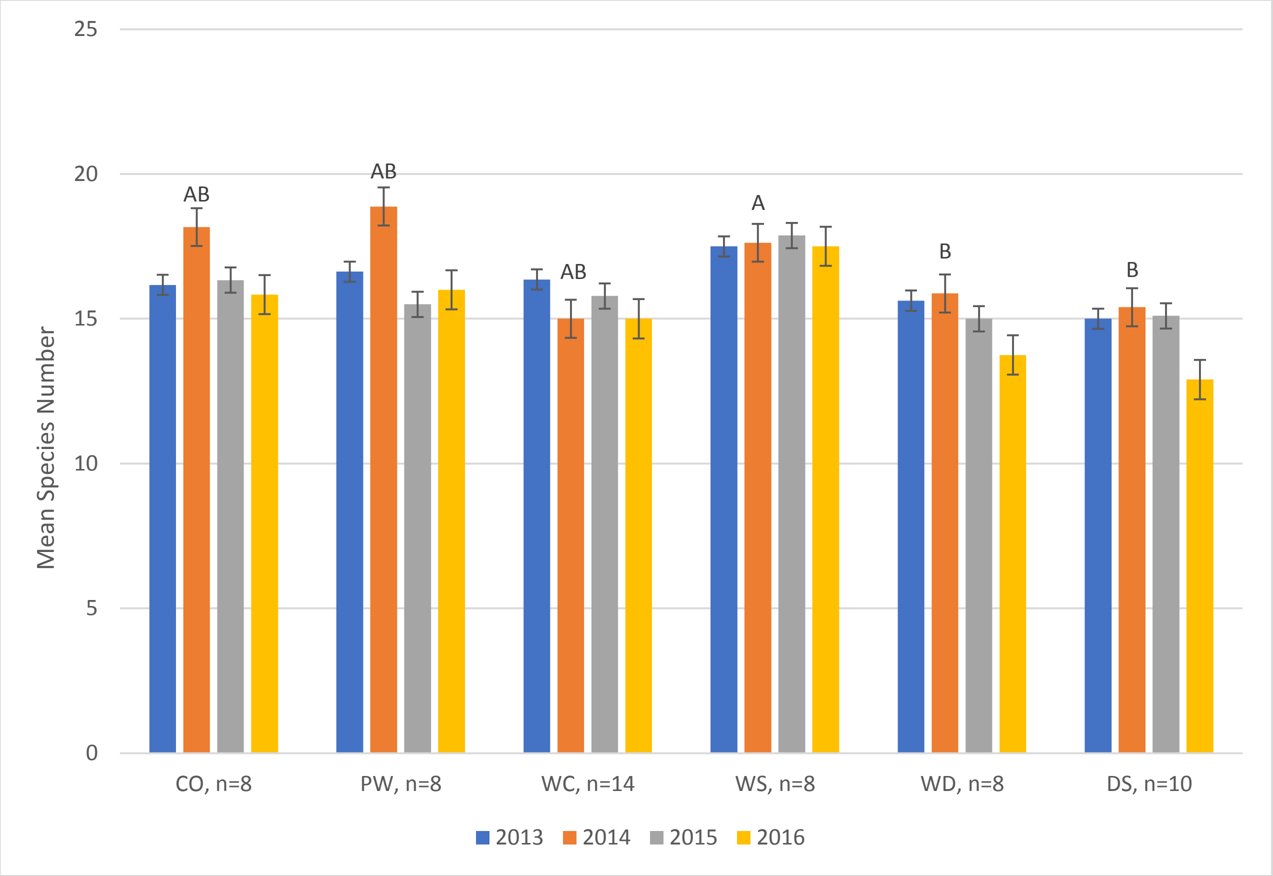


Figure 23: The mean species richness of reference sites (with and without *H. ranunculoides)* and those with management treatments undertaken over four years, with standard error bars. CO = No *H. ranunculoides* un-cut, PW = *H. ranunculoides* un-cut, WC = Weed-cut only, WS = Weed-cut and sprayed, WD = Weed-cut and de-silted, DS = Weed-cut, de-silted and sprayed. Significant differences between treatments for all four years combined (ANOVA, P=0.004, n=56) are represented by letters A, B and C following a Tukey pairwise comparison and means that do not share a letter are significant (P<0.05).

4.4.1.2. Effect of management treatment on diversity index values

For 2016, where fifty-six sites had been managed for three years, Table 18 compares both diversity index values described above. Four reference sites without *H. ranunculoides* have been excluded due to un-expected de-silting taking place in 2016. The monthly analysis in section 4.3.2. suggested a strong association with *H. ranunculoides* and the month September. In 2016 the average survey month was mid-July so the highest abundance of *H. ranunculoides* was unlikely to have been reached. The highest diversity for both index values was found in the uninvaded sites (CO) (Table 18).

Table 18: Diversity index values of surveyed sites, n=56, for 2016 for all management treatments.

Management Measure 2016	Shannon-Wiener index of diversity 2016	Simpson 1-D index of diversity 2016	No. sites	Treatment Codes
No H.r.	3.114	0.946	8	CO
Un-Cut	2.600	0.881	8	PW
Cut Only	2.535	0.825	14	WC
Cut-Spray	2.607	0.879	8	WS
Cut-Desilt	2.402	0.811	8	WD
Desilt-Spray	2.633	0.863	10	DS

4.4.1.3. Shannon-Weiner index

Using the Shannon-Wiener diversity index data, arcsine transformed to provide normal data, an ANOVA gave a P-Value of 0.033 which indicates a significant difference. Figure 24 shows that the significant difference was between the sites without *H. ranunculoides* (CO) and the management treatment weed-cutting and de-silting (WD). These weed-cutting and de-silted sites had a significantly lower Shannon-Wiener diversity index .

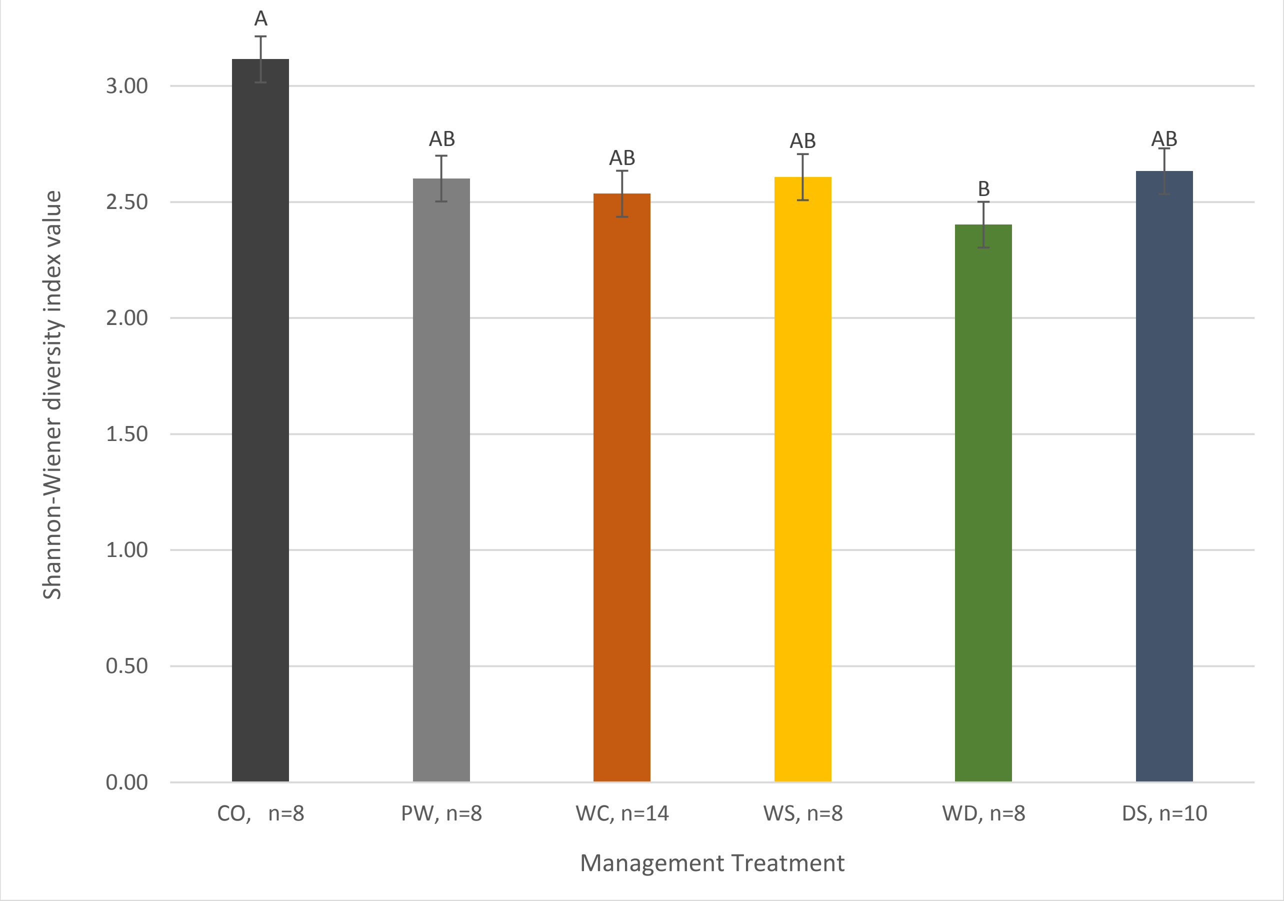


Figure 24: Mean Shannon-Wiener index of diversity values for all surveyed sites, for 2016, after three years of management treatments, with standard error bars. CO = No *H. ranunculoides* un-cut, PW = *H. ranunculoides* un-cut, WC = Weed-cut only, WS = Weed-cut and sprayed, WD = Weed-cut and de-silted, DS = Weed-cut, de-silted and sprayed. Significant differences between treatments (ANOVA, P=0.033) are represented by letters A and B following a Tukey pairwise comparison and means that do not share a letter are significant (P<0.05), n is described for each treatment in the graph.

4.4.1.4. Simpson 1-D index

An ANOVA of the Arcsine transformed data using the Simpson 1-D index values, had a P-value of 0.102 showing that diversity was not significantly different across sites with different management treatments. Figure 25 shows the diversity index scores vary very little under all management treatments.

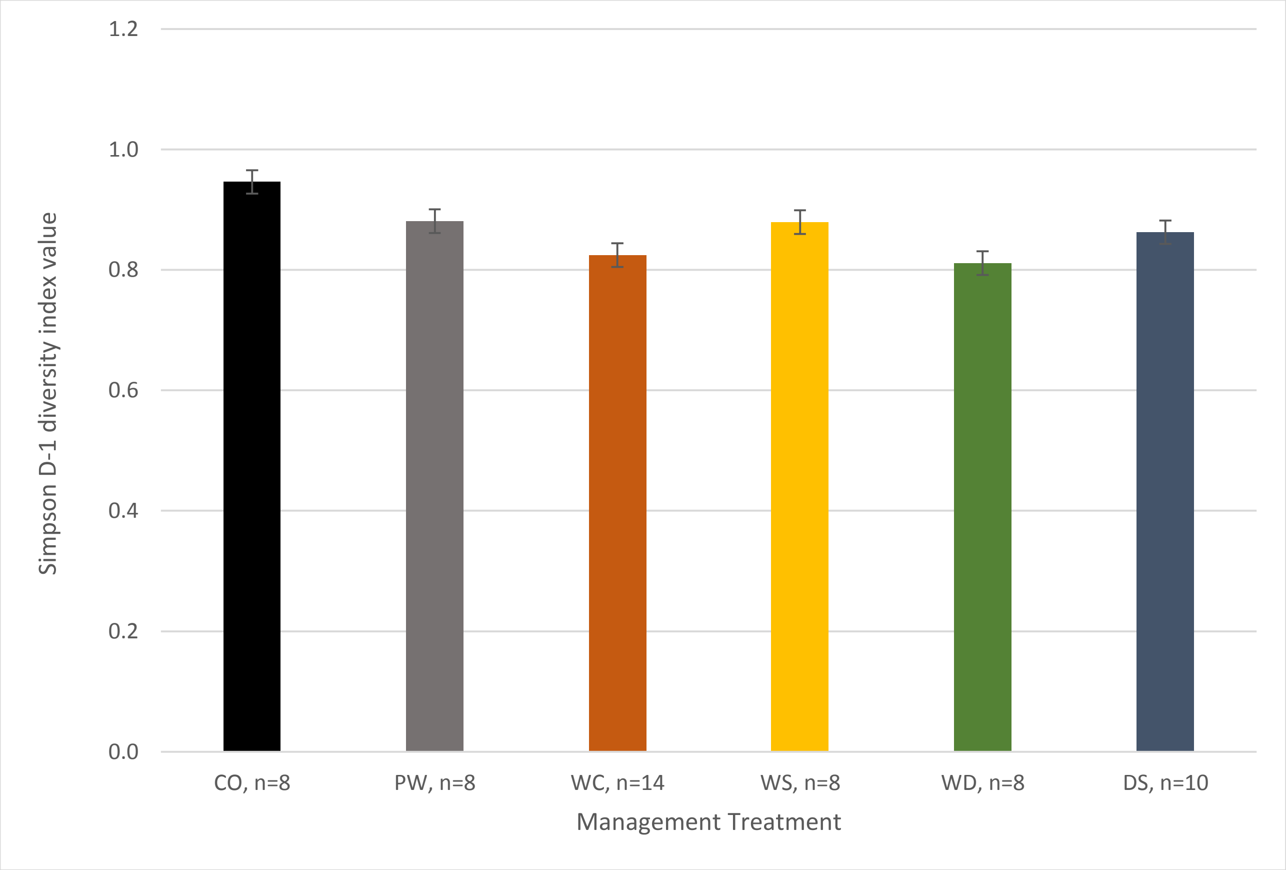


Figure 25: Mean Simpson 1-D index of diversity values of all surveyed sites, for 2016, after three years of management treatments, with standard error bars. CO = No *H. ranunculoides* un-cut, PW = *H. ranunculoides* un-cut, WC = Weed-cut only, WS = Weed-cut and sprayed, WD = Weed-cut and de-silted, DS = Weed-cut, de-silted and sprayed. There were no significant differences (ANOVA, P=0.102)**.**

4.4.2. Relationships between management treatment and *H. ranunculoides.*

4.4.2.1. Management treatment and community composition.

All 60 sites were subject to an RDA using all species for all four years of the study to investigate the impact of treatment on community composition. 292 plant species records were included with the 20 plant species, with best fit to the displayed ordination axes, illustrated against the management treatment. The first gradient (horizontal axis) explains 7.32% of the total variability in species composition with the 4.82% explained by the second (vertical axis) gradient (Table 19). The permutation test results gave a pseudo-F=2.2 with a P=0.002 indicating a significant result. The cumulative variation is not a high figure, which means there may be other factors explaining the plant community better than management treatment, but this P-value is significant.

Table 19: Summary of RDA results of all species and management treatment for all years of the study.

Statistic	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	0.0732	0.0482	0.0226	0.0154
Explained variation (cumulative)	7.32	12.14	14.40	15.94

The plant species and management treatments biplot (Figure 26) suggests three directions of influence. The *H. ranunculoides* vectors are strongly aligned with the management treatment weed-cutting (WC) in all four years. Weed-cutting and spraying (WS) and the reference sites without *H. ranunculoides* (CO) have the native species vectors clustered between, with *Berula erecta* and *Lemna trisulca* represented for three out of the four years. The weed-cutting and de-silting (WD), weed-cutting, de-silting and spraying (DS) management treatments show *Glyceria maxima* and *Phalaris arundinacea* vectors associated. These are marginal ditch species which are unlikely to be removed by de-silting and would return rapidly following a weed-cut as their root systems would remain intact.

The classified plot diagram in Figure 27 displays the grouping of the management treatments in relation to the 60 sites and can be used to evaluate the separation of the sites resulting from the management treatments. The distance between the plot envelopes represents the average dissimilarity of plant species composition. The reference sites without *H. ranunculoides* (CO) are set apart from most management treatments and the un-cut *H. ranunculoides* sites (PW). However, the most apparent dissimilarity in species composition is between the reference sites without *H. ranunculoides* (CO) and the weed-cut (WC) only sites with *H. ranunculoides* infestation suggesting that the weed-cutting only treatment is changing community composition.

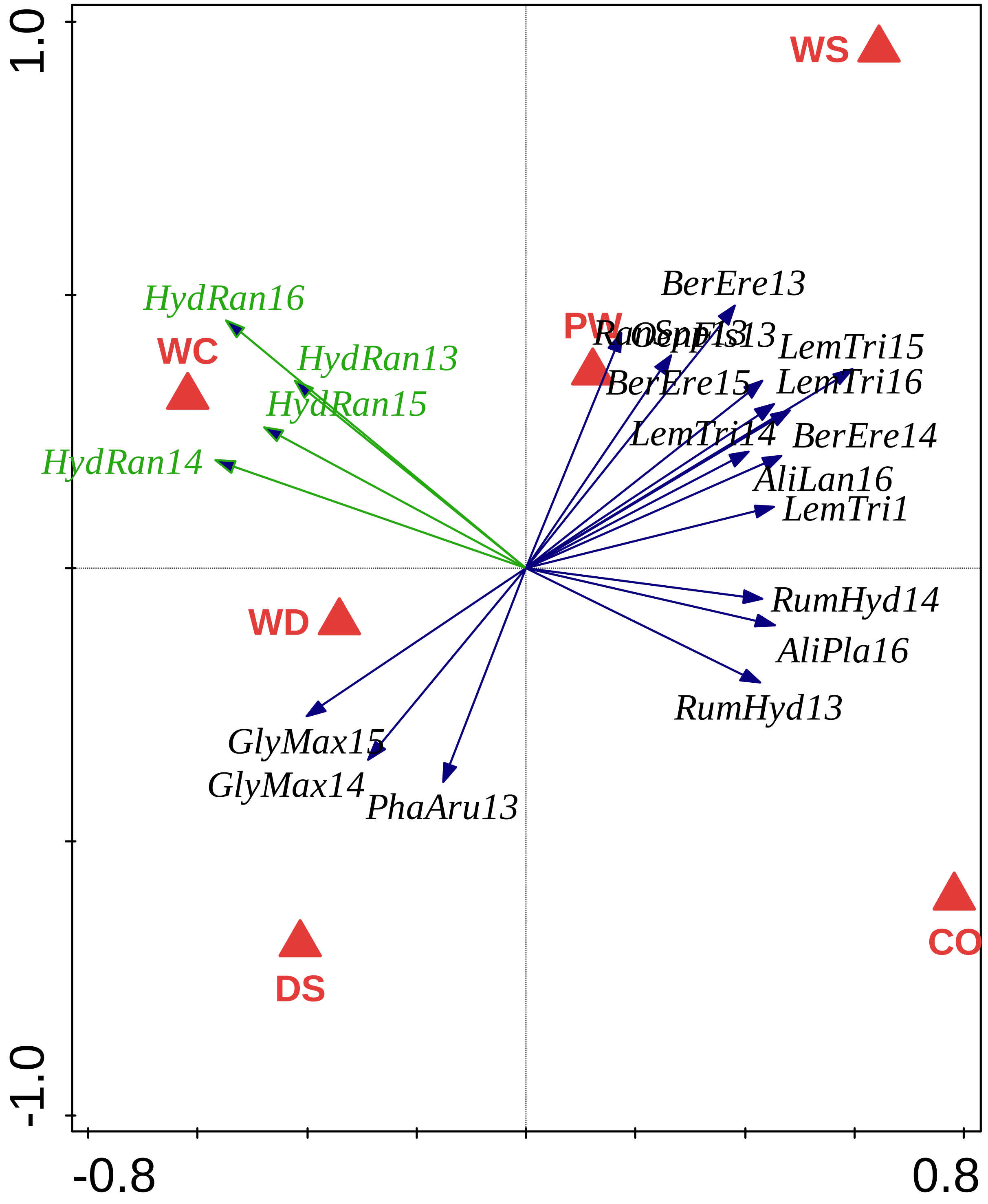


Figure 26: RDA plant species and management treatments biplot of all sites, for all four years, with the 20 species with the best fit to the ordination axes shown. The triangles represent the management treatments. Plant names are shortened versions of the plant latin binomials with the year suffixed (Table 11). CO = No *H. ranunculoides* un-cut, PW = *H. ranunculoides* un-cut, WC = Weed-cut only, WS = Weed-cut and sprayed, WD = Weed-cut and de-silted, DS = Weed-cut, de-silted and sprayed. The first two axes are plotted.

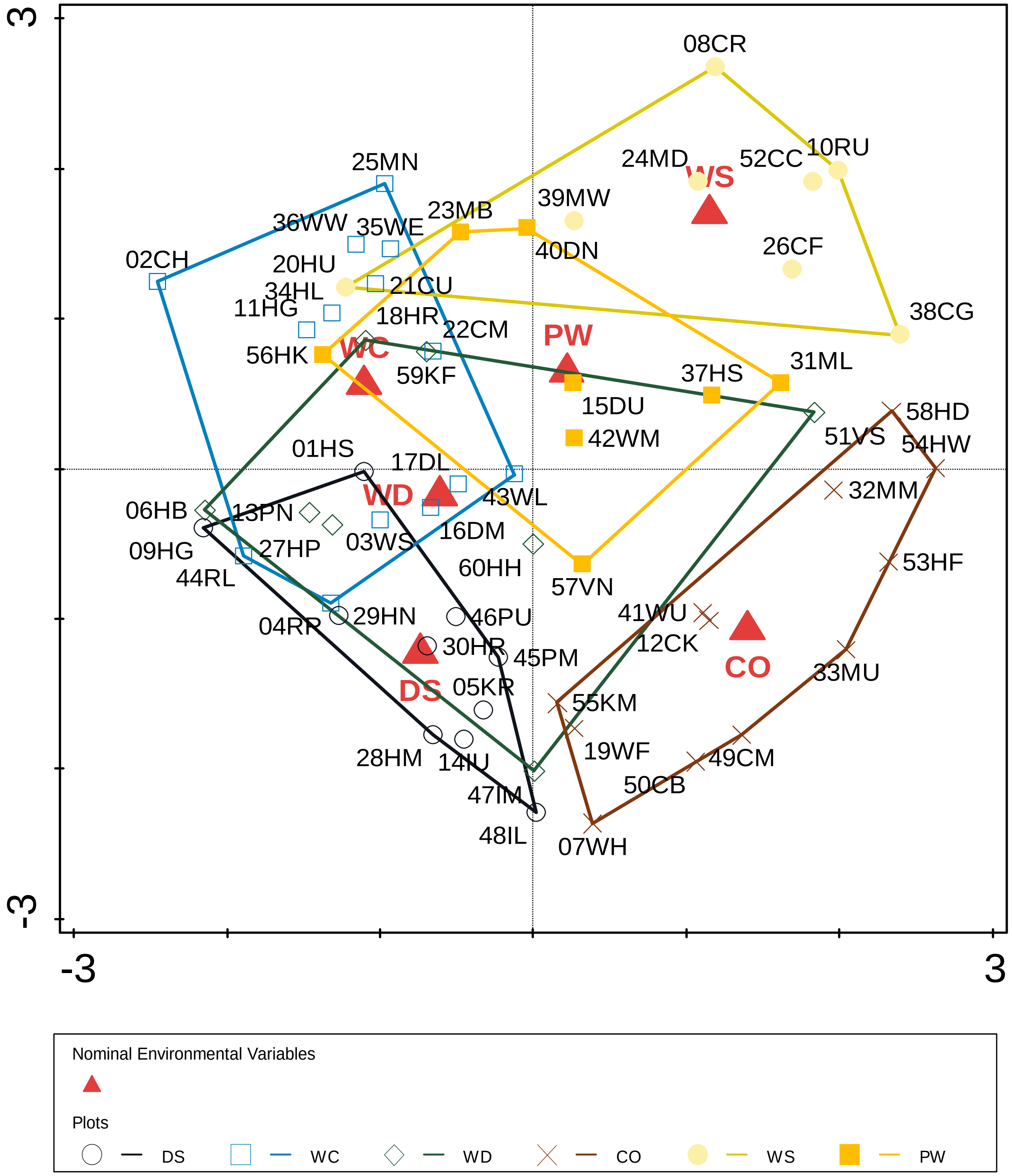


Figure 27: Classified plot diagram for all 60 sites, all species and management treatments for all four years, 2013 -2016. The triangles represent the management treatments. CO = No *H. ranunculoides* un-cut, PW = *H. ranunculoides* un-cut, WC = Weed-cut only, WS = Weed-cut and sprayed, WD = Weed-cut and de-silted, DS = Weed-cut, de-silted and sprayed. The sites are represented by codes using two numbers and two letters. The plot envelopes are colour coded by management treatment. The first two axes are plotted.

Using 2016 data after three years of management treatments, an RDA gave a P=0.002, indicating a significant difference between management treatments. The 20 species with the best fit to the most important ordination axes are plotted in Figure 28.

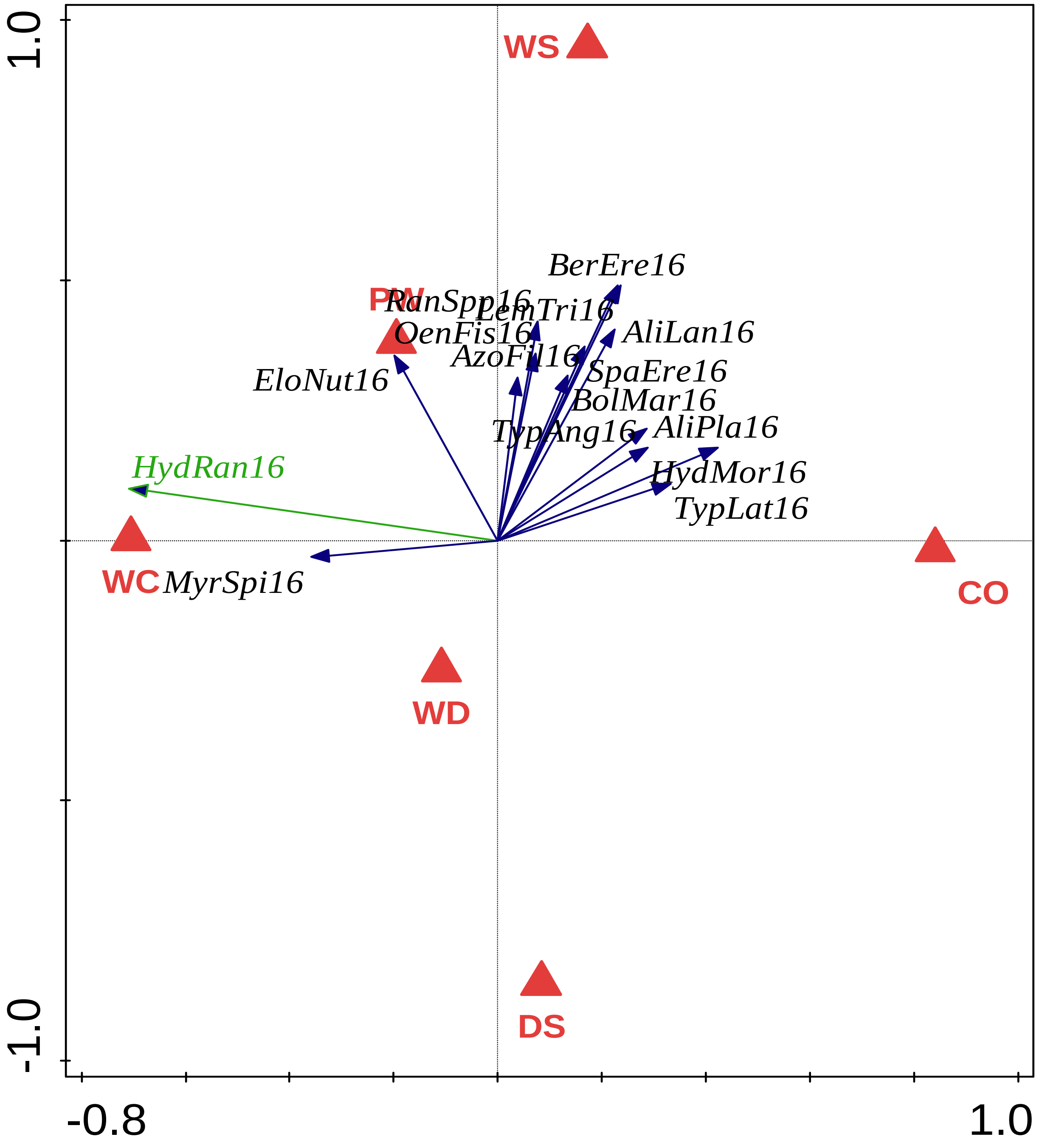


Figure 28: RDA biplot of all plant species, and management treatments for 2016, with the 20 species with the best fit to the ordination axes shown. Triangles represent the management treatments. Plant names are shortened versions of the plant latin binomials with the year suffixed (Table 11). CO = No *H. ranunculoides* un-cut, PW = *H. ranunculoides* un-cut, WC = Weed-cut only, WS = Weed-cut and sprayed, WD = Weed-cut and de-silted, DS = Weed-cut, de-silted and sprayed.

Table 20 shows the first gradient (axis) explained 9.40% of the total variability in species composition with a total of 19.12% explained. Although the P value was significant, these are low values and do not explain a high percentage of the variation.

Table 20: Summary of RDA results of all species, n=82, for 2016, P=0.002.

Statistic	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	0.0940	0.0622	0.0223	0.0127
Explained variation (cumulative)	9.40	15.62	17.85	19.12

In Figure 28 the *H. ranunculoides* vector is the longest, along the horizontal, indicating a strong positive correlation with the weed-cut only management treatment (WC) and with the un-cut, infested sites (PW). The native species vectors are clustered between, and positively associated with the weed-cut and spray (WS) and un-infested sites (CO). The *H. ranunculoides* vector is negatively associated with the majority of the native plant species (more than 90 degrees). Except for *Myriophyllum spicatum* (MyrSpi16) no native species were positively associated with either of the de-silting management treatments (WD and DS).

An RDA interactive forward selection option was chosen to select the best environmental variables to summarize the variation in plant species composition (Šmilauer and Lepš, 2014). The weed-cutting (WC) treatment was most significant, P=0.002, with weed-cut and spray (WS), P=0.004, and de-silting and spraying (DS) also significant, P=0.022. The plant species and management biplot (Figure 29) shows that these three management treatments are distinctly separated. Although herbicide spraying was used in both the weed-cut and spray (WS) and weed-cut, de-silt, and spray (DS) treatments, the de-silting appears to be the treatment affecting the species composition most. The *H. ranunculoides* vector remains positively associated with the weed-cut (WC) only treatment .

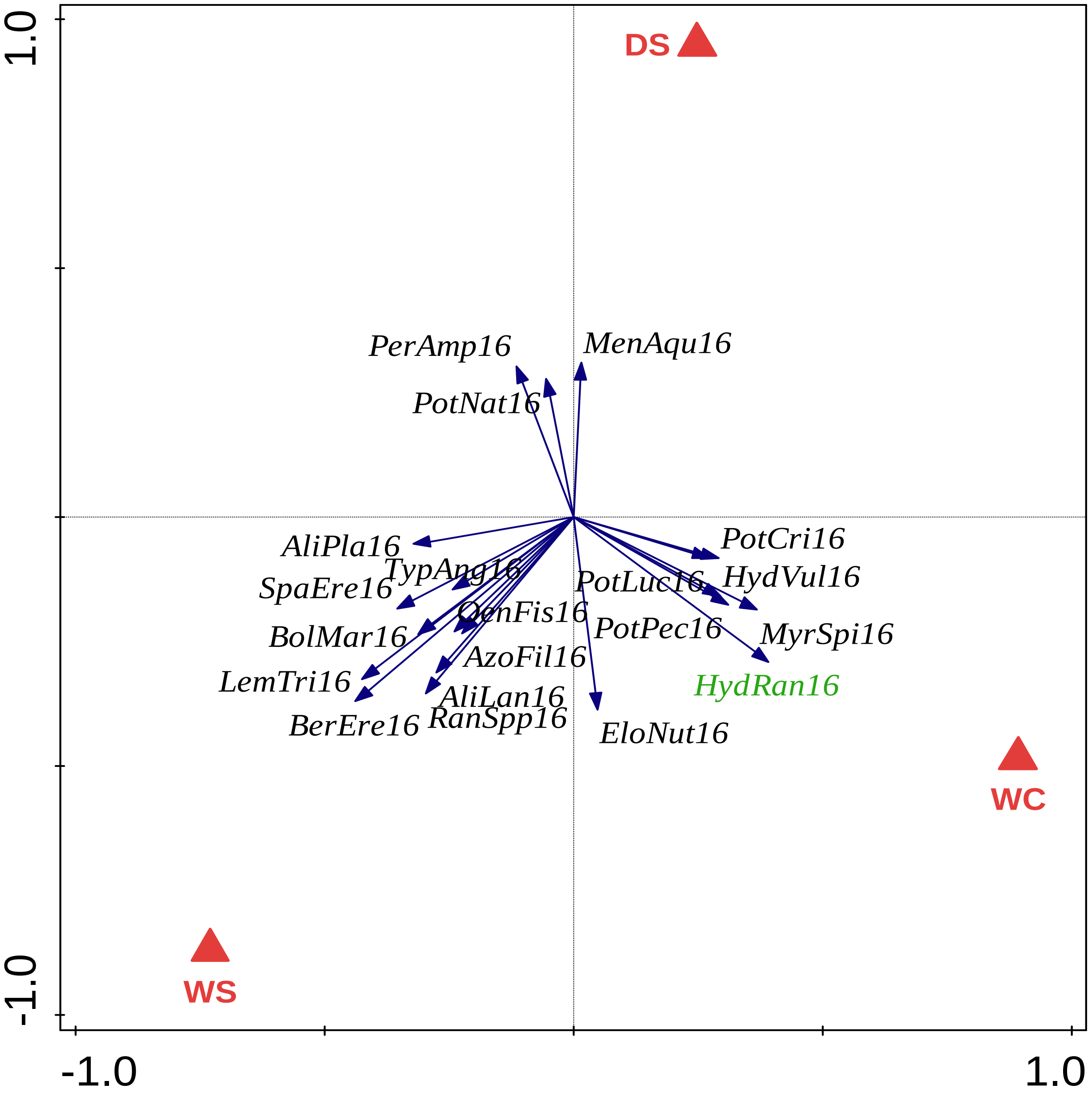


Figure 29: RDA interactive forward selection of plant species, and management treatments, biplot for 2016, with the 20 species with the best fit to the ordination axes shown. Plant names are shortened versions of the plant latin binomials with the year suffixed (Table 11). Triangles represent the management treatments. WC = Weed-cut only, WS = Weed-cut and sprayed, DS = Weed-cut, de-silted and sprayed.

These results infer that weed-cutting alone may favour the presence of *H. ranunculoides* rather than controlling it. The position of the weed-cut, de-silt, and spray (DS) treatment in both graphs (Figures 28 and 29) may indicate a negative impact on native plant species from this management.

In addition to management, the environmental variables, watercourse width, shade, land use and silt depth were also measured for each of the 60 sites. In addition to the management treatment, width and silt depth were significant in influencing species abundance, at P=0.002 (Table 21).

Table 21: Summary of RDA results of all species and environmental variables, n=60, for all years, P=0.002.

Statistic	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	0.0599	0.0382	0.0247	0.0111
Explained variation (cumulative)	5.99	9.811	2.28	13.39

Figure 30 illustrates the relationship between these environmental variables and plant species. The explained cumulative variation was very low at a total of 13.39 % for the first four axes (Table 21), probably because there is so much variation in the plant community of the numerous ditches and their physical environment over the four years. *H. ranunculoides* only appeared as one of the strongest explanatory species for 2016. The native plant species were negatively correlated with width and silt depth, with smaller ditches and shallower ditches, due to deeper silt, being more species rich.

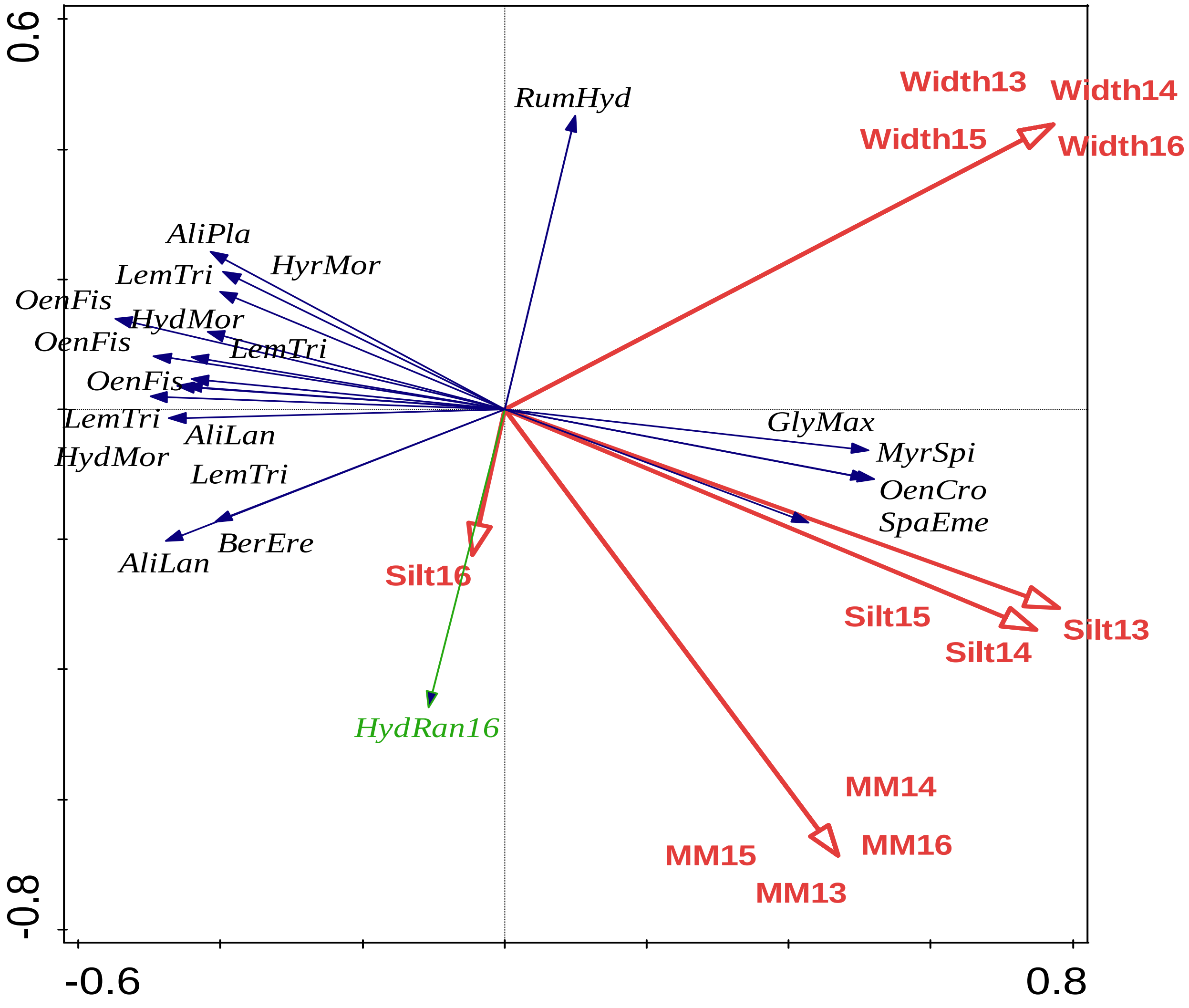


Figure 30: RDA biplot of all plant species, and environmental variables for all years, with the 20 species with the best fit to the ordination axes shown. Plant names are shortened versions of the plant latin binomials with the year suffixed (Table 11). Width = width of the watercourse, Silt = silt depth, MM = all management methods combined.

4.4.2.2. Effect of management treatment on community change over time

An analysis of repeated observations of the species composition of the 60 sites over a period of four years was carried out. This provided baseline data (from 2013) to compare with the following three years (2014, 2015 and 2016) to show any differences in the dynamics of the managed and reference sites with and without *H. ranunculoides*. Here the response of the macrophyte communities to the management treatment was tested as temporal dynamics.

The display in Figure 31 is limited to the 20 species with the best fit to the shown ordination axes as there are too many species to interpret the charts effectively if all are displayed. Figure 31 shows the weed-cut (WC) only sites are aligned with the *H.*

ranunculoides vectors in all years, suggesting that weed-cutting alone favours the presence of *H. ranunculoides.* The reference sites without *H. ranunculoides* (CO) are placed opposite to the WC sites in all which suggest that they may have different community compositions than that of the CO sites. Their chart positions remain relatively consistent for three of the four years with 2015 being quite different. The weed-cut and sprayed sites (WS) and de-silted and sprayed (DS) sites are placed well apart from each other in all years suggesting these management treatments produce different community compositions. The weed-cut and de-silt (WD) sites are placed near to both the weed-cut (WC) and de-silted and sprayed (DS) sites in all years and away from the un-infested (CO) sites. Interestingly, the sites, un-cut with *H. ranunculoides* (PW) are not strongly associated with the *H. ranunculoides* vectors in any year.

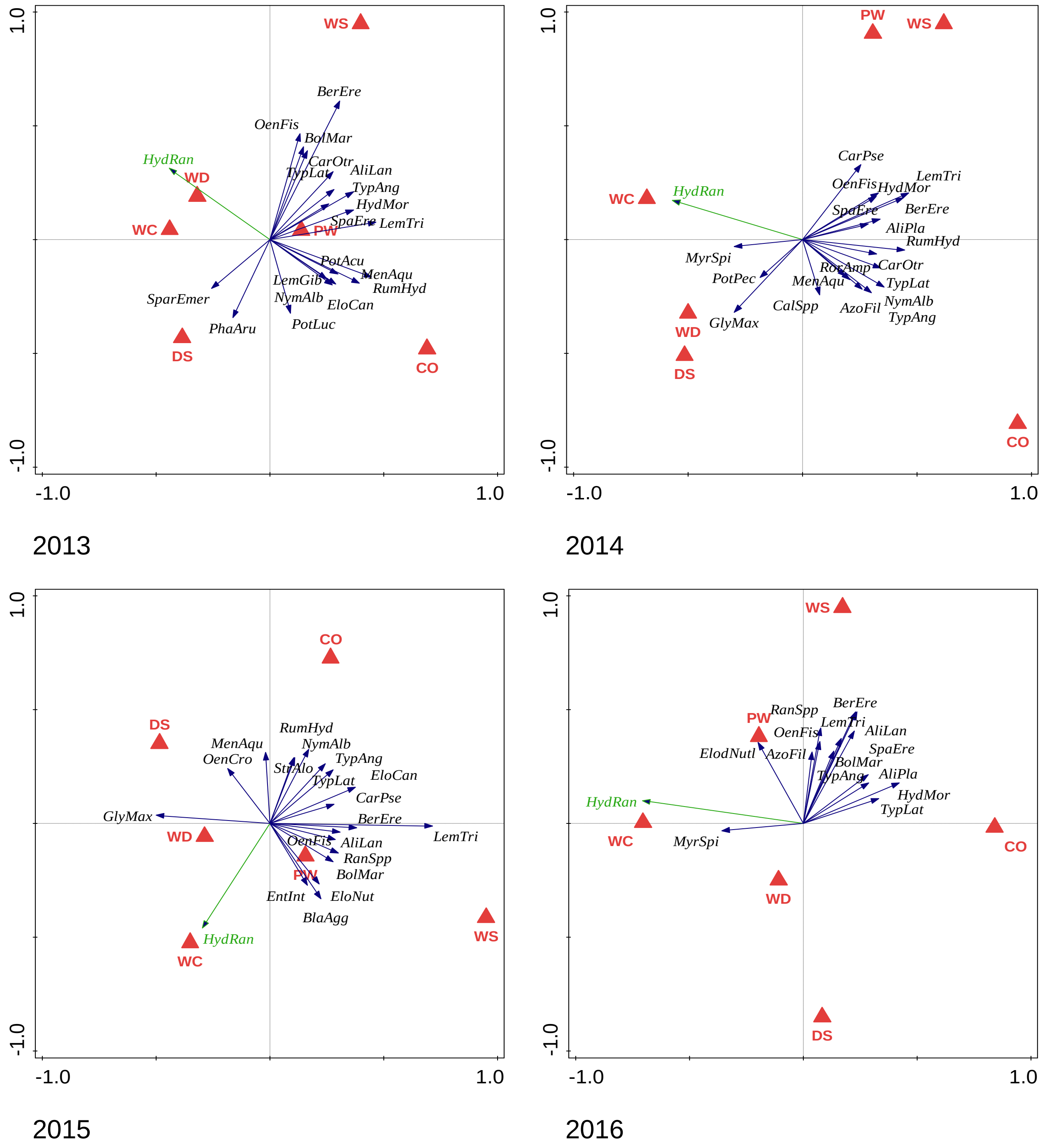


Figure 31: RDA charts for each of the study years with management treatment and the 20 species with the best fit to the ordination axes shown. Plant names are shortened versions of the plant latin binomials (Table). CO = No *H. ranunculoides* un-cut, PW = *H. ranunculoides* un-cut, WC = Weed-cut only, WS = Weed-cut and sprayed, WD = Weed-cut and de-silted, DS = Weed-cut, de-silted and sprayed.

4.4.2.3. Efficacy of management treatments

The mean percentage cover of *H. ranunculoides* for each treatment, was plotted by year, for 2014, 2015 and 2016, for the 48 infested sites. Reference sites without *H. ranunculoides* were excluded. The data were Arcsine transformed to provide normal data, and subject to an ANOVA. The management treatments included were un-cut and unsprayed with *H. ranunculoides* (PW), weed-cut only (WC), weed-cut and sprayed (WS) and weed-cut, de-silted and sprayed (DS). The ANOVA, on the mean percentage cover of all three years combined, gave a significant difference between treatments at P=0.031. The Tukey pairwise comparisons with 95% confidence showed that there was a significant difference between the un-cut, with *H. ranunculoides* (PW), and weed-cut and sprayed (WS) management treatments. The abundance of *H. ranunculoides* was significantly reduced in the weed-cut and sprayed (WS) management treatment compared to the un-cut infested sites (PW). Figure 32 shows the abundance of *H. ranunculoides* by year, 2014, 2015 and 2016, in sites with different management treatments. As each years' growing conditions are different, varying levels of *H. ranunculoides* were present, with 2016 having particularly high levels.

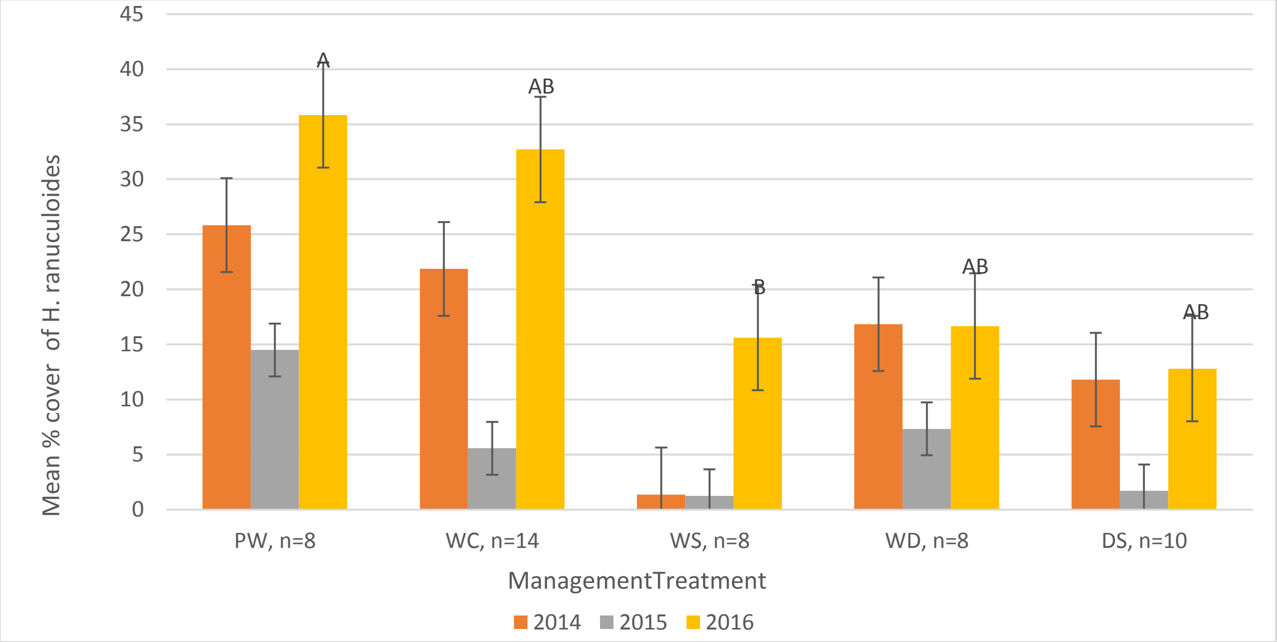


Figure 32: Abundance as mean percentage cover of *H. ranunculoides* under different management treatments over three years, with standard error bars. CO = No *H. ranunculoides* un-cut, PW = *H. ranunculoides* un-cut, WC = Weed-cut only, WS = Weed-cut and sprayed, WD = Weed-cut and de-silted, DS = Weed-cut, de-silted and sprayed. Significant differences between treatments for all three years combined (ANOVA, P=0.031) are represented by letters A and B following a Tukey pairwise comparison and means that do not share a letter are significant (P<0.05), n is described for each treatment in the graph.

4.5. Discussion

4.5.1. Relationships between H. *ranunculoides* and macrophyte community composition

4.5.1.1. Community classification

Most of the sites were placed in the NVC traditional swamp (S) communities rather than true aquatic (A) communities. Community composition, as NVC community, changed at most sites with only 30% of these remaining the same throughout the survey period 2013 to 2016, and these were not specific to either the reference or management treatment sites. The changes in NVC are likely to be attributed to the varying abundance of the species, which does affect how the NVC is calculated, although type and species richness recorded at the monthly sites, remained relatively consistent over the year.

The data show that *H. ranunculoides* can invade a range of NVC communities and these include those with submerged, floating, and emergent species. In its native regions, there is no comparable phytosociological description but Milne (2003) found that *H. ranunculoides* was unique to an *Eichornia azurea – Limnobium laevigatum* sub-community in Brasil. In Argentina it co-exists with macrophytes that include submerged and floating species as well as emergent (Walsh et al. 2013). Walsh et al. (2013) observed *H. ranunculoides* was continually present at sites in Argentina but exhibited patch dynamics with other macrophytes replacing it as it disappeared but with no specific succession. Patch dynamics may be occurring in the invaded regions as abundance did vary in un-cut reference sites and could partly account for why NVC communities still changed at these sites.

There is little or no data available on NVC communities in other invaded regions such as the Netherlands, Germany, and Belgium where *H. ranunculoides* has been studied in Europe. However, a study of four lakes in Minnesota, USA, was undertaken by Kovalenko et al. (2010) using the herbicide 2,4-D amine to control the invasive *Myriophyllum spicatum*. Kovalenko's (2010) research followed a similar methodology to this study with the macrophyte communities being surveyed pre-treatment and for three years following treatment and twice a year in June and September to eliminate seasonal variations. The results indicated a negative association between the invasive *M. spicatum* and native macrophytes as with this study between *H. ranunculoides* and native species but that communities with *Ceratophyllum demersum* and *Nymphaea odorata* recovered well post-treatment.

4.5.1.2. Annual macrophyte community dynamics

Species richness remained relatively high throughout the year, although fluctuating, across the months except in September where there was a clear dip in numbers. This corresponds with a positive correlation between abundance of *H. ranunculoides* and month indicating a peak in September, chapter 4, 4.3.2. Hussner and Lösch (2007) report that *H. ranunculoides* peak growth rate in Germany took place in June and July but this study indicates that growth might peak during the warm June and July period but that peak biomass is likely to be later in the season during September. Macrophyte species composition appears to vary throughout the season with species associated with certain months of the growing season, which is to be expected as plant communities do have seasonal niches (Kunii and Maeda, 1982).

The EA's macrophyte monitoring for the WFD, OI 131_07, (Environment Agency, 2016c) states that monitoring should take place from June to September. These results suggest that May is as species rich and has some species associated with it that could be missed in later season surveys. However, for *H. ranunculoides,* surveys in the early part of the season, even within the accepted WFD period June to July are not likely to record the full abundance *H. ranunculoides* is likely to attain.

The data shows that, even at sites heavily infested, most species do not disappear completely but remain at very low percentage numbers. This may be due to two reasons. Early season native macrophytes may be able to gain sufficient biomass to reproduce before *H. ranunculoides* cover increases in the latter part of the growing season. But it could also be due to the patch dynamics of *H. ranunculoides* (Walsh et al. 2013) which provides niches for native species to re-colonise, even in heavily infested sites. Nijs et al., (2012) noted that submerged species may disappear completely, but this study suggests that they persist in low numbers in infested sites and did re-colonise in subsequent years following treatment. An example would be site 45PM, which was desilted in 2015. The baseline P. obtusifolius in 2013 was 3% cover, which fell to 0% observed following the de-silt but had recovered to 10% cover in 2016.

4.5.1.3. *H. ranunculoides* abundance and species richness

A study by Stiers et al. (2011) in Belgium found that *H. ranunculoides* reduced native plant species richness on average by 70%, in heavily invaded sites (75%+) compared to uninvaded. Stiers et al. (2011) also found that the abundance of an aquatic invasive species needed to be 50% or more to show a decline in species number. In all four years of this study, although the sites infested with *H. ranunculoides* had lower species richness, they were not significantly lower compared to the reference sites without *H. ranunculoides*. However, the diversity index values clearly showed a decline in diversity as a combination of species richness and abundance as abundance of *H. ranunculoides* increased. Both index values showed that the level of infestation was 50% or higher before a significant reduction in diversity. Therefore, these and Stiers et al. (2011) results suggest 50% cover of INNS is the critical level at which diversity either as species number or diversity index values are affected. *H. ranunculoides* was shown to be negatively affecting six species; submerged, floating and emergent. With *H. morsus-ranae* being impacted in three out of four years this study supports Stiers et al. (2011) study which found that that *H. morsus-ranae* was absent from heavily infested sites.

4.5.1.4. Plant community change and infestation

A study by Pyšek et al. (2012) on the impact of plant invasions around the world investigated the significant impacts of invasive plants at the community level. Resident plant species abundance, richness and diversity all declined dramatically at the community level, but the significance of the impact depended on interactions between species traits and the biological community. The enormous regeneration capacity and rapid stoloniferous growth rate of *H. ranunculoides* (Hussner and Lösch, 2007) are traits that differ from the native macrophytes found in the studied sites. The community change results showed that *H. ranunculoides* was distinctly separate from submerged and floating species but also from *P. australis* indicating that there could be a shift in plant community because of infestation. Stiers et al.'s (2011) work showed that native community composition remained similar between uninvaded and semi-invaded (25%) sites, but that uninvaded and heavily invaded sites differed. The floating and submerged species being most affected by the invasions.

The results in this study show that certain native species appear more impacted than others, *H. morsus-ranae, L.* trisulca*, P. lucens, S. erectum*, and *B erecta.* The floating species *H. morsus-ranae* and *L. trisulca*, and the submerged species *P. lucens* would conform with Stiers et al.'s (2011) results. However, the species of note, as they are species present in some of the rarer NVC communities that are only found on a few sites in the UK, are *H. morsus-ranae* and *L. trisulca*. *H. morsus-ranae* is found in many of the NVC communities in this study; A11 – *Potamogeton pectinatus-Myriophyllum* spicatum, S8 – *Scirpus lacustris* spp. Swamp, S14 – *Sparganium erectum* swamp and S16 – *Sagittaria sagittifolia* swamp. *L. trisulca* is also found in A11, S14 and S16.

The results described show that, in three of the years (except 2015 which had very low *H. ranunculoides* cover) there were significant negative effects of *H. ranunculoides* on *H. morsus-ranae. H. morsus-ranae* is a constant species in the local and declining NVC A3 *Spirodela polyrrhiza-Hydrocharis morsus-ranae* and A4 *Hydrocharis morsus-ranae-Stratiotes aloides* communities (Rodwell, 1998). A marked decline in *H. morsus-ranae,* and the NVC associated *Stratiotes aloides*, has been recorded in the last century (Online Atlas of the British and Irish flora, 2019). *H. morsus-ranae* prefers still or very slow-flowing, neutral to base-rich water with moderate levels of nitrogen and other nutrients (Online Atlas of the British and Irish flora, 2019) which is also the preferred habitat for *H. ranunculoides. H. morsus-ranae* is closely associated with *L. trisulca* (which is also negatively associated with *H. ranunculoides*) and *S. aloides*.

For WFD status, the following metrics are used: river macrophyte nutrient index, number of macrophyte taxa, number of functional groups and cover of green filamentous algae (Water Framework Directive, 2014). These results would indicate that the presence of *H. ranunculoides* may not affect the WFD status because macrophyte species number does not decline. However, the river macrophyte nutrient index may be affected as several macrophyte species scoring highly (above 8.00) are significantly impacted by *H. ranunculoides*, *L.trisulca, H. morsus-ranae, B. erecta, P. lucens* and *S. erectum.*

The Pevensey Levels SSSI status is at risk because it requires less than 5% cover of INNS and the weed-cutting only management treatment does not effectively control *H. ranunculoides.* In addition, *L. trisulca*, *P. lucens, H. morsus-ranae* and *Sparganium erectum* are all listed in the SSSI citation (Natural England, 2019) and have been shown in this study to be negatively impacted by *H. ranunculoides.*

P. australis was a very dominant species where found present at the field sites and is likely to shade out other native macrophyte species which might explain the distinctive placement in some of the diagrams, for example Figure 19. Shade is a management method used to control *H. ranunculoides* (Duenas and Newman, 2010), and so *P. australis* could be used to suppress *H. ranunculoides*. However, as P. australis would also suppress native macrophytes, this would not be an option for the Pevensey Levels if it were to retain its' citations for macrophyte diversity (Joint Nature Conservation Committee, 1999, Natural England, 2013a). However, *P. australis* could be used as a 'fire-break' species to prevent further spread, especially at confluences of watercourses.

Watson (2002) noted that the rare snail *Anisus vorticulus*, for which the Pevensey Levels is designated a Special Area of Conservation (SAC), is found mainly on floating or submerged vegetation and *L. trisulca* and *H. morsus-ranae* are specifically mentioned as important. The negative impact of *H. ranunculoides* on *L. trisulca* and *H. morsus-ranae*, both of which are constant species in the NVC A3 and A4 communities, could therefore have a significant impact on the ability of *A. vorticulus* to survive on the Pevensey Levels. The traditional management treatment of controlling *H. ranunculoides* using annual mechanical removal, which is the only treatment permitted in the organic farming areas, is not suitable for allowing the development of diverse plant communities or the A3 and A4 communities that are favoured by the snail. Hence, the presence of *H. ranunculoides* controlled mechanically, or uncontrolled, is likely to put the SAC status of this site at risk.

4.5.2. The role of management treatments and their efficacy

4.5.2.1. Management treatments used to control *H. ranunculoides* abundance and their effect on species richness

Weed-cut and sprayed ditches retained the most diversity as species richness. De-silted and sprayed ditches had the lowest richness with cut and de-silted ditches having the lowest diversity using the Shannon Wiener diversity index. However, the weed-cut and sprayed sites had the highest percentage change in community composition over the four years. This may not be detrimental to the Pevensey Levels SSSI designations, as they require successional change in combination with high diversity (Natural England, 2013a).

The key native plant species for the SAC (Joint Nature Conservation Committee, 2016b) were positively associated with the reference sites without *H. ranunculoides* and those managed using the weed-cut and sprayed treatment, with *Berula erecta* and *Lemna trisulca* represented for three out of the four years. *H. ranunculoides* was negatively associated with these native plant species but positively associated with the weed-cut treatment in all four years. 2015 had a different configuration of management treatments along the ordination axes (Figure 31). This may be because the average survey month during 2015 was earlier than the other years, being mid-July, whereas 2013 and 2014 it was early August and 2016 late July.

4.5.2.2. Management treatments and their effectiveness at reducing abundance of *H. ranunculoides*.

The weed-cutting and spraying management treatment was shown to be more effective in reducing the abundance of *H. ranunculoides* compared to the un-cut, unsprayed reference sites. This treatment and the reference sites without *H. ranunculoides* were associated with the key native species *B. erecta* and *L. trisulca* noted in the SSSI citation for Pevensey Levels. As this was shown to be the management treatment least likely to impact on species richness, it is suggested that not only is it the most effective management treatment for *H. ranunculoides*, but it is also the least likely to impact on native macrophyte diversity. Weed-cutting alone allows *H. ranunculoides* to re-grow unchecked as the roots are not removed by this method. But targeted spraying of *H. ranunculoides* as it re-grows following weed-cutting, and avoiding spraying native species, permits native species to re-establish. Spraying was undertaken in this study two to four weeks after mechanical removal, in line with other practical studies (Sims, 2010). Weed-cutting only has historically been used on the Pevensey Levels, where the watercourses infested have never been completely controlled and the spread of *H. ranunculoides* continues (Natural England, 2013a). This study supports this historical evidence, suggesting that weed-cutting alone is not an effective control treatment and results in reduced native plant diversity.

4.5.2.3. Recovery of communities from management treatment

The comparison of the 2016 NVC communities with the 2013 baseline over the 60 sites in this study, showed that only 30% remained unchanged. NE’s Pevensey Levels SSSI citation (2019) states that maintenance and dredging (de-silting) is necessary for the successional progression that is required to maintain the diversity of native macrophytes and aquatic invertebrates that rely on these plant communities. However, the management treatment weed-cutting and de-silting reduced diversity in the short term. The weed-cutting only treatment used annually to control *H. ranunculoides* and keep the watercourses open in the organic farmed areas (not permitted to use herbicides), would not permit native macrophyte community recovery as there would be regular disturbance.

Three years are too short a length of time to evaluate recovery fully particularly as management was ongoing on many of the sites over the study period. However, a study by Hulme and Bremner (2010) on the impact of the INNS *Impatiens glandulifera* on riparian habitats showed that plant communities recovered well following removal and that plant species diversity increased. Kovalenko's (2010) study, found the 2,4-D amine herbicide treatment on *M. spicatum* had little impact on the native macrophytes because the spray was timed for early in the season before the natives had established for the season and recolonised rapidly post-control. However, Bakker et al. (2013) studying lake macrophyte restoration on a number of European projects, found that macrophyte return can take anything from a few weeks to several years but often was not as diverse as before. Therefore, careful control at the right time of year can permit native plant recovery but there remains the risk that other invasive or non-native species may be more able take advantage of the cleared environment more rapidly than desired native species.

4.6. Conclusion

The aim of these surveys on 60 sites over four years in the field was to quantify the impact of *H. ranunculoides* on native macrophyte communities. Stiers et al. (2011) showed that *H. ranunculoides* reduces native plant species diversity but Nijs et al., (2012) states that studies quantifying the community level effects are scarce. In this study the reduction in diversity index values indicates that *H. ranunculoides* infestation alters alter community composition. The abundance of *H. ranunculoides* was above 50% before any statistically significant impact was demonstrated. *H. ranunculoides* abundance in this study peaked around September. Therefore, early season native macrophyte species are less likely to be impacted but later season species are at risk. Higher abundance of *H. ranunculoides* was correlated to a reduction in certain native macrophytes which underpin the designations of the Pevensey Levels. Two later season species, *L. trisulca* and *H. morsus-ranae,* that support the Pevensey Levels SAC snail species *A. vorticulus* were affected so, if *H. ranunculoides* is left uncontrolled, it could threaten the site's status for this designation. The sites SSSI status is at risk as several native macrophyte species listed on the citation are negatively impacted by *H. ranunculoides.* Five species found to be negatively impacted by *H. ranunculoides* are high scoring for the WFD assessment criteria, *L.trisulca, H. morsus-ranae, B. erecta, P. lucens* and *S. erectum* so the WFD status could also be threatened.

The traditional management treatment for controlling *H. ranunculoides*, and the only one that is permitted in the organic farmed areas, weed-cutting, does not effectively control *H. ranunculoides* and reduces native macrophyte diversity. The Pevensey Levels SSSI criteria requires less than 5% cover of INNS so, the weed-cutting only management treatment would not reduce the current level of invasion and may even promote further spread of *H. ranunculoides* as it fragments the plant producing more vegetative propagules (Centre for Agriculture and Biosciences International, 2020b). The management treatment weed-cutting followed by spraying was most effective in reducing abundance of *H. ranunculoides and* did not impact on species diversity. No treatment used in this study eliminated *H. ranunculoides.*

Bakker et al.'s (2013) study showed that nutrient loading can reduce macrophyte diversity and Hussner and Lösch (2007) showed that increasing levels of nutrients promoted the growth of *H. ranunculoides.* So, alongside the field macrophyte surveys, water and sediment samples were taken from the same study sites to investigate the impact of nutrient availability on the growth capacity of *H. ranunculoides* in the field. A greenhouse growth experiment was conducted to ascertain which of the specific nutrients, ammoniacal nitrogen, nitrate and orthophosphate might limit the growth of *H. ranunculoides.* The results of these field samples and growth experiment are presented in chapter 5.

Chapter 5 - The Effects of nutrient availability on the growth of *H. ranunculoides.*

5.1. Introduction

Chapter 2 reviewed the literature on the nutrient preferences and growth capacity of *Hydrocotyle ranunculoides* as an INNS. Drawing on this body of research, chapter 3 then described the methods used in this study to investigate the nutrient levels in the field in its invaded region and a controlled experiment to investigate the effect of differing levels of nutrients on the relative growth rate. Chapter 4 examined the abundance of *H. ranunculoides* and its impact on native plant communities. This chapter will describe the results of the water and sediment field sampling and a greenhouse experiment to evaluate the impact of nutrient availability on the growth capacity of *H. ranunculoides.*

5.1.1. Macrophyte responses to nutrients in water and sediment

Nitrogen and phosphorus are two of the essential nutrients for plant growth and are the most likely to be capable of limiting macrophyte growth (Barko et al., 1991). Phosphorus is utilised by plants in the form of soluble reactive phosphate (orthophosphate), with two forms of nitrogen commonly used, ammonium and nitrate (Gosselin et al., 2018). These three nutrients were thought to be principally taken up by submerged macrophytes from the sediment (Barko et al., 1991), however more recent research has challenged this, reporting that macrophytes may obtain some nutrients from the substrate but that when nutrient concentrations are higher in the water this source can be utilised (Gosselin et al., 2018). Gosselin et al. (2018) also suggest that there is still uncertainty about which nutrients are taken up from substrate or water (or both) and mechanisms for uptake vary amongst macrophytes and may differ between species. The ability to respond to high nutrient levels is important for an invasive species as communities become invasible when depleted resources, especially nutrients, increase (Grime, 2006). pH is also important regarding phosphorus uptake as it facilitates the fixation of the nutrient to the root surface (Barrow, 2017). Ullrich-Eberius et al. (1981) and Garbey et al. (2004) found that phosphate uptake in Lemna was optimal at pH6 but decreased above this.

Gosselin et al. (2018) confirm that it is now accepted that aquatic macrophytes do obtain some nutrients from the substrate, but it is still not clear which nutrients are taken up from substrate and which from water, or both. Barko et al. (1991) noted that there is a large available pool of phosphorus in sediments (to submersed macrophytes) but that experiments to stimulate macrophyte growth by adding phosphate or reduce growth by limiting it had not been successful. Gosselin et al. (2018) studying submersed macrophytes rooted in sediment, showed that levels of nitrogen over 150mg per kg restricted growth. Nitrogen limiting was not accurately determined as plants in substrates with no added nitrogen still grew but Gosselin et al. (2018) could not exclude nitrogen present in the water column. Although Gosselin et al.'s (2018) research can be used to inform this study, *Hydrocotyle* is the only aquatic genus from the family Araliaceae and is emergent, so may have different nutrient requirements and uptake routes compared to the studied submersed species from other plant families. Basílico et al. (2017), investigating *H. ranunculoides* for phytoremediation, found remarkably high removal of nitrogen as ammoniacal nitrogen from water (nitrate was at too low a level to investigate). Barko et al. (1991) explains that nitrogen is used in two different forms by plants and states that rooted submerged macrophytes preferred ammonium to nitrate as the source of nitrogen and that the sediment seems to be the main source. Based on Barko et al. (1991), Basilico et al. (2017) and Gosselin et al.s' (2018) findings, it was decided that ammoniacal nitrogen and nitrate be investigated separately in addition to a combined high nitrogen level.

5.1.2. Nutrient requirements of *Hydrocotyle ranunculoides*

H. ranunculoides prefers eutrophic conditions (Fried et al., 2009). Fried et al. (2009) describe the nutrient ranges found where *H. ranunculoides* is present in the field in Belgium as orthophosphate: 0.005-0.21 mg/l and dissolved inorganic nitrogen (DIN): 0.018-4.14 mg/l of water. Hussner & Lösch (2007), again under field conditions, found *H. ranunculoides* with 6.2 - 11.5 mg of nitrate and 2.9 - 61.9 mg of total phosphate per 100 mg/kg of sediment. Hussner & Lösch (2007) under controlled laboratory conditions showed that increasing nutrient availability promoted increasing relative growth rates of *H. ranunculoides.* Fried et al. (2009) concluded that there is likely to be no upper nutrient limit to the growth of *H. ranunculoides*. Hussner & Lösch (2007) did not establish whether there is a lower nutrient limit that would reduce growth rate or which nutrient might be the limiting factor, or whether they are co-limiting. However, the Centre for Agriculture and Biosciences International (2020b) suggest the rapid growth rate of *H. ranunculoides* is linked to nitrate availability, allocating it to shoots and leaves under high nutrient levels. Moss et al. (2013) note that phosphorus may become scarcer in summer after rapid spring growth by macrophytes but that nitrogen would be more available in summer due to nitrogen fixation which coincides with the most rapid growth phase of *H. ranunculoides* in June and July (Hussner and Lösch, 2007). Basilico et al. (2017) investigating the use of *H. ranunculoides* for phytoremediation, found that this plant could remove high levels of nitrogen in the form of ammonium thus raising a further question about the form of nitrogen enabling the rapid growth of this species.

5.1.3. Mechanisms of growth limitation

Fernández-Aláez et al. (1999) established that both phosphorus and nitrogen are aquatic plant growth limiting chemical elements and Garbey et al. (2004) confirmed that there is robust evidence that these nutrients are important for the management of macrophytes. Moss et al. (2013) suggest that there may be a tendency to co-limitation so that there is a good reason to control both nutrients. But for communities dominated by rooted submerged macrophytes, which can obtain phosphorus from the substrate, there is an association between reduced diversity and nitrogen loading (Moss et al., 2013). In order to understand how the mechanism of limitation works, Garbey et al. (2004) note that a plant has to have both the ability to extract and retain the nutrients and then have the ability to use it for rapid growth. They suggest that morphological plasticity could be a strategy that affects the ability to acquire nutrients, so the phenotypic plasticity exhibited by *H. ranunculoides* (Robert et al., 2013) may well be linked to the species ability to obtain nutrients better than native macrophytes. Therefore, it is important to understand the key nutrients, and their sources, *H. ranunculoides* is using and the levels linked to its growth and biomass accumulation. In addition, recent research has linked high pH to reduced growth in low nutrient systems (Bellinger and Davis, 2017) so being tolerant of higher pH levels could be an important factor in *H. ranunculoides* success.

5.1.4. Aims and objectives

The aim of this study was to combine water and sediment sampling across 60 sites over four years in the field with a greenhouse experiment to investigate the impact of nutrient availability on the abundance and invasiveness of *H. ranunculoides*. The objectives were to:

- Relate the abundance of *H. ranunculoides* to levels of nitrogen and phosphorus in sediment and water in the field.
- Examine the influence of the nutrients, phosphate, nitrate and ammoniacal nitrogen in sediment on the growth of *H. ranunculoides* under controlled conditions.

5.2. Methods

5.2.1. Site selection for field sampling

The research combines studies in the field with a controlled greenhouse experiment, which is a novel approach but practically effective because the, often variable, findings in the field can be supported by evidence from replicable experiments which source material from the same field sites. It had been thought that the phosphate loading from discharges may drive the growth of this species on the Levels but stringent phosphate stripping was introduced from 2003 which reduced the level to below the WFD requirements of 0.069 mg/l (UK Technical Advisory Group on the Water Framework Directive, 2013) but the level of *H. ranunculoides* infestation has continued to increase. This may be due to nutrient input from other sources. The majority of the Pevensey Levels is used for raising livestock, cattle, and sheep (Atkins, 2015). The watercourses, especially the farm ditches, are used for ‘wet fencing’ and drinking water (Atkins, 2015), so manure is likely to be entering the system on a regular basis adding nutrients. Additionally, some of the peripheral areas are under arable crops (Atkins, 2015) and would have fertiliser inputs with the potential for run-off into the watercourses. Even the organically farmed areas may have some form of fertiliser input, as there are Soil Association approved products available (Soil Association, 2021).

5.2.2. Field methods

Field measurements of nutrients in sediment and water were taken as sediment samples pre, 2013, and post de-silting, 2016, and water samples annually, for each of the four years 2013 – 2016. Sixty sites overall were surveyed and sampled during the study but twelve of these were reference sites that did not contain the invasive *H. ranunculoides,* so these nutrient results focus on the 48 infested sites. These were analysed at an Environment Agency UKAS accredited laboratory: National Laboratory Services (NLS), Starcross, Exeter. For both sediment and water analyses, the samples were required to be received within twenty-four hours so were sent on the day of collection by overnight courier.

Macrophyte surveys were undertaken in accordance with the EA’s macrophyte OI 131_07, over a 100m survey length (reach) detailed in chapter 3.2.1.2. The timing of these surveys was randomised within years. The macrophyte abundance data were recorded as percentage cover.

5.2.2.1. Sediment sampling methodology

Sediment samples were collected at the start of each of the 60 100m macrophyte survey reaches illustrated in chapter 3, Figure 10), in 2013 and 2016, in summer. A direct manual sample collection technique was employed using a 1.4m long, 6cm diameter, clear, heavy duty plastic tube fitted with an open-close suction valve at the top. The samples were taken to the depth of the sediment at the margin of each ditch because *H. ranunculoides* roots marginally. An appropriate portion of the sediment, avoiding anoxic surface layers or lower clay layers, was immediately used to fill a clean, new, 1 litre (1L) EA sediment sampling container. All sample containers were labelled with prescheduled labels stating site name and number, sample type and date. At the end of each sampling day, these containers were refrigerated for courier collection.

5.2.2.2. Sediment analyses

Determinands ammoniacal nitrogen, total oxidised nitrogen (TON) and nitrite (KCI extract) in sediment were analysed using potassium chloride extraction with discrete colorimetric analysis using a Konelab 30 Discrete Analyser. The NLS LE I Nutrients Method Summary v012 can be found in Appendix 2. Determinands orthophosphate and total phosphate were analysed using sodium hydrogen carbonate extraction with discrete colourimetric analysis, also using a Konelab 30 Discrete analyser. The NLS LE I Phosphate Method Summary v010 can be found in Appendix 3.

5.2.2.3. Water sampling methodology

Water samples were collected, once annually for each of the 60 reaches, over a four-year period from 2013 to 2016, at the same time as the macrophyte surveys. A clean, new, 1L EA water sample bottle was filled directly from the watercourse at the start of each of the 60 macrophyte survey sites. The sample was taken at a depth of 30cm to be consistent with the YSI (Xylem Inc., 2019) sampling instrument, monitoring depth. Where possible all samples were taken in the morning to minimise diurnal influence. All sample bottles were labelled with prescheduled laboratory labels stating site name and number, sample type and date. These were kept in a cool box during transit prior to refrigeration for courier collection.

5.2.2.4. Water analyses

Determinands Alkalinity as mg/l CaCO3, ammonia as ammoniacal nitrogen, soluble chloride, reactive phosphorus as orthophosphate, nitrite, total oxidised nitrogen (TON) and nitrate (obtained by calculation) in water were analysed using a Konelab 30 Discrete Analyser. pH was also determined for each sample by an automated electrode probe system. The NLS SX I Nutrients Sum - New method summary v17 can be found in Appendix 4. Biological oxygen demand was obtained using the five-day method and a robotic analyser. The NLS SX I BOD Sum - New method summary v11 can be found in Appendix 5. In addition, environmental data were collected as observational variables and measured using a handheld water quality measuring YSI Pro Plus Multiparameter Instrument (Xylem Inc., 2019).

5.2.3. Greenhouse methods

5.2.3.1. Introduction

An important practical finding of Gosselin et al. (2018) was that sand could be successfully used (without the addition of micronutrients) in combination with water soluble fertilisers containing individual macronutrients to study macrophyte substrate nutrient requirements. This study on substrate nutrient uptake by a macrophyte will eliminate the influence of nutrients supplied from the water element because, although *H. ranunculoides* grows best in fully aquatic conditions, it can tolerate and phenologically adapt to low water conditions (Hussner and Meyer, 2009).

The control substrate was a pure, washed horticultural sand, mixed, and sieved through a 4mm sieve as three 20kg bags were required. Prior to commencing the experiment, three dry sand samples from the control substrate and from each treatment were homogenised, air-dried for 48 hours, and analysed over the next 24 hours at the EA's accredited Starcross laboratory (Environment Agency, 2016b) to check the levels of nutrients added were accurate for the control, and each of the five treatments, prior to the experiment commencing. The following nutrients were analysed: ammoniacal nitrogen (NH_4-N), inorganic nitrate (NO_3-N), orthophosphate (PO_4-P which is expressed as P_2O_5 for soil analyses) and P_2O_5-P_{tot} (total phosphate). The extraction methods were the same as used for the analysis of the field sediment samples by the EA's accredited Starcross laboratory (Appendix 3 and 4.).

5.2.3.2. Experimental design

The experiment was conducted in a Hartley Botanic horticultural greenhouse. The aim was to maintain temperature and light within *H. ranunculoides* optimal growth parameters according to Hussner & Lösch (2007); above 25^{o}C and with a minimum photon flux density of ~800 µmol photons m^{-}2 s^{-}1 during growing hours.

Heating levels were maintained using a Bio Green Phoenix 2.8Kw output electric greenhouse heater designed for heating from 0^{o}C to 26^{o}C in a 2.4 x 3.0m greenhouse. The experiments were run from January to March in the UK as, during a trial undertaken during the peak growing season of June to July in 2018, the temperatures became too high (over 40^{o}C for nearly a week) and 11 of 36 of the plants had died. Maximum and minimum temperatures were recorded using two ThermoPro TP50 Digital Indoor thermometers. Due to10 days of sub-zero external temperatures, the actual average temperatures maintained were 12.9^{o}C at night and 22.9^{o}C, in January, during the day. Climate data were not collected as these plants were grown under controlled laboratory conditions.

Natural sunlight and day length were augmented, when necessary, by white light generated by a 160w Sylvania growing light bulb, with a light intensity of 43.3 µmol m-2 s-1 (micromol per square meter per second of light in the photosynthetically active radiation range) to provide 12 hours from 7:00 to 19:00. The average light levels were measured using a Precision Gold NO9AQ Environmental Meter. The range was 1868 to 1146 µmol photons m^{-}2 s^{-}1 during the day and 1073 m^{-}2 s^{-}1 to 351 m^{-}2 s^{-}1 µmol photons when augmented in the early morning and late afternoon. The light levels at midday on a bright summer day, at sea level, would be 2000 m^{-}2 s^{-}1 (Hull, 2019). The experimental light levels were lower than the growing season but still above Hussner & Lösch (2007) optimum of above 800 m^{-}2 s^{-}1 µmol photons for the majority of the day.

Set-up took place on 11th January 2019 and plants were monitored every10 days until 21st March 2019. Pots containing the plants were placed in controlled greenhouse conditions in a randomized block design to avoid position effects (Figure 33). The random numbers were generated using the Randomise List Function in Excel™ (Table 22) using number 6 for the sand control. Each pot was numbered within the block and each block randomised separately.

A.

B.

C.

D.

Figure 33: Greenhouse design for the substrate growth experiment. A. Six of the initial cuttings for batch 1. nutrient treatment 5 (all-nutrients). B. Batch 1 at the start (11/01/2019), nutrient treatment 5. C. Batch 1 at the end of the experiment (21/03/2019). D. Randomised block design in the greenhouse (Photographs Birch, J.).

Table 22: Randomised numbers for the greenhouse nutrient experiment block design.

3	6	5	5	2	3
4	5	6	1	1	1
5	4	4	6	4	6
2	1	3	4	3	2
6	3	1	2	5	4
1	2	2	3	6	5

The pots were fitted with drip trays that were checked daily and kept filled with water to 1cm to ensure the horticultural grade sand was constantly wet to replicate natural waterlogged conditions. The water used was tap water with a mean pH of 7.5 which compares favourably with the mean field pH for 2016 of 7.48 both for the hand-held YSI sampling in field and the EA laboratory water analyses.

5.2.3.3. Nutrient treatments

The nutrients used were ammoniacal nitrogen (NH_4-N), inorganic nitrate (NO_3-N), Orthophosphate (PO4-P) and total phosphate (P_2O_5-P_{tot}). Although plants also use Potassium for growth, they obtain this primarily from water and it is relatively poorly mobilised from sediment (Barko et al., 1991) so was not included as a nutrient. This may have reduced potential growth, but excluding it removed the possibility that it could influence uptake of other nutrients, particularly Ammonium, as Potassium and Ammonium compete for exchange sites on roots (Barko et al., 1991).

Single nodes of *H. ranunculoides*, with adventitious roots, (fresh weight of 0.30g ± 0.02g, dry weight of 0.02g ± 0.005g) were potted in 36 no. 1 litre (L) pots (Figure 33b). 1L pots were used because it is equivalent in volume to I kilogram which is the standard EA sediment analysis volume. The pots were filled with a sand substrate with five different nutrient treatments and a control (Table 23) and there were 6 replicates per substrate.

Hussner & Lösch (2007) experiments on *H. ranunculoides* growth were used to inform the levels of nutrients used. However, the levels of nutrients in the field experiment sediment samples, where *H. ranunculoides* was present, and evidence from other studies discussed, were used to refine the nutrient levels used in the experiments. The level of ammoniacal nitrogen was increased, as the field levels were much higher than Hussner &

Lösch (2007) experimental levels. The field orthophosphate levels were lower than Hussner & Lösch (2007), so these were reduced.

Table 23: Mean substrate nutrient contents for the greenhouse experiment, for each treatment, n=6. C, control; treatments; 1, ammoniacal nitrogen, 2, nitrate, 3, ammoniacal nitrogen and nitrate, 4, orthophosphate, 5, ammoniacal nitrogen, nitrate, and orthophosphate.

Substrate treatments	NH_4-N (mg/kg sand)	NO_3-N (mg/kg sand)	P_2O_5 (mg/kg sand)	P_2O_5-P_{tot} (mg/kg sand)
	Amm. Nitrogen	Nitrate	Ortho-P	Phosphate
C – Sand	2.0	2.8	1.0	396.0
1 – Amm N	60.2	2.8	1.0	380.0
2 - Nitrate	2.0	31.0	1.0	389.7
3 - High N	57.0	78.0	1.0	309.0
4 - Ortho-P	2.9	7.1	210.0	480.7
5 – All high	82.0	72.0	140.0	442.0

Horticultural grade nutrients were added to the sand to produce the experimental substrates; nitrate as Garden Direct Nitrate of Soda, Na NO3, 16-0-0 (N-P-K), ammoniacal nitrogen as Proctors Sulphate of Ammonia, $(NH_4)2$ SO4, 20-0-0 and phosphorus as Proctors Single Superphosphate, P_2O_5, 0-17-0 to make up the five treatments. The nutrients were each weighed out for the whole experiment then ground as small as possible using a pestle and mortar. From this main source these were then divided for each treatment, with six replicates and three samples for analysis. The six replicates were added to 1L pots using a round of laboratory filter paper to prevent the sand from washing out. The three samples were added to 1L pots and sent for analysis at the EA Starcross laboratory.

Determinands ammoniacal nitrogen, and nitrate (KCI Extract) in sediment were analysed using potassium chloride extraction with discrete colorimetric analysis using a Konelab 30 Discrete Analyser according to the EA LE I Nutrients Method Summary v012 (Appendix 2). Determinands orthophosphate and total phosphate were analysed using sodium hydrogen carbonate extraction with discrete colourimetric analysis, also using a Konelab 30 Discrete analyser according to the EA SX I Nutrients Sum - New method summary v17 (Appendix 3). The results for the nutrient treatment samples prior to the experiment are presented in

Table 23. The control substrate (C) had very low levels of all three nutrients. The ammonium treatment (1) had high levels of ammonium only. The nitrate treatment (2) was high in nitrate only. The high nitrogen treatment (3) had high levels of both nitrogen components, although nitrate levels were higher than the nitrate only treatment (2). The orthophosphate only treatment (4) had high levels of orthophosphate but low levels of the nitrogen components. The overall high nutrient treatment had high levels of all-nutrients although the ammonium was higher than both the ammonium only and the combined high nitrogen treatment, and the orthophosphate was lower than the orthophosphate only treatment. Overall, the treatments achieved the desired experimental differences in nutrient levels.

5.2.3.4. Plant monitoring

Measurements were taken every 10 days for each *H. ranunculoides* plant using a laser digital caliper with a resolution of 0.1mm and accuracy of ±0.2mm to measure the variables; leaf diameter, petiole length and shoot length. Leaf number and number of shoots were also counted. The biomass of each pot was harvested after a growth period of 70 days, allowing 14 days for establishment and 56 days for growth. Typically, nutrients in compost are sufficient for up to eight weeks (Horticultural Trades Association, 2019) but the experiment was terminated when the leaves began to senesce. Biomass was determined separately for leaves, petioles, shoots, and roots. The 36 samples with roots, shoots, leaves and petioles separated were air dried to a constant DW in an OP250 LTE Thermoscientific muffle furnace for 48 hours at 105^{o}C. The initial DW was determined by using six sample cuttings of similar size and weight, from the same host plants, as those used in the experiment (Figure 33a). The dried samples were then weighed using an enclosed, laboratory calibrated, balance technology scale. The DWs were used for all subsequent calculations.

5.2.4. Statistical methods

5.2.4.1. Introduction

The statistical analyses were influenced by the large size of the environmental dataset, but with small counts of each species, over a period of four years. Multivariate statistical analyses were performed to highlight interactions between environmental variables and the target species (*H. ranunculoides*), which were then tested using classical statistics.

Classical analyses were performed using Minitab 19. Data were tested for normality in Minitab and transformed in Excel™ using the formula =ASIN(SQRT(cell/100)) if non-normal.

5.2.4.2. Field data analysis

The Principal Component Analyses (PCA) and RDA functions in Canoco 5 were used to highlight any interactions between nutrient levels, environmental variables, and the abundance of *H. ranunculoides*.

Using the results from the water and sediment sample analyses, the relationships between *H. ranunculoides* and nitrogen and phosphorus were examined. There were two years of sediment data and four years of water data for the 60 sites. Large ecological and environmental datasets are usually not normally distributed but Canoco 5 was used to log transform data to approximate a normal distribution (Šmilauer and Lepš, 2014). PCA and RDA functions in Canoco 5 were chosen to investigate interactions between nutrient levels, pH, and the abundance of *H. ranunculoides*.

PCA's were completed using all macrophytes and environmental variables to identify if *H. ranunculoides* emerged as a dominant species with a relationship to any of the variables. In sediment samples, only nutrients were measured but in water samples chlorine, conductivity and pH were measured in addition to nutrients. This was important to evaluate the level of influence of nutrients on the abundance of *H. ranunculoides* in comparison with other environmental influences and species. The RDA function in the Canoco 5 programme was used as a linear regression option by selecting a single response variable (*H. ranunculoides*) and a single predictor (Šmilauer and Lepš, 2014). Linear regressions were completed with *H. ranunculoides* as a single response variable and each of the environmental variables that had been identified as single predictor variables. This was done for both sediment and water parameters.

To investigate the relationships identified by the multivariate analyses, classical statistics were used. Correlation was measured using the Spearman Rank correlation, a nonparametric measure of statistical dependence between the ranking of two variables. Spearman Rank was chosen rather than Pearson product-moment correlation because the latter benchmarks linear relationships whereas Spearman benchmarks monotonic relationships and where data may not fit a normal distribution (Minitab, 2019). Spearman Rank Correlations using Minitab were completed for each year, with the predictors being ammoniacal nitrogen, nitrate, nitrite, TON, orthophosphate and total phosphate in sediment and the response being abundance of *H. ranunculoides*. For water, the predictors were ammoniacal nitrogen, TON, orthophosphate, and total phosphate. pH was also investigated as interactions between ammoniacal nitrogen, nitrogen and orthophosphate and pH levels have been demonstrated, and the use of ammoniacal nitrogen fertilisers can increase absorption of orthophosphate (Riley, 1971).

5.2.4.3. Greenhouse data analysis

Following the analyses of the field results, the impact of nutrients on the growth of *H. ranunculoides* were investigated using controlled greenhouse experiments. The impact of the nutrients nitrogen, ammoniacal nitrogen and phosphorus on the dry weight (DW), relative growth rate (RGR) and root to shoot ratio (R:S) of *H. ranunculoides* used classical statistics, ANOVA and Tukey tests, if significant. Final biomass data for each treatment were tested for normality and arcsine transformed to provide normal data. A one-way ANOVA was used with *H. ranuculoides* DW being the dependent variable and the nutrients being the independent variables. Any significant differences were analysed using a post-hoc Tukey grouping with a 95% confidence level.

Relative Growth Rate (RGR) was calculated as in Hussner & Lösch (2007). RGR was also examined using leaf number over the seventy-day growth period as leaf number was counted weekly. Data were tested for normality, arcsine transformed to provide normal data, and any significant differences analysed using a post-hoc Tukey grouping with a 95% confidence level.

$$\mathrm{s} = (\ln \mathrm{S2} - \ln \mathrm{S1})/(\mathrm{t2} - \mathrm{t1})$$

Equation 3: Relative Growth Rate, where ln = natural log, S1 and S2 are plant DWs at times t1 (start of experiment) and t2 (end of experiment) for each nutrient treatment.

Root to shoot ratio (R:S) was calculated by combining the DWs of the leaves, shoots and petioles and dividing the DW of root biomass by the DW of the combined shoot biomass, leaves, petioles, and shoots, to provide the ratio R=x:S=1. The data were tested for normality, arcsine transformed to produce normal data and a one-way ANOVA completed with a post-hoc Tukey grouping, with a 95% confidence level, to investigate any significant differences.

5.3. Results of field studies

5.3.1. Nitrogen and phosphorus in water

Table 24 shows the range of concentrations of nutrients measured in water over the four years of the study, 2013 to 2016 in the 48 infested sites. The mean ammoniacal nitrogen level is below that of the WFD target status for Good at between 0.3 – 0.6 mg/l. There is no WFD target for TON in water. The mean Phosphate level is well above the WFD target of 0.069 mg/l for Good status. These results indicate that the Pevensey Levels study sites overall are generally in an eutrophic condition.

Table 24: Maximum and minimum range, and means, of nutrients in mg/l measured in the water samples taken from the 48 infested field survey sites over four years from 2013 to 2016, n=192.

Nutrients mg/l	Maximum	Minimum	Mean
Ammoniacal Nitrogen	1.68	0.03	0.07
TON	26.80	0.20	3.36
Orthophosphate	1.91	0.01	0.13

A PCA of all macrophyte species abundance and nutrient data for the 48 infested sites, for all four years, did not place *H. ranunculoides* amongst the 20 species with the best fit to the ordination axes shown. However, if the number of species included is increased to 30 the *H. ranunculoides* vectors for 2014 and 2016 appear and are positively aligned with the TON (nitrate and nitrite combined) vectors which are closely clustered (Figure 34). The ammonium and orthophosphate vectors are weaker, more dispersed, and not all positively associated with the *H. ranunculoides* vectors. The native plant species are principally clustered away from the nutrient vectors indicating a negative association. The strong native species, *Phragmites australis* (*PhrAus*), vectors are represented for all four years, closely clustered and distinct from both the *H. ranunculoides* and other native plant species. The first two axes explain 25.62% of the variation with a total of 41.84% for all four axes (Table 25). RDAs were then performed with *H. ranunculoides* infested sites (n=48) and then infested sites that had not been de-silted (n=30) to investigate the three measured environmental variables in water. The reason for removing the de-silted sites and comparing them with the overall infested sites is because de-silting removes rooted macrophytes. Rooted macrophytes can affect phosphate release from the sediment by increasing it, thus removing them may affect the results (Stephen et al., 1997b).

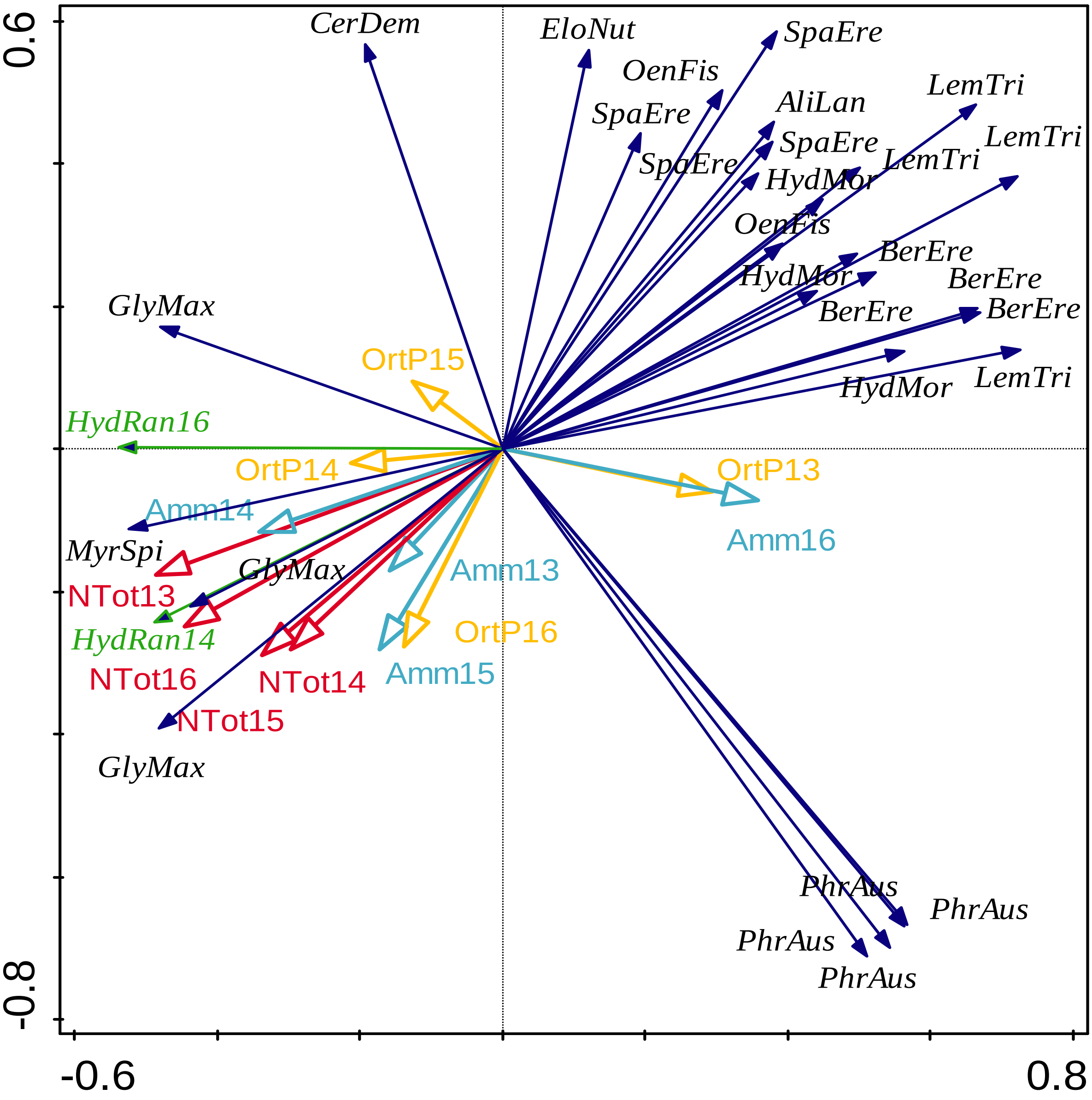


Figure 34: PCA of plant abundance and environmental variables diagram of all plant species and nutrients in water, mg/l, for *Hydrocotyle ranuculoides* infested sites, n=48. The first two axes are plotted. Plant names are shortened versions of the plant latin binomials, *H. ranunculoides* with the year suffixed (Table 11). Amm = ammonium, mg/l, NTot = total nitrogen, mg/l, and OrtP = orthophosphate mg/l, with years suffixed.

Table 25: Summary of PCA results for *H. ranunculoides* infested sites and Ammoniacal nitrogen and total nitrogen in water (n=48) for all four years, with abundance of *H. ranunculoides*.

Statistic	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	0.1374	0.1188	0.1009	0.0614
Explained variation (cumulative)	13.74	25.62	35.70	41.84

5.3.1.1. Nitrogen

Table 26 illustrates the range of TON in the water of the 48 infested sites over the four years of surveying. TON decreased after the first year and the number of sites with extremely low levels (0.02 mg/l, or below) increased.

Table 26: Range of TON in water for *H. ranunculoides* infested sites, n=48, in mg/l, in all four years of surveying.

TON in water mg/l	2013	2014	2015	2016
Maximum	26.8	20.6	19.5	21.6
Minimum	0.20	0.20	0.20	0.20
Mean	6.61	2.42	1.69	2.73

The target species *H. ranunculoides* and TON were extracted for an RDA. For TON in water the RDA was significant at P = 0.032 but the explained variation was a weak fit at only 14.36% for the first two axes (Table 27). However, the *H. ranunculoides* and TON parameters biplot (Figure 35) does show the close alignment of TON and *H. ranunculoides* vectors along the horizontal axis indicating a positive relationship between abundance of *H. ranunculoides* and nitrogen in water.

Table 27: Summary of RDA results for *H. ranunculoides* infested sites, n=48, and TON, for all four years, with abundance of *H. ranunculoides* and TON included in the analysis.

Statistic	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	0.1113	0.0323	0.0072	0.0018
Explained variation (cumulative)	11.13	14.36	15.08	15.26

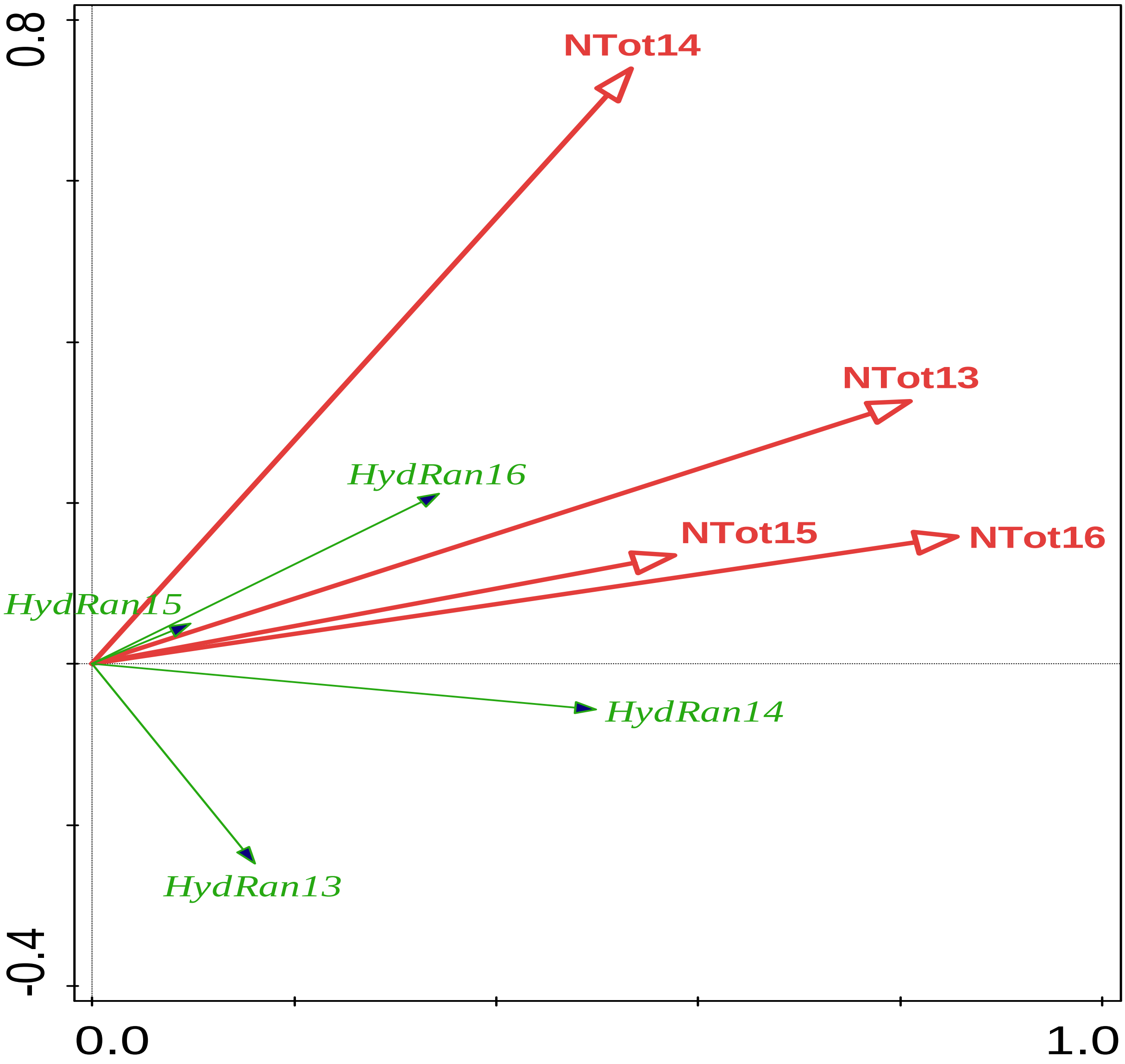


Figure 35: RDA biplot of abundance of *H. ranunculoides* and TON in water, mg/l, for all four years using *H. ranunculoides* infested sites, n=48. Plant names are shortened versions of the plant latin binomials with the year suffixed (Table 11). The first two axes are plotted.

An RDA analysis between abundance of *H. ranunculoides* and ammoniacal nitrogen did not show significant results.

5.3.1.2. Phosphorus

Table 28 illustrates the levels of orthophosphate in the water over the four years of surveying in the 48 infested sites. The levels of orthophosphate are higher overall in 2016 than at the start of the survey period in 2013 and, for the period studied, half the sites (24) exceeded the WFD threshold for ‘Good status of 0.069 mg/l.

Table 28: Range of orthophosphate reactive as P in water, mg/l, for *Hydrocotyle ranunculoides* infested sites, n=48, for all four years of surveying. WFD target for Good status is 0.069 mg/l.

Ortho-P in water mg/l	2013	2014	2015	2016
Maximum	0.60	1.73	0.63	1.91
Minimum	0.02	0.02	0.01	0.01
Mean	0.10	0.16	0.12	0.17
No. sites below WFD	30	23	17	24

An RDA using all 48 infested sites did not show any significant associations between *H. ranunculoides* and orthophosphate. As de-silting can alter the phosphate loading (Stephen et al., 1997a) the eighteen de-silted sites were removed from the analysis and the RDA then showed a significant result, P=0.022, and the explained variation is a good fit at 26.15% with only a further 1.02% explained by the second axis (Table 29).

Table 29: Summary table of RDA results for *H. ranunculoides* infested sites that were not de-silted, n=30, and orthophosphate mg/l in water or all four years, 2013, 2014, 2015 and 2016.

Statistic	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	0.2615	0.0102	0.0031	0.0002
Explained variation (cumulative)	26.15	27.17	27.49	27.51

The *H. ranunculoides* and orthophosphate parameters biplot (Figure 36) shows the orthophosphate and *H. ranunculoides* vectors placed at more than 90^o angles, indicating strong negative relationships in all years.

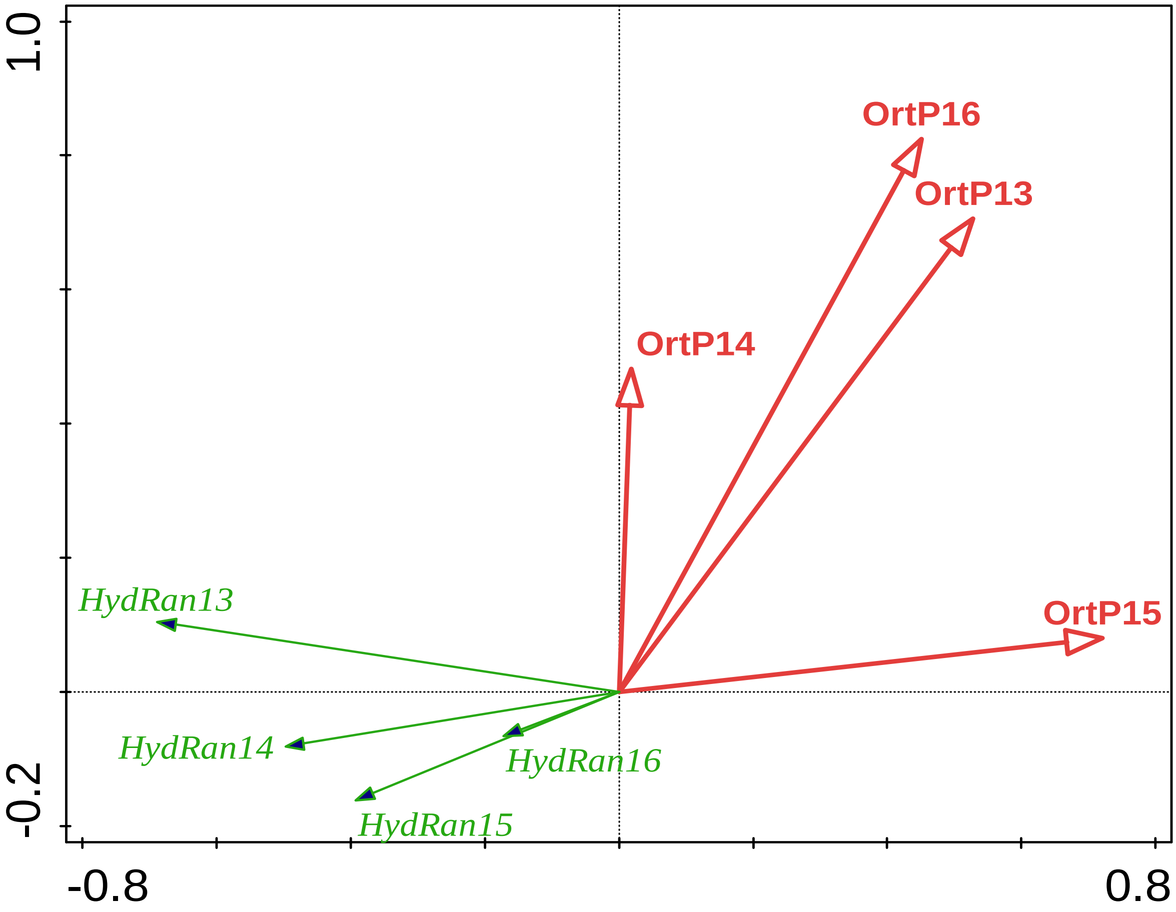


Figure 36: RDA biplot of abundance of *H. ranunculoides* and orthophosphate in water, mg/l, using *H. ranunculoides* infested sites that were not de-silted, n=30, for all four years 2013, 2014, 2015 and 2016. Plant names are shortened versions of the plant latin binomials with the year suffixed (Table 11). Orthophosphate is shortened to OrtP with the year suffixed. The first two axes are plotted.

5.3.1.3. Correlations between *H. ranunculoides* and nutrients

Correlations were used to identify specific years in which relationships between abundance of *H. ranunculoides* and the nutrients, ammoniacal nitrogen, TON, and orthophosphate were significant. None of the correlations were strong but a significant positive correlation was found between abundance of *H. ranunculoides* and ammoniacal nitrogen in water for two of the four years, 2014 and 2016 (Table 30). In 2014 there was also a significant positive correlation with TON in water. In 2015 there was a significant negative correlation between abundance of *H. ranunculoides* and orthophosphate.

Table 30: Spearman Rank Correlation (Rho) between abundance of *Hydrocotyle ranunculoides* and values for nutrients in water of the infested sites, n=48, over four years. Significance is indicated by the P value (P) with significant values in bold.

Nutrient	2013 Rho/P	2014 Rho/P	2015 Rho/P	2016 Rho/P
Amm. Nitrogen	0.245/0.059	0.272/**0.035**	0.196/0.134	0.296/**0.039**
TON	0.121/0.357	0.295/**0.002**	0.211/0.106	0.276/0.055
Orthophosphate	-0.187/0.153	-0.105/0.428	-0.319/**0.014**	0.013/0.931

5.3.2. Nitrogen and phosphorus in sediment

Table 31 illustrates the minimum and maximum range of nutrients in sediment in all 48 *H. ranunculoides* field sites. Sediment samples were taken in 2013 before any de-silting took place and in 2016 after de-silting. The maximum ammoniacal nitrogen is extremely high in comparison with Hussner and Lösch’s (2007) field study on *H. ranunculoides* growth, which recorded zero mg/kg. Hussner and Lösch’s (2007) nitrate measurements of 6.2 – 11.5 mg/kg are more comparable to this study’s 4.80 – 20.0 mg/kg. This study’s field sites orthophosphate range of 6.45 – 87.00 mg/kg is much lower than Hussner and Lösch (2007) measurements of 29.0 – 619.0 mg/kg.

Table 31: Minimum and maximum range of nutrients in mg/kg, in sediment, in the *Hydrocotyle ranunculoides* infested field sites, n=96, in 2013 and 2016.

Nutrients mg/kg	Minimum	Maximum
Ammoniacal Nitrogen	3.00	443.00
TON	5.00	20.00
Nitrate	4.80	20.00
Nitrite	0.20	1.35
Orthophosphate	6.45	187.00
Total Phosphate	257.00	4480.00

5.3.2.1. Nitrogen

Table 32 illustrates the range of nitrate in the sediment at the start and end of surveying for *H. ranunculoides* infested sites. This did not vary much despite eighteen sites being de-silted during the study period.

Table 32: Range of Nitrate in sediment for *H. ranunculoides* infested sites, n=48, in mg/kg, at the start of surveying in 2013 and at the end in 2016.

Nitrate in sediment mg/kg	2013	2016
Maximum	20.00	19.50
Minimum	5.00	4.80
Mean	7.85	7.93

5.3.2.2. Phosphorus

Table 33 illustrates the range of orthophosphate in the sediment pre-management treatment in year one of surveying, 2013, and the final year, 2016 after eighteen sites had been de-silted. Levels of orthophosphate rose by 30% over the period.

Table 33: Range of orthophosphate in sediment for *H. ranunculoides* infested sites, n=48, in mg/kg, at the start of surveying in 2013 and at the end in 2016.

OrthoP in sediment mg/kg	2013	2016
Maximum	132.00	187.00
Minimum	9.03	6.45
Mean	38.05	49.81

A PCA of nutrients and all macrophyte species in sediment for all 48 infested sites showed no associations between any nutrients and *H. ranunculoides* for either year. If the de-silted sites are removed from the analysis, the *H. ranunculoides* vectors now appear (Figure 37). The nutrient vectors point in the direction of the steepest increase of each variable's value, and they are all short indicating they contribute little to the explanation of the macrophyte distribution. The orthophosphate vectors are the steepest and the 2016 vector aligns strongly with the 2016 *H. ranunculoides* vector indicating a positive relationship. The 2013 orthophosphate vector is at more than 90^{o} angle with the 2013 *H. ranunculoides* vector indicating a negative relationship.

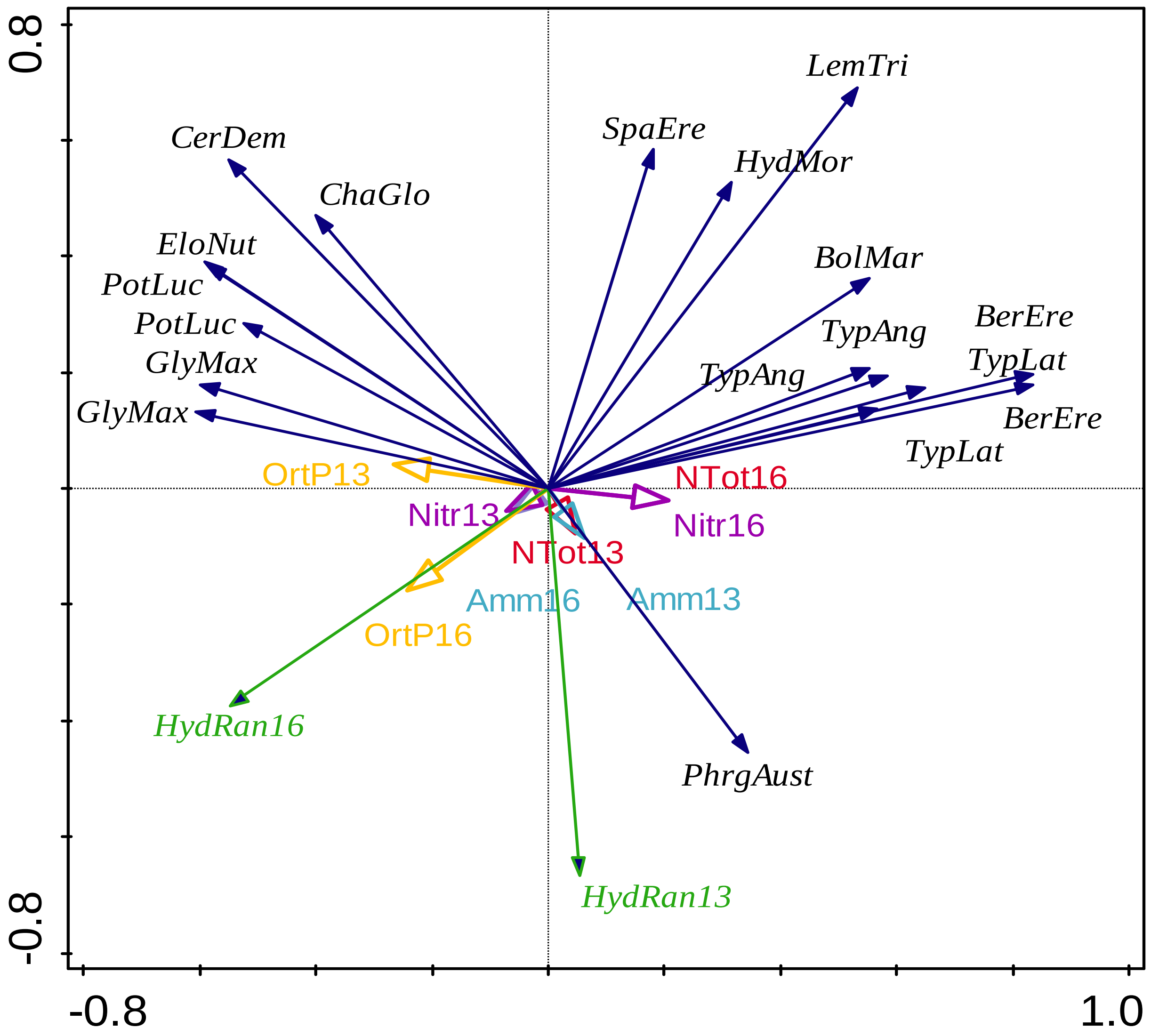


Figure 37: PCA plant species abundance and environmental variables diagram of all plant species and nutrients in sediment for 2013 and 2016, with de-silted sites excluded, n=30. Plant names are shortened versions of the plant latin binomials with the year suffixed for the *H. ranunculoides* and nutrient vectors. Orthophosphate is shortened to OrtP, ammoniacal nitrogen to Amm, nitrate to Nitr and total nitrogen to NTot, mg/kg, with the years suffixed. Axes 1 and 2 are plotted.

Table 34 summarises the explained variation with axis 1 explaining 16.69% and axis 2 contributing a further 14.30%. In figure 37 the native plant species (except for *Phramites australis*) are all clustered above the horizontal axis away from the nutrient vectors which are predominantly below this axis. The H. ranunculoides vectors are both below the horizontal axis, along with most of the nutrient vectors. An RDA with *H. ranunculoides* and nutrients in sediment was not significant, P=0.44.

Table 34: Summary of PCA results for *H. ranunculoides* infested sites that were not de-silted (n=30), in sediment for 2013 and 2016.

Statistic	**Axis 1**	**Axis 2**	**Axis 3**	**Axis 4**
Eigenvalues	0.1669	0.1430	0.1250	0.0794
Explained variation (cumulative)	16.69	30.99	43.49	51.43

There were no significant correlations between the abundance of H. ranunculoides and nutrients in sediment (Table 35). The 2013 positive correlations between nitrate / nitrite and *Hydrocotyle ranunculoides* were close to the significance level of 0.05, P=0.058.

Table 35: Spearman Rank Correlation between *Hydrocotyle ranunculoides* abundance and nutrients in sediment of the *Hydrocotyle ranunculoides* infested sites, n=48, in 2013 and 2016.

Nutrient	**2013 Rho/P**	**2016 Rho/P**
Ammoniacal Nitrogen	0.035/0.813	0.211/0.149
Nitrate	0.276/0.058	-0.030/0.840
Nitrite	0.256/0.058	0.010/0.948
TON	0.264/0.069	-0.040/0.789
Orthophosphate	0.115/0.436	0.114/0.440
Total Phosphate	-0.104/0.481	0.038/0.800

5.3.3. pH in water

Table 36 illustrates the range of pH found in the water of the 48 infested sites over the four years of sampling. The means show that the sites are alkaline, being over pH of 7.0 (Hill et al., 2004). A PCA of all macrophytes and pH for the 48 infested sites, did not place *H. ranunculoides* in the 20 species with the best fit to the ordination axes. However, an RDA with all species and pH was significant at P=0.002 and the *H. ranunculoides* 2013 vector appeared with a strong negative association with the 2013 pH vectors (Figure 38). The 2013 pH vectors are placed along the horizontal axis separate from the other three years suggesting the pH data is different for 2013. The species vectors clustered around the 3013 pH vectors are all submerged species and a different assemblage to those aligned with the other three years

Table 36: Mean, maximum and minimum pH of the infested sites, n=48, over the four years of surveying, 2013 to 2016. A suffix of Y indicates the samples were taken using a handheld YSI instrument, the others were collected as samples, on the same date, and analysed at EAs UKAS accredited Starcross laboratory.

pH in water	2013Y	2013	2014Y	2014	2015Y	2015	2016Y	2016
Maximum	8.79	8.55	8.12	9.04	9.71	9.10	8.61	8.75
Minimum	6.66	6.93	6.90	6.97	6.89	6.92	6.52	6.77
Mean	7.27	7.39	7.30	7.45	7.52	7.59	7.50	7.50

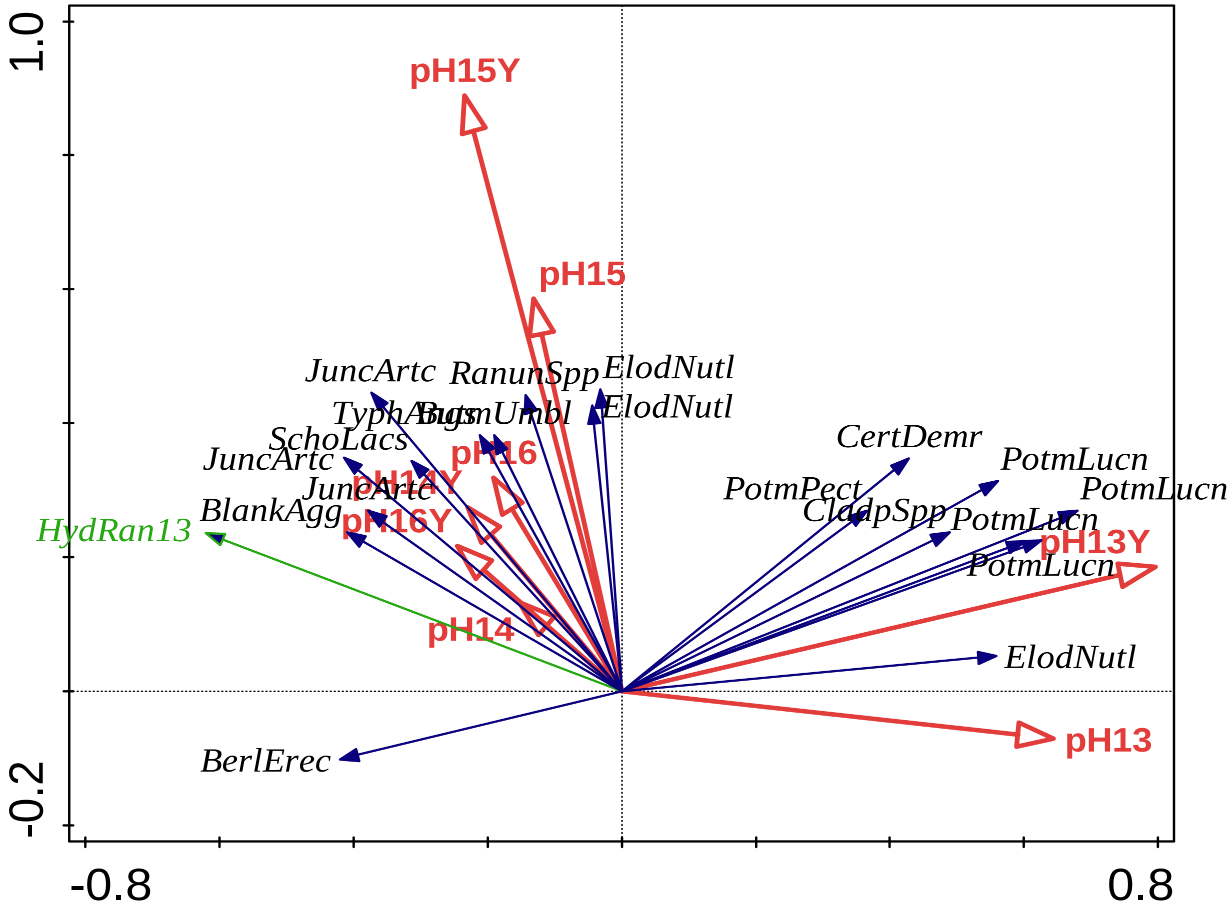


Figure 38: RDA of all plant species abundance and water pH parameters biplot for all four years using *H. ranunculoides* infested sites (n=48) for all four years 2013, 2014, 2015 and 2016. pH suffixed with Y indicate samples taken with a handheld YSI instrument, the others were collected as samples, on the same date, and analysed at EAs UKAS accredited Starcross laboratory, and the year is suffixed. Plant names are shortened versions of the plant latin binomials (Table 11). The first two axes are plotted.

Table 37 shows the results of the RDA with pH, using data from the water analyses and YSI pH data (Y) taken at the time of survey. The first gradient explains 7.26% of the total variability in species composition, which is a low expression. The second gradient explains a further 5.14% of the variation. The P value of 0.002 is significant with an explained cumulative variation of 18.98%, for all four axes. Although the P value is significant, the low explained cumulative variation suggests that other factors may be influencing the species composition.

Table 37: Summary of RDA results for *H. ranunculoides* infested sites, using abundance of al plant species, and pH in water, n=48, for all four years.

Statistic	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	0.0726	0.0515	0.0352	0.0306
Explained variation (cumulative)	7.26	12.40	15.92	18.98

Figure 39 illustrates the negative association between *H. ranunculoides* abundance and pH values in water, as the higher the pH, the lower the abundance of *H. ranunculoides.* This negative association is particularly evident in 2013 and 2016 with the *H. ranunculoides* vectors situated directly opposite to the pH vectors along the vertical axis for 2016 and the horizontal for 2013.

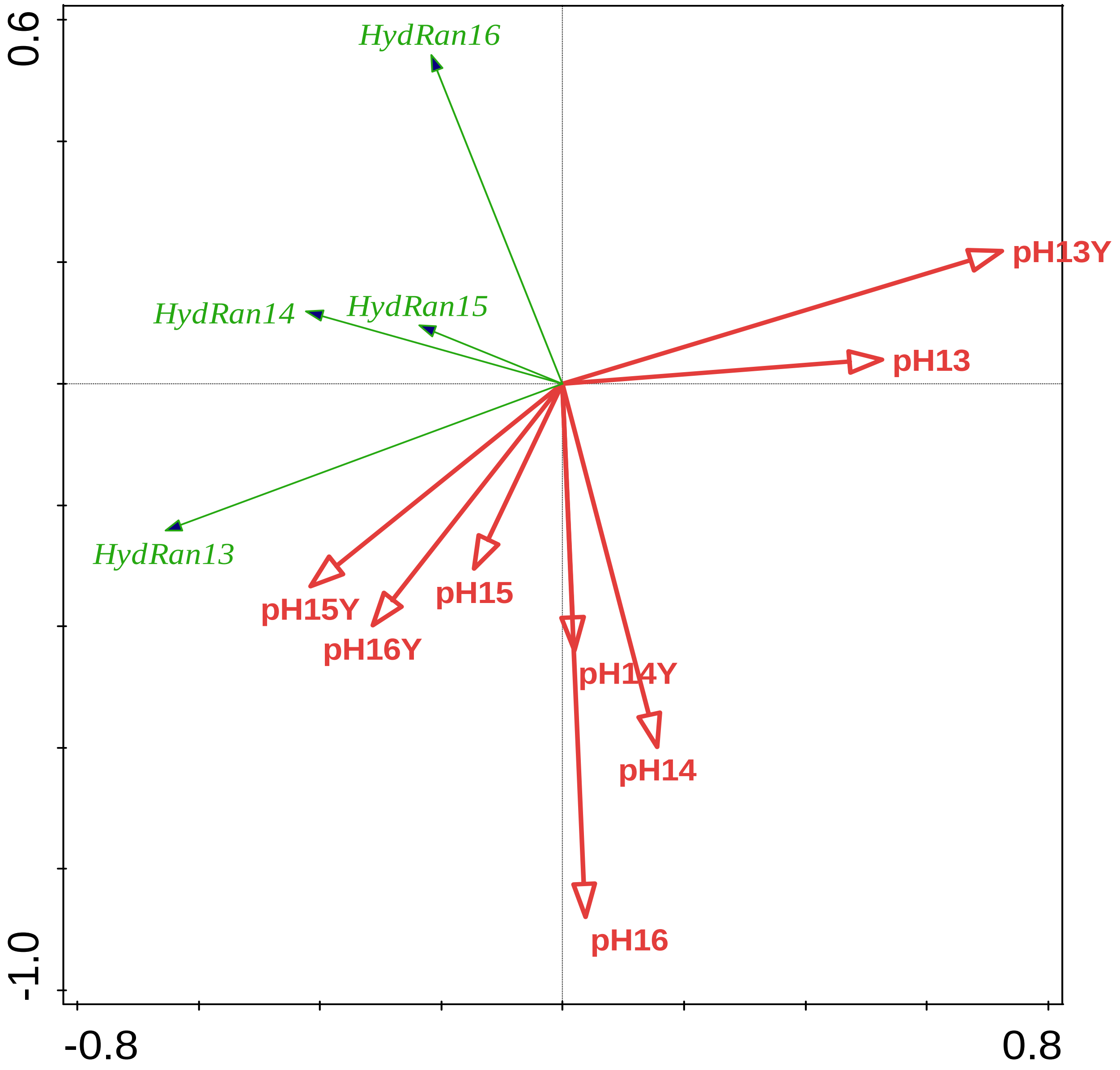


Figure 39: RDA of biplot *H. ranunculoides* and pH in water, using *H. ranunculoides* abundance in infested sites, n=48, for all four years 2013, 2014, 2015 and 2016. pH suffixed with Y indicate samples taken with a handheld YSI instrument and the year is suffixed. Plant names are shortened versions of the plant latin binomial for with the year suffixed (Table 11). The first two axes are plotted.

Table 38 shows the results of the RDA of *H. ranunculoides* abundance with pH, using data from the water analyses and YSI pH data (Y) taken at the time of survey. The first gradient explains 20.39% of the total variability in species composition, which is a good percentage. The second gradient explains a further 10.29% of the variation. The P value of 0.002 is significant with an explained variation for the first two axes of 30.68%.

Table 38: Summary of RDA results for *H. ranunculoides* infested sites, using abundance of *H. ranunculoides* and pH in water, n=48, for all four years.

Statistic	Axis 1	Axis 2	Axis 3	Axis 4
Eigenvalues	0.2039	0.1030	0.0386	0.0140
Explained variation (cumulative)	20.39	30.68	34.54	35.94

Correlations were used to confirm the two years in which negative relationships between abundance of *H. ranunculoides* and pH were indicated. None of the correlations were strong but all were negative and two were significant, 2013 and 2016 (Table 39).

Table 39: Spearman Rank Correlation (Rho) between abundance of *Hydrocotyle ranunculoides* and pH in water of the infested sites, n=48, over four years. Significance is indicated by the P-value, with significant values in bold.

pH in water	2013	2014	2015	2016
Rho	-0.480	-0.127	-0.156	-0.362
P-value	**0.001**	0.390	0.290	**0.012**

5.4. Results of greenhouse experiment

For the greenhouse experiment the nutrient levels used reflected the field sediment parameters and the two nitrogen elements, ammoniacal nitrogen and nitrate, were separated as well as including a treatment using both combined. Of the 36 plants grown for the experiment all rooted, survived, and grew.

5.4.1. Biomass

The mean DWs of each of the plants' component parts, for each nutrient treatment, are shown in Figure 40. The all-nutrients treatment gave the highest biomass compared to the control, but the biomass in the phosphate treatment was also high. The combined ammoniacal nitrogen and nitrate treatment gave the lowest DW.

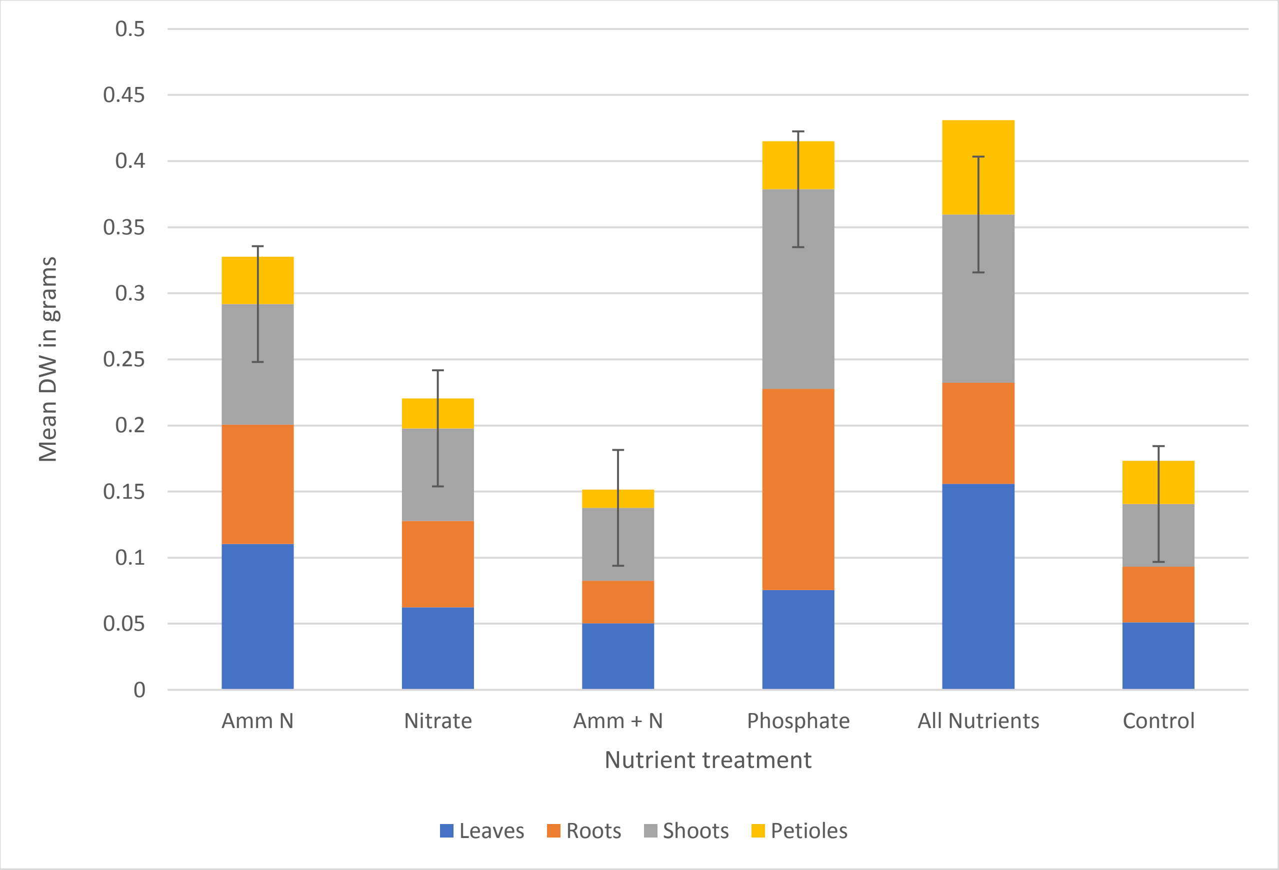


Figure 40: Mean dry weight, n=6, of *Hydrocotyle ranunculoides* after growth in sand for 70 days with different nutrient treatments, (for details of nutrient levels see Table 23), with standard error bars relating to total biomass. ANOVA indicated a significant difference for total biomass between treatments (P=0.029), but Tukeys test did not identify any significant differences at P=0.05.

ANOVA gave a significant difference in total biomass between nutrient treatments at P=0.029 (n=6). However, the Tukey pairwise comparison indicated that no treatments were significantly different to the control. But, to test which treatments the significant differences were between, a further ANOVA using treatments ammoniacal nitrogen and nitrate combined, all-nutrients and the control was used and gave a significant difference P=0.007. The graph in Figure 41 shows these significant differences and that the component parts differ under different treatments.

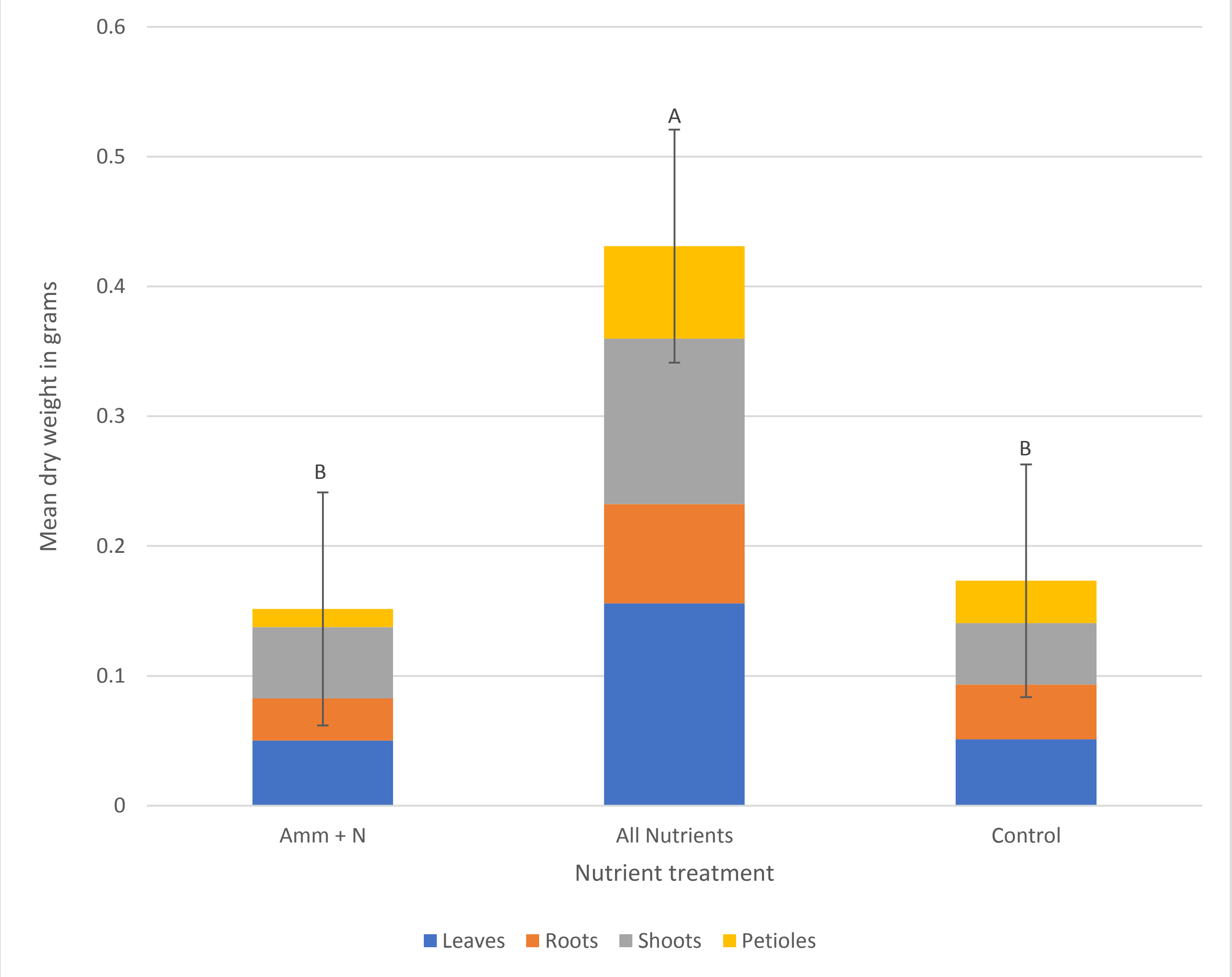


Figure 41: Mean dry weight, n=6, of *Hydrocotyle ranunculoides* after growth in sand for 70 days with different nutrient treatments, (for details of nutrient levels see Table 23), with standard error bars relating to total biomass. The letters A and B represent the Tukey pairwise comparison results and mean values that do not share a letter are significant for total biomass.

Each biomass component was tested separately across all treatments and all produced significant results from the ANOVAs; leaves P=0.001, roots P=0.002, shoots P=0.001 and petioles P=0.013. For the leaf component, the all-nutrients treatment had a significantly greater biomass than the ammoniacal nitrogen plus nitrate and control treatments (Figure 42). The root component had a significantly greater biomass in the phosphate treatment than the control and ammoniacal nitrogen plus nitrate treatment (Figure 42). The shoot component had a significantly greater biomass under the phosphate treatment than the control and ammoniacal nitrogen plus nitrate treatments. The all-nutrient treatment also had a significantly greater biomass than the control for the shoot component (Figure 42). For the petiole component, the all-nutrient treatment had a significantly greater biomass than the ammoniacal nitrogen plus nitrate treatment (Figure 42).

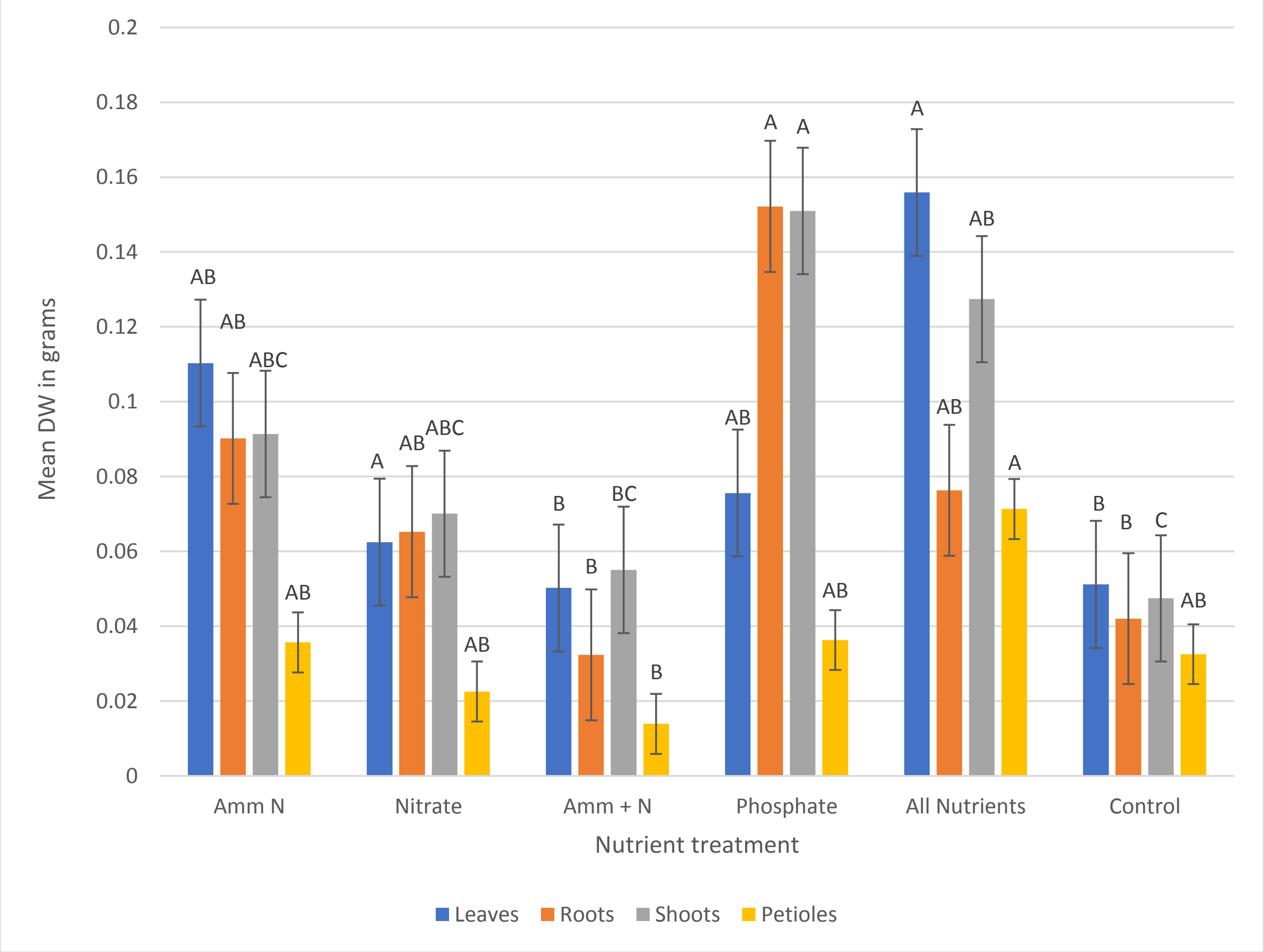


Figure 42: Comparison of *Hydrocotyle ranunculoides* plant component parts as mean dry weight, after growth in sand for 70 days with different nutrient treatments, n=6 (for details of nutrient levels see Table 23). The letters A, B and C represent the Tukey pairwise comparison results for each component part across treatments, and mean values that do not share a letter are significant for total biomass.

5.4.2. Relative growth rate (RGR)

The RGR between treatments (Figure 43) mirrored the final DWs of the plants but the Tukey post-hoc groupings gave different results. The ANOVA gave a significant result with a P<0.001. Here the phosphate and all-nutrients treatments had a significantly greater RGR to the control and combined ammoniacal nitrogen and nitrate treatment. The RGR for the ammonium treatment was significantly greater than the combined ammoniacal nitrogen and nitrate treatment. The phosphate only treatment gave the highest RGR and the combined ammoniacal nitrogen and nitrate the lowest.

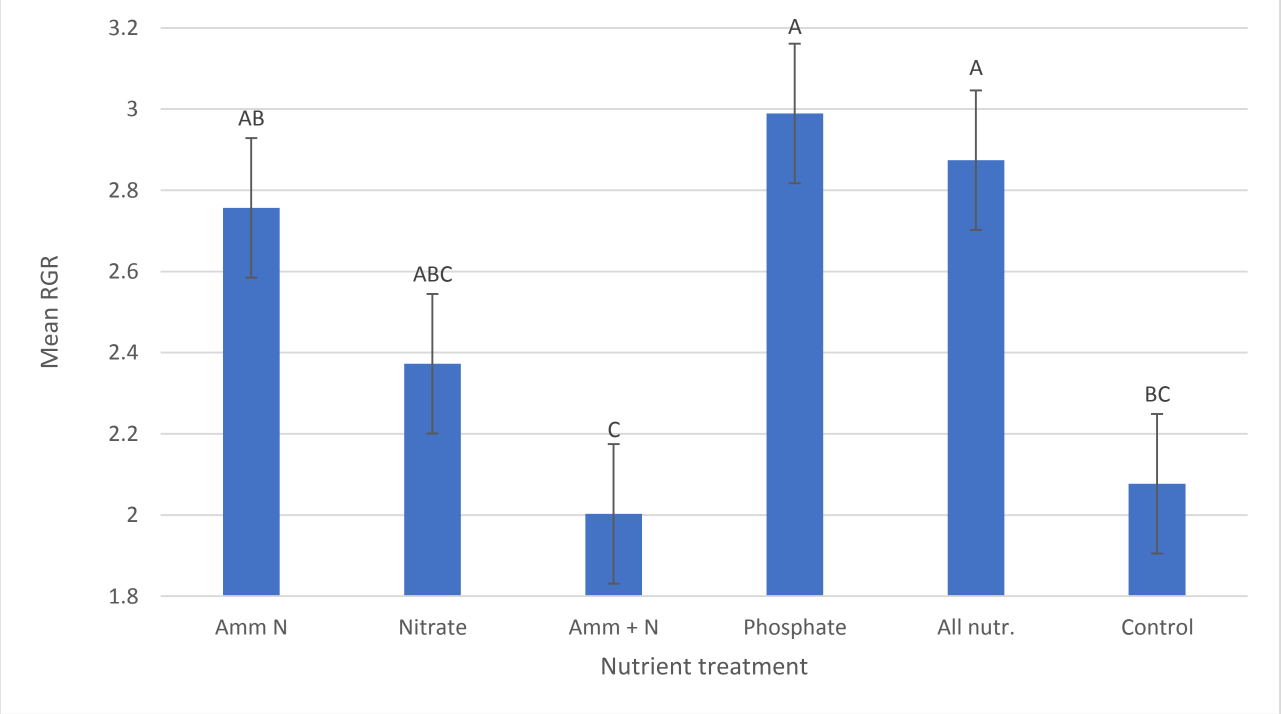


Figure 43: Mean RGR of *Hydrocotyle ranunculoides* under different nutrient treatments, n=6, (for details of nutrient levels see Table 23) with standard error bars. The letters A, B and C represent the Tukey pairwise comparison results and mean values that do not share a letter are significant for RGR.

The similarity between the RGR and the final DW was borne out by a correlation analysis, there being a strong correlation of 0.943 with a P=0.005.

5.4.3. Root to shoot ratio (R:S)

The R:S ratios for each treatment are shown in Figure 44. ANOVA gave a significant result with a P=0.012. The Tukey pairwise comparison illustrates the significant differences between the plants receiving the phosphate treatment and the all-nutrients treatment. Most biomass is allocated to the roots under the phosphate only treatment and least biomass is allocated to the roots under the all-nutrients treatment. The plants receiving the all-nutrients and the combined ammonium and nitrate treatments had lower R:S ratios than the control.

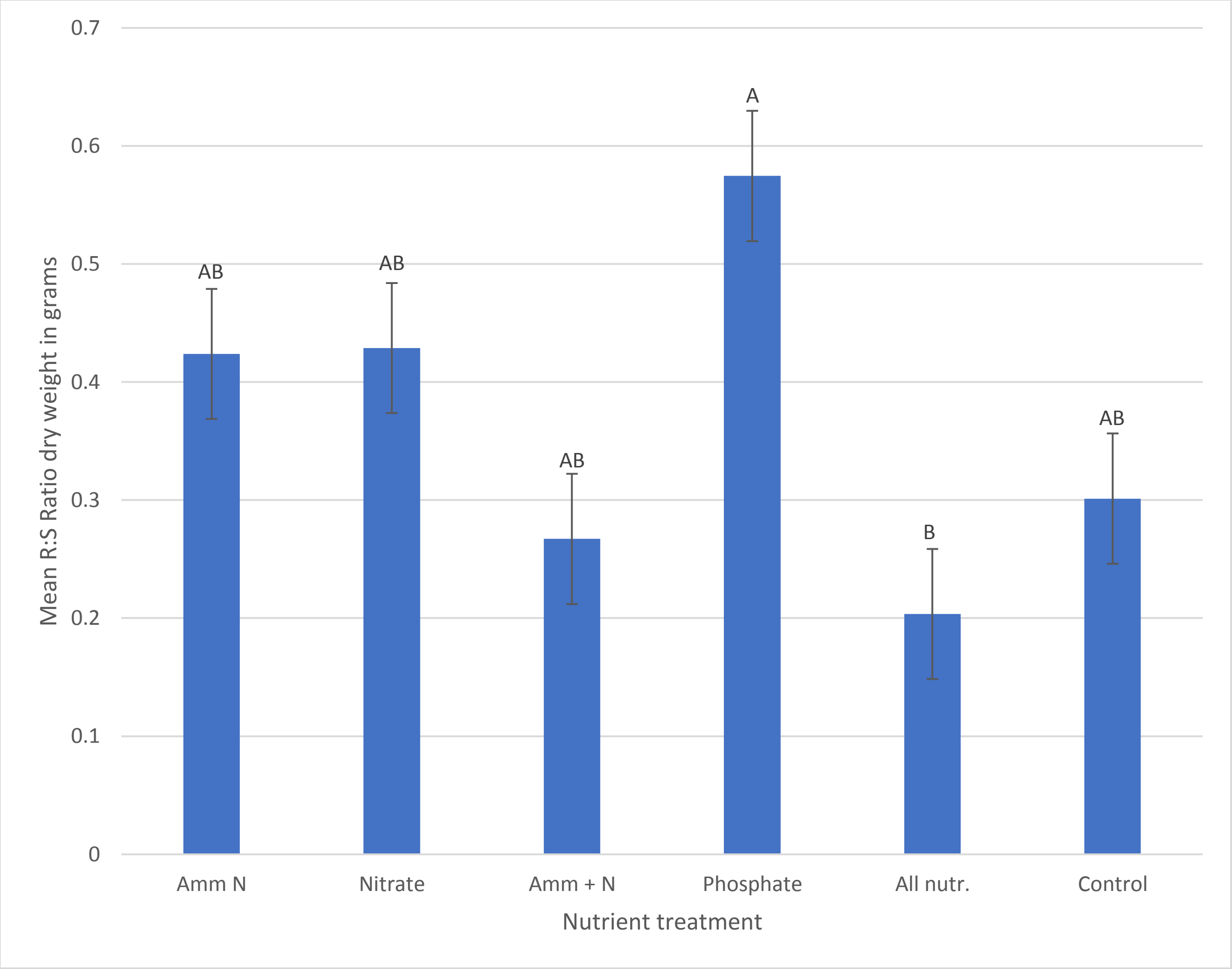


Figure 44: Mean root to shoot ratio, n=6, of *Hydrocotyle ranunculoides* under different nutrient treatments, (for details of nutrient levels see Table 23) with standard error bars. The letters A and B represent the Tukey pairwise comparison results and mean values that do not share a letter are significantly different.

5.5. Discussion

In this study the abundance of *H. ranunculoides* in ditches over a period of four years was recorded along with water and sediment samples taken to investigate the effects of nutrient and pH. In addition, growth of *H. ranunculoides* was monitored in a controlled greenhouse experiment, under different nutrient levels and the final growth was reported as DW, RGR and R:S ratio in comparison to a control with no added nutrients.

5.5.1. Relationships between *H. ranunculoides* and nutrients in water

Fried et al. (2009) concluded that *H. ranunculoides* had no specific preference for water nutrient parameters. However, this study has found that, in water, TON (nitrate and nitrite) was positively associated with *H. ranunculoides* abundance in all years. Two out of four study years showed a positive correlation between abundance of *H. ranunculoides* and ammoniacal nitrogen. The tolerance parameters of *H. ranunculoides* to TON on the Pevensey Levels are wider than previously reported in the literature (Fried et al., 2009, Hussner and Lösch, 2007), with a TON maximum of 26.80 mg/l.

Barko et al. (1991) suggest that rooted submerged macrophytes are likely to take up most of their nitrogen and phosphate from sediment rather than water but the results from the current study suggest it is more likely that *H. ranunculoides* is obtaining nitrogen from water. This is supported by the results from chapter 4, where *H. ranunculoides* accumulated most biomass later in the season when algal blooms are more likely to have fixed atmospheric nitrogen into the water column (Paerl, 1990). Basílico et al. (2017), studying *H. ranunculoides* capacity for bioremediation of sewage effluent, found it was ammonium that was the preferred nitrogen and that *H. ranunculoides* was capable of almost completely removing it from the sewage polluted water. This study concurs with Basílico et al. (2017), as there are positive associations between *H. ranunculoides* abundance and both TON and ammoniacal nitrogen in water.

For phosphorus there was a negative association between *H. ranunculoides* and orthophosphate in one year only for all infested sites, but when the de-silted sites were removed then there was a much stronger negative association. Many of the watercourses on the Pevensey Levels are affected by phosphate loading from the sewage treatment works (Diston et al., 2007). This phosphate can become bound to the sediment and released under summer conditions (Gerhardt et al., 2010) which is when the surveying and sampling was undertaken. Desilting would remove the surface layers of sediment containing the phosphate so less is likely to be released into the water from these sites. Bellinger and Davis (2017) found that as growth rates increased, phosphorus contents increased in the macrophytes they studied and Diston et al. (2007) suggested *H. ranunculoides* growth was driven by phosphate from the sewage treatment works on the Pevensey Levels. Basílico et al. (2017) did find some orthophosphate removal from sewage by *H. ranunculoides* but this was not significant. However, some orthophosphate would be removed and as *H. ranunculoides* abundance increases this could lead to a negative association with *H. ranunculoides* and orthophosphate in water. These field results suggest that orthophosphate in water is not driving the growth of *H. ranunculoides*. Fried et al.(2009), in Belgium, found *H. ranunculoides* on sites with orthophosphate 0.005-0.21 mg/l and total phosphate of 0.066-0.82 mg/l. Although orthophosphate levels varied over the years, five infested sites were consistently above Fried et al.'s (2009) orthophosphate maximum of 0.21 mg/l. Less than half (seventeen) sites had orthophosphate levels of below 0.069 mg/l in water for WFD 'Good' status for the four-year period. For the Pevensey levels SSSI to meet the common standards monitoring guidance for alkaline rivers the orthophosphate level would need to be lower than the EU WFD standard, below 0.05 mg/l (Joint Nature Conservation Committee, 2016a). Only eleven sites would meet this criterion for all four of the studied years. Therefore, in most of the studied ditches, the Pevensey Levels would not meet either the WFD phosphate target or the SSSI phosphate target so its status would be at risk. However, this study suggests that whilst the phosphate levels may not be contributing to the abundance of *H. ranunculoides,* which is a threat to the SSSI status, nitrogen as ammoniacal nitrogen and TON could be driving its growth.

Ullrich-Eberius et al. (1981), studying phosphate uptake by *Lemna gibba* (a free-floating macrophyte), found that optimum pH was 6.0 with uptake dropping off rapidly above this, almost halved by pH 7.5 and by 8.5 was almost zero. Fried et al.(2009) recorded the *H. ranunculoides* pH range as between 6.7 to 7.5. The range of pH in the infested study sites was between 6.52-9.10, an average of 7.44 (Table 36). These field results show that pH in the infested ditches at Pevensey is above optimal for the growth of macrophytes (Bellinger and Davis, 2017) and many are above the accepted range for *H. ranunculoides*. This study has suggested a negative association between orthophosphate and abundance of *H. ranunculoides*. So, to flourish as it has done over the four years of the study in the infested sites with the higher orthophosphate and above optimal pH levels, *H. ranunculoides* may have an alternative method of obtaining orthophosphate from the water, or it may be that it is preferentially obtaining it from the sediment. Studies show Ammoniacal Nitrogen can help increase the uptake of phosphate by plants from nutrient solutions (Rayar and Van Hai, 1977). Thus, the positive correlation between *H. ranunculoides* and ammoniacal nitrogen in water suggests that *H. ranunculoides* may be able to utilise ammoniacal nitrogen at the higher pH sites to continue obtaining phosphate, even though the pH is no longer optimal.

5.5.2. Relationships between *H. ranunculoides* and nutrients in sediment/substrate

In Germany, field measurements showed that monospecific stands of *H. ranunculoides* could occur in sediment with 6.2 to 11.5 mg of nitrate/ kg in the sediment (Fried et al., 2009). In this study, in both years, the range of nitrate in sediment could support the extensive infestations observed on the Pevensey Levels study sites. Hussner and Lösch's (2007) experiment used a maximum of 3.9 mg/kg of ammoniacal nitrogen and all field sites were well above this level.

The same study recorded monospecific stands of *H. ranunculoides* in sediment with between 2.9 to 61.9 mg of orthophosphate per 100g sediment (29-619 mg/kg) (Fried et al., 2009). For this field study of the Pevensey Levels, orthophosphate sediment levels were predominantly in the lower range recorded by Fried et al. (2009) with all 48 sites below the 619 mg/kg level. Hussner and Lösch's (2007) experiments showed that *H. ranunculoides* growth was restricted only when orthophosphate was as low as 2.0 mg/kg. Thirteen sites remained below 29 mg/kg in both 2013 and 2016 but no site had orthophosphate levels low enough to restrict growth. De-silting is likely to mobilise phosphate into the water column in the short term (Gerhardt et al., 2010) which was why de-silted sites were removed from the analyses. However, as the remaining sediment settles there may be less phosphate available in subsequent growing seasons (Gerhardt et al., 2010). Combined with the higher levels of pH found in water in the field sites, these two factors might reduce overall phosphate uptake (Bellinger and Davis, 2017).

Despite there being sediment nutrient ranges capable of supporting abundant *H. ranunculoides* growth in the field study sites, there were no significant associations between any nutrient and abundance of *H. ranunculoides.* However, in the greenhouse experiment, the addition of phosphate alone produced a significantly higher root and shoot biomass than the control with no added nutrients, and the combined nitrogen treatment. The RGR gave similar results to the biomass results but accentuated the poor growth under the combined nitrogen treatment in comparison to the phosphate and all-nutrients treatments. However, the R:S ratio gave different results to the total biomass and RGR with more root mass produced under the phosphate treatment. Here, the all-nutrient treatment produced the lowest root mass with the combined nitrogen treatment root mass being comparable to the control. These results suggest that phosphate is an important nutrient in all three analyses.

Research on root growth, nitrate and phosphate (using *Arabidopsis* plants) shows that primary root length decreases as nitrate increases but that it lengthens as phosphate increases (Linkohr et al., 2002). Gastal et al. (2015), noted that nitrogen deficit increases

R:S ratio in agricultural crops. Pedersen et al. (2019), working with maize plants, reported that nitrogen fertilisers had a root inhibiting effect if used without a phosphate input. The greenhouse experiment results concur with these studies indicating that, as a macrophyte, *H. ranunculoides* is responding in a similar way to terrestrial plants. Here the phosphate only treatment produced a higher R:S ratio and a greater root biomass compared to the ammoniacal nitrogen and nitrate combined treatment, which gave a much lower R:S ratio and the lowest root biomass. However, the response of plant growth to the availability of one particular resource, such as nitrogen, also depends on the availability of other resources and co-limitation often occurs (Gastal et al., 2015).

Reddy and Tucker (1985) used ammonium nitrate to investigate the effect of nitrogen on the growth of *Hydrocotyle umbellata* (Manyflower marsh-pennywort) a plant which favours waterlogged conditions. They found that concentrations in the substrate of up to 20 mg/l increased growth but above this there was no increase in biomass. They also found that root length was inversely related to nitrogen level but that shoot length was not affected by any level of nitrogen addition (4-40 mg/l). This accords with the findings of this study where the combined ammoniacal nitrogen and nitrate treatment gave a much lower R:S ratio than phosphate alone. Their findings were comparable to this study in that phosphate uptake increased when ammoniacal nitrogen was increased to 20mg/l but, above this, phosphate accumulation decreased. RGR was much lower when plants only obtained nutrients from the substrate (Reddy and Tucker, 1985).

An experiment by Hussner and Lösch (2007) on *H. ranunculoides* showed that with combined nutrients there was a significant increase in RGR and DW when ammoniacal nitrogen was 3.1 mg/kg, nitrate 14.0 mg/kg and orthophosphate 21.0 mg/kg, compared to no added nutrients, and that both RGR and DW continued to increase steadily as the nutrients were increased. The greenhouse experiment results have illustrated the effects of these nutrients on *H. ranunculoides* growth both in-combination but also independently. Orthophosphate alone promoted root and shoot growth and increased RGR compared to the combined ammoniacal nitrogen nitrate and nitrate treatment and the control. Ammoniacal nitrogen and nitrate combined produced a lower biomass and RGR compared to the control which lacked nutrients.

5.5.3. Mechanisms of growth and stoichiometry for *H. ranunculoides*

Growth experiments by Hussner and Lösch (2007) found that very low substrate nutrient levels (nitrate at 6.07 mg/kg, orthophosphate at 2.7 mg/kg and ammoniacal nitrogen at 1.97 mg/kg) produced very limited growth in *H. ranunculoides*. The greenhouse results from this study support these results; the control contained nitrate at 2.8 mg/kg, orthophosphate at 1.0 mg/kg and ammoniacal nitrogen at 2.0 mg/kg, which produced significantly less growth than substrates with added nutrients. However, the results of the sediment and water analyses from the field study sites showed that the majority of sites had nutrient levels well above both the experimental levels and that, even if current WFD and SSSI targets are met, this would not reduce levels low enough to restrict *H. ranunculoides* growth. In Germany, Hussner and Lösch (2007) recorded nitrate levels of 6.2 – 11.5 mg/kg, and orthophosphate levels of 29.0 – 619.0 mg/kg, in the sediment of their field sites which is far higher for orthophosphate than the limiting factors in their experiment. The higher field nutrient levels, found at the experimental sites on the Pevensey Levels, were not used for the growth experiment as the aim was to determine the lower nutrient levels that might restrict growth. Phosphate stripping at the wastewater treatment works feeding into the Pevensey Levels has not reduced the cover of *H. ranunculoides as* Diston et al. (2007) had predicted, even though phosphate levels dropped considerably in the period from 2003 to 2015 (Environment Agency, 2016a). So, the indications are that growth limitation by restricting nutrients, in the field, is unrealistic.

The pH of the ditch water was investigated as this can have an influence on uptake of nutrients, particularly phosphate, with uptake decreasing above pH6 (Ullrich-eberius,1981). Bellinger and Davis (2017) studying the macrophytes *Hydrilla verticillata* and *Cabomba caroliniana* in the US*,* found that high pH was one of the most important determining factors of growth with optimal growth between pH 4-6, but that pH needed to be over 8 before it caused senescence. There was a strong negative association between pH and abundance of *H. ranunculoides* in water in all years of the field studies. However, the water analyses also showed that *H. ranunculoides* has a much wider tolerance range of pH than has previously been recorded. Fried et al. (2009) put *H. ranunculoides* pH range; 6.7 – 7.5. But on the Pevensey Levels, although the overall mean was within this range at pH 7.4, 15 of the 48 infested sites had a mean pH of greater than 7.5 in all four years of the study. These results suggest that despite the negative association between *H. ranunculoides* and pH, it can tolerate a higher pH than has previously been proposed.

Riley (1971), found that using ammonium fertiliser increases the availability of orthophosphate from the soil because the ammonium ion decreases the pH of the soil surrounding the root. So, if this principle also applies in water, the high levels of ammoniacal nitrogen in the water may be allowing *H. ranunculoides* to obtain orthophosphate under the relatively high pH conditions because the ammonium ion is lowering the pH nearer to optimal levels at the root interface. This is particularly because *H. ranunculoides* has large root masses suspended in the water as well as rooting into sediment. Smith (2014), using *Lemna* species, found that floating plants did not exhibit linear responses to TON or total phosphate. Smith (2014) found that nitrogen initially drove floating plant growth but that there were multifaceted interactions between nutrients and pH, which needed further investigation.

Plant stoichiometry includes the multiple interactions that occur between available resources, and their ecological interactions with the environment (de Mello Prado and da Silva, 2017). It is useful in explaining a plants' response to changing resources and how it adapts to different environments (de Mello Prado and da Silva, 2017). Stephen et al. (1997b) noted that rooted macrophytes tend to increase phosphorus release when present compared to when absent in a waterbody. These results illustrating the influence of nutrients and pH on the growth of *H. ranunculoides*, demonstrate that one of its key invasive plant traits, adaptability (Robert et al., 2013), may extend further than previously thought. It may be capable of utilising the resources available more effectively, potentially modifying the nutrient balance of its invaded community.

5.6. Conclusion

The field results show that in water there appears to be a positive association between nitrogen and *H. ranunculoides*; but a negative association between orthophosphate and *H. ranunculoides.* In sediment there were no significant associations between the nutrients and *H. ranunculoides.* In water, *H. ranunculoides* was recorded under a much wider range of pH than previously reported, though there was a significant negative relationship between pH and *H. ranunculoides*.

The greenhouse experiments showed that phosphate, as orthophosphate, in the substrate produced a higher biomass than a control treatment with no nutrients but combined high nitrogen as ammoniacal nitrogen and nitrate produced poorer growth than the control.

RGR showed similar results, highlighting the poor growth of *H. ranunculoides* in the combined high nitrogen substrate. R:S ratio produced different results showing that the substrate with phosphate only produced a much higher root proportion than the control and combined nitrogen substrates with the all-nutrients substrate having the lowest root component. It is possible that the all-nutrients treatment promotes shoot growth more than the nitrogen components, either separately or in combination, thus reducing the root ratio below that of the control. EPPO (2010) note that biomass is more likely to be allocated to roots in low nutrient conditions and shoots under higher nutrient availability. This would be supported by the evidence of extensive floating mats formed under eutrophic conditions (Centre for Agriculture and Biosciences International, 2020b) but the roots, although profuse are small and hairlike (Newman and Duenas, 2010).

Thus, while there does not seem to be a specific nutrient driving the growth of *H. ranunculoides,* it appears to preferentially use nitrogen from water and phosphate from sediment. There are indications that there are more complex interactions between nutrient resources, especially regarding uptake and release of nutrients, the effect of pH and the ability of *H. ranunculoides* to obtain nutrients efficiently under sub-optimal conditions. A combination of nitrogen elements, without available phosphate, does restrict the growth of *H. ranunculoides* particularly by limiting root development but this is a situation unlikely to occur naturally. These factors may add additional information to explain some of the reasons behind *H. ranunculoides* invasive capacity, adaptability, and ability to out-compete native macrophtyes.

This chapter has illustrated the growth potential of *H. ranunculoides* and investigated which nutrients are key drivers of growth and spread of *H. ranunculoides*. Its success and spread is thought to be due to its extremely efficient reproduction (Robert et al., 2013), mainly due to its exceptional vegetative regeneration capacity. It is now recognised that it can reproduce sexually and has been noted to flower and produce seed in its invaded regions (Centre for Agriculture and Biosciences International, 2020b). However, it is not known whether this seed is viable and can germinate in its European invaded territories. This is important as the current management methodology and biosecurity described in chapter 4 does not take regeneration as germination from seed into account. Thus, seed germination and growth experiments were undertaken and are presented in chapter 6.

Chapter 6 - The influence of temperature on growth, seed production, germination, and invasive potential of *H. ranunculoides*.

6.1. Introduction

The superior vegetative regenerative capacity of *H. ranunculoides* is well described in the literature and is one of the main reasons for its ability to spread in its invaded regions (Robert et al., 2013). This rapid growth has been noted in north-western Europe in Belgium, The Netherlands, and the UK by EPPO (2010), and since then has spread to France, Belgium, Germany and Italy (CABI, 2020b). The ability of *H. ranunculoides* to produce large quantities of viable seed (Newman and Dawson, 1999) and for these seeds to germinate in its native regions (Walsh, 2016) is also documented. However, despite being observed to flower and produce seed in its invaded regions (Centre for Agriculture and Biosciences International, 2020b), it is not known whether this seed is capable of germination outside its native regions. This chapter will examine the potential impact of climate warming on the sexual reproductive capacity of *H. ranunculoides,* as seed production, germination, and growth in its European invaded regions. It will also discuss the implications of sexual reproduction on the invasive potential, management, and ability to control *H. ranunculoides.*

6.1.1. Climate change

There is scientific consensus that global temperature rise and other climatic changes will occur as a result of anthropogenic greenhouse gas emissions (Karl and Trenberth, 2003; Committee on Climate Change, 2019). It is now generally agreed that the world is facing a climate emergency and that, despite the UKs original 2008 aim towards limiting emissions to restrict global warming to a 1.5^{o}C rise, as in the Paris agreement, it is highly likely this threshold will be breached in the next 20 years (Committee on Climate Change, 2019). The consequences of releasing stored CO_2 is that there is an almost linear relationship between the stock of CO_2 and global temperature (Committee on Climate Change, 2019). A 1.0^{o}C warming above pre-industrial levels has regularly been exceeded in the last decade (The Chartered Institute of Eology and Environmental Management (CIEEM), 2019) and current, longer term predictions show that, globally, the trajectory is more likely to deliver a 3^{o}C rise by 2050, which would have destructive consequences (Committee on Climate Change, 2019).

The 2010's were a notable decade for high temperature records with 2014 ranked as the warmest since 1910 (Met Office, 2020a). The minimum UK average temperature in summer 2014 was 11.7°C and the maximum 21.8°C giving an average of 16.75°C (Met Office, 2019), 0.9 °C warmer than the 1961-1990 averages. The minimum UK average in summer 2016 was 12.4°C and the maximum 21.6°C giving an average of 17°C (Met Office, 2019) and Winter 2016 (December 2015 to February 2016) was the mildest on record since 1910, with a mean temperature more than 2°C above the long-term average for southern UK. The 2017 UK Climate Change Risk Assessment (Humphrey and Murphy, 2016) predicts a very definite 2^{o}C rise for the UK and models now predict that even drastic global action only has a 50% chance of preventing an average temperature rise of 2°C above pre-industrial levels (The Chartered Institute of Eology and Environmental Management (CIEEM), 2019).

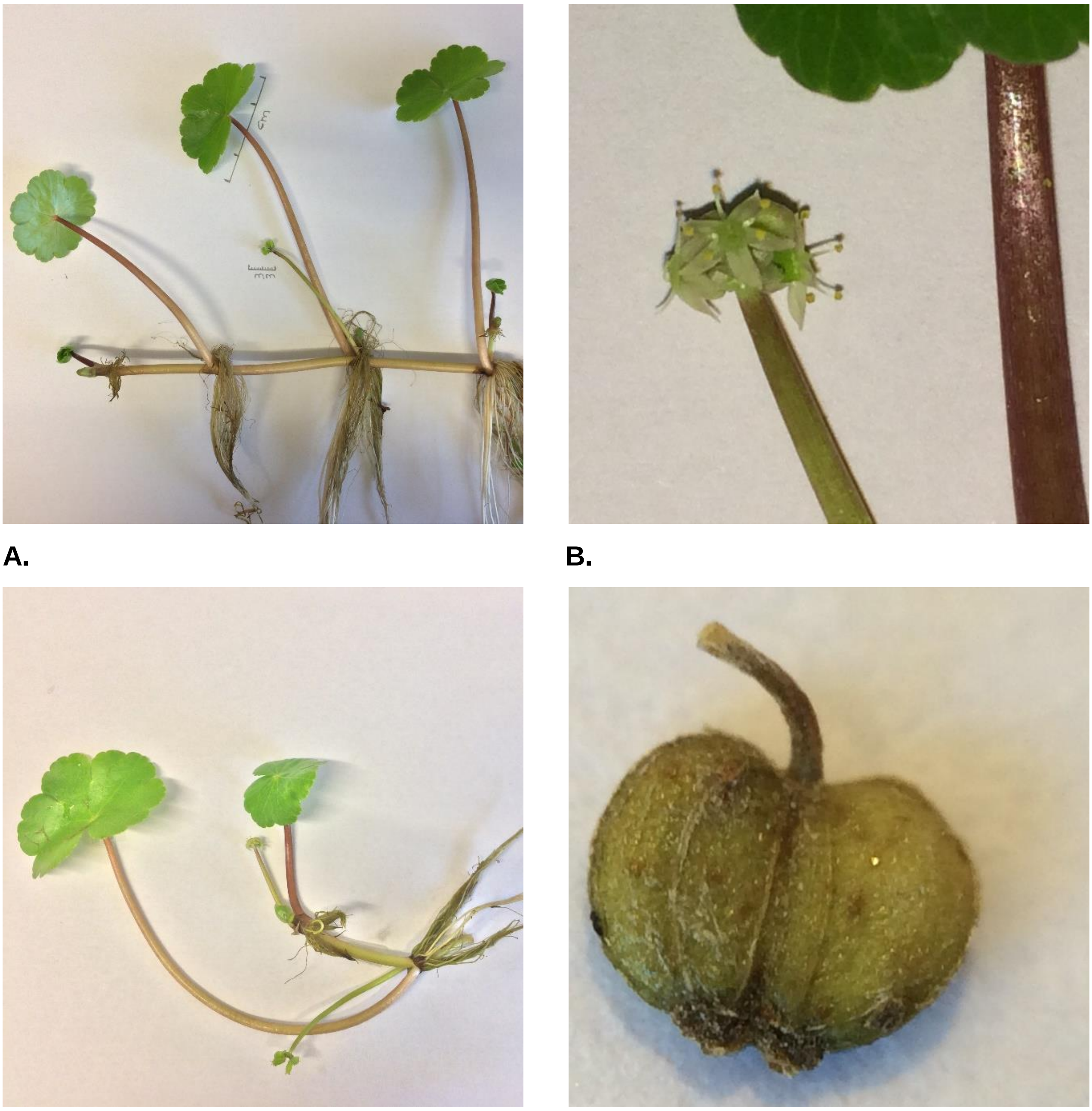
For invasive species in Europe, which have predominantly arrived from the warmer south eastern regions (Gallardo and Aldridge, 2015) climate warming is likely to provide more favourable conditions (Rahel and Olden, 2008). Climate warming will also influence the likelihood of new species arriving and establishing (Rahel and Olden, 2008). For aquatic organisms there are many ways in which warming may alter conditions; a change in physiological optima, less ice cover increasing winter light levels, and increased rates of evapotranspiration leading to lower flows (Rahel and Olden, 2008). For *H. ranunculoides* this may take the form of warmer water which could extend the growing season and thus increase competitive superiority, allowing encroachment into native plant niches, and increasing opportunities to expand its range as more areas fall in to the invasible climatic parameters. Warming may also provide environmental conditions more favourable for sexual reproduction in addition to the already extremely efficient vegetative method (Centre for Agriculture and Biosciences International, 2020b).

Temperature is considered the most important regulator of the germination response (Gillard et al., 2017a), so the predicted global temperature rise (defined as combined surface air and sea surface temperatures (First, 2019)) may have a considerable impact on seed dormancy and germination potential (Gillard et al., 2017a). Gillard et al. (2017a) postulated that climate warming may promote a shift from clonal to sexual reproduction. These increasing temperatures might improve germination potential as time to germination and final germination percentage, and might also reduce length of dormancy (Gillard et al., 2017a, Gillard et al., 2017b). Benvenuti et al. (2001) found that a greater fluctuation in temperature promoted increasing germination in *Rumex obtusifolius* and Wagner and Oplinger (2017) found that higher temperature fluctuations improved germination in four macrophyte species, the fluctuating temperatures providing the cue that conditions had become favourable for germination. Ter Heerdt et al. (2017) found that emergence of the macrophyte seedlings, *Typha latifolia* and *Phragmites australis,* increased with increasing temperature. Increasing temperature also increased relative growth rates of three aquatics, *Hydrilla verticillata*, *Egeria densa* and *Lagarosiphon major* (Hussner et al., 2015). This increased growth may be linked to increased seed production in annual aquatics, (Li, 2014). Increased germination capacity, combined with other plant responses to warmer conditions, is particularly concerning with regard to the potential expansion of invasive aquatic species (Gillard et al., 2017a).

6.1.2. *H. ranunculoides* reproductive capacity

H. ranunculoides has a huge capacity for spread due to its high growth rates and ability to grow vegetatively from only a single stem node (Robert et al., 2013). These small fragments of a minimum of one centimetre, with a growth node, break off easily from the main plants, usually due to disturbance from water flow, animal interaction or mechanical control methods, and spread rapidly downstream and to adjacent watercourses (Robert et al., 2013). Newman and Duenas (2010) reported that, in Europe, *H. ranunculoides* has only ever been observed to reproduce by this vegetative method. Rapid establishment and colonisation is ideally facilitated by such vegetative means but seed production is important for re-establishment after extreme events and to maintain genetic capacity to respond to temporal changes (Li, 2014). If *H. ranunculoides* could produce viable seed in its European invasive range because of warmer temperatures in the growing season due to climate warming this may have significant consequences for the future spread and impact in its invaded regions. Given that *H. ranunculoides* already exhibits phenotypic plasticity in its vegetative growth response, the addition of genetic variability resulting from sexual reproductive ability would raise considerably more concerns regarding its invasive capacity.

H. ranunculoides produces seed from flowers that emerge from the leaf nodes towards the tips of the shoots and just above the water (Figure 45). They are greenish-white, hermaphrodite flowers grouped in a small umbel of five to 10 (EPPO, 2006). They are small and open with no sepals, five unconnected petals, five stamens, an inferior twolobed ovary, and two styles. The fruits, when ripe, are flattened and rounded, dark brown and divided in two halves with a short stalk (EPPO, 2006). There are at least four distinct genetic strains of *H. ranunculoides* in the UK population (Newman, 2004), so there may already be genetic variability in other invaded regions. Although DNA barcoding was undertaken by van de Wiel et al., in 2009, there is no other published work on the genetics of *H. ranunculoides*.



A.

B.

C.

D.

Figure 45: Photographs (Birch. J.) of stages of seed formation in *H. ranunculoides.* Material collected from the wild in the UK (Pevensey Levels Latitude 50^{o} 50' N, Longitude 0^{o} 18' E). A. flower emerging from leaf node, B. flower showing petals and stamens, C. seed set (lower leaf node) and beginning to bend down towards the water (see Figure 53a. for close-up of this ripening seed), D. ripening seed (fully ripe seed see figure 58d.).

6.1.3. Impact of climate warming on seed viability

The Intergovernmental Panel on Climate Change (IPCC) (IPCC, 2014) estimated that global atmospheric temperature, driven primarily by anthropogenic CO_2 emissions, was likely to rise over the 21st century by at least 2°C compared to pre-industrial temperatures, under all emissions scenarios but more recent estimations raise this by a further degree (Committee on Climate Change, 2019). It is likely that *H. ranunculoides* will take advantage of increasing temperatures and become more invasive in Central and North Europe (Robert et al., 2013) and warmer temperatures during the growing season could improve the chances of viable seed production (Millane and Caffrey, 2014).

In *H. ranunculoides* native regions, southern and central North America and South America, the summer (December – February) growing temperature range is 18-28^{o}C (Weather and Climate, 2019a), mostly below the 25-35^{o}C optimal range for growth suggested by Hussner and Lösch's (2007) experiment. In southern UK (Eastbourne) the summer temperatures (June to August) are well below the Hussner and Lösch optimum at 10-22^{o}C (Weather and Climate, 2019b), but a 2^{o}C rise would take these within 1^{o}C of the lower limit of this optimum range and a 1^{o}C rise has already been experienced in the last decade (The Chartered Institute of Eology and Environmental Management (CIEEM), 2019). The minimum average in summer 2014 was 11.7°C and the maximum 21.8°C (average 16.75°C). The minimum average in summer 2016 was 12.4°C and the maximum 21.6°C (average 17°C). It is possible that these higher temperatures might enable more suitable growth conditions which, in turn might favour both the production of more biomass and accumulation of resources necessary to produce seed.

The Representative Concentration Pathway (greenhouse gas concentrations and aerosol emissions) RCP scenario 4.5 shows a 2.1 - 4.2°C (Humphrey and Murphy, 2016) rise for 2080-2099 compared to 1860-1899. The lowest RCP 2.6 range of 1.4 - 3.2°C was not used because annual average UK land temperature have already increased by 0.9°C in 2005 – 2014 compared to 1961 – 1990, with 2014 being the warmest individual year, and previously the central England average temperature rose about 1°C since the 1880's (pre-industrial) (Humphrey and Murphy, 2016). The Met Office climate summaries (2020b) show that for England south east and central (where the field sites are) the mean average summer temperature in 2014 was 16.8°C a 1.1°C rise in 1961 to 1990 averages. In 2016 the summer temperature was 17°C, a 1.3°C rise (Met Office, 2020b).

The aim of the 2015 Paris Agreement's is to keep the rise in global mean surface temperature 'well below' 2°C above pre-industrial levels, 1850 – 1900 (Humphrey and Murphy, 2016). Medium emissions scenario for UKPC09 projections give a global average warming by the end of the century of approximately 4°C relative to pre-industrial condition. The UK Climate Change Risk Assessment 2016 (Humphrey and Murphy, 2016) seems to focus on 2^oC and 4^oC rises.

6.1.4. Aims and objectives

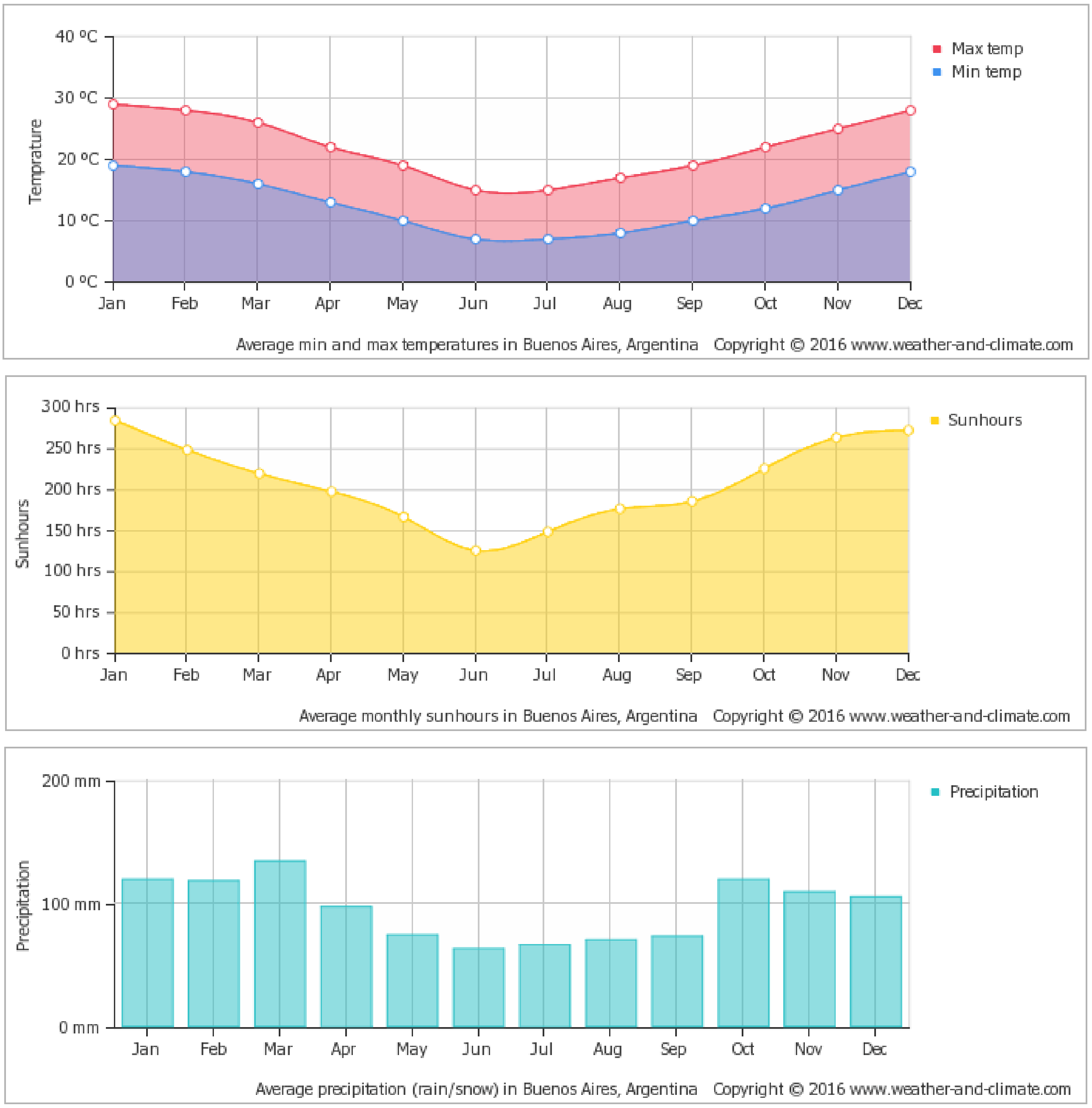
The aim of this greenhouse experiment was to examine the potential impact of the UK Climate Projections prediction of a minimum rise in 2^oC (Humphrey and Murphy, 2016) during the growing season and germination period on the reproductive and invasive capacity of *H. ranunculoides*. The objectives were to:

- Establish whether there is a relationship between temperature and germination of *H. ranunculoides* seed.
- Determine the impact of a rise in mean late summer/autumn minimum temperature of 2^oC on the growth and seed production of *H. ranunculoides.*
- Investigate the effects of a rise in temperature on the invasive capacity of *H. ranunculoides* in its European invaded regions.

6.2. Methods

6.2.1. Climate in native range

All the regions in which *H. ranunculoides* grows have four seasons: in the southern hemisphere winter (June–August), spring (September–November), summer (December–February) and autumn (March–May) and in the northern hemisphere winter (December-February), spring (March-May), summer (June-August) and autumn (September-November) (World Weather and Climate Information, 2016). The temperature in *H. ranunculoides* native regions, southern North America, and South America, is a bit below the 25-35^oC summer range suggested for its optimum growth (Hussner, & Lösch, 2007) (Figures 46-47). Light levels are high during the growth period, over 250 hours of sunshine in the native regions during summer (Figures 46-48) providing the bright, sunny conditions in which *H. ranunculoides* grows best (Hussner & Lösch 2007). Precipitation is regular, with over 100mm per month, during the summer growing seasons (Figures 46-47).



Average min and max temperatures in Buenos Aires, Argentina Copyright © 2016 www.weather-and-climate.com

Average monthly sunhours in Buenos Aires, Argentina Copyright © 2016 www.weather-and-climate.com

Average precipitation (rain/snow) in Buenos Aires, Argentina Copyright © 2016 www.weather-and-climate.com

Figure 46: Average minimum and maximum temperature (Celsius), sunshine and rainfall in Buenos Aires, Argentina where *H. ranunculoides* is native (Millane and Caffrey, 2014). (Weather and Climate, 2019a, Weather and Climate, 2020a).

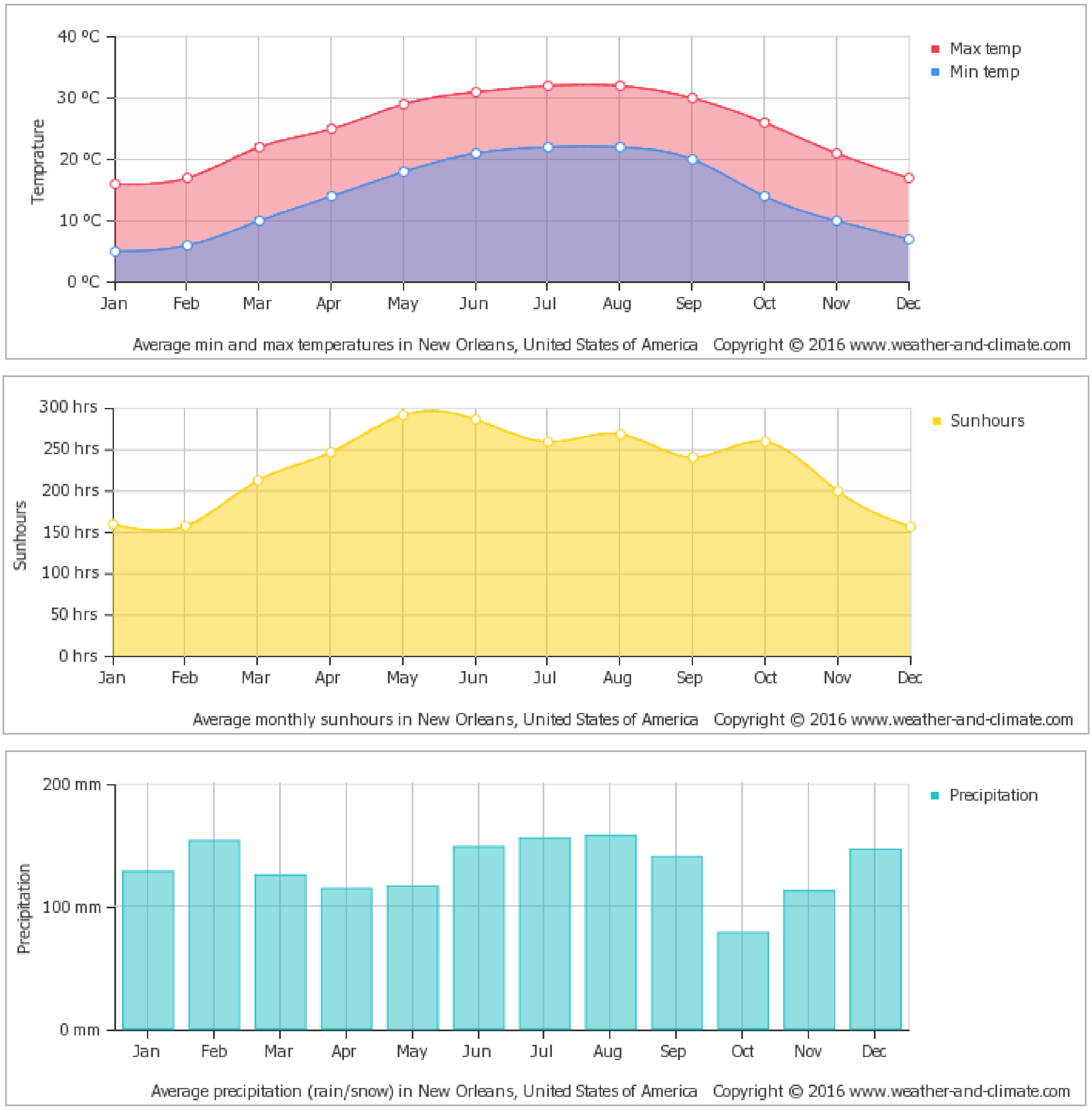
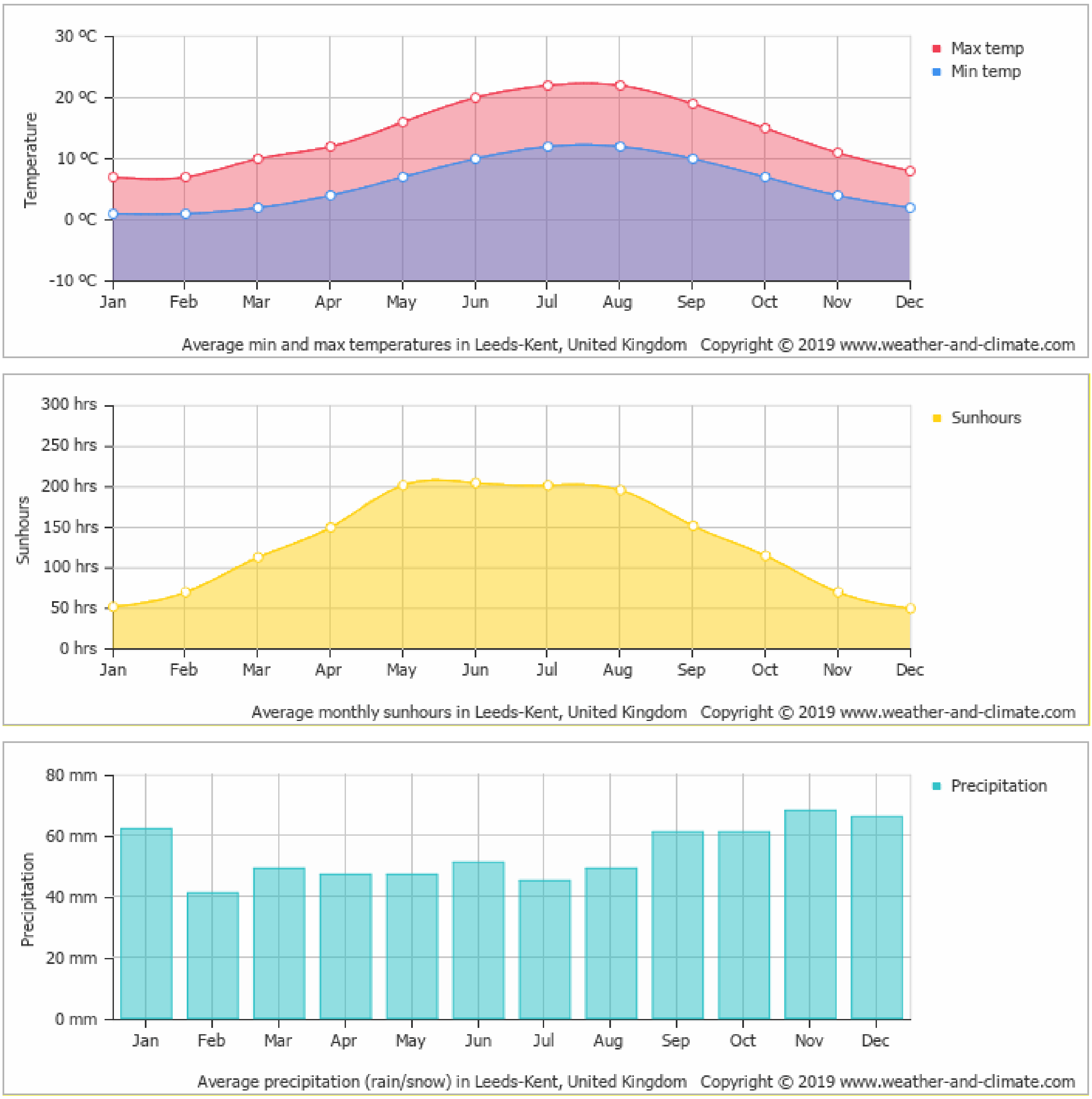


Figure 47: Average minimum and maximum temperature (Celsius), sunshine and rainfall in New Orleans, Louisiana, USA, where *H. ranunculoides* is native (Centre for Agriculture and Biosciences International, 2020b). (Weather and Climate, 2020e).

6.2.2. Climate in invaded ranges

However, in the European invaded regions, the summer temperatures are considerably lower, on average not reaching *H. ranunculoides* minimum optimum growth temperature of 25^{o}C (Hussner and Lösch, 2007) (Figures 48-49). Light levels are also much lower during the summer growth period (Figures 48-49) between 150 and 200 hours of sunshine compared to over 250 hours in the native regions. Precipitation is regular (Figures 48-49) although not as high as the 100mm per month, during the summer growing seasons in the native regions. The Leeds weather station in Kent was the nearest available and no graphs were available for Pevensey.



Average min and max temperatures in Leeds-Kent, United Kingdom Copyright © 2019 www.weather-and-climate.com

Average monthly sunhours in Leeds-Kent, United Kingdom Copyright © 2019 www.weather-and-climate.com

Average precipitation (rain/snow) in Leeds-Kent, United Kingdom Copyright © 2019 www.weather-and-climate.com

Figure 48: Average minimum and maximum temperature Celsius), sunshine and rainfall in Eastbourne, England (nearest weather station, Leeds-Kent). (Weather and Climate, 2020c).

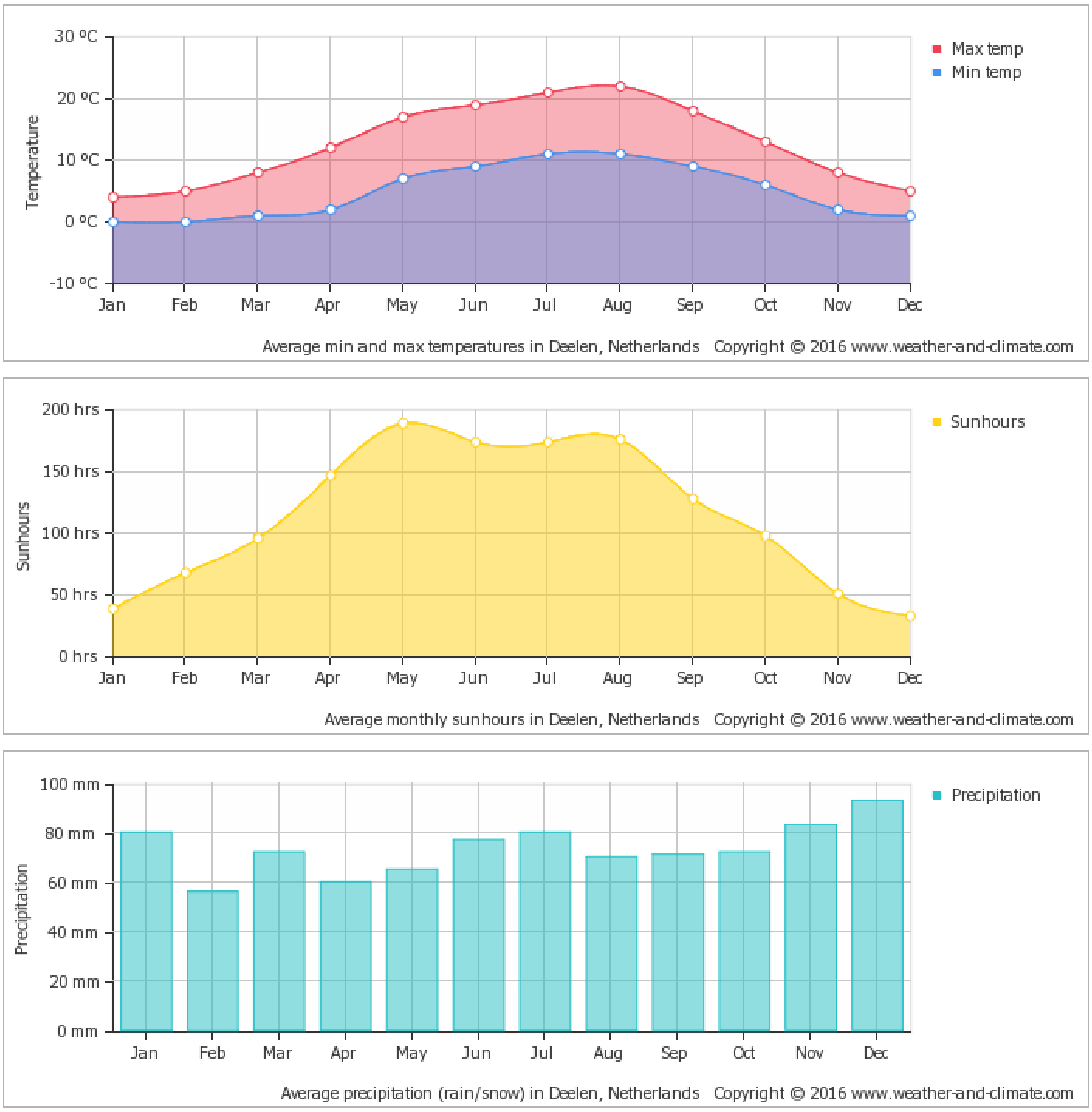
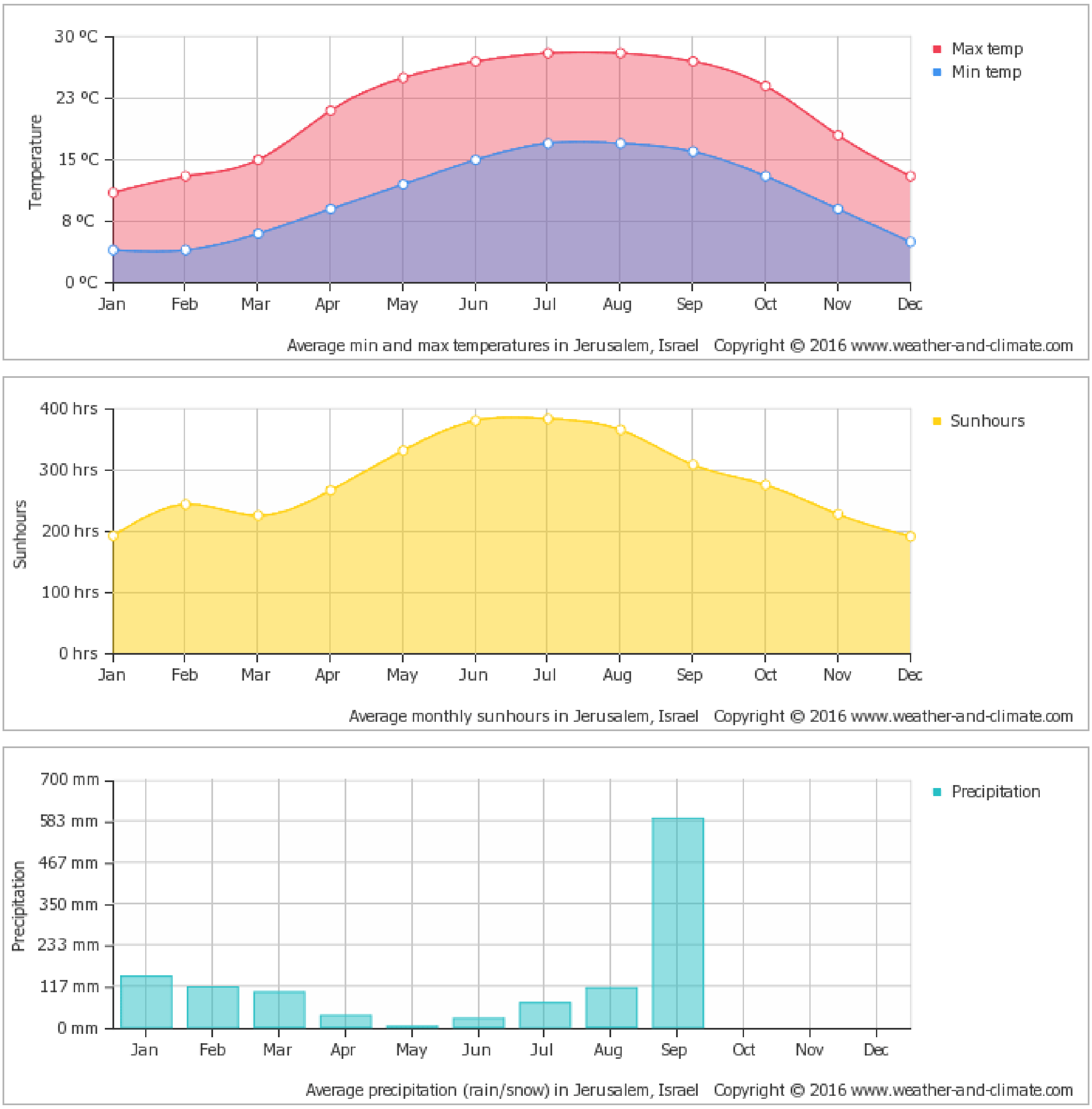


Figure 49: Average minimum and maximum temperature (Celsius), sunshine, and rainfall in Deelen (near Arnhem), Netherlands. (Weather and Climate, 2020b).

The only seed available from a seed-bank for this study was from Israel. Seed from a seed-bank was used so that any differences between fresh and stored seed could be taken into consideration. The temperature in the invaded Israel region is a little cooler than that of the native regions, being over 25°C in the summer period (Figure 50). It is similar to that in Australia where the rapid growth rate (Robert et al., 2013) and viable seed production capacity (Newman and Dawson, 1999) reflects these optimal conditions. Light levels are high, over 300 hours of sunshine during summer (Figure 50) and more than the native region. Precipitation is, however, much lower during the summer growing seasons (Figure 50).



Average min and max temperatures in Jerusalem, Israel Copyright © 2016 www.weather-and-climate.com

Average monthly sunhours in Jerusalem, Israel Copyright © 2016 www.weather-and-climate.com

Average precipitation (rain/snow) in Jerusalem, Israel Copyright © 2016 www.weather-and-climate.com

Figure 50: Average minimum and maximum temperature (Celsius), sunshine and rainfall in Jerusalem, Israel. (Weather and Climate, 2020d).

These climate comparisons would indicate that, in the European invaded regions, the current European average maximum summer temperatures have reached the minimum of *H. ranunculoides* native regions (Table 40). Sun hours are approaching the minimum summer levels in native regions (Table 40). Rainfall in the native regions is more than double that of the invaded regions (Table 40). In the invaded Australian region precipitation is less than 10mm during the summer season, so is unlikely to be a liming factor providing there is sufficient to maintain the water levels in the invaded waterbodies, but the warmth of the water may have a bearing on growth of *H. ranunculoides* (Patrick et al., 2012).

Table 40: Comparison of minimum average summer temperatures, sun hours and rainfall in *H. ranunculoides* native regions with average maximums in European invaded regions.

Influence	Native, minimum	Invaded, maximum
Temperature	17-23 ^{o}C	18-22 ^{o}C
Sun Hours	225 approx.	175 approx.
Rainfall	At least 100mm	At least 45mm

6.2.3. Climate change predictions

H. ranunculoides prefers a 5^{o}C higher temperature for optimal growth, with net photosynthesis at 25-35^{o}C, in comparison to *Hydrocotyle vulgaris* (which is the European native species) at 20-30^{o}C (Hussner and Lösch, 2007). Light saturation for *H. ranunculoides* net CO_2 gas exchange is much higher at 800 μmol photons m^{-2} s^{-1} than *H. vulgaris* 350 μmol photons m^{-2} s^{-1}. The potential distribution of *H. ranunculoides*, based on the climate in its native range is shown in Figure 51 (Robert et al., 2013). A 2^{o}C rise in temperature would bring the July and August temperatures in the UK (Figure 51) nearer to the 25^{o}C minimum required for optimal growth. There is also evidence that warmer water because of this climate change might extend the growing season for aquatic invasive species (Patrick et al., 2012).

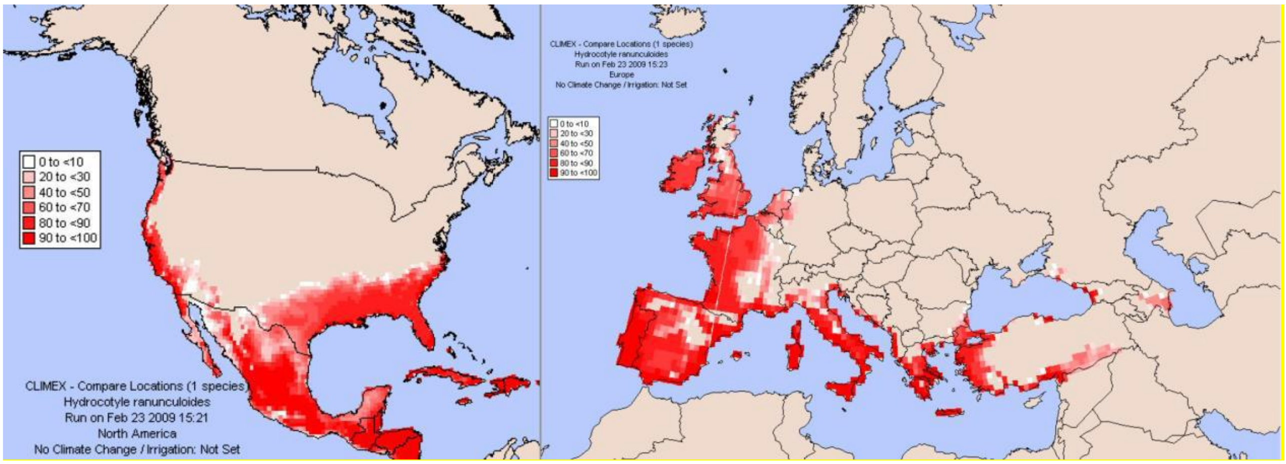


Figure 51: Potential distribution of *H. ranunculoides* based on the climate in its native N. American range (Robert et al., 2013).

The overall mean temperatures in England for the growing period in 2014 were: Spring 2.1°C, Summer 1.0°C and Autumn 1.9°C above the 1961 to 1990 averages (Met Office, 2020c). The 2015 temperatures were not as high but still above average: Spring 0.9°C, Summer 0.4°C and Autumn 1.0°C (Met Office, 2020c). However, the 2016 temperatures were also above the long-term average: Spring 0.6°C, Summer 1.3°C and Autumn 1.8°C (Met Office, 2020c). All these years trend towards the higher late season temperatures (July, August and September) that favour the growth (Hussner and Lösch, 2007) and the seed germination capacity of *H. ranunculoides* (Millane and Caffrey, 2014). So, there is potential for further vegetative expansion of the species as temperatures increase but the potential for sexual reproduction (viable seed production), in the European invaded regions, as a result of these increasing temperatures, is not known.

6.2.4. Selection of materials and seed sources

6.2.4.1. Pilot project

It is hypothesised that a 2^{o}C increase in temperature, during the growing season in the UK, will allow *H. ranunculoides* to reproduce sexually as well as vegetatively, enhancing its invasiveness and ensuring survival under fluctuating or extreme conditions due to climate change.

To inform the design of a controlled experiment to germinate *H. ranunculoides* seed, a pilot project was undertaken to establish whether any seed from the UK invaded region would germinate and gauge the approximate percentage gemination. Although *H.*

ranunculoides seed production had been observed in Europe (The Netherlands), this had not been recorded in the UK and seed viability was unknown (Centre for Agriculture and Biosciences International, 2020b). Temperature does regulate germination response in plants generally (Gillard et al., 2017a), and increasing temperature favours growth of invasive species (Gillard et al., 2017a, Gillard et al., 2017b) which could provide more favourable conditions for seed ripening. As the 2014 autumn season progressed in the UK, there was potential for the mean average temperature to be close to the predicted 2°C warming compared to the 1961 to 1990 averages (Met Office, 2019), so *H. ranunculoides* seed was collected for a germination trial (Table 41).

Table 41: Seed sample I.D., seed number, sources, and dates of collection for the pilot germination project.

Sample I.D.	Seed No.	Grid Reference	Location	Date Collected
1.	56	TQ6257806294	Hankham Gut (River), Pevensey Levels, East Sussex	31/10/2014
2.	47	SJ4052977206	Ellesmere Port Boat Museum Basin,Shropshire Union Canal, Cheshire	20/10/2014
3.	24	SJ6405770080	River Weaver, Royal Vale Locks, Old Arm, Cheshire	20/10/2014
4.	26	SJ6513468724	River Weaver Upstream, main river, Cheshire	20/10/2014

As seeds were collected by several different people, a collecting protocol was issued. Photographs of the seed heads and how to find the ripe seeds was issued to the collectors unfamiliar with the seed (Figure 52). The collectors were asked to collect shoots, with the seed heads attached,, with the ripest seed possible. The shoots should be dry and placed in clean, sealed plastic bags for posting. Shoots were requested to confirm identity of the plants. The seed samples from the three Cheshire locations, were in transit for up to a week so seed from local sites was also kept in plastic bags for one week before the seed tips were taken off and put in unsealed sterile plastic tubes to dry out. To avoid damage, the individual seeds were separated from the remaining peduncle only when dry. The seeds were then placed in dry paper, sealed, and stored at ambient temperature, in the dark, ready for the germination experiment.

Figure 52: Photograph (Birch, J.) of growing tips of *H. ranunculoides* with flowers and developing seed labelled.

The final overall mean 2014 seasonal temperatures in England were: Spring 2.1°C, Summer 1.0°C and Autumn 1.9°C (no growth was expected in the winter season) above the 1961 to 1990 averages (Met Office, 2019). Seed was collected as late as possible in the growing season, in October 2014 from four sites in the UK. This was to allow the longest period available for seed ripening (Table 42) and, if germination takes place would be the first recorded viable seed production in the UK.

Table 42 shows the UK temperatures in 2014 during the pilot study for the growing and germination periods. The maximum average temperature during the summer was still below the optimal growth temperature for *H. ranunculoides* of 25°C (Hussner and Lösch, 2007).

Table 42: Temperature during seed production, sowing and period to germination for *H. ranunculoides* in the UK for the 2014 germination pilot project (Met Office, 2020b).

Month (2014)	Max. Temp.	Min. Temp.	Seed Stage
June	18.0	9.9	Growing
July	21.2	11.5	Growing
August	18.0	9.9	Flowering
September	18.2	9.7	Seed set
October	14.3	7.9	Ripening
November	10.4	4.8	Incubation
December	7.4	1.5	Incubation
January (2015)	6.6	0.8	Germination

A germination pilot project was undertaken using seed collected in October 2014 from plants growing at sites in two locations: the Pevensey Levels, grid reference TQ 62578 06294, and the Shropshire Union Canal, grid reference SJ 40529 77206 in the UK.

6.2.4.2. Germination experiment

It has been noted that viable seed has not yet been found in the European invaded range (Robert et al., 2013). In the native regions, the average lowest minimum summer temperature in the growing season is 18°C in Buenos Aires and in New Orleans 22°C (Weather and Climate, 2020e). In the UK and The Netherlands, the average minimum summer temperature in the growing season is 10°C but the average highest maximum summer temperature is 20°C (Weather and Climate, 2020b). During the pilot project the UK summer temperature was 1°C and the autumn temperature 1.9°C above the 1961 to 1990 averages (Met Office, 2020c). An experiment was therefore designed to replicate these temperatures under controlled conditions and to determine if the predicted 2°C rise in temperature (IPCC, 2014) during the growing season in Europe allows production of viable seed. Comparing germination rates and percentages from European seed with that produced in the native region may also allow prediction of the impact rising temperatures might have in the European invaded regions as a change in reproductive capacity.

This experiment aimed to test both the influence of temperature during the seed production period, and the viability of those seeds under different germination temperatures. To do this, seed was sourced from four locations, one native and three invaded regions with different climate regimes (Table 43). These were southern USA (S.E, Louisiana) to represent the climate in the native region (as seed cannot be imported from South America where the species is thought to originate); Israel as this was the only available seed stored in a UK seedbank; the Netherlands (Wageningen) to represent mainland Europe; and the UK (from the same plant material source where the field surveys were undertaken on the Pevensey Levels). These provided a range of temperatures for the seed production period, June to August, from warmest in the native region to coolest in the Netherlands: USA (native)18-28^{o}C, Israel 15-28^{o}C, UK 10-22^{o}C and the Netherlands 9-22^{o}C.

For the greenhouse experiments, seed was collected in the UK (from the Pevensey Levels) and Europe (Wageningen, Netherlands) in autumn (October) 2016 from growing material in situ. It was also sourced from a seed bank from Israel. Seed was not able to be sourced from the native region in South America due to export restrictions (Walsh, 2016). However, it was sourced from North Louisiana, southern North America in June 2016, where it is also native, and which has a warm climate, similar to its likely original source in South America. It was collected earlier here as it becomes very rare by the end of summer due to high levels of herbivory and disease (Harms, 2017) however, the temperature during June is still high with a minimum of over 20^{o}C (World Weather and Climate Information, 2016).

The seed was folded in dry paper and labelled with collector's name, site grid reference and date of collection. Confirmation of identification of the plant material from which the seeds were collected was not requested, as all the collectors were experienced researchers or staff working on control of *H. ranunculoides*.

No collecting protocol was issued for the germination experiment as the seed was collected or supplied by researchers that were studying *H. ranuculoides*; Nathan Harms (U.S.A.), Kew seedbank (Israel), the author (U.K.) and Johan van Valkenberg (the Netherlands). The choice of germination temperatures was influenced by the climate of the autumn germination period in the native region and that of the European invaded region and is outlined in the greenhouse methods section.

Table 43: Seed sample I.D., number, sources, and dates of collection for the germination experiment.

Sample I.D.	Seed No.	Geographic Co-ordinates	Location	Date Collected
a.	200	Latitude 32^{o} 28’ N Longitude 91^{o} 29’ W	Poverty Point Reservoir, N.E. Louisiana, USA	02/06/2016
b.	60	Latitude 32 ^{o} 35’ N Longitude 35 ^{o} 29’ E	South Golan, Israel	Unknown date, from seedbank
c.	100	Latitude 50^{o} 50’ N Longitude 0^{o} 18’ E	Rickney, Pevensey Levels, UK	06/10/2016
d.	100	Latitude 51^{o} 58 N Longitude 5^{o} 40’ E	Outdoor research tank, Wageningen, Netherlands.	20/09/2016

6.2.4.3. Growth and seed production experiment

As the results of the germination experiment were unexpected, in that the seed from the European invaded regions germinated but not that from the native region, a further experiment was established. Instead of replicating the previous experiment, as it clearly showed viable seed could be produced from European invaded regions, plants from a UK invaded region were grown under different temperature regimes to investigate whether temperature during the growing period affects growth, flowering, and seed production.

An experiment was run to investigate how much seed might be produced under different climate scenarios. To investigate the influence of climate warming, as increasing temperature, during the growing season in the European invaded regions, on the growth and seed production potential of *H. ranunculoides,* plant material was sourced from the UK. One of the sites used both for the field surveys and where seed was collected for the germination experiment, namely Rickney, Pevensey Levels, was selected to allow comparisons between experiments. Plant material was collected in bulk by rake and sorted to provide 3 x 30 (90) tip cuttings (one leaf and node) with the uniform size and weight required for the three temperatures regimes.

6.2.5. Greenhouse methods

6.2.5.1. Pilot project

For the pilot project, seeds were collected by several different people, so a collecting protocol was issued. Photographs of the seed heads and how to find the ripe seeds was issued to the collectors. The seed clusters were taken from live material, in situ, with the growing tip and at least one leaf for confirmation of identity. The seed samples were placed in clean sealed plastic bags for posting. On receipt of the sample, one leaf node, with a leaf and shoot tip, was taken with each seed cluster to allow the leaf material to senesce and provide nutrient supply to the seed for final ripening.

Four clean new, 1L, plastic tubs, without drainage, were labelled with a site name and date and a circle of new blotting paper placed in the bottom of each one. All the leaf nodes and seed clusters collected from each of the four sites were then placed onto the blotting paper in their site tub with 1cm depth of previously collected rainwater and left uncovered. The tubs were then placed outdoors on 31/10/2014, on a sheltered paved area away from other vegetation, to minimize weed seed contamination, but where they could receive natural rainfall and light. They were checked weekly, to ensure they did not dry out and for signs of germination, but otherwise left to senesce naturally as the autumn progressed.

Seeds were considered to have germinated when the radicle had emerged and measured 1mm (Gillard et al., 2017a). On 3rd January 2015, one seedling and two germinating seeds were observed and placed, with the decaying plant material on which they were growing, in a new 0.15L plastic horticultural growing pot filled with John Innes No. 1 seed compost and the pot fitted with a saucer to retain water. This was placed on a cool (6^oC minimum night and 12^oC maximum day), south facing indoor windowsill and the soil was kept waterlogged with rainwater. The remaining seeds were put back onto the blotting paper in the 1L tub on the same windowsill and kept wet with 1cm depth of rainwater.

On 11th March 2015 one leaf each from seedlings 2 and 3 (the two larger germinating seeds) was collected, seedling 1 having been damaged during photography. This fresh leaf material was placed in a sealed plastic bag with damp cotton wool for transit to the

Nederlandse Voedsel-en Warenautoriteit (The Netherlands Food and Consumer Product Safety Authority) for molecular analysis. The fresh leaf material was dried, and DNA was extracted from the dry leaves following tissue disruption in a Retsch MM300 bead mill according to the manufacturer's instructions. It was then analyzed by means of the barcoding protocol using a single plastid DNA sequence, trnH-psbA described by van de Wiel et al., (2009).

6.2.5.2. Germination experiment

Building on experience from collection and identification of seed for the pilot project, it was possible to differentiate ripe from still developing seed, so the most viable seed was selected from those supplied from each region. To test germination for all populations at the current temperatures in native and invaded regions plus a predicted 2°C rise over the invaded UK region, there were four groups of five replicates per temperature regime; regime 1a – current invaded temperature, regime 2a – invaded +2°C and regime 3a – native temperature. These were placed in heated propagators set with three diurnal ranges to represent the autumn germination temperatures in the native region, in the invaded region and a 2°C rise. Regime 1a had a temperature range of 8-14°C, giving an average of 11°C. Regime 2a had a temperature range of 10-16°C, giving an average of 13°C. Regime 3a had a temperature range of 12-18°C, giving an average of 15°C.

In the warm conditions of Australia, *H. ranunculoides* is capable of producing possibly more than 9,000 seed/m^2 (Newman and Dawson, 1999) so the expectation was that germination would occur from the seed from the native region under regime 3a (average 15°C), as this should be viable seed and the temperatures during its natural germination season are replicated. Under regime 1a (average 11°C). and 2a (average 13 °C), seed sourced from the native regions might also germinate, as it would be expected to be viable, but the germination rate may decline at the lower temperatures if temperature is a limiting factor to germination. Regime 1a was below the minimum average temperature range of 13°C in the germination period in the native regions, so germination was unlikely.

The germination experiment was carried out over the period 01/05/2017 - 30/07/2017. There is little information on germination of *Hydrocotyle* species, but *H. asiatica* is recorded as taking between 60 to 90 days and *H. bonariensis* 60 days (Hackbart and Cordazzo, 2003). The pilot project suggested germination may take approximately 60 days, so ninety days was allowed in line with the existing knowledge of the genus.

Three identical, Geopod 37w horticultural propagators with controlled lighting and digitally controlled heating thermostats were used (Harrod Horticultural Ltd, 2019) to provide the climate regimes. These could regulate heating between 5–30^{o}C and with lid vents to prevent excessive condensation. Two ThermoPro, TP50 maximum / minimum thermometers per propagator were used to measure temperature. The lighting for each propagator was 2 x 6400k grow lamps producing 2,232 initial lumens specifically in the photosynthetically active radiation range, housed in a nanotech enhanced reflector, which increases the lumen availability by 300% to 6,696 (Harrod Horticultural Ltd, 2019). The day length was set at twelve hours, as this is the maximum daylength at the beginning of the autumn germination period in the native range exemplified by Buenos Aires, Argentina (Weather and Climate, 2019a).

A diurnal range was used because the literature suggests that wider fluctuations may lead to expansion and contraction of the embryo and be the signal that conditions are favourable for germination (Wagner and Oplinger, 2017, Centre for Agriculture and Biosciences International, 2020b). Maximum and minimum temperatures were recorded daily, and daily checks were done to ensure the water level remained at +0.5cm and for signs of germination.

For each of the regions from which seed was collected, fifteen new, sterile plastic petri dishes (35cm in diameter) were labelled with the site name and date and a 35cm circle of, general purpose grade, Simple Filtration filter paper was placed in the bottom. 10ml of distilled water was added, using a calibrated plastic pipette, to bring the level of water to depth of 0.5cm as the seeds have been observed to germinate on waterlogged soil or floating on water (Walsh, 2016). The petri dishes were checked daily, and water was topped up as required.

The most viable looking seed (largest and darkest in colour) were selected first and evenly distributed across all three climate regimes so that all regimes received seed of all sizes and potential viability. Six seeds were placed onto the filter paper in each petri dish (ninety seeds per source) and left uncovered. Only four seeds per dish were used for the seed from Israel as only a total of 60 seed were available per request. To test germination for all populations at the temperatures in native and invaded regions plus a 2^{o}C rise, these were split into three groups of five replicated petri dishes per population, numbered and positions randomized three times at 20-day intervals, to avoid position effects, in each propagator. The random numbers were generated using the Randomise List Function in Excel™. Each propagator (regime) was randomised separately.

To test germination for all populations at the temperature in the native region, invaded UK region and invaded plus a 2°C rise, seeds were split into three groups of five replicated petri dishes per population, numbered and with positions randomized three times at 20-day intervals, to avoid position effects, in each propagator. The random numbers were generated using the Randomise List Function in Excel™. Each propagator (regime) was randomised separately.

As with the pilot trial, the seeds were considered to have germinated when the radicle had measured 1mm (Gillard et al., 2017a). As seeds germinated, they were removed from the petri dish and placed on to new 7cm plastic horticultural growing pots filled with John Innes No. 1 seed compost and the pot fitted with a saucer to retain water. These were placed in controlled greenhouse conditions, as described for the nutrient experiments in chapter 5, greenhouse methods, and the soil was kept waterlogged with distilled water. They were monitored daily for a further 45 days, to compare the stages of development in the seed from native versus invaded regions. The stages to be monitored were emergence of the radicle, emergence of the cotyledon, true leaf production and shoot extension growth.

6.2.5.3. Growth and seed production experiment

The growth and seed production experiment was carried out over the period 26/05/2019 to 04/08/2019, to include *H. ranunculoides* peak growth period of June and July (Hussner and Lösch, 2007). Six identical, Geopod 37w thermostatically controlled, horticultural propagators with controlled lighting and heating, and lid vents to prevent excessive condensation (Harrod Horticultural Ltd, 2019) were used. There were two propagators for each of the temperature regimes. Each propagator contained fifteen, 10cm pots with one plant per pot. Two ThermoPro, TP50 maximum / minimum thermometers per propagator were used to measure temperature. The lighting for each propagator was 2 x 6400k grow lamps producing 2232 initial lumens in the photosynthetically active radiation range, housed in a nanotech enhanced reflector which increases the lumen availability by 300% to 6,696 (Harrod Horticultural Ltd, 2019).

Due to the volume of water required, tap water was used as it was readily available and meant that the plant saucers could be regularly filled up to maintain the waterlogged status required. Tap water contains a sterilizing agent, chlorine, but this evaporates from water within 24 hours. The water was stored in a galvanised metal watering can before application so that the chlorine could off-gas. The tap water had an average pH of 7.5 during the experiment and was within the field study parameters where *H. ranunculoides* was present. No alterations were made to the tap water in line with other growth studies (Nygaard and Ejrnæs, 2009).

H. ranunculoides tip cuttings (a piece of plant including the growing tip) of 0.18 – 0.22g fresh weight were used, with one node and one leaf, and two weeks were allowed for the cuttings to root. DiTomaso et al. (2013) suggest that seed is more likely to be produced from plants rooted in the substrate (rather than floating plants) so the plants were grown in a proprietary John Innes No. 2 compost (volume 0.5L per pot) suitable for growing on plants. The propagator positions were randomised twice, at four and eight weeks. The pots were checked daily, to ensure they remained waterlogged, and weekly for production of buds, flowers, and seeds and recorded for each plant. Root establishment was tested by resistance to gentle pressure to avoid disturbance from lifting out of the soil. *H. ranunculoides* is monoecious with the capacity for self-fertilisation, so isolation within the propagators was not expected to be a barrier to seed formation even if insects were not able to enter.

To test the effect of the temperature during the growing season on the ability of *H. ranunculoides* to produce viable seed, the climate of one of its native regions (Buenos Aires), that of the UK (where it is invasive) and a further 2°C rise above the UK temperature were replicated. A diurnal temperature range was used (Table 44). For the reference regime 3b, the propagator was set to a night temperature of 20°C and a day of 30°C. This was to replicate native growing conditions, and achieve an average of 25°C, the minimum for optimum growth (Hussner and Lösch, 2007). In 2014 and 2016, the years in which UK viable seed was collected, the 1.4^{o}C rise predicted by the UK Climate risk assessment RPC 2.6 range of 1.4 - 3.2 ^{o}C (Humphrey and Murphy, 2016) had already been exceeded. So, to represent this current European invaded region (2014 average 16.75^{o}C and 2016 average 17^{o}C), regime 1b was set at a night temperature of 12°C and a day of 22°C, which produced an average of 17°C. For the UKP09 medium emissions scenario of a 2°C rise (Humphrey and Murphy, 2016) the propagators were set to 14°C at night and 24°C during the day providing an average of 19°C, regime 2b. This also meant both treatment propagators were below the optimal 25°C all the time. To comply with the derogation, once the experiment finished, any remaining plant material was cut up and dried at 105°C, then burned in a furnace at 550°C degrees for 6 hours. Once this had cooled it was disposed of as inorganic material.

Table 44: Temperature regimes, for the growth and seed production experiment.

Source	Regime 1b	Regime 2b	Regime 3b
Day	22°C	24°C	30°C
Night	12°C	14°C	20°C
Average	17°C	19°C	25°C

6.2.6. Statistical methods

All data were tested for normality, and if not normal, were arcsine transformed in Excel™ prior to analysis to obtain a normally distributed dataset.

6.2.6.1. Pilot project

Final germination percentage only was calculated for this experiment as the results were principally observational data. FGP was calculated using the following equation 4.

$$\mathrm{FGP} = \mathrm{N}/\mathrm{Nl}\ \mathrm{x}100$$

Equation 4: FGP=N/NIx100 where *N* is total seeds germinated and *NI* is the total number of seeds tested (Gillard et al., 2017a)

6.2.6.2. Germination experiment

To investigate the effect of different temperatures during the growing season on seed viability, germination indices were determined. These were calculated as final germination percentage (FGP), mean time to germination (MTG) and germination index (GI). FGP was calculated using the equation 4,

$$\mathrm{MTG} = (\Sigma \mathrm{nD})/\mathrm{N}$$

Equation 5: Mean Time to Germination, where *n* is the number of seed germinated on day *D*, *D* is the incubation period until counting in days and *N* is the total number of seed germinated during the experiment (Gillard et al., 2017a).

GI was calculated according to the Association of Official Seed Analysts (Gillard et al., 2017a) (Equation 6). The GI reflects both the amount of germination and the germination rate (Gillard et al., 2017a). In this study, the maximum GI possible is 10. A high GI means that many seeds germinated early, and a low GI suggests a late germination or few germinated seeds (Gillard et al., 2017a).

$$\mathrm{GI} = \Sigma(\frac{\mathrm{n}}{\mathrm{D}})$$

Equation 6: Germination Index, where n is the number of seeds which germinated on day, and D is the incubation period until counting, in days.

To investigate the impact of the different temperatures during the growing season and the germination period, statistical analysis was done using Minitab19 software. Then, a two-way ANOVA was used with *H. ranunculoides* FGP as the dependent variable and the range of temperatures during the growing (seed source) and germination periods (propagator) the independent variables. This compares the effect of two categorical independent variables on a continuous dependent variable to establish if there is an interaction between the two independent variables on the dependent variable. Tukey's HSD test was used when P-values were significant.

6.2.6.3. Growth and seed production experiment

To test *H. ranunculoides* biomass production under different temperature regimes, DW's, RGR and R:S rations were calculated. Relative Growth (RGR) rate was calculated as RGR=(ln S2 – ln S1)/(t2 – t1). Where *ln* = natural log, *S1* and *S2* are plant dry weights (DW) at times *t1* (start of experiment) and *t2* (end of experiment) for each temperature. RGR was also tested using leaf number over the ninety-day growth period.

Root to shoot ratio (R:S) was calculated by combining the DWs of the leaves, shoots and petioles and dividing the DW of root biomass by the DW of the combined shoot biomass (leaves, petioles, and shoots) to provide the ratio R=x:S=1.

To investigate the effects of temperature on mean seed number, mean leaf number, final dry weight (DW), relative growth rate (RGR and root to shoot ratio (R:S) one-way ANOVAs were used. Any significant differences were analysed using a post-hoc Tukey grouping with a 95% confidence level. Spearman rank correlations were used to test the relationships between temperature and mean seed number, mean leaf number, final DW, RGR and R:S. Although Spearman rank does not rely on normality, it was chosen in preference to Pearson product-moment correlation because the latter evaluates linear relationships whereas Spearman evaluates monotonic relationships where variables change together (Minitab, 2019). This analysis was also used to test if there were any relationships between mean leaf number and mean seed number, final DW, RGR and R:S. A Spearman rank value of 0.5 would indicate a moderate relationship and above 0.7, a strong relationship.

6.3. Results

6.3.1. First recorded germination in European invaded regions

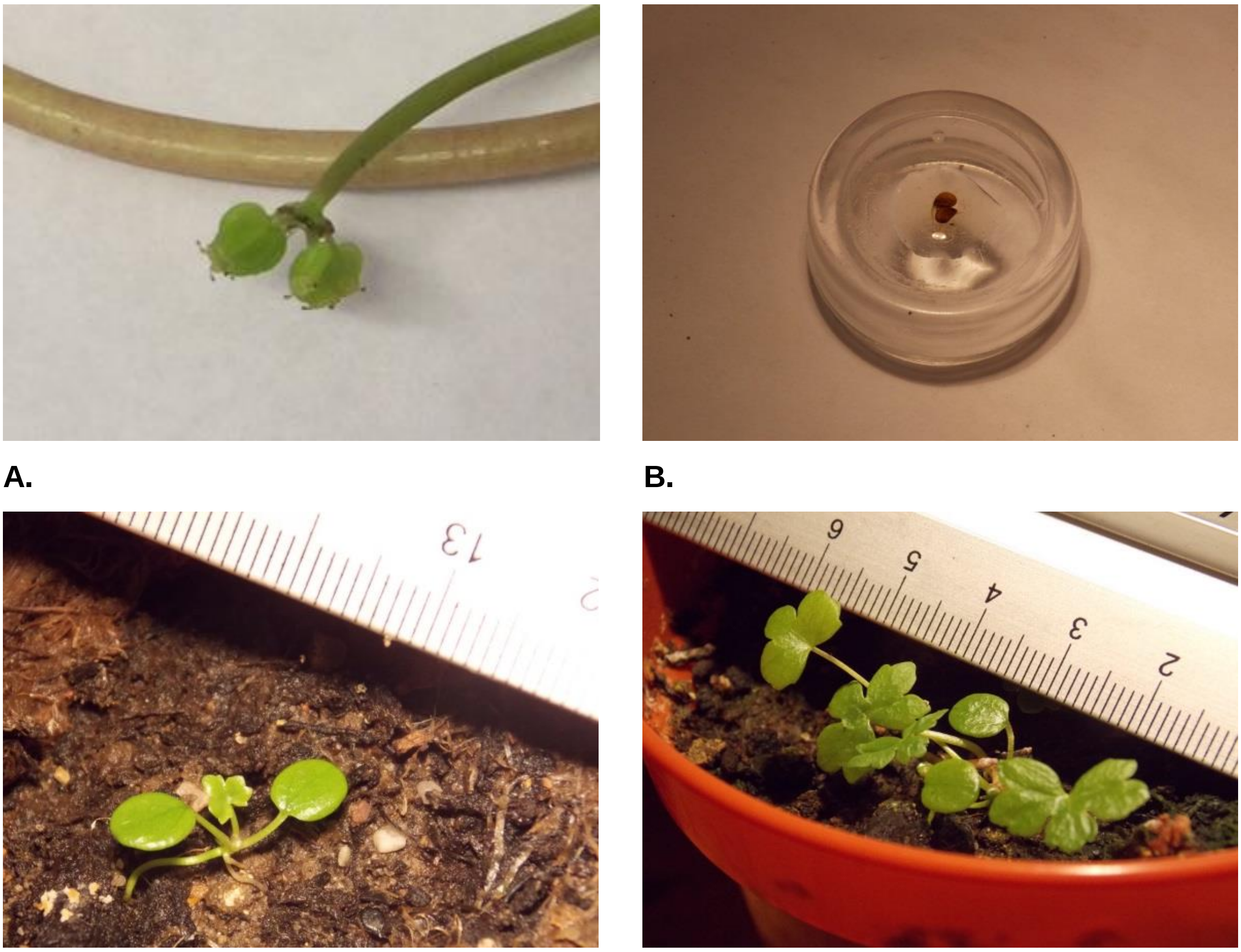
This pilot project illustrated a timeline of seed germination and seedling development of *H. ranunculoides,* collected from the UK, to show that the species can produce viable seed under an average of 1.6°C increase in temperature (over the UK 1961-1990 averages) in its invaded range. The temperatures during the incubation period were, November 2014 0-17^{o}C, with an average of 10^{o}C, and December 2014 -3-12^{o}C, with an average of 6^{o}C. This gave an overall average of 8^{o}C which is well below the native region average minimum of 13^{o}C during its germination period.

On 3rd January 2015, at 64 days (Table 45), one seedling (1.) on the decaying plant material and two germinating seeds (2. and 3.) were observed in sample site 2 from the Shropshire Union Canal, Cheshire. By 14th February 2015, at 107 days, seedling 1. had produced one true leaf but had no visible roots, remaining attached to its seed coat. The two germinating seeds (seedlings 2. and 3.) (Table 45) had both produced at least one true leaf and seedling 3. had already produced adventitious roots. On 9th March 2015, at 130 days (Table 45), seedling 3. had grown to over 4.5cm long and had produced four true leaves (Figure 53). Rooting was observed from the nodes and prostrate, trailing extension growth had begun. At 130 days, seedling 2. had produced three true leaves and was approximately 4cm long, also showing adventitious root production from the nodes. Seedling 1. became detached from its seed during photography but survived and rooted from adventitious roots, also producing three true leaves, but these were much smaller.

Table 45: Key dates for collection and timeline of the germination and establishment of *H. ranunculoides* seed collected and grown in the UK.

Date	Milestone	No. of days
31/10/2014	Collection and sowing	0
03/01/2015	Germination (2. & 3.)	64
03/01/2015	Cotyledon emergence (1.)	64
14/01/2015	Cotyledon emergence (2. & 3.)	75
14/02/2015	First true leaf	107
09/03/2015	Extension growth with 3-4 leaves	130
27/03/2015	DNA confirmation	148

The seedlings were confirmed by Johan van Valkenberg (pers. comm.) as *H. ranunculoides* on 27th March 2015 using the Van De Wiel (2009) technique. This involved the analysis of 553 nucleotides of rbcl and 331 nucleotides of trnH in the National Center for Biotechnology Information GenBank (a collection of all the most up to date DNA sequences) and the Nederlandse Voedsel- en Warenautoriteit data. The total germination was in very low proportions compared to the number of seed collected; 6.5% of the sample from site 2 and 2% overall.



A.
B.
C.
D.

Figure 53: Photographs (Birch, J.) illustrating the development of *H. ranunculoides* during the germination pilot, from seed to germination, production of first true leaf, extension growth and adventitious rooting. A. developing seed, B. seed ‘3’. germinating (radicle emergence) at 64 days, C. seedling ‘3’. (true leaf emerging) at 107 days and D. seedling ‘3’. at 130 days (in mm).

The results from the pilot project demonstrated that viable seed can be produced from *H. ranunculoides* in the UK. This is a significant outcome for this study because it is the first confirmed record of the production of viable seed production from this species in its European invaded regions, and the germination event of this species could be examined in a controlled environment.

6.3.2. Germination from native and invaded regions

No seed from the USA and Israel germinated even though all appeared fully ripened. The seed from the cooler invaded European sites (UK and Netherlands) did germinate under all three temperature regimes, albeit at very low percentages.

6.3.2.1. Final germination percentage

The FGP for the UK seeds was 2.2% (2 of 90 seeds). The UK seed only germinated under regime 2a (10-16^{o}C), the 2^{o}C rise over the UK 1961-1990 averages. The FGP for the seeds from the Netherlands was 11.1% (10 of 90 seeds). The Netherlands seed germinated under all three regimes (Figure 54). The seed from the USA and Israel did not germinate.

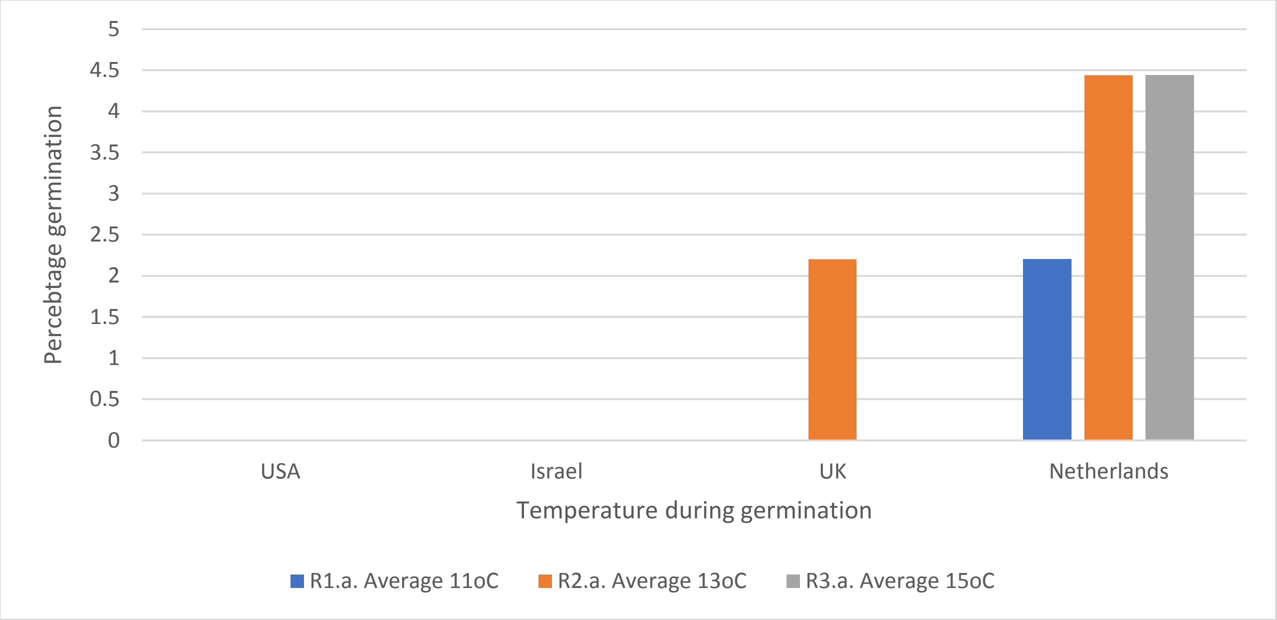


Figure 54: Percentage germination of *H. ranunculoides* seed collected from the UK and the Netherlands under different temperature regimes after a period of 77 days. Regime 1.a. 8-10^{o}C (average 11^{o}C), Regime 2.a. 10-15^{o}C (average 13^{o}C). Regime 3.a. 12-17^{o}C (average 15^{o}C). The seed from the USA and Israel did not germinate.

6.3.2.2. Mean time to germination

The first observed germination was at day 40. The MTG overall was 45 days. The MTG for seed from the Netherlands was 48 days. Only two seeds from the UK germinated and these were on days 42 and 43. This was at least two weeks slower than the pilot project under natural UK conditions but the average temperature in that study was 8^{o}C, which was 3^{o}C lower than that of the lowest regime 1a (11^{o}C) during its germination experiment. Germination slowed and appeared to cease after day 59 but then two more seeds from the Netherlands germinated on days 74 and 77. No further germination was observed, and the experiment was terminated after 90 days.

6.3.2.3. Germination index

The GI describes the germination percentage/speed relationship; the timing that most seeds germinate. In this experiment, the maximum GI possible for the *H. ranunculoides* seed from the invaded regions was 0.17, between day 52 and 59. A high GI shows that a high percentage of seeds germinated early, and a low GI suggests a late germination or few germinated seeds (Gillard et al., 2017a). 0.17 is a very low germination index score which reflects the low percentage of germination in this experiment. Given the low GI for the European seed and that the seed from the USA and Israel did not germinate a comparison of GI's between source and across the temperature regimes was not possible. Similarly, a statistical investigation of the interaction of the different temperatures, seed sources and the germination period, was not possible due to insufficient data.

All germinated seedlings survived and were grown on in proprietary horticultural compost, in greenhouse conditions, to observe establishment. For all germinated seeds, rooting was observed 2-3 days after emergence of the radicle. After 7-8 days cotyledons had emerged and at 10-12 days the first true leaf emergence was observed. By 20-23 days three or more leaves were observed and extension growth had begun.

6.3.3. Growth and seed production under different temperature regimes

6.3.3.1. Growth under three temperature regimes

Root establishment of the ninety *H. ranunculoides* plant tip cuttings took place within two weeks (tested by resistance to gentle pressure to avoid disturbance from lifting out of the soil), and all survived. The actual average temperatures achieved were, regime 1.b. – (12 - 22^{o}C) average 18^{o}C regime 2.b. (14 - 24^{o}C) average 20^{o}C and 25^{o}C regime 3.b. (20 - 30^{o}C) average 25^{o}C.

The mean leaf number of the plants, at the end of the experiment, in each of the three temperature regimes are shown in Figure 55. ANOVA gave a significant difference in leaf number between temperature regimes at P=0.017. The Tukey post-hoc pairwise comparisons showed that there were significantly more leaves in regime 3.b. compared to regime 1.b. plants. There was an increase in mean leaf number from regime 1.b. to regime 2.b. (a 2^{o}C rise in temperature) and an increase in mean leaf number from regime 2.b. to regime 3.b. (a 5^{o}C rise in temperature).

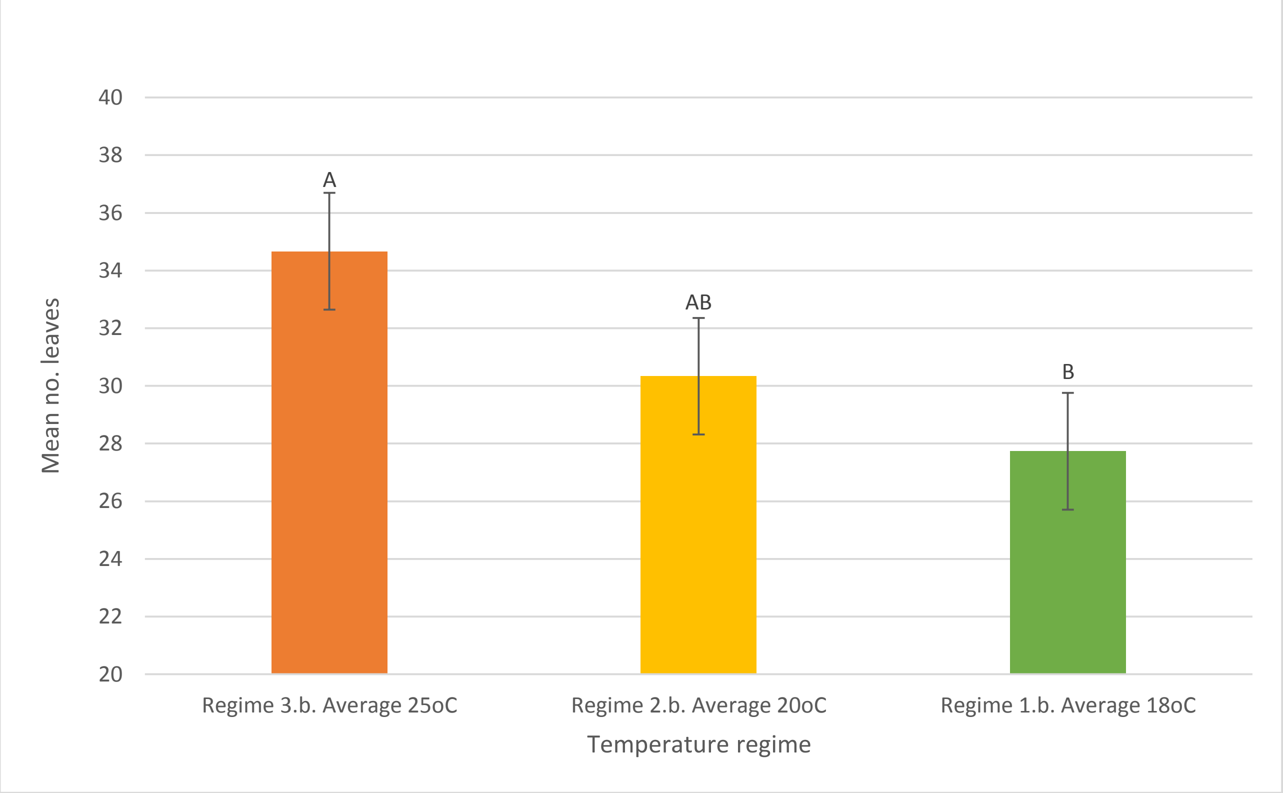


Figure 55: Growth of *H. ranunculoides* as mean (n=30) number of leaves per plant after ninety days under different temperature regimes, with standard error bars. The letters A and B represent the Tukey post-hoc test results and mean values that do not share a letter are significantly different.

The mean DWs of *H. ranunculoides* component parts as leaves, petioles, shoots, and roots are shown in each of the three temperature regimes (Figure 56). Although the differences for total biomass between treatments were not significant, there are similar trends in the data if compared to the mean leaf number data, as the temperature increases, the DWs proportionally increase. There was an increase from regime 1.b.to regime 2.b. of 6.64g and from regime 2.b. to 3.b. of 15.93g.

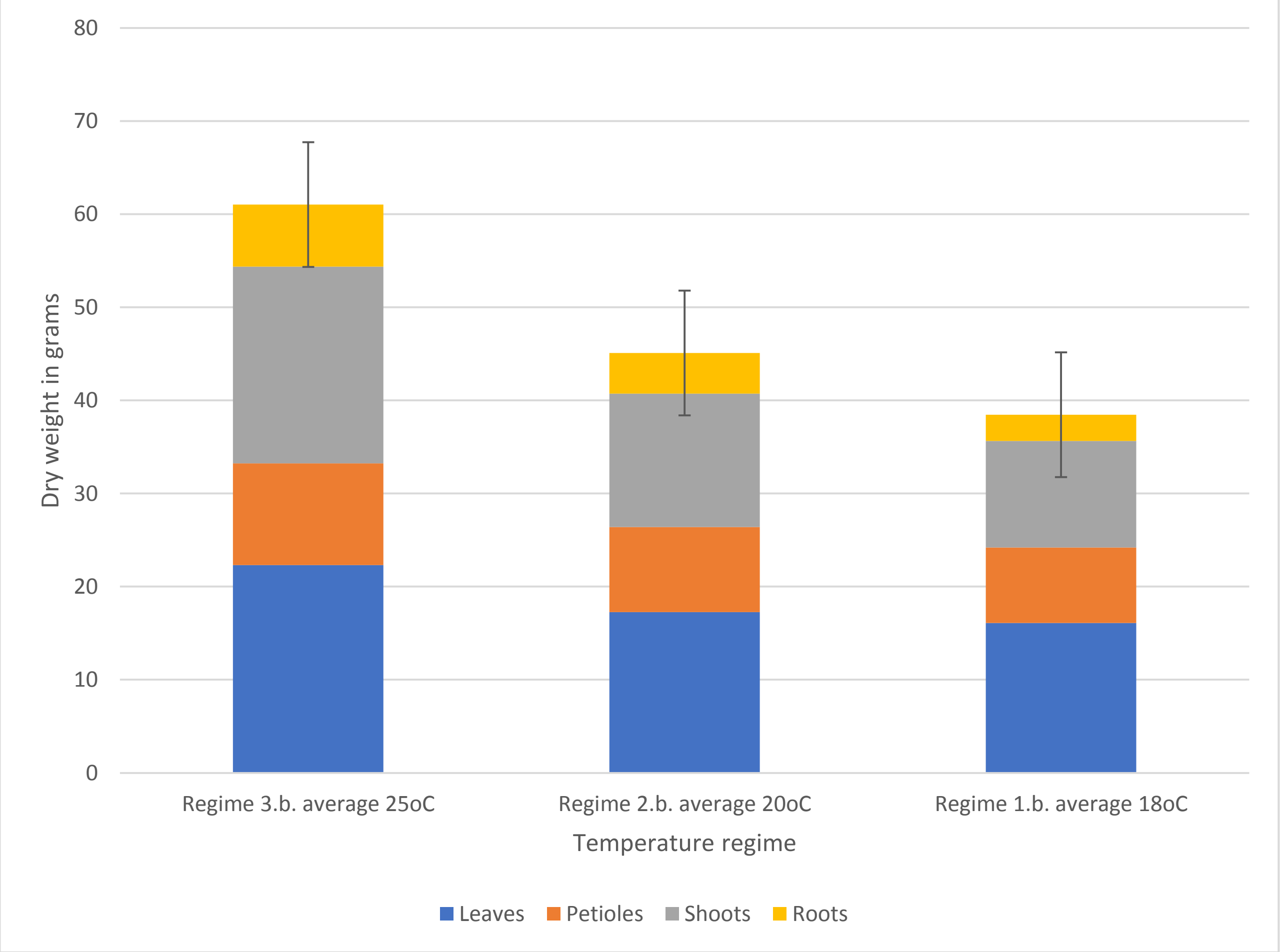


Figure 56: Mean dry Weights of *H. ranunculoides* components after 90 days under different temperature regimes, with standard error bars, n=30. The composition of the plants is shown in proportions as leaves, petioles, shoots, and roots and the standard error bars relate to total biomass values. There was no significant difference in total biomass between regimes.

ANOVA indicated that there was no significant difference in RGR between the temperature regimes with P=0.243 (Figure 57), although there was a similar trend to the number of leaves of *H. ranunculoides*, because as the temperature increased, the RGR increased.

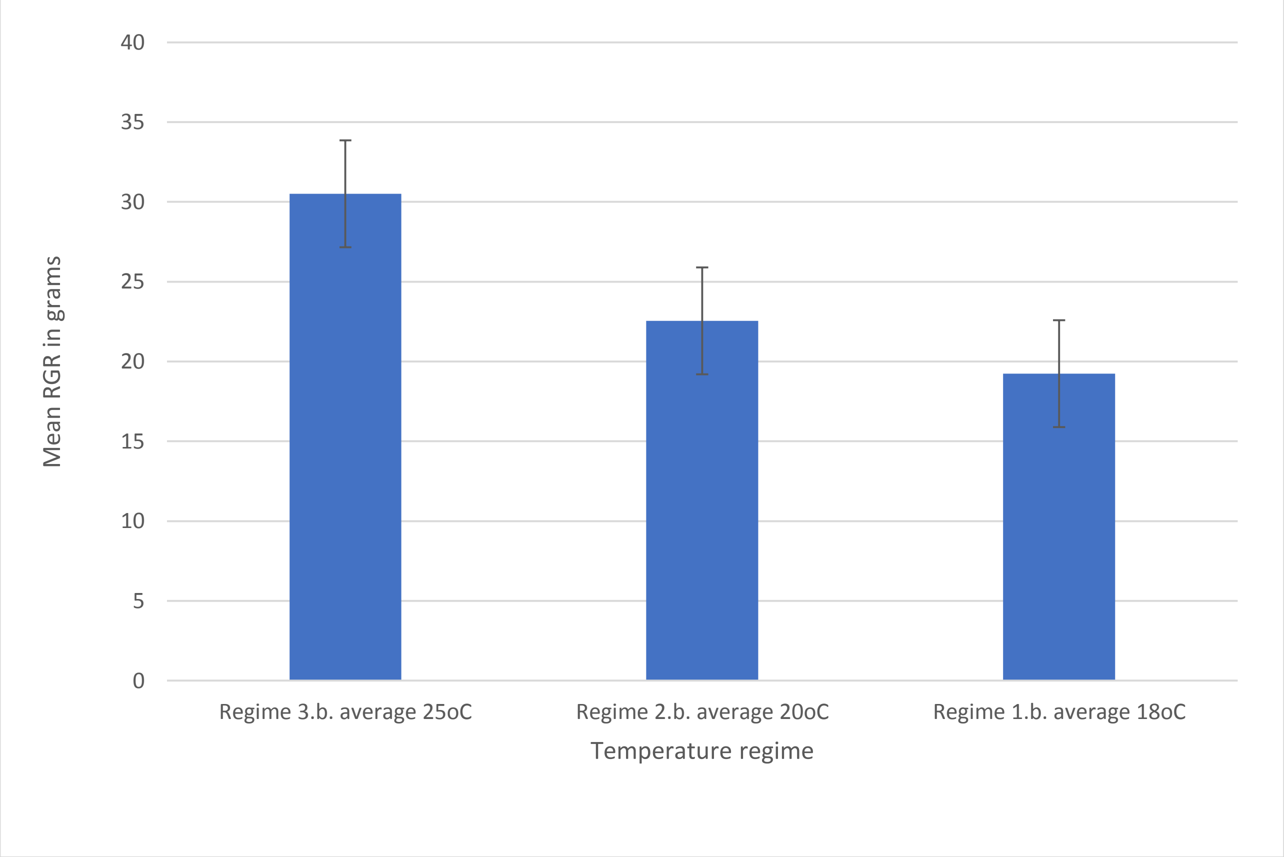
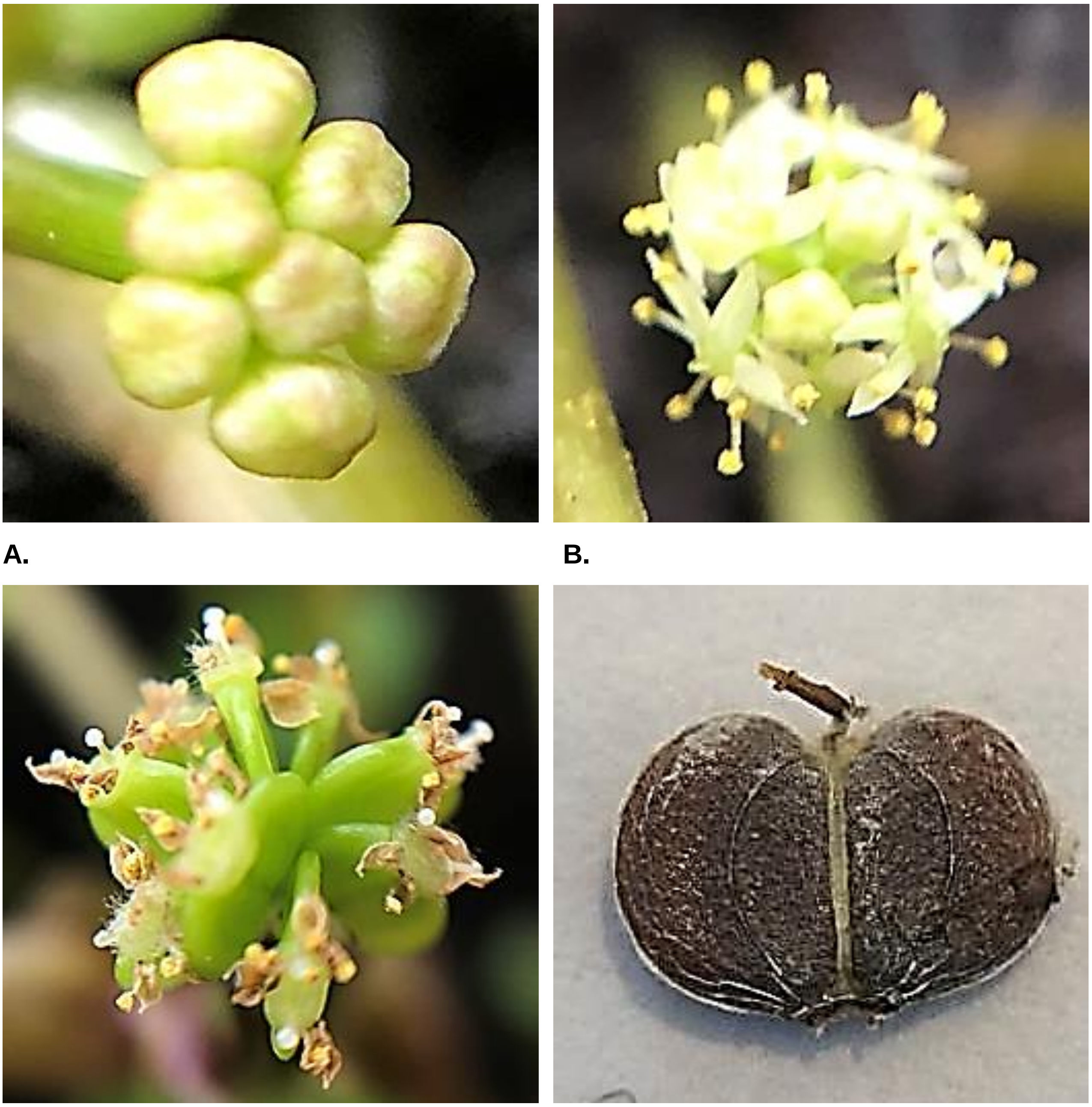


Figure 57: Mean, n=30, RGR in grams of *H. ranunculoides* after 90 days under different temperature regimes, with standard error bars. There was no significant difference between regimes, ANOVA, P=0.243.

The ANOVA results also showed no significant differences in the R:S ratio between the different temperature regimes, P=0.401, but a weak significant, positive Spearman correlations between number of leaves and temperature, Rho=375, P=<0.001 and R:S ratio and temperature, Rho=0.245, P=0.020 There was a significant, negative correlation between DW and temperature regime Rho=-0.231, P=-0.028 and R:S and temperature, Rho=-0.330, P=0.001. There were no significant correlations between temperature regime and RGR, however, Figures 56 and 57 show a trend towards increasing total DW and RGR as temperature increases.

6.3.3.2. Seed production under three temperature regimes

Buds were first observed after four weeks of growth in both temperature regimes 2.b. (20^{o}C) and 3.b. (25^{o}C) (Figure 58A). In regime 1.b. (18^{o}C) buds were first observed after six weeks. One plant was also observed to have flowers at four weeks in regime 3.b. but most of the plants producing buds had flowers from five weeks onward in regime 2.b. and 3.b., with regime 1.b. plants flowering at week seven. This pattern was consistent throughout the temperature regimes, with flowers opening a week after the buds were observed and seed formation starting a week after flowers opened (Figure 58B). Where a second flower head was produced, this occurred approximately a month later than the first.



A.

B.

C.

D.

Figure 58: Photographs (Birch, J.) of the stages of seed production of *H. ranunculoides* under controlled temperature conditions in a propagator (30^{o}C day and 20^{o}C night). A. bud formation, B. open flowers (with unopened buds in background), C. seed formation, D. ripe seed.

The mean number of seeds produced by all plants at each of the three temperature regimes is shown in Figure 59. ANOVA gave a significant difference between temperature regimes (P=0.029). The Tukey post-hoc pairwise comparisons showed that there was significantly more seed produced from regime 3.b. (25^{o}C) plants compared to regime 1.b. (18^{o}C) plants.

Plants in regime 2.b. (20^{o}C), produced more seeds than those is regime 1b(18 ^{o}C), but this was not significant. Plants in regime 3.b. (25^{o}C) produced more seed than those in regime 2.b. (20^{o}C), but, again, this was not significant.

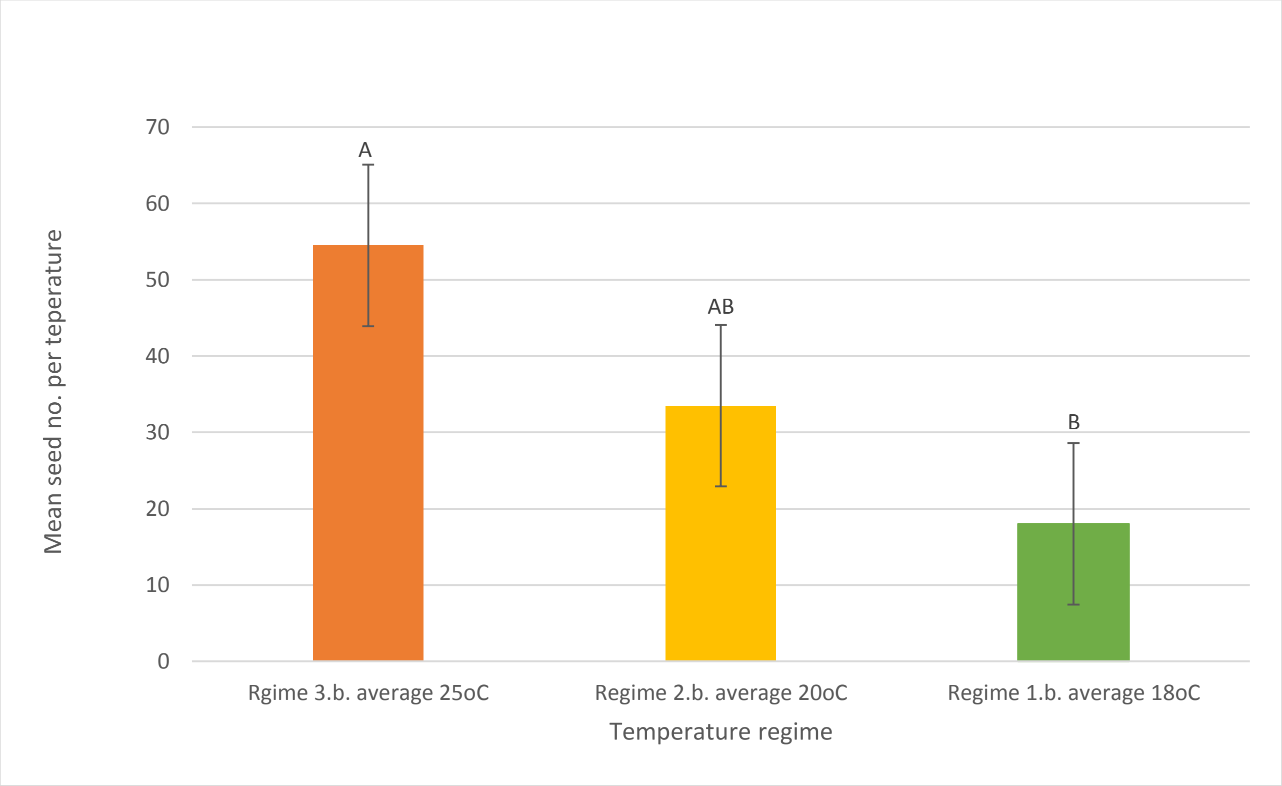


Figure 59: *H. ranunculoides* mean seed production per temperature, n=30, after 90 days under different temperature regimes with standard error bars. The letters A and B represent the Tukey pairwise comparison results and mean values that do not share a letter are significantly different.

This trend in increasing seed production as temperature rises was investigated further using a Spearman rank correlation which gave an Rho=0.655 indicating a positive relationship, which was significant at P=0.001 (n=22) (Table 46). There were no other significant correlations for seed.

Spearman correlations were also completed between leaf number, DW, RGR, R:S ratio and temperature. The correlation between leaf number and temperature, was weak but positive at Rho=0.655, and significant, P=<0.001 (n=90). A Spearman rank correlation showed a negative relationship between temperature regime and DW Rho=-0.231, and this was significant <0.028 (n=90). However, there was a weak positive correlation between R:S and temperature regime, Rho=0.245, P<0.020, n=(90). The correlation results are shown in Table 46.

Table 46: Spearman rank correlation values for associations between measured values in the greenhouse experiment growing *H. ranunculoides* for seed production. DW = dry weight, RGR = relative growth rate, R:S = root to shoot ratio. The significance of the relationship is indicated by the P-value, with significant values in bold.

Correlation between:	Rho	P-value
Leaf no. and temperature	0.375	**<0.001**
Leaf no. and mean seed no.	-0.126	0.576
DW and temperature	-0.231	**0.028**
RGR and temperature	-0.190	0.073
R:S ratio and temperature	0.245	**0.020**
Seed no. and temperature	0.655	**0.001**
Seed no. and DW	0.208	0.353
Seed no. and RGR	0.208	0.353
Seed no. and R:S ratio	0.245	0.353

6.4. Discussion

In this study the impact of a 2^{o}C rise in temperature on the seed production and germination and growth of *H. ranunculoides* was investigated, using controlled experiments with three treatments; a native temperature regime, an invaded temperature regime and a 2^{o}C rise over invaded temperature regime. The impact of climate warming on the reproductive capacity, the invasive potential of *H. ranunculoides*, and the implications for its future management are considered.

6.4.1. Seed production and germination in European invaded regions

Given that the pilot project collected *H. ranunculoides* seed naturally produced in the wild under existing climatic conditions in the UK, and that at least some of this seed germinated under current, natural winter temperature conditions, it is now known that viable seed is already being produced in the UK. The germination experiment then confirmed the low proportion of seed produced that are likely to be viable and these seeds were produced in more than one location in Europe. The study presented has established that viable seed can be produced in its European invaded regions. However, the study also shows that, under current climatic conditions, viable seed remains a very small percentage of the total potential seed *H. ranunculoides* is actually capable of producing (Newman and Dawson, 1999); 2.2% of UK sampled seed and 11.1% of Netherlands sampled seed. It is interesting to consider that the percentage germination may have been affected by the ripeness of the seed. The UK seeds all appeared only half-fertilised, that is, only one of the two mericarps had enlarged (Queensland Government, 2020) and ripened to fully brown so only 25 of 100 seed may have been capable of germination. The remaining seed being either still un-ripe (green) or not large enough to have sufficient resources to germinate. For the Netherlands seeds, approximately 50 of the 100 seed appeared fully ripe. If all the seed from the invaded regions had been fully ripe, the UK seed germination percentage could have been 8.8% and that of Netherlands 22.2%, which is a much higher percentage.

The seed production experiment illustrated that increasing temperatures along a gradient towards those present in its native regions produced an increase in the number of seeds. Interestingly, the seeds from the Netherlands (which has a slightly cooler summer than the UK (Weather and Climate, 2020b, Weather and Climate, 2019b), gave a higher percentage germination than that of the UK, which endorses the finding that seeds can already be produced in Europe and a projected 2^{o}C rise in temperature is likely to improve the percentage of viable seed produced in Europe.

The results show that as temperature increases the mean leaf number, dry weight, relative growth rate and mean seed number increase. Although significant results were obtained, the exact temperature rise at which a significant increase in mean leaf number, or mean seed production, occurs was not detectable. The increased leaf number and seed number were correlated with temperature, which is likely to be an important driver, however, an increase in this one environmental factor (in isolation) may not be sufficient to drive the seed production alone. These results were from plants grown in a system where nutrient input was fixed, so total biomass may have been limited by the nutrient availability at the higher temperature (Barko and Smart, 1981) due to the higher relative growth rate, although neither were significant. So, it is possible that nutrient limitation may have been a factor in there being an insignificant increase in overall biomass and mean seed number from 20^{o}C to 25^{o}C. Nevertheless, this suggests that an increase of 2^{o}C is likely to improve the chances of viable seed production still further.

All collected seed was sown in the spring of the year following collection so it could reasonably be expected to have some germination, if viable. The Israeli seed, which did not germinate, had been stored in a seedbank prior to receipt and the date of collection and length of storage was not known. This seed may well have been affected by the duration and conditions of storage. The reason for non-germination of the native seed from the U.S.A. is not known. Potential influences could be the that it was irradiated during transport through customs, affected by cabin pressure during air transport or the length and conditions of storage. This seed was stored from the spring (June) when it was collected, until sowing in May the following year compared to the European seed which was collected in autumn (late September and early October). However, the conditions of storage from date of collection to date of receipt was not known. All seed, once received, was stored in dry, dark conditions between 5-15^{o}C, similar to the autumn / winter conditions in the native regions (New Orleans) (Weather and Climate, 2019a), prior to sowing. Considering that the European seed was stored dry for a period, and germinated, dry storage for six months (November to April) does not seem to prevent germination. The UK pilot seed was not dried, and this produced a 2% germination (very close to the main germination experiment of 2.2% for UK seed). Bakker et al. (2013), studying restoration of macrophytes in shallow temperate lakes found that seed longevity was very short in the 13 studied macrophyte species, none more than one year. However, further work would be needed to investigate seed viability over time in *Hydrocotyle* species.

From these studies, it may be possible to infer that a rise in air temperature has contributed to the ability of *H. ranunculoides* to reproduce sexually in western Europe, albeit in very low numbers compared to the potential numbers recorded in its native regions or that of regions with similar temperature regimes (Newman and Dawson, 1999). It appears plausible that further climate warming may exacerbate this.

6.4.2. Impact of sexual reproduction on *H. ranunculoides* invasive capability

The ability to reproduce sexually as well as asexually confers additional benefits to a species with regards to establishment, survival and persistence (Li, 2014). Asexual reproduction, in the case of *H. ranunculoides,* is a process of fragmentation and dispersal, and facilitates rapid expansion and colonisation of the immediate surroundings and downstream stretch of water using these vegetative ramets (Robert et al., 2013). However, sexual reproduction facilitates genetic diversity leading to variation (Lei, 2010).

This adaptability is an evolutionary advantage leading to resilience to dynamic changes from extreme events, (Li, 2014) disease and chemical resistance but it could also improve invasive potential as it is an additional method of dispersal. Seed is much more easily dispersed either by hydrochory, birds, animals or on clothing, machinery and equipment being used to control *H. ranunculoides* than vegetative ramets, as they are much smaller and require better biosecurity. This could facilitate invasions in new locations and those further away than vegetative ramets are capable of travelling. Whereas vegetative reproduction produces clearly visible mats of vegetation, the small seed is extremely difficult to find and young seedlings hard to identify so new plants can establish very quickly before control methods can be used.

Reproductive allocation in aquatic plants is not well studied but Li (2014) notes that seed production is proportional to total biomass and it has been suggested that seed is more likely to be produced from plants rooted in the substrate than floating plants (DiTomaso et al., 2013). This study on *H. ranunculoides* concurs with this, as the number of seeds produced increased with biomass, but future studies could compare seed production from rooted plants (as in this study) with free-floating plants under different temperature regimes.

Growth under the different temperature regimes indicates that, other than increased mean leaf and seed number, there was no significant alteration in root to shoot ratio. So, the increase in temperature seems to be the main factor affecting seed production. This is an interesting concept in relation to *H. ranunculoides* because its phenological adaptation to drainage conditions results in a reduction in above ground biomass (Hussner and Meyer, 2009). Thus, although *H. ranunculoides* plants (which root in the substrate) would be more vulnerable to stressors such as fluctuating water levels, and drought, the resultant reduction in biomass would imply it is less likely to produce seed. A shift in resource allocation to sexual reproduction could be an investment, such as to ensure long term persistence, or an adaptation to disturbance and stress like an increase in the frequency of extreme events triggered by higher temperatures (Havel et al., 2015).

Aquatic plant seed productivity is less than for terrestrial species so less energy may be available for the energy-expensive sexual reproduction process (Li, 2014). Sexual reproduction requires the additional creation of floral and reproductive structures (Lei, 2010), which, unless adapted by hydrophily (Du and Wang, 2014), need to be produced above water, a method *H. ranunculoides* employs. Once seed is produced, germination, seedling establishment and growth are all very susceptible to biotic and abiotic influences (Lei, 2010). Thus, there needs to be some ‘reward’ for the additional investment. Whilst not being as rapid, or potentially as extensive a method of reproduction as vegetative, benefits of seed production include genetic diversity and improved ability to disperse to new locations. This could be by hydrochory but also by mechanical / human and animal transport. Although all these dispersal methods can be applied to the vegetative ramets, *H. ranunculoides* seed is much smaller than its vegetative ramets and more easily transported by water, assimilated into mud, onto machinery, equipment, or animals, and is likely to be more persistent. Regular vegetative reproduction leads to depletion of genotypic variability and is likely to lead to susceptibility to pests, diseases and environmental stochasticity over time (Lei, 2010) so the ability to reproduce using both types of reproduction is a distinct advantage to *H. ranunculoides’* invasive capability.

As all the *H. ranunculoides* vegetative plant material was obtained from the same source it is highly likely to be the same genetic composition. In addition, the experimental propagators were isolated for most of the time so there was minimal opportunity for insect access for pollination, so it is very probable the *H. ranunculoides* flowers are dioecious and self-fertile. Being self-fertile does not exclude the ability to cross-pollinate, but to utilise the full benefit of sexual reproduction, there needs to be cross-pollination and genetic variation in populations. Future work on molecular analysis of plants across the UK and Europe might give an insight into the extent of current genetic variability and potential diversity.

6.4.3. Invasive potential of *H. ranunculoides*

Figure 60 illustrates the key plant invasive species traits required by a non-native species; a very rapid growth rate, adaptability and extremely efficient vegetative reproduction as well as resistance to chemical control and winter quiescence, all of which *H. ranunculoides* exhibits (Robert et al., 2013).

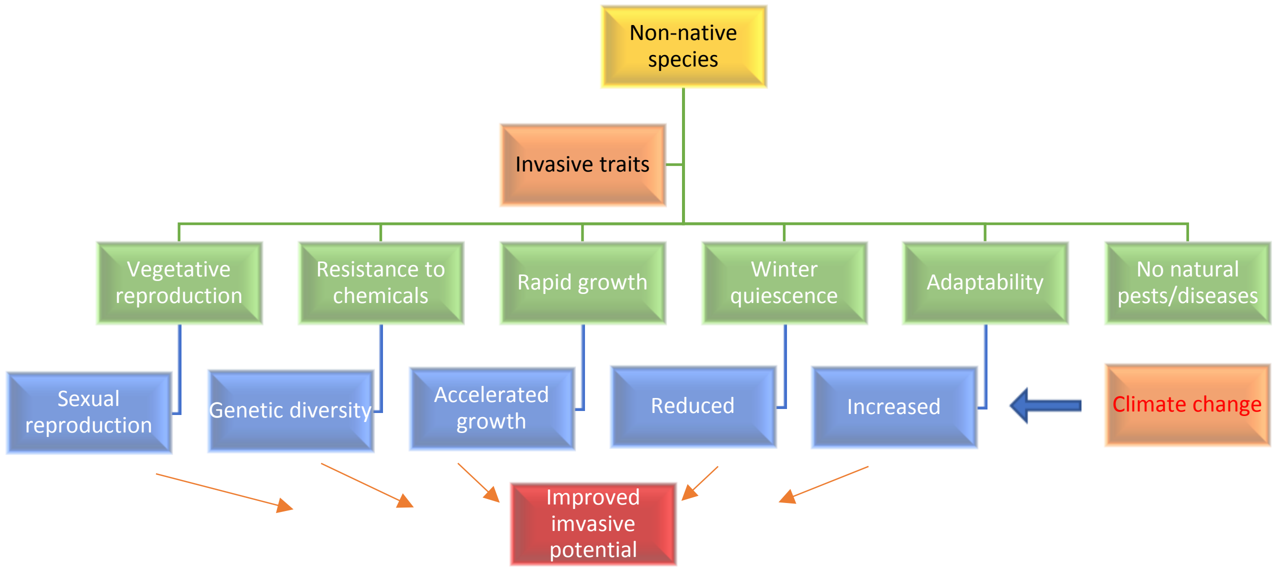


Figure 60: Plant invasive species traits (in green) and how the non-native species' response to climate change (in blue) might ameliorate their invasive potential.

Biological invasions are linked to global change (Arim et al., 2006), with climate warming increasing water temperature and increasing the growth rate of invasive aquatic plant species, giving them a more competitive edge (Patrick et al., 2012). Figure 60 illustrates how climate change in the form of rising temperature might confer benefits to plant invasive species.

Sexual reproduction may to lead to a more genetically diverse and resilient population of *H. ranunculoides* (Figure 60), allowing the species to recover from extreme events, which are an increasing feature of climate warming, and adapt to chemical control methods through resistance. Currently, although it is able to survive periods of drying and lowering of water levels, it adapts by reducing biomass (Hussner and Meyer, 2009). Increased temperatures as result of climate change could also lead to periods of drought and reduction in summer flows. Genetic diversity could favour selection of plants better able to survive these drier conditions, or grow more effectively during them, permitting extension growth to re-connect with the water and continue spreading. Sexual combined with vegetative reproduction means *H. ranunculoides* now has two methods available to establish, colonise and spread in Europe. This may confer the ability to utilise different ecological conditions that vegetative reproduction alone could not. Seedlings could establish in areas cleared of all vegetation where no vegetative ramets remain. *H. ranunculoides* has been noted as exhibiting patch dynamics in its native regions, where its behaviour as an opportunistic annual cannot be ruled out (Walsh et al., 2013). Hence, the ability to reproduce from seed would allow colonisation of new sites periodically throughout the season even in areas where vegetation is not routinely disturbed either by management or other natural methods. The extent to which this could occur would not be known until seed viability over time is established.

Genetic variability could also lead to selection of strains resistant to chemical herbicides (Figure 60). Currently only one chemical is permitted for control of vegetation in or near water in the UK and Europe, Glyphosate, in various formulations (Centre for Agriculture and Biosciences International, 2020b). *H. ranunculoides* is already suspected of having some tolerance to glyphosate, translocating it poorly through its system and being able to excrete the chemical through its roots (Centre for Agriculture and Biosciences International, 2020b). Thus, further resistance to this chemical might eliminate one method of controlling *H. ranunculoides*.

Hussner and Lösch (2007) showed that optimum photosynthesis in *H. ranunculoides* occurs above 20°C and this study shows warmer growing conditions are likely to lead to more rapid growth. As *H. ranunculoides* is already able to out-compete native flora in its invaded regions, the ability to grow even faster would put additional stress on the native plant communities, enable faster colonisation of new areas and more rapid spread. This, combined with a shorter quiescence period (Figure 60), would mean an earlier start and later finish to the growing season. Chapter 4 discussed the current impact of *H. ranunculoides* on native macrophyte communities, and this longer growing season may mean that the early season native plant species surviving in the infested areas may not have their current window of opportunity to grow and maintain presence, even at the lower levels of *H. ranunculoides* found in the surveys. The implications of this are a potential change in the plant communities and possibly loss of some native plant species because of *H. ranunculoides* infestation.

6.4.4. Implications for *H. ranunculoides* management

The implications for the future spread and management of *H. ranunculoides* in Europe will need reviewing. Climate change, as an increase in temperature, could lead to more rapid growth, shorter periods of winter quiescence, regular seed production and increased adaptability (Figure 60). These results question whether existing management methods are sustainable and resilient to climate change. The methods most used to control *H. ranunculoides* are mechanical and chemical, although manual and environmental techniques are used on smaller populations (Duenas and Newman, 2010) and biological control is under development (Centre for Agriculture and Biosciences International, 2020a).

H. ranunculoides already has a very rapid growth rate, even in its invaded regions, but a further increase in growth rate would mean mechanical control methods are likely to be less effective in controlling the spread and more frequent interventions may be required. Additionally, a warmer climate with shorter cold periods could lead to a reduced winter quiescence period, effectively lengthening the growing season and further impairing control efforts. Additional mechanical and chemical control would have cost implications which, if not met, is likely to allow further spread and additional impact on native plant communities and their associated aquatic fauna.

Sexual reproduction may lead to a more genetically diverse and resilient population, which could allow *H. ranunculoides* to recover from extreme events (an increasing feature of climate change) and adapt to chemical control methods through resistance. Eradication of new, small, and localised infestations may no longer be effective if seed is present, particularly as seed viability and longevity is unknown. Current timing of mechanical control may need reviewing. Spring and autumn growth are less likely to produce viable seeds as the warmer temperatures needed for seed ripening are unlikely to be present. Early spring chemical control, providing growth is enough for absorption of Glyphosate, could target seedlings that mechanical control would miss, as well as controlling young vegetative growth. However, if genetic diversity leads to chemical resistance control using herbicides will no longer be an effective option. Chapter 4 suggested that mechanical control alone was ineffective, and potentially damaging to native plant communities, so the loss of the chemical control option may have serious consequences for the management of *H. ranunculoides* in its invaded regions and permit much more extensive invasion.

Later season mechanical clearance may reduce overwintering biomass and retard spring vegetative re-growth, but it may expedite germination of any seeds already produced and dispersed by exposing bare substrate and providing light (Clout and Williams, 2009). Other riparian management activities to control *H. ranunculoides* such as de-silting and re-profiling would be ineffective if seed were present and may promote spread, especially if sediment were taken off-site for disposal. So, although vegetative reproduction will likely remain the principal method of spread, management efforts will need to consider how to prevent or reduce viable seed formation to limit further range expansion and establishment.

6.5. Conclusion

The aim of this study was to examine the potential impact of the UK Climate Projections prediction of a minimum rise in 2^{o}C during the growing season and germination period on the reproductive capacity of *H. ranunculoides*. Since the inception of the IPCC in 1988 all three subsequent UK decades have had more high temperature records than low (Met Office, 2020a) indicating an increase in temperature has already been experienced. Any findings linked to a warmer climate are likely to be experienced in Europe in the coming decade. Effective invasive species management depends on a comprehensive knowledge of its ecology. So, filling the knowledge gap regarding the germination dynamics of *H. ranunculoides* and how it responds to a warming climate will inform management methods and invasive species risk assessments.

These results have confirmed that sexual reproduction of *H. ranunculoides*, as viable seed production and germination, can occur under a 2°C temperature warming scenario for Europe. Germination of seed from European invaded regions was demonstrated under natural and controlled conditions. *H. ranunculoides* seed collected from wild UK populations was observed to germinate under natural winter conditions. Germination capacity of four *H. ranunculoides* populations were tested at average temperature regimes of 9°C, 11°C and 13°C. Only two of the tested populations germinated, reaching a final germination percentage of 2.2% for UK seed and 11.1% for Netherlands seed. There were insufficient results to determine if temperature influenced final germination percentages or time to germination. As seed from two of the sources did not germinate there were no results to compare germination from different populations of *H. ranunculoides* from native (USA) and invaded regions (Europe). However, *H.*

ranunculoides' ability to produce viable seed in the European invaded regions is an important finding both regarding its invasive potential and for effective and sustainable management.

There was a significant correlation between increasing temperature and increased leaf number and increased seed number under controlled conditions so there are indications that rising temperatures will influence these factors. Increasing the vegetative regenerative growth of *H. ranunculoides*, in its invaded regions, further increases its invasiveness and combined with the ability to produce seed, may facilitate survival under fluctuating or extreme conditions due to climate change. Increased growth would also pose additional problems for management and make eradication less likely. There may also be implications for other plant traits that *H. ranunculoides* possesses, regarding climate warming and its invasive capacity.

Chapter 4 discussed the impact of *H. ranunculoides* on native macrophytes communities and the effectiveness of management under current climate conditions. Chapter 5 investigated the effect of nutrients and pH on the growth potential of *H. ranunculoides.* This chapter has assessed the influence of climate warming on the reproductive and invasive potential of *H. ranunculoides.* Chapter 7 will synthesise these results and further consider the implications of climate change and nutrient effects. It will also reflect on the novel contribution to the existing body of knowledge, future research opportunities, implications for wetland conservation and practical applications for invasive species control.

Chapter 7 - Discussion

7.1. Introduction

This chapter integrates the findings from the previous three results chapters and discusses how they address the overall aims of the thesis. The chapter critically assesses the practical aspects of the research and reflects on how the study might influence future research directions. It concludes by considering the applications of the research, implications of future spread of *H. ranunculoides* and for native macrophyte conservation.

7.2. Key Results

7.2.1. *H. Ranunculoides*, plant community composition and management

In chapter 4 the results from field studies on Pevensey Levels were reported. This included annual surveys of 60 ditch sites for four years from 2013 to 2016, and monthly surveys of eight sites (May to October) for three years, 2014 to 2016, which included those with different levels of *H. ranunculoides* infestation, six management treatments and un-infested sites.

It was found that *H. ranunculoides* infestation of >50% cover altered community composition by reducing native macrophyte diversity index values but not species richness. Using species richness as a measure, Stiers et al. (2011) study on three INNS, found that >50% INNS infestation reduced submerged species by 94% and floating species cover by 68%. For *H. ranunculoides* the reduction in species richness was an average of five species in uninvaded plots to 2.5 species in semi-invaded plots (25% cover) to 1.5.species in heavily invaded plots (>75% cover). Stiers et al. (2011) did not investigate community composition but both these results indicate that >50% cover is the threshold at which impact occurs. Stiers et al. (2011) only sampled macrophytes during one month, in August 2007 and 2008, but this study sampled over a four year period, and over six monthly periods, to ascertain the level of impact over the whole growing season and not just when *H. ranunculoides* was most abundant. Highest levels of *H. ranunculoides* infestation were recorded in September which resulted in significantly lower abundance of native macrophytes that require warmer, late season, conditions (Patrick et al., 2012, Zhang et al., 2015).

L. trisulca and *H. morsus-ranae* were two species particularly affected (Joint Nature Conservation Committee, 1999) but others that make up part of the assessment criteria for WFD status were also impacted, *B. erecta, P. lucens* and *S. erectum* (Water Framework Directive, 2014). The snail, *A. vorticulus*, for which the Pevensey Levels is designated a SAC, is at risk due to NVC changes from *H. ranunculoides* infestation because the macrophytes *L. trisulca* and *H. morsus-ranae* form part of the community it depends on. This snail species is also at risk from habitat destruction, as the late successional communities it inhabits are unable to develop under a constant annual weed-cut regime (Natural England, 2017).

Mechanical clearance (weed-cutting) of H. ranunculoides did not effectively control it and it is postulated that it contributes to further spread by fragmenting the plant which produces many viable, vegetative propagules (Newman and Duenas, 2010). Weed-cutting, followed by a glyphosate-based herbicide application did reduce abundance of H. ranunculoides but no method eliminated the plant from the infested sites. Weed-cutting alone and weed-cutting and de-silting also impacted on native macrophyte diversity. The most effective management treatment, weed-cutting and herbicide spraying did not affect native macrophyte species diversity. Newman and Dawson (1999), found that the herbicide 2,4-D amine was more effective in controlling H. ranunculoides than glyphosate but 2,4-D amine was withdrawn from use and glyphosate only has temporary approval to 2022 (Health and Safety Executive, 2020). This raises concerns over the efficacy of future control measures as biological control, which is likely to be a sustainable method, is not yet available (Centre for Agriculture and Biosciences International, 2020b).

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7.2.2. Effects of nutrients on *H. ranunculoides*

The field studies also provided information to determine the role that nutrients play in the growth and development of *H. ranunculoides*. These, in combination with the results from the greenhouse experiments using controlled levels of nutrients were reported in chapter 5. The field and greenhouse results showed several common features but there were also conflicting results which indicated alternative stoichiometric influences and potentially altered ecological conditions. Both phosphate and nitrogen influenced growth, but in different ways, and the effect of pH on the abundance of *H. ranunculoides* and its utilisation of these nutrients was examined. Combined higher levels of nitrogen as ammoniacal nitrogen and nitrate reduced relative growth rate compared to control plants.

The biomass of roots, an increase of which may enhance survival potential as a strong root system favours persistence (Christiansen et al., 2016), was the plant growth metric that responded positively to the nutrient phosphate.

This study found a positive association with *H. ranunculoides* and nitrogen in water, which agrees with the findings of Fried et al. (2009) and Basilico (2017). Barko et al. (1991) observed that nitrogen was readily taken up by macrophytes from sediment as well as water and that the macrophytes studied preferentially obtained phosphate from sediment. This study agrees with Barko et al. (1991) that there is a positive association with phosphate in sediment, which promoted root growth in the nutrient experiment. The results from the field studies indicated a negative association between *H. ranunculoides* and phosphate in water, which is interesting because, during summer, *H. ranunculoides* develops large floating rafts with dense mats of roots suspended in the water column (Centre for Agriculture and Biosciences International, 2020b). However, pH can affect phosphate uptake and many of the field sites were above optimum pH for phosphate uptake (Ullrich-Eberius et al., 1981). So, *H. ranunculoides* may not be able to obtain phosphate from the water under these high pH conditions leading to lower abundance. However, as discussed in chapter 5, 5.2.2., *H. ranunculoides* may be compensating for this difficulty by utilising ammoniacal nitrogen to help increase the phosphate uptake (Rayar and Van Hai, 1977), a suggestion supported by the positive association found between *H. ranunculoides* and ammoniacal nitrogen in water in two of the study years.

Based on these results, it would be better to prevent phosphate entering the watercourse in the first case. In the case of the Pevensey Levels, phosphate stripping has already been introduced at the two wastewater treatment works feeding into the system. However, phosphate can enter the catchment in a variety of other ways, the use of fertilisers both for arable crops and to improve grazing pasture, allowing livestock to graze near, and drink from, watercourses and allowing the removed cut vegetation to decompose on the bankside which returns the nutrients to the water in run-off. Changing farming practises such as reducing fertiliser use, using cover crops to reduce winter run-off (and add nitrogen), leaving vegetated buffer strips and fencing watercourses to exclude stock are some options.

7.2.3. Climate change and germination

Chapter 6 considered the response of *H. ranunculoides* to the possible effects of climate change through a rise in temperature. The ability of *H. ranunculoides* to produce viable seed in its European invaded regions was demonstrated under current climatic conditions. The effect of a 2^oC average rise in temperature during the *H. ranunculoides* growing period was increased seed production as temperature rose. Marinho et al, (2010) demonstrated that emergent macrophytes contribute to nutrient enrichment in the limnotic zone with regard to both nitrogen and phosphorus as a result of carbon accumulation. This study supports these findings as the 2^oC rise in temperature increased overall biomass of *H. ranunculoides*. There were unexpected results from the germination experiment that did not permit comparison of germination from the invaded regions with that of seed produced from the native region. However, the novel evidence that *H. ranunculoides* can produce viable seed and germinate in the European invaded regions is important for predicting the future invasive capacity of the species and for the potential control and eradication of the species.

The ability of *H. ranunculoides* to establish and perform effectively under conditions currently regarded as sub-optimal regarding temperature (Hussner and Lösch, 2007) for the species is suggested. However, Van Kleunen et al. (2010) noted that key traits for invasiveness are performance related citing leaf area, shoot allocation and growth rate. The results from the growth and seed production experiment suggest that increasing temperatures do facilitate production of more leaves, an increase in shoots and growth rate so enhancing *H. ranunculoides* invasive capacity. Arim et al. (2006) suggest that successful invaders can minimise the lag phase during establishment, so increasing temperatures which facilitate a better growth rate might allow *H. ranunculoides* to reach its propagation and dispersal phase faster.

7.2.4. Future management

Given that the higher levels of *H. ranunculoides* could affect the native macrophyte communities supporting the designations and WFD status, an effective control measure is essential for the future survival of these native macrophyte communities. As weed-cutting followed by a glyphosate spray was the most effective at reducing abundance of *H. ranunculoides* and did not impact on native macrophyte diversity, this is the best option for future control. The results indicating the higher levels of *H. ranunculoides* abundance in

September, combined with the potential for viable seed production in the later part of the growing season, provide evidence to support control methods starting earlier in the season prior to *H. ranunculoides* rapid growth in June & July (Hussner and Lösch, 2007). This could have the multiple benefits of reducing the chances of the detrimental >50% accumulation of *H. ranunculoides* due to higher temperatures, possibly nitrogen fixation, and preventing seed formation. Later season mechanical clearances are unlikely to capture all the biomass as it begins to form submerged leaves and leafless stolons sink below the surface in autumn, from which it can regrow in spring Hussner and Lösch, 2007). Even if glyphosate herbicides were not used, the move to earlier season mechanical clearances would be beneficial for the reasons outlined.

One key point for INNS management, of which *H. ranunculoides* is an example, is that under Section 14(1) of the Wildlife and Countryside Act it is 'illegal to plant or otherwise cause to grow in the wild any plant listed in Schedule 9 to the Act' (GB Non-native Species Secretariat, 2020c). The seed viability results raise new challenges for disposal of spoil when de-silting watercourses. The cut vegetative material and seed are not likely to establish on banksides, where they are commonly disposed of, because even though it can survive short periods of drying out, they are too dry for *H. ranunculoides* to persist (Hussner and Meyer, 2009). However, heavy rainfall, drinking and grazing livestock and wading birds could transfer the vegetative material and seeds back into the watercourse. The use of a long-reach excavator to place the material further back from bank top, where possible, may minimise this risk. For smaller infestations, making compost heaps away from the bank top would encourage faster decomposition of the vegetative material and keep the seeds away from the damp mud banks they prefer for germination (Centre for Agriculture and Biosciences International, 2020b). Burial is a more time-consuming option but would prevent both vegetative material and seeds from re-entering the watercourse.

If the watercourse containing *H. ranunculoides* is de-silted the waste sediment could contain seeds so would now be classified as controlled waste (Wade, 2014). This could still be disposed of bankside on the landowners own property and be compliant with the law but, if taken off-site, it would need to be disposed of under the Environmental Protection Act, 1990 (UK Government, 2020a) at an appropriately licensed landfill site (Wade, 2014) which has additional cost consequences. It may be better to incur the cost of disposal to a landfill site if it prevented the costs of long-term control and continued spread as seen on the Pevensey Levels. However, a programme of monitoring and hand-pulling or spot-spraying with herbicide is likely to be necessary as it is unlikely all material would be removed in one clearance. All control methods would need to be considered at a catchment level as re-infestation from upstream sources is always possible and a collaborative approach is recommended.

Future control of *H. ranunculoides* is likely to be more sustainable and economically viable using biological control, currently under development by (Centre for Agriculture and Biosciences International, 2020a). This method might permit restoration of a natural successional process of communities, as Walsh et al., (2013) observed other macrophytes replacing it following disappearance during the season, probably due to predation by insects.

7.3. Practical considerations

7.3.1. Field site sampling

Timing of field sampling and surveys were subject to practical restrictions and weather. All surveys were undertaken within the accepted WFD survey season (June to September) (Water Framework Directive, 2014). The 60 annual surveys were randomised, by month, within and between years to minimise any seasonal effects. Practically, due the health and safety requirement of double-manning because of entry to water, reliance on volunteers, studying part-time and the length of time required to access the sites, the surveys were undertaken over a period of several months instead of focussing on one month. However, additional monthly surveys were taken at eight sites (including one month outside each end of the survey season, May to October) and six of these (one for each management treatment and the two reference sites) were subject to statistical analysis to determine any seasonal survey influence. Some surveys had to be re-scheduled due to heavy rain and high-water levels, but these occasions were rare due to the water level management on the Pevensey Levels. Nevertheless, the water environment is dynamic with erosion, transport and deposition (Barko et al., 1991) plus human and animal influences being difficult to spot when sites are only being visited once a year. To visually assess any potential physical changes (such as unexpected de-silting), each site was photographed on every visit and compared over the four years. The sites remained remarkably consistent in appearance over the years with little or no evidence of interventions, an example of which is shown in Figure 61. The water sample results should be interpreted with care as water samples can be very variable (Diston et al., 2007) and there was only one sample per site per year due to financial constraints. However, variation was minimised by the number of samples, 60 sites over four years (240 samples in total) and sampling at the same time of day.

2013

2014

2015

2016

Figure 61: Site 54HG, an un-infested control reach, photographed annually over the four years of the study.

Several reference sites, without *H. ranunculoides*, became infested during the period. For future studies, the infestation pathways and vectors should be considered to reduce this risk. Freshwater systems have multiple pathways and potential transport vectors for invasive species (Anderson et al., 2014, Havel et al., 2015). These include human; as a result of fishing, boating, use of wet suits, waders and watercourse management (Anderson et al., 2014) and via natural means; birds, animals and extreme events such as flooding (Havel et al., 2015). Siting reference sites upstream on watercourses would minimise the risk of downstream propagule transport, should the watercourse become infested. Some of the water courses surveyed had fishing rights and this may have contributed to the spread. Advising landowners that invasive species control should be undertaken from upstream to downstream would also reduce the risk of infestation. Moving stock from fields containing watercourses invaded with *H. ranunculoides* to uninvaded areas was another possible source of infestation. The need for better biosecurity is critical but, despite the benefits to native species, the economy and protected sites, barriers to implementing biosecurity have been identified (Sutcliffe et al., 2018). In spite of the potential cost savings that preventing invasions in the first place might bring, the costs of implementing biosecurity measures, entrenched attitudes, lack of consistent clear guidance and problems with partnership working mean that biosecurity is often not prioritised (Sutcliffe et al., 2018) or may be targeted to areas where legislation forces action (UK Government, 2020c). However, reducing risk of infestation is easier if sites with infested proximal and connected ditches are not used as reference sites, as spread dynamic studies illustrate that these closer sites are likely to be within the dispersal kernel and thus at higher risk of invasion in a shorter period of time compared to isolated ditches (Arim et al., 2006).

Increasing sample size to anticipate loss of sample sites, would be better for future studies, although this was implemented as much as practicable using many replicates. In addition, the survey lengths were based on the EA’s WFD OI 131_07 100m protocol to allow comparison with other survey results across the country. However, this relatively large area may contain more than one plant community or sub-communities so detailed analysis of impact on specific communities may not yield statistically sound results.

7.3.2. Annual weather conditions

Annual weather conditions influence plant growth and flowering period and should be taken into consideration when interpreting the results between years. These conditions can vary considerably so it is important that data are collected over a period of years to ensure that the influence of warmer or cooler years, or those with above or below average rainfall, can be considered in the analysis and interpretation of the results. The10 years from 2010 to 2019 hold eight highest daily temperature records with the year 2014 during the study period being the warmest since 1910 (Met Office, 2020a). However, these field studies took place over four years from 2013 to 2016, which was considered a long enough period to reveal any effects of annual weather. The annual weather conditions during the study can be found in the methodology chapter, 3.4.1.1. as monthly temperature, rainfall, and sunshine graphs. Table 47 compares the survey years with historic data for Eastbourne (Met Office, 2020d, Weather and Climate, 2020c). This shows temperature during the study period across all four years was 1°C higher than the historic average, rainfall 1mm lower but with 20 more sunshine hours. 2015 was cooler and wetter than the other three study years. This may have negatively affected the abundance of *H. ranunculoides* as in chapter 4, 4.4.2.3.

Table 47: Averages for the survey months June - September for the study years 2013-2016, with historic comparisons, temperature, rainfall, and sunshine hours for Eastbourne (Met Office, 2020d, Weather and Climate, 2020c).

Year	Temperature, °C	Rain, mm	Sun, hours
2013	17	33	219
2014	17	46	218
2015	16	83	205
2016	17	40	193
Average, during study	17	50	209
Average, historic	16	51	189

The temperature and rainfall through the growing season will also influence abundance, growth rate and flowering time for some species, as will that of the previous season (Brotherton, 2017). This study included monthly surveying, during the growing season, over three of the four surveyed years to record the abundance of *H. ranunculoides* and demonstrate there was no between year effects. As the Pevensey Levels is an artificial hydrological system with managed water levels, the effects of varying rainfall between years were not considered likely to have an impact on results. Storms may lead to short term, temporary raises in water levels but the pump drainage can rapidly restore water level to within 10cm of the 30cm below mean ground level required by the WLMP (Atkins Ltd, 2015). Temperature is an important influence on plant growth (Brotherton, 2017) and this was discussed in chapter 6 with reference to warmer conditions.

7.3.3. Phenotypic plasticity

Even though the plants used in the greenhouse experiments were taken as cuttings from plants studied in the field sites, the performance of these plants grown under greenhouse conditions may have varied from those in the field due to phenotypic plasticity. Phenotypic plasticity is a change in the phenotype allowing a single genotype to respond to different environments (Gratani, 2014). Phenotypic plasticity is often exhibited in aquatic plants (Garbey et al., 2004). This manifests as differences in size, form, and phenology in *H. ranunculoides* (Robert et al., 2013). The environmental stress factors studied include invasiveness (as a stress on the native macrophytes), nutrient availability and temperature rise and the functional plant traits recorded related to flowers, seeds, leaves, stalks, and roots, but there are other factors that can influence phenotypic plasticity, such as resource acquisition and allocation, and photosynthetic capability (Gratani, 2014). This may account for some of the differences between the results from those plants studied while investigating the sediment nutrient levels in the field and those plants in the nutrient experiment.

Phenotypic plasticity should not have affected the germination experiment, especially as the percentage germination from the UK germination pilot study outdoors (2%) was remarkably similar to that in the main experiment for the UK seed (2.2%), chapter 6. 6.3.2 and 6.3.3. However, for the experiment involving growing plants for seed, the restrictions in pot size and nutrients will have affected the biomass and leaf size so the number of flowers and seeds may also have been lower than field conditions may permit.

7.4. Future research recommendations

7.4.1. Refining the field experiments

7.4.1.1. Invasion in specific communities

The field study sites were selected using a stratified sampling technique to ensure a range of watercourse sizes were included. NVC communities were only identified once the baseline surveys had been undertaken. The results from chapter 4 identified certain native macrophyte species (Table 16) that showed a negative abundance relationship with *H. ranunculoides* in the field, which are found in specific, and declining aquatic NVC communities, A3 *Spirodela polyrrhiza-Hydrocharis morsus-ranae* and A4 *Hydrocharis morsus-ranae-Stratiotes aloides* communities (Rodwell, 1998). There should be sufficient invaded areas on the Pevensey Levels, and other UK and European wetland sites (Hussner et al., 2017), to select sites based on these NVC communities to investigate how invasion changes these and whether these communities can recover if *H. ranunculoides* was eliminated. Use of smaller reaches and the recommended MAVIS (Centre for Ecology and Hydrology, 2016) minimum of five samples to ascertain the NVC community would give statistically robust results.

7.4.1.2. Studying the types of watercourse

For this investigation, although different widths of watercourse were included, *H. ranunculoides* was studied in an artificial, human-made and managed environment where infestation has been shown to develop quickly and extensive (Ryland, 2008). Nearly all watercourses in the UK have been influenced by humans (Dawson and Holland, 1999) but how native macrophyte communities respond to invasion in re-naturalised watercourses or wetland systems, has not been investigated.

7.4.1.3. Using herbicides under controlled conditions

The herbicide applications were done in dry weather, and with light or no wind to prevent spray drift to non-target plant species. However, there will have been natural variations in local climate, flow, and water chemistry between sites. Newman and Dawson (1999) carried out a controlled field experiment using sub-blocks of a 65m stretch of watercourse to compare 2,4-D amine with Glyphosate but there have been no mesocosm or greenhouse experiments investigating herbicide effectiveness. Intentionally growing *H. ranunculoides* under controlled conditions and applying herbicide to measured densities has not been done and would permit detailed analysis of the newer Glyphosate formulations and adjuvants action and efficacy on this species. This information could inform management planning, timing of applications and whether applications on small early-stage growth might eliminate the plant.

7.4.1.4. Monitoring recovery after management treatments

Monitoring is a long-term activity that would need to be undertaken over a period of several years, but this research has indicated that aquatic communities change under infestation and management over a four-year period. Successional change is a natural process (Grime, 2006) but understanding how infestation might modify this is important for conservation reasons and to determine the potential severity of future infestations particularly as climate warming is likely to increase the potential for *H. ranunculoides* expansion (Gallardo and Aldridge, 2020). It is also important to identify if communities can recover from the heavy infestations that this study has shown to have an impact on diversity and regain a level of diversity comparable to pre-infestation status.

7.4.2. Developing the greenhouse experiments

7.4.2.1. Responses of *H. ranunculoides* to nutrients in water

The effect of nutrients on the growth of *H. ranunculoides* was studied in substrate under greenhouse conditions but during the peak growth phase in the field, in summer, there is an extensive network of floating stems with root systems suspended in the water column as well as being rooted into bank margins (Centre for Agriculture and Biosciences International, 2020b). The nutrients used in the substrate experiment were water soluble and could be used in sand, as it is naturally extremely low in nutrients (Gosselin et al., 2018), in the same quantities under aquaculture conditions. Gosselin et al. (2018) found that rooted submerged aquatic plants were able to grow well in sand impregnated with water soluble fertilisers such as those used in this study. This might resolve the query raised by this study with respect to the roles of the nutrients and whether *H. ranunculoides* does obtain different, and / or differing amounts, of nutrients from water and sediment.

7.4.2.2. Responses of *H. ranunculoides* to nutrients under different pH levels

This research has shown that *H. ranunculoides* can grow successfully under a wider range of pH than previously recorded. Recent studies have investigated the interactions between pH and nutrient uptake (Smith, 2014, Bellinger and Davis, 2017). Smith (2014) found that nitrogen initially drove the growth of floating macrophytes but that as pH rose growth of floating species growth declined, possibly due to pH negatively affecting the bioavailability of nutrients, and submerged species growth increased due to complex inter actions between pH and nutrient availability. Bellinger and Davies (2017) found that >pH8 reduced the growth of the submerged species *Cabomba caroliniana* and that this was linked to a preference for CO_2. Where *C. caroliniana* was grown in systems with similar phosphorus levels, water pH was one of the most important determinants of growth (Bellinger and Davis, 2017). Chapter 5 discusses the possibility that part of *H. ranunculoides'* success may be due to being able to utilise the available nutrient resources more effectively than previously thought and further work on how pH might affect *H. ranunculoides* uptake of nutrients may help to inform management of environmental conditions to restrict the growth of *H. ranunculoides*.

7.4.2.3. Germination of native vs invaded seed to investigate germination potential

Given the failure of the seed from the native region to germinate in the germination experiment discussed in chapter 6, repeating this experiment using seed from multiple native and invaded locations would be useful. Determining the temperature where germination potential of *H. ranunculoides* in invaded regions reaches that of the native regions would help establish the potential scale of the risk posed by germination in the invaded regions. Having demonstrated that *H. ranunculoides* can produce viable seed in Europe, a next step is to determine seed longevity. It may be that the native seed, and that from the seed bank, did not germinate in this research, because it has a short lifespan. There is little in the literature on macrophyte seed longevity, but Alderton et al., (2017) studying ghost ponds found that, even when restored up to 150 years after being in-filled, some macrophyte species were still viable, *Juncus spp*., *Potamogeton natans* and *Ranunculus aquatilis*. Sullivan and Wood (2012) demonstrated that seed from the floating invasive aquatic *Eichhornia crassipes* can survive for over 28 years. Kauth and Biber (2015) studying the submerged aquatic *Vallisneria americana* found that seed could survive at least four years of cold storage. The European seed was sown in the spring following collection in the previous autumn whereas the native seed was collected in the early summer of the previous year. Nakashima and Oki (2017) germinating *H. ranunculoides* seed produced in southern Japan, found that seed collected in the autumn had a higher percentage germination than spring collected seed. All seed was dried, so this process is less likely to have influenced variation in germination rates, but Kauth and Biber (2013) found differing effects of drying on related species of *Vallisneria*, drying negatively affecting *V. americana* germination but doubling germination percentage of *V. australis*. The difference between *H. ranunculoides* spring and autumn germination rates might also indicate that seed has a very short lifespan, but it could be that the winter period triggers a second dormancy phase (Nakashima and Oki, 2017). Seed longevity is important for development of future management programs because, if an infestation is cleared of all vegetative growth, it is no longer certain that *H. ranunculoides* has been controlled. So, as the seed might be viable for more than one year, the site may have to be re-visited for several years to ensure no seed has geminated from the seed bank.

7.4.3. Investigating the genetic code

An unpublished study by Newman (2004) established four genetic types of *H. ranunculoides* in the UK population (Centre for Agriculture and Biosciences International, 2020b). One is similar to the native *Hydrocotyle vulgaris* suggesting potential hybridisation and one was possibly tetraploid which means the *H. ranunculoides* in Europe may be different to that of the native regions (Millane and Caffrey, 2014). Thus, seed production in Europe may have been occurring un-noticed for many years and genetic diversity could already have progressed further the four genetic types identified sixteen years ago (Newman, 2004). Although tetraploid plants are likely to have broader niche breadths than diploids, diploids may be better colonisers (Grewell et al., 2016). Therefore, it would be important to establish if any of the European population of *H. ranunculoides* is tetraploid as this would assist with horizon scanning and predicted spread. It may also have an impact on genetic diversity if tetraploid *H. ranunculoides* were unable to cross-pollinate with diploid strains.

7.5. Applications of the research

7.5.1. Diversity, structure, and function of aquatic communities

Wetlands are highly valuable ecologically for plant and animal diversity and economically for ecosystem services so understanding the impact that INNS have on these is critical for future protection and management. Species and community outcomes, as community complexity, decline after invasion (Stiers et al., 2011, Nijs et al., 2012). This research has confirmed that macrophyte communities invaded by *H. ranunculoides* are altered and, for the first time, quantified the reduction in diversity. There could be loss of rare and diverse communities thus threatening local, national, and international designations in European invaded regions. This has implications for conservation management. Using the results of this study to inform management programmes to keep *H. ranunculoides* below the 50% cover threshold could help conserve native macrophyte communities and the protected vertebrate and invertebrate protected species that depend on them.

7.5.2. Climate warming and spread prediction

The impact of a 2°C rise in temperature, compared to the current average in the UK, on the enhanced growth and seed production capacity of *H. ranunculoides* has been demonstrated. Charting northern European regions where a 2°C rise in temperature would bring new areas within invasible parameters, would inform the affected regions, and allow them to raise awareness of identification, reporting and the importance of enhanced biosecurity in these regions.

7.5.3. Risk assessments and targeting new invasions

An understanding of how *H. ranunculoides* responds to nutrients and pH in addition to rising temperatures means existing UK heat maps, which illustrate the areas suitable for invasion, (Gallardo and Aldridge, 2020) need to be re-visited to assess whether these new data on ecological tolerances might put new areas at risk. Mapping current invaded areas using traditional surveying methodology, but also using more rapid recently developed remote sensing techniques, and horizon scanning of potential new suitable areas could highlight invasion pathways and transmission vectors. This would enable prioritisation of preventative measures and the location, and control, of small new invasions before they establish.

7.5.4. Paradigm shift in management and control

This research should assist in challenging the current way of thinking about control measures for *H. ranunculoides* but also how other emerging invaders respond to climate warming. There are numerous methods available for controlling *H. ranunculoides*; mechanical, physical (hand pulling), de-silting, dyes, biological, chemical, nutrient reduction and shade (Hussner et al., 2017). This research has illustrated that mechanical management alone does not reduce abundance of *H. ranunculoides,* in the long term, but does negatively impact on native macrophyte diversity. Therefore, mechanical management alone should not be undertaken without considering herbicide use in combination, as mechanical management alone may encourage further spread, but if followed up by herbicide use would reduce abundance of *H. ranunculoides.* In organically-farmed areas, where herbicides are not permitted, additional biosecurity measures would be required to prevent spread of vegetative fragments. *H. ranunculoides* ability to produce viable seed in Europe, means sites should not be considered clear of the plant until checked the following season and possibly for several years. Although the longevity of macrophyte seed is variable (Sullivan and Wood, 2012, Kauth and Biber, 2015, Alderton et al., 2017), *H. ranunculoides* may have a shorter seed longevity as it was previously placed in the plant Family Apiaceae (Stace, 2019) which are typically short-lived (Walters et al., 2005), although it is now in Araliaceae which is in the same plant Order as Apiaceae

(Nicolas and Plunkett, 2009). Timing of management becomes more important as earlier intervention would help prevent the development of ripe seed. An integrated approach should be adopted, with other tactics such as preventing algal blooms which fix nitrogen in *H. ranunculoides* peak growth period being considered. Finally, considering the wider ecological tolerances of *H. ranunculoides* than previously reported shown by this research, and the impact climate warming will have on *H. ranunculoides* ability to become even more successful, support for sustainable biological control should be prioritised (Centre for Agriculture and Biosciences International, 2020a).

Chapter 8 - Conclusion

The aims of this research were to:

1. Determine the impact of *H. ranunculoides* and its management methods have on native macrophyte community composition.

2. Establish the influence of nutrients on its abundance and invasiveness.

3. Examine the impacts of rising temperatures on growth and reproduction and investigate the effects of climate warming on *H. ranunculoides* invasive capacity.

This research investigated how *H. ranunculoides* affects UK native macrophyte communities, what impact the management of the species has on these communities and how effective that management is. The study then examined the influence of nutrients and increasing temperature on the growth of *H. ranunculoides* and how temperature might affect seed production and germination. *H. ranunculoides* is under-represented in certain research areas, such as autecology, impacts in its invaded environment and effectiveness of management. It is known that it has an impact on native macrophyte communities (Stiers et al., 2011) but the communities and species affected have not been described. There are a number of management methods currently in use (Centre for Agriculture and Biosciences International, 2020b) but the efficacy of these methods in controlling *H. ranunculoides* and what impact these methods have on the native macrophyte communities have not been documented in detail. Hussner and Lösch (2007) reported the influence of combined nutrients on the growth of *H. ranunculoides* but did not investigate if a specific nutrient was driving the growth. Hussner and Lösch (2007) and Hussner (2009) studied the effects of temperature on *H. ranunculoides* photosynthesis but not on growth and there have been no studies on germination in cooler temperate regions.

Here, a novel approach was used, combining field and greenhouse studies, using genetic material from one of the study field sites for the greenhouse experiments, which has given a rare insight into the ecology and impacts of this INNS. Four years of field surveys of watercourses, recording macrophyte communities and different management treatments, using multiple metrics, has produced exceptionally detailed data sets. *H. ranunculoides* responses to nutrients were investigated under natural field, and in controlled greenhouse, conditions using the same clonal plant material. Observation of the first germination of *H.*

ranunculoides seed in Europe and measures of the responses of *H. ranunculoides* growth and seed production to increasing temperatures in its invaded regions has not been undertaken previously.

The results show that *H. ranunculoides* negatively impacts native macrophyte communities and that some management methods are also detrimental to these communities. However, one management method, weed-cutting and herbicide spraying, effectively controlled *H. ranunculoides* without damaging the native macrophyte communities. The nutrients, nitrogen in water and phosphate in substrate, promoted growth of *H. ranunculoides.* Additionally, an association between high pH and reduced abundance of *H. ranunculoides* was found. Rising temperatures were found to permit development of viable seed in *H. ranunculoides* invaded European regions and be related to more biomass.

8.1. Synthesis of key findings

The results of four years of field research have shown that there are changes to aquatic plant (macrophyte) communities related to *H. ranunculoides* infestation, and these may be species specific. In this study, macrophyte diversity as measured by diversity index values, but not species richness, decreased significantly when *H. ranunculoides* infestation exceeded 50% cover. However, plant community dynamics are highly complex (Grime, 2006) and the macrophyte communities studied appear resilient to lower levels of infestation.

The study of community dynamics identified the months in which *H. ranunculoides* abundance peaked, August – September, which indicated that later season native macrophyte species might be more affected than early to mid-season species. Five key indicator species for WFD assessment were affected, *L.trisulca, H. morsus-ranae, B. erecta, P. lucens* and *S. erectum* (Water Framework Directive, 2014) and two later season species, *L. trisulca* and *H. morsus-ranae,* forming the basis for rare NVC communities underpinning the Pevensey Levels SAC (Joint Nature Conservation Committee, 2016b), were particularly at risk.

Some of the treatments used to manage *H. ranunculoides* in watercourses impacted on plant community composition. These were evaluated as a reduction in plant diversity as species richness, or diversity index values, or a shift in composition. However, the community composition of sites receiving weed-cutting alone was shown to be different in composition to reference sites that received no treatment. This suggests that the regular disturbance, as weed-cutting, required to control *H. ranunculoides* alters communities. However, if weed-cutting is used in combination with an herbicide spray, which aims to kill or reduce plant viability, this may prevent the >50% cover threshold of *H. ranunculoides* that reduces diversity being reached.

All the management treatments monitored reduced cover of *H. ranunculoides* to some degree. However, weed-cutting followed by spraying was the only method that significantly reduced cover. No treatment eliminated *H. ranunculoides* during this four-year study. It was established that the only method that is permitted in the watercourses of land farmed organically, weed-cutting, does not effectively control *H. ranunculoides*. This method alone is likely to favour the spread of *H. ranunculoides* as fragmenting the plant during the mechanical removal produces many vegetative propagules.

Existing literature suggests that *H. ranunculoides* prefers high levels of nitrate and phosphate (Diston et al., 2007, EPPO, 2010, Millane and Caffrey, 2014). There was no evidence from this study that nitrogen-based nutrients in sediment influenced the growth of *H. ranunculoides* in the infested field watercourses but there was an indication that phosphate in sediment might positively influence abundance of *H. ranunculoides*. There were indications that nitrogen in water may positively influence growth and the rapid growth period of *H. ranunculoides* in the latter summer months would coincide with nitrogen being fixed into the water column by algal blooms (Paerl, 1990). For the first time, the importance of pH in water and the presence and abundance of *H. ranunculoides* was examined. There was a negative association between *H. ranunculoides* and water pH, as the more alkaline the water, the less abundant *H. ranunculoides*. However, *H. ranunculoides* was found under a wider range of pH than previously recorded (Centre for Agriculture and Biosciences International, 2020b). This is important because research has suggested that pH may be a key factor in nutrient uptake (Ullrich-Eberius et al., 1981, Bellinger and Davis, 2017, EPPO, 2006). Bellinger and Davis (2017) studying submerged macrophytes and Smith (2014) studying floating macrophytes, both found that >pH8 levels reduced nutrient bioavailability, particularly phosphate. The interactions between pH and nutrient availability are complex but a combination of higher pH and lower nutrient levels in both water and sediment may limit *H. ranunculoides* abundance.

Under controlled conditions, phosphate alone in the growing medium improved relative growth rate and overall biomass of *H. ranunculoides*. In particular, the root system benefitted which would enable the plant to firmly establish and possibly withstand mechanical clearance better than weak-rooting or floating native macrophyte species. Using combined high nitrate and ammoniacal nitrogen, with an absence of phosphate, was a new treatment in the study of *H. ranunculoides* and was found to considerably reduce the relative growth rate compared to a phosphate only treatment and significantly reduce it in comparison to an all-nutrient treatment.

For the first time, germination of *H. ranunculoides* seed, produced under current, natural, climatic conditions in its European temperate invaded regions, was observed. The temperature regime under which this was observed was lower than its native regions (EPPO, 2006) and the results indicated that between 2.2% and 11.1% of *H. ranunculoides* seed produced in its European invaded regions, the UK and the Netherlands (Weather and Climate, 2019b, Weather and Climate, 2020b), are viable under these cooler conditions. Seed production and germination can occur under a 2°C temperature warming scenario and results demonstrated that more seed was produced as temperatures rise. Additionally, rising temperatures appear to stimulate *H. ranunculoides* to flower earlier and produce more leaves, biomass, and seeds.

8.2. Implications of research

Some prior research has been done on *H. ranunculoides* and community composition, nutrients, and temperature but these studies had broad remits (Newman and Dawson, 1999, Hussner and Lösch, 2007, Stiers et al., 2011). These results have made a more detailed and original contribution to scientific knowledge and this new information has implications for the sustainability of some macrophyte communities, the invasive potential of *H. ranunculoides* under current and future climate conditions, and the options for managing the species.

Phenotypic plasticity can evolve when there is a sufficient genetic variation (Gratani, 2014) and the implication of seed production is that *H. ranunculoides,* which already exhibits considerable plasticity (Robert et al., 2013), is likely to become more adaptable. Overall, the results have highlighted how adaptable *H. ranunculoides* is, and indicate that it is likely to be capable of invading more regions given the appropriate pathways and distribution methods. They also illustrate how detrimental the species can be, to native macrophyte communities and biodiversity. The results have also provided new knowledge to inform professional practice, highlighting the need for better biosecurity, indicating the level of infestation that causes loss of diversity and enabling management methods to target the communities that are likely to be most affected.

8.2.1. Plant communities

A wide range of macrophyte communities were invaded in this study, including floating, submerged, and emergent species. The overall reduction in diversity and decline in native macrophyte species underpinning rare communities and hosting protected fauna, could threaten national, European, and international designations at the Pevensey Levels. The national SSSI designation does not permit more than 5% total cover of all INNS across the whole site (Natural England, 2013a). The cover of *H. ranunculoides* in 2006 was already at 10% of the SSSI site, 45km of watercourses and 352 hectares (Environment Agency, 2006). The study results indicate that the management of weed-cutting alone, which was exclusively used from 2008 to 2013 (when this study began) and continued to be the sole method of control for the organic farms, does not reduce cover of *H. ranunculoides*, so the site is currently unable to meet the SSSI designation criteria. For the European SAC designation, the macrophyte communities favoured by the designated feature (the snail *Anisus vorticulus*) have been shown to be threatened by the higher cover of *H. ranunculoides*. The protected snail, *A. vorticulus,* is susceptible to a deterioration in water quality associated with nutrient input, and to inappropriate ditch management. The management protocol requires low intensity grazing, low fertiliser and occasional or partial ditch clearance (Natural England, 2017). The snail requires the mid to late successional ditch habitat which is unable to develop under a regular weed-cutting regime where *H. ranunculoides* is present. The evidence presented shows that *H. ranunculoides* is more likely to become abundant under these regular mechanically managed conditions. The Ramsar international designation may also be under threat as the citation highlights the nationally rare and scarce macrophytes and invertebrates; *Lemna trisulca*, *Hydrocharis morsus-ranae* and *A. vorticulus* are notable species (Joint Nature Conservation Committee, 1999) and have been shown to be affected by *H. ranunculoides* presence.

The impact of *H. ranunculoides* on the WFD status of waterbodies may be missed if surveys are carried out in the early months of the recommended WFD survey period of

June to September as the peak biomass period for *H. ranunculoides* seems to be August to September. As most native plant species did not disappear completely, functionality at community and ecosystem services level may not be lost if *H. ranunculoides* can be kept below the 50% cover threshold shown to be detrimental. Early growing macrophyte species may be able to survive because they can complete their life cycle before *H. ranunculoides* reaches its peak, but later season species are likely to be under threat. However, survival of some rarer macrophyte communities is likely to be dependent on the susceptibility of individual species to these higher levels of *H. ranunculoides* infestations.

The physical management, weed-cutting and de-silting, used to reduce *H. ranunculoides* abundance also reduced the native macrophyte species richness. However, if weed-cutting was combined with a follow-up, chemical spray which targeted just *H. ranunculoides*, native macrophyte diversity was retained. De-silting of watercourses is only used infrequently, every 10 to 20 years, although an important part of the Pevensey Levels SSSI management regime (Natural England, 2013a). This would result in a reduction in diversity but is deemed acceptable in order to retain the successional habitat stages (Natural England, 2013a). These results suggest that annual weed-cutting alone, currently undertaken by the Environment Agency to reduce *H. ranunculoides* cover, would not support native macrophyte diversity and recovery of these diverse communities is unlikely under this regime.

8.2.2. Climate and invasive capacity

This research has shown that viable seed is already being produced by *H. ranunculoides* in European invaded regions. Incremental climate change is certain and a 2°C rise in global temperature by the end of the 21st century, is a likely scenario (IPCC, 2014). Seeds confer many advantages to a plant in addition to vegetative growth and regeneration. They permit survival through periods of unsuitable or extreme conditions, provide an additional overwintering opportunity, allow germination under optimal conditions and confer additional dispersal opportunities (Li, 2014). Additional germination or dormancy benefits would not be known until seed viability over time was established but opportunities for colonisation of disturbed areas within the first season of seed production would be immediately available. Seeds are well suited to hydrochory (Eckert et al., 2016) and, *H. ranunculoides* seed, being smaller than its vegetative ramets, could readily be transferred to machinery or equipment in mud splashes. For the same reason, seed is more likely to be transported by birds, aquatic animals or grazing livestock (Li, 2014).

Sexual reproduction leads to genetic variability which can allow adaptation to changes in the environment (Lei, 2010). Rising temperatures, are becoming ‘very likely’ (IPCC, 2014) and adaptation to, and recovery from, these is a distinct advantage to a species’ establishment (Li, 2014). Genetic diversity may permit selection of seedlings better able to cope with environmental stressors (Li, 2014) such as periods of drought or excess flows. Possessing both sexual and vegetative reproductive methods would enable *H. ranunculoides* to take advantage of a wider range of environmental conditions to invade, establish and thrive. Current partial tolerance of *H. ranunculoides* to glyphosate, which is currently the only available herbicide for aquatic use (Centre for Agriculture and Biosciences International, 2020b), could result in resistance strains as a result of genetic variability, with subsequent impacts on control methods. In addition to the invasive traits of a rapid growth rate, efficient vegetative reproduction, adaptability and resistance to chemical control *H. ranunculoides* also exhibits winter quiescence, having small submerged shoots (Hussner and Lösch, 2007). For riparian ecology the greatest impact from climate warming is likely to be warmer water and changes to hydrological patterns (Rahel and Olden, 2008). This could lead to a shorter quiescence period, because of an earlier start and later finish to the growing season, which is likely to give *H. ranunculoides* an even greater competitive advantage. This research has shown that native macrophyte communities are already negatively impacted by *H. ranunculoides.* It has also shown that rising temperatures are likely to enhance its growth and reproductive capacity and that this is likely to place increasing pressure on some key aquatic communities and rarer species because of heavier and more prolonged infestations.

8.2.3. Horizon scanning

Gallardo and Aldridge (2020) included *H. ranunculoides* in their assessment of risks to the water industry from aquatic invasive species, because of predicted 2050 climate scenarios. Climate warming increased the bioclimatic suitability in the UK for *H. ranunculoides* by between 12-18%. This research indicates that *H. ranunculoides* is likely to be able to capitalise on existing suitable habitats by growing and reproducing faster under warmer conditions but, in combination with Gallardo and Adridge’s (2020) heat maps, may also help to assess how well it will establish and spread in any new locations based on temperature. In Europe there was an increase of 76% in INNS between 1980 – 2010 (Bellard et al., 2013) and this coincides with a consistent rise in temperature over these 30 years (Met Office, 2020a). Whilst projected climate change might expand distribution for some species, others may shrink. For aquatic invasive plants, however, the range is likely to expand by 12% and the areas are likely to be northern temperate regions, with north America and Europe being hotspots (Bellard et al., 2013).

As well as geographic range, environmental tolerance is a valuable predictor of invasive potential (Havel et al., 2015). This research shows that *H. ranunculoides* favours warmer conditions which improve its chances of producing seeds Increased propagule pressure contributes to a shift from submerged to floating plants and is often caused by nutrient enrichment (Havel et al., 2015). These results have shown that there are links between increasing nutrients and greater growth of *H. ranunculoides* and that it can tolerate and utilise nutrients under a wider range of pH than previously known (EPPO, 2010). This may allow it to thrive outside optimal growth parameters thus contributing to its invasive capacity.

Additionally, planning of management options may be more effective and less labour intensive if infestations could be mapped more accurately. The use of drone technology would enable mapping of larger scale infestations, whereas the development of an eDNA identification technique could allow timely detections of new, small infestations of *H. ranunculoides* and aid rapid response efforts (Gantz et al., 2018).

8.2.4. Management options

In areas where herbicides are not permitted, such as organic holdings, the only realistic option available to control *H. ranunculoides* is physical removal, either mechanically or by hand. Hand-pulling has been effective at new locations or smaller infestations (Centre for Agriculture and Biosciences International, 2020b) where there is sufficient labour available but is unlikely to be practical on extensive infestations or sustainable over the longer term. A biological control option is under investigation (Centre for Agriculture and Biosciences International, 2020a) but has not yet been given approval. A weevil, *Listronotus elongatus*, was imported from Argentina for testing against non-target species (Centre for Agriculture and Biosciences International, 2020a). Initial results have shown this to be very effective at reducing *H. ranunculoides* biomass. Following a successful pest risk analysis submission, approval for test releases has been given for autumn 2021

(Djeddour, 2020). Mechanical control in the form of weed-cutting alone has been shown in this study to be ineffective in reducing *H. ranunculoides* cover so managers unable, or unwilling, to use herbicides are unlikely to control or eliminate the species. However, the weevil could be used on both organic and traditionally farmed holdings thus providing continuity of control across catchments. Historically, reservations about the non-intended effects of biological control agents have led to the development of risk averse, expensive, and lengthy regulatory processes which has restricted the development and use of this method (Barratt et al., 2018). But there are economic, environmental, and social benefits of biological control for weeds (Barratt et al., 2018).

However, weed-cutting accompanied by an herbicide spray was shown to reduce *H. ranunculoides* cover in this research. Using this new information on the effectiveness of management treatments, the time of year at which *H. ranunculoides* reaches its peak biomass and its ability to produce viable seed, management may be modified to be more effective. Where chemical control is possible, an integrated approach is proposed. Mechanically removing the biomass prior to the peak growth period, in August-September, may prevent the species achieving >50% cover, which has been shown in this research to be detrimental to native macrophyte communities. Additionally, as it is now known that seed can be produced, and that as temperature increases seed is likely to be produced earlier, controlling the growth earlier in the season becomes more critical. The gradual adaptation of native biota to become pests and diseases of *H. ranunculoides* and reduce its vigour, is less likely to occur if genotypic variability due to sexual reproduction (seed production) occurs. Herbicide treatment, either early in the season on small patches, or on re-growth following mechanical removal, is then likely to be more effective as the leaf area (which absorbs the chemical) to overall biomass ratio is greater and therefore the chemical is more likely to be taken back to the root system and kill the plant (Newman and Dawson, 1999). This also reduces the risk of oxygen depletion due to large areas of decaying plant material, which is harmful to aquatic fauna (Thomas, 2010).

Traditional control programmes for H. ranunculoides have not included the possibility of re-establishment from seed. So, where infestation has been reduced, and in some cases thought to have eliminated *H. ranunculoides,* follow-up monitoring needs to be included to check for localised re-infestations. As seed viability over time is not yet known (Walsh, 2016), the period over which this might prove necessary is a subject for further research. Li (2014) notes how sparse information on aquatic plant seed longevity is, but Walters et al. (2005) suggest that the Family Apiaceae, in the same plant Order as *Hydrocotyle* species, are characteristically short-lived. Additionally, disposal of spoil from de-silting may now be subject to the provisions of the Environmental Protection Act, 1990 (UK Government, 2020a), as controlled waste containing propagules, which has additional handling and cost implications (Wade, 2014). Routine biosecurity measures, the check, clean and dry protocol, should eliminate the clearly visible vegetative ramets. However, these measures may require re-assessing as *H. ranunculoides* seeds, being much smaller and less obvious than the vegetative ramets, may be more widely dispersed in the environment and require different or additional cleaning methods. Traditional washing down might require upgrading to pressure washing or hot pressure washing, as heat has been shown to be more effective in decontaminating (Anderson et al., 2014).

This research has highlighted the need to re-evaluate management options and the invasive capacity of *H. ranunculoides* as a result of rising temperatures, its adaptability to nutrient supplies and seed production in temperature European invaded regions, in order to conserve native macrophyte communities and enable long term control in a sustainable and ecologically acceptable way.

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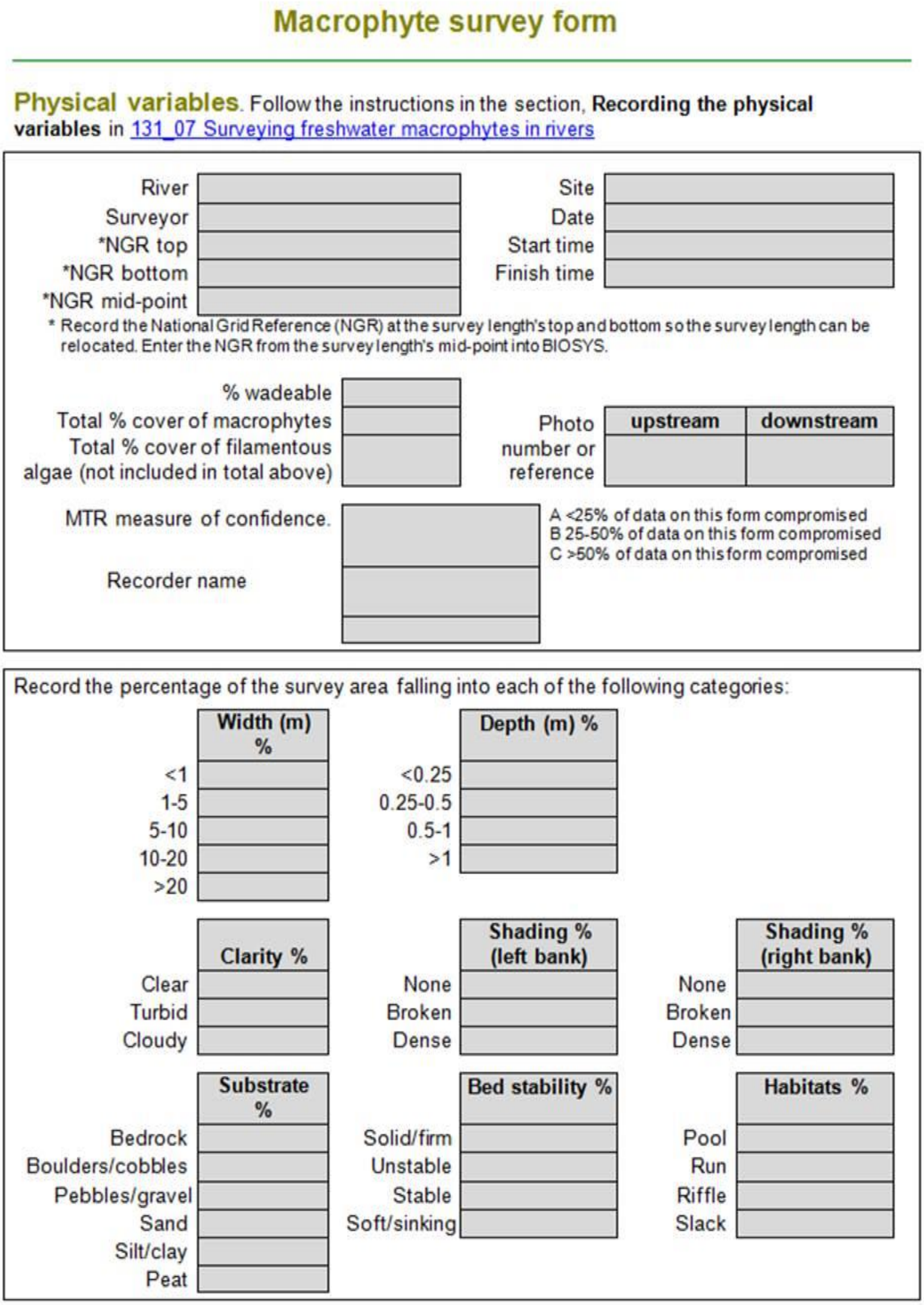
Appendices

Appendix 1: Macrophyte survey form in accordance with the Environment Agency operational instruction 131_07



Macrophyte survey form

Physical variables. Follow the instructions in the section, **Recording the physical variables** in 131_07 Surveying freshwater macrophytes in rivers

River		Site	
Surveyor		Date	
*NGR top		Start time	
*NGR bottom		Finish time	
*NGR mid-point			

* Record the National Grid Reference (NGR) at the survey length's top and bottom so the survey length can be relocated. Enter the NGR from the survey length's mid-point into BIOSYS.

% wadeable	
Total % cover of macrophytes	
Total % cover of filamentous algae (not included in total above)	

Photo number or reference	**upstream**	**downstream**

MTR measure of confidence.	
Recorder name	

A <25% of data on this form compromised
B 25-50% of data on this form compromised
C >50% of data on this form compromised

Record the percentage of the survey area falling into each of the following categories:

	Width (m) %
<1	
1-5	
5-10	
10-20	
>20	

	Depth (m) %
<0.25	
0.25-0.5	
0.5-1	
>1	

	Clarity %
Clear	
Turbid	
Cloudy	

	Shading % (left bank)
None	
Broken	
Dense	

	Shading % (right bank)
None	
Broken	
Dense	

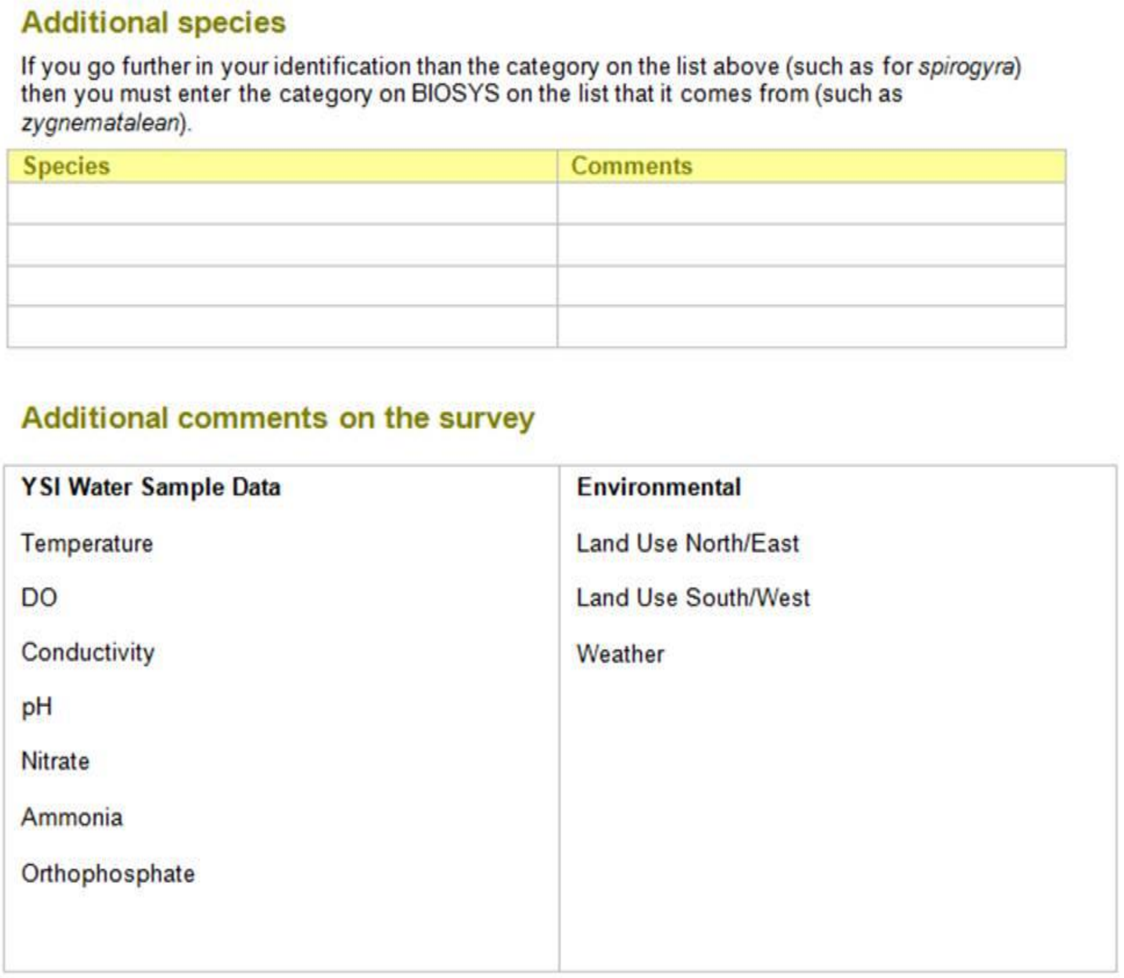
	Substrate %
Bedrock	
Boulders/cobbles	
Pebbles/gravel	
Sand	
Silt/clay	
Peat	

	Bed stability %
Solid/firm	
Unstable	
Stable	
Soft/sinking	

	Habitats %
Pool	
Run	
Riffle	
Slack	

Taxa (synonym)	TCV	Notes and actions
Macroalgae		
chara sp*		specimens required
cladophora aegagropila		
blanketweed agg		
nitella sp*		specimens required
enteromorpha intestinalis (ulva flexuosa / intestinalis)		
Vascular plants		
azolla filiculoides		
equisetum fluviatile		
equisetum palustre		
alisma lanceolatum		
alisma plantago-aquatica		
apium inundatum		
apium nodiflorum		
berula erecta		
bidens cernua		
bidens tripartite		
bolboschoenus maritimus		
butomus umbellatus		
callitriche hamulata		
callitriche hermaphroditica/truncata		specimens required
callitriche stagnalis/platycarpa/obtusangula		specimens required
caltha palustris		
carex acuta		
carex acutiformis		
carex aquatilis		
carex paniculata		
carex riparia		
carex rostrata		
carex vesicaria		
catabrosa aquatica		
ceratophyllum demersum		
eleocharis palustris		
elodea canadensis		
elodea nuttallii		
glyceria fluitans agg		includes any small glyceria
glyceria maxima		
hottonia palustris		
hydrocharis morsus-ranae		
hydrocotyle ranunculoides		
iris pseudacorus		
juncus articulatus		
lemna gibba		
lemna minor		
lemna minuta		
lemna trisulca		
lotus pedunculatus		
lythrum salicaria		
mentha sp*		specimens required
mentha aquatic		
myosotis scorpoides		

myriophyllum alterniflorum		
myriophyllum spicatum		
nuphar lutea		
nymphaea alba		
nymphoides peltata		
oenanthe aquatica		
oenanthe crocata		
oenanthe fistulosa		
oenanthe fluviatilis		
persicaria amphibia		
persicaria hydropiper		
phalaris arundinacea		
phragmites australis		
potamogeton acutifolius		
potamogeton berchtoldii		
potamogeton crispus		
potamogeton gramineus		
potamogeton lucens		
potamogeton natans		
potamogeton obtusifolius		rare
potamogeton pectinatus		
potamogeton perfoliatus		
potamogeton polygonifolius		
potamogeton praelongus		
potamogeton pusillus		
potamogeton trichoides		rare
potentilla palustris		
potentilla erecta		
ranunculus sp		
ranunculus circinatus		
ranunculus flammula		
ranunculus sceleratus		
rorippa amphibia		
rorippa nasturtium-aquaticum agg.		
rorippa palustris		
rumex hydrolapathum		
sagittaria sagittifolia		
schoenoplectus lacustris		
sium latifolium		
sparganium angustifolium		
sparganium emersum		
sparganium erectum		
spirodela polyrhiza		
typha angustifolia		
typha latifolia		
utricularia sp(p)		specimens required
veronica anagallis-aquatica		
veronica beccabunga		
veronica catenata		
veronica scutellata		



Additional species

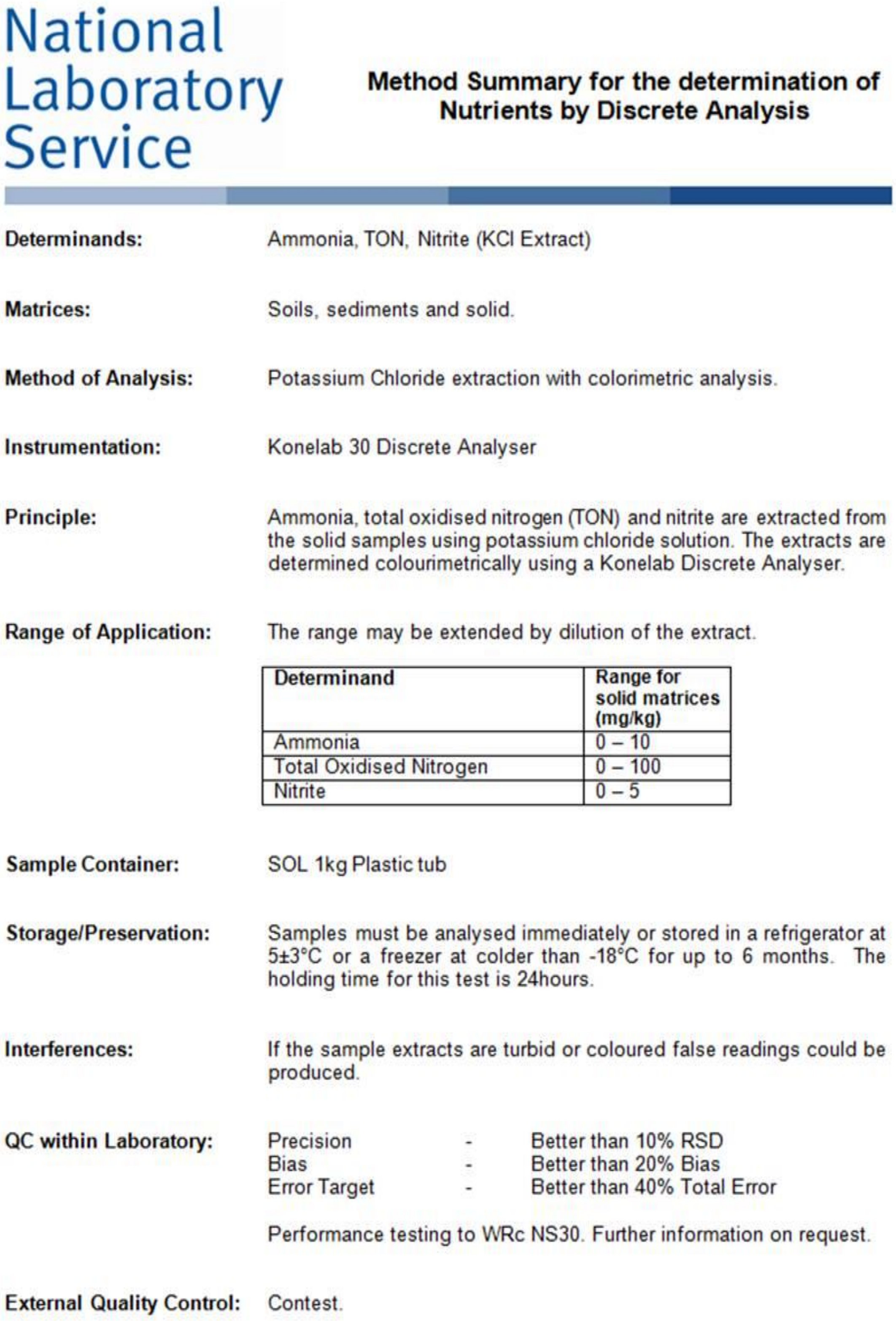
If you go further in your identification than the category on the list above (such as for *spirogyra*) then you must enter the category on BIOSYS on the list that it comes from (such as *zygnematalean*).

Species	Comments

Additional comments on the survey

YSI Water Sample Data	**Environmental**
Temperature	Land Use North/East
DO	Land Use South/West
Conductivity	Weather
pH	
Nitrate	
Ammonia	
Orthophosphate	

Appendix 2: The NLS LE I Nutrients Method Summary v012



National
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Method Summary for the determination of Nutrients by Discrete Analysis

Determinands: Ammonia, TON, Nitrite (KCl Extract)

Matrices: Soils, sediments and solid.

Method of Analysis: Potassium Chloride extraction with colorimetric analysis.

Instrumentation: Konelab 30 Discrete Analyser

Principle: Ammonia, total oxidised nitrogen (TON) and nitrite are extracted from the solid samples using potassium chloride solution. The extracts are determined colourimetrically using a Konelab Discrete Analyser.

Range of Application: The range may be extended by dilution of the extract.

Determinand	Range for solid matrices (mg/kg)
Ammonia	0 – 10
Total Oxidised Nitrogen	0 – 100
Nitrite	0 – 5

Sample Container: SOL 1kg Plastic tub

Storage/Preservation: Samples must be analysed immediately or stored in a refrigerator at 5±3°C or a freezer at colder than -18°C for up to 6 months. The holding time for this test is 24hours.

Interferences: If the sample extracts are turbid or coloured false readings could be produced.

QC within Laboratory:

Precision - Better than 10% RSD
Bias - Better than 20% Bias
Error Target - Better than 40% Total Error

Performance testing to WRc NS30. Further information on request.

External Quality Control: Contest.

Appendix 3: The NLS SX I Nutrients Sum - New method summary v17

National
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Service

Method Summary for The Determination of Alkalinity, Ammonia, Chloride, Nitrite, Orthophosphate, Silicate and Total Oxidised Nitrogen by Discrete Analysis

Determinand:

Alkalinity (methyl orange) expressed as mg/L $CaCO_3$
Ammonia reported as Ammoniacal Nitrogen as N
Soluble Chloride
Reactive phosphorus (generally that in the form of orthophosphate)
Nitrite ion
Silicate
Total Oxidised Nitrogen
Nitrate (obtained by calculation)

Matrix: Freshwaters and Effluents

Instrumentation: Konelab Discrete Analyser

Principle:

Alkalinity

The reagent used is methyl orange buffered with potassium hydrogen phthalate. Reduction in the red acid component of the indicator by carbonate/bicarbonates present in the sample is measured as a decrease in absorbance at 550nm.

Ammonia

Ammonia reacts with salicylate and dichloroisocyanurate in the presence of sodium nitroprusside to form a blue colour, the intensity of which is proportional to the amount of ammonia present. Sodium citrate is added to mask possible interference from cations.
The colour produced is measured at 660nm.

Chloride

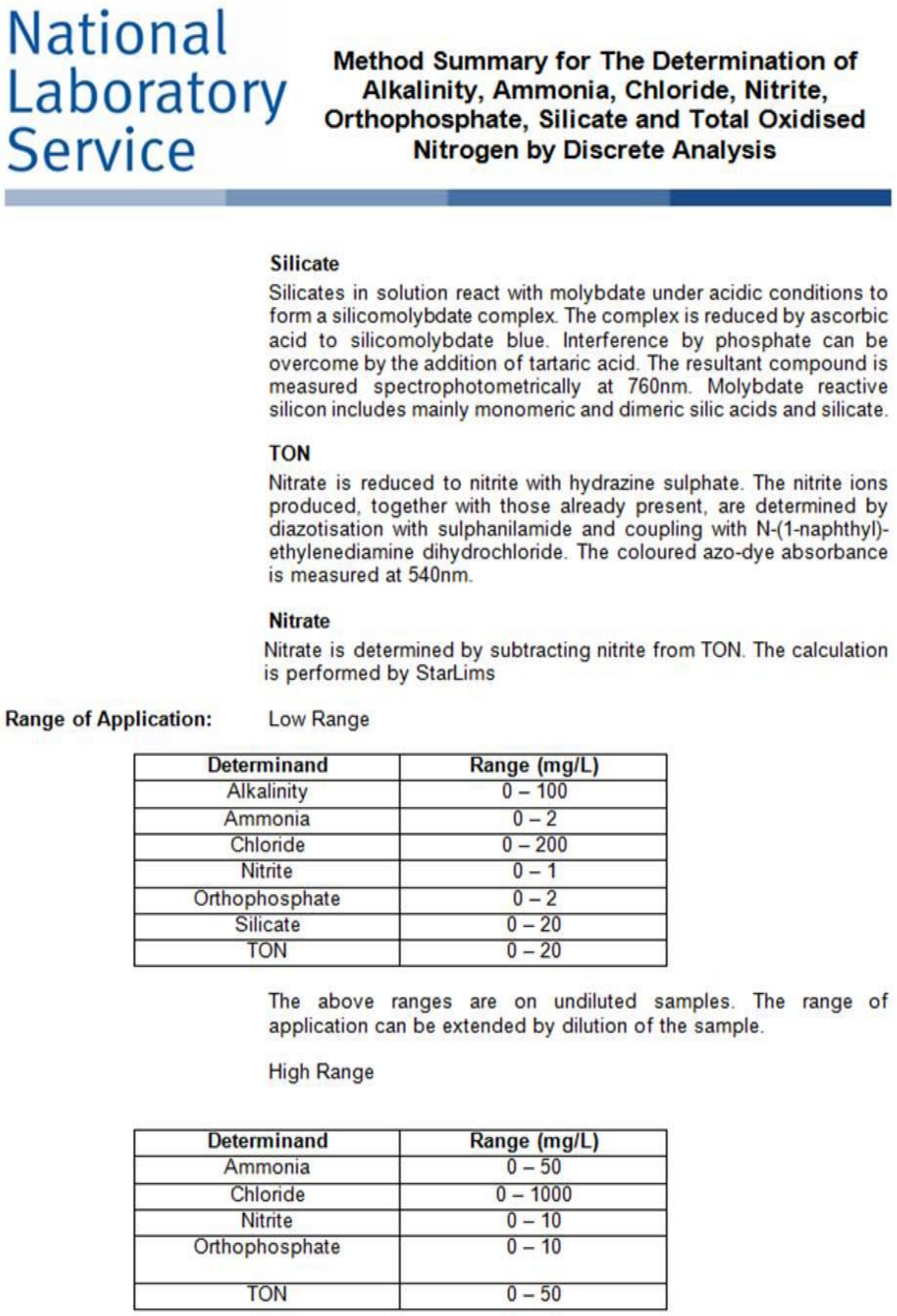
Chloride reacts with mercuric thiocyanate forming a mercuric chloride complex. Released thiocyanate reacts with iron (III) forming a red ferric thiocyanate complex. The intensity of colour produced, measured at 510nm, is proportional to the chloride concentration.

Nitrite

Nitrite ions, when reacted with a reagent containing sulphanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride, in the presence of acid, produce a highly coloured azo dye that is measured photometrically at 540nm.

Orthophosphate

Orthophosphate reacts with ammonium molybdate and antimony potassium tartrate under acidic conditions to form a complex which, when reduced with ascorbic acid produces an intense blue colour, the absorbance of which is measured at 880nm.



National Laboratory Service

Method Summary for The Determination of Alkalinity, Ammonia, Chloride, Nitrite, Orthophosphate, Silicate and Total Oxidised Nitrogen by Discrete Analysis

Silicate

Silicates in solution react with molybdate under acidic conditions to form a silicomolybdate complex. The complex is reduced by ascorbic acid to silicomolybdate blue. Interference by phosphate can be overcome by the addition of tartaric acid. The resultant compound is measured spectrophotometrically at 760nm. Molybdate reactive silicon includes mainly monomeric and dimeric silic acids and silicate.

TON

Nitrate is reduced to nitrite with hydrazine sulphate. The nitrite ions produced, together with those already present, are determined by diazotisation with sulphanilamide and coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride. The coloured azo-dye absorbance is measured at 540nm.

Nitrate

Nitrate is determined by subtracting nitrite from TON. The calculation is performed by StarLims

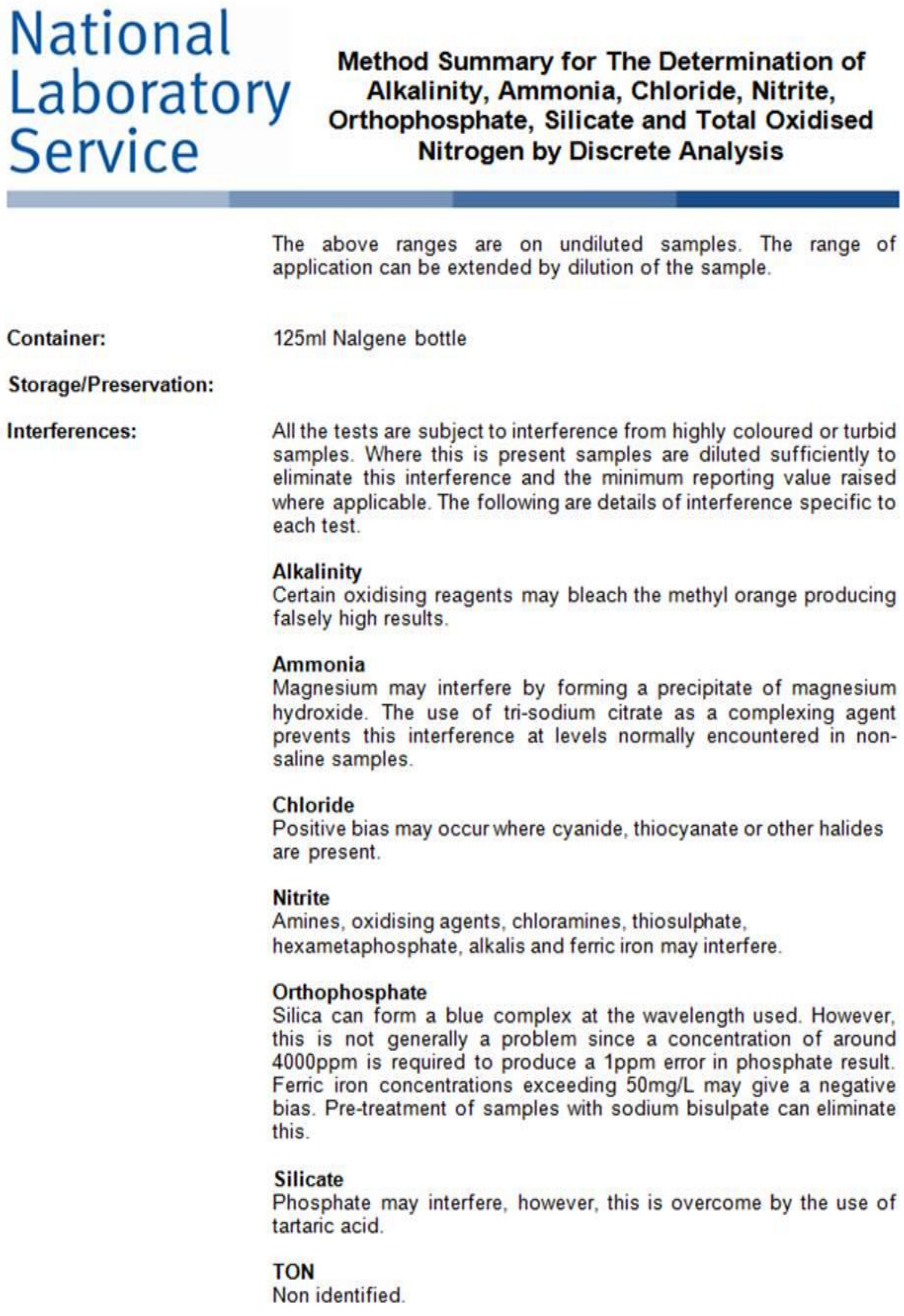
Range of Application: Low Range

Determinand	Range (mg/L)
Alkalinity	0 – 100
Ammonia	0 – 2
Chloride	0 – 200
Nitrite	0 – 1
Orthophosphate	0 – 2
Silicate	0 – 20
TON	0 – 20

The above ranges are on undiluted samples. The range of application can be extended by dilution of the sample.

High Range

Determinand	Range (mg/L)
Ammonia	0 – 50
Chloride	0 – 1000
Nitrite	0 – 10
Orthophosphate	0 – 10
TON	0 – 50



National
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Method Summary for The Determination of Alkalinity, Ammonia, Chloride, Nitrite, Orthophosphate, Silicate and Total Oxidised Nitrogen by Discrete Analysis

The above ranges are on undiluted samples. The range of application can be extended by dilution of the sample.

Container: 125ml Nalgene bottle

Storage/Preservation:

Interferences: All the tests are subject to interference from highly coloured or turbid samples. Where this is present samples are diluted sufficiently to eliminate this interference and the minimum reporting value raised where applicable. The following are details of interference specific to each test.

Alkalinity
Certain oxidising reagents may bleach the methyl orange producing falsely high results.

Ammonia
Magnesium may interfere by forming a precipitate of magnesium hydroxide. The use of tri-sodium citrate as a complexing agent prevents this interference at levels normally encountered in non-saline samples.

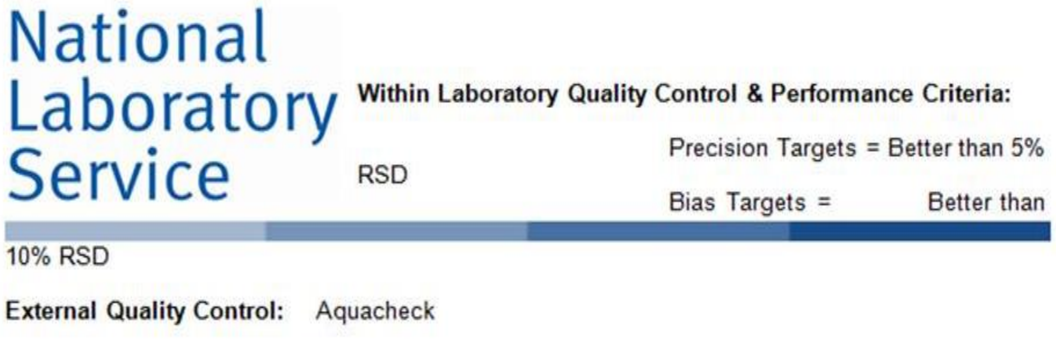
Chloride
Positive bias may occur where cyanide, thiocyanate or other halides are present.

Nitrite
Amines, oxidising agents, chloramines, thiosulphate, hexametaphosphate, alkalis and ferric iron may interfere.

Orthophosphate
Silica can form a blue complex at the wavelength used. However, this is not generally a problem since a concentration of around 4000ppm is required to produce a 1ppm error in phosphate result. Ferric iron concentrations exceeding 50mg/L may give a negative bias. Pre-treatment of samples with sodium bisulpate can eliminate this.

Silicate
Phosphate may interfere, however, this is overcome by the use of tartaric acid.

TON
Non identified.



National Laboratory Service

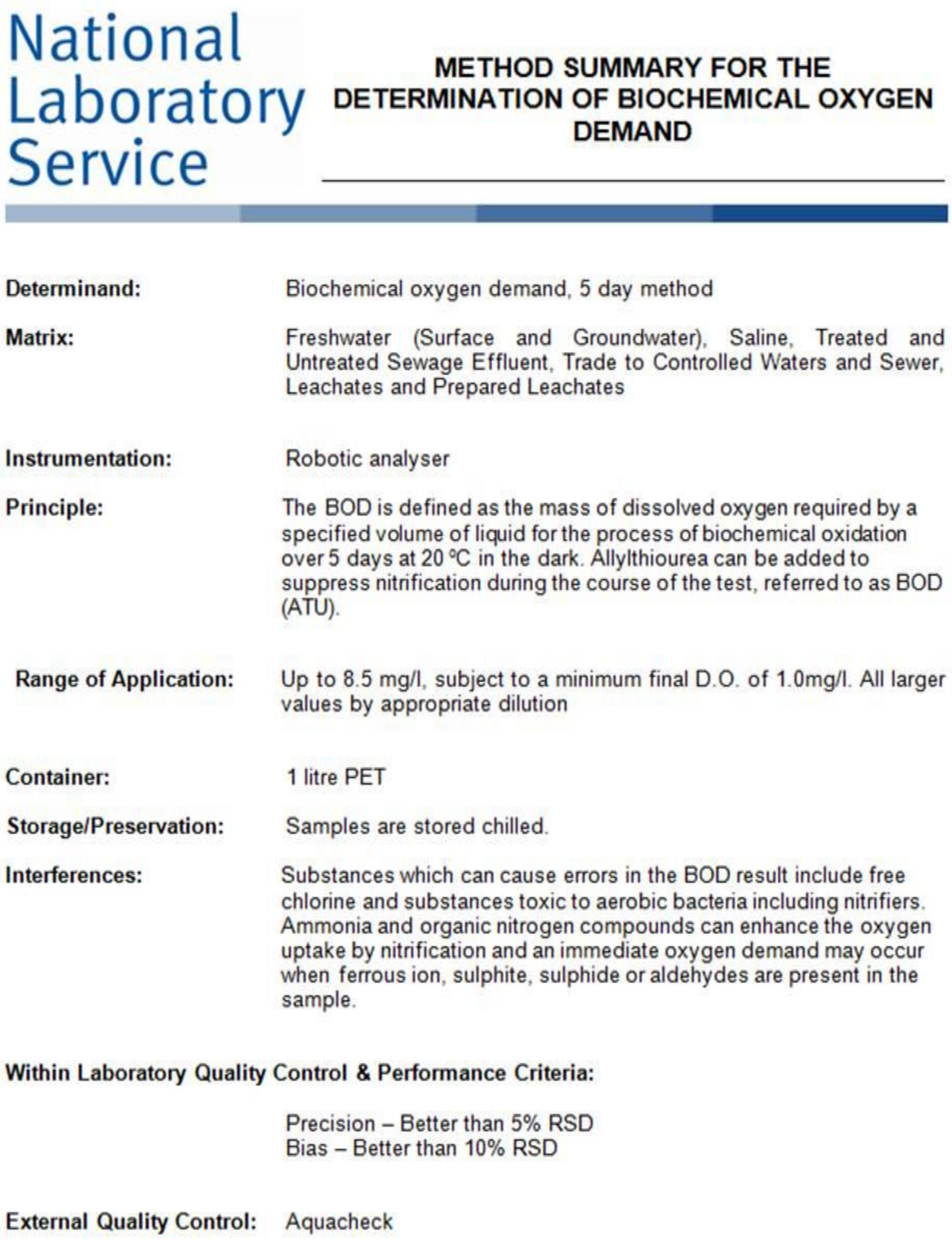
Within Laboratory Quality Control & Performance Criteria:

Precision Targets = Better than 5%
RSD

Bias Targets = Better than
10% RSD

External Quality Control: Aquacheck

Appendix 4: The NLS SX I BOD Sum - New method summary v11



National Laboratory Service

METHOD SUMMARY FOR THE DETERMINATION OF BIOCHEMICAL OXYGEN DEMAND

Determinand:	Biochemical oxygen demand, 5 day method
Matrix:	Freshwater (Surface and Groundwater), Saline, Treated and Untreated Sewage Effluent, Trade to Controlled Waters and Sewer, Leachates and Prepared Leachates
Instrumentation:	Robotic analyser
Principle:	The BOD is defined as the mass of dissolved oxygen required by a specified volume of liquid for the process of biochemical oxidation over 5 days at 20 °C in the dark. Allylthiourea can be added to suppress nitrification during the course of the test, referred to as BOD (ATU).
Range of Application:	Up to 8.5 mg/l, subject to a minimum final D.O. of 1.0mg/l. All larger values by appropriate dilution
Container:	1 litre PET
Storage/Preservation:	Samples are stored chilled.
Interferences:	Substances which can cause errors in the BOD result include free chlorine and substances toxic to aerobic bacteria including nitrifiers. Ammonia and organic nitrogen compounds can enhance the oxygen uptake by nitrification and an immediate oxygen demand may occur when ferrous ion, sulphite, sulphide or aldehydes are present in the sample.

Within Laboratory Quality Control & Performance Criteria:

Precision – Better than 5% RSD
Bias – Better than 10% RSD

External Quality Control:	Aquacheck