Feasibility study for the biocontrol of *Elodea nuttallii* and *E. canadensis*

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Foreword

Invasive alien plants have serious economic and ecological impacts, for example, by displacing native plants and invertebrates, and their management is often costly and ineffective in the long term. Classical biological control (biocontrol) is advocated as an alternative to conventional invasive species management that has the potential for long term, self-perpetuating and effective control, especially in more sensitive environments such as protected areas or riparian habitats.

CABI was commissioned by Natural England to conduct a biocontrol feasibility assessment for *Elodea nuttallii* and *E. canadensis*. This current study improves our current knowledge on the natural enemies for both species in their invasive and native ranges.

Natural England commission a range of reports from external contractors to provide evidence and advice to assist us in delivering our duties. The views in this report are those of the authors and do not necessarily represent those of Natural England.

Executive summary

This report presents information on the North American aquatic plant species *Elodea canadensis* (Canadian pondweed) and *Elodea nuttallii* (Nuttall's waterweed), in the context of:

- their presence and spread in the UK;
- the negative impacts they bring to the aquatic habitats they dominate and;
- the current management options available to control them at sites where they have become problematic.

A summary of current knowledge on the natural enemies (i.e. invertebrates or pathogens) that cause damage to and can reproduce on these plants is given, and the findings of several natural enemy surveys conducted in England in 2022 are also presented. These show little evidence of any significant damage or deterioration caused by either invertebrates or pathogens within the introduced range of the plants. Although the surveys were not extensive, they suggest that the lack of control exerted by natural enemies may be a factor in the invasiveness of these plants.

The feasibility of biological control – a management tool that is becoming widely accepted in the UK as a sustainable alternative to conventional methods – is considered for both species of *Elodea*. This approach identifies natural enemies that are host-specific (feed and develop exclusively on the target plant) and that exert control on the plants in their native range (i.e. North America). While little research has been conducted into the natural enemies in North America, expert opinion suggests that there is likely to be a co-evolved natural enemy present in nature that could be found and tested for the purposes of biological control in the UK.

Finally, research into a rapid and inexpensive method of identification of the two *Elodea* species and two other very morphologically similar aquatic plant species is presented. The results show that it is possible to easily discriminate between these species. While the experiments presented here were conducted using fresh plant samples, this baseline study has the potential to be adapted for more practical use in the field.

Contents

Introduction

The North American waterweeds *Elodea canadensis* and *Elodea nuttallii* are both problematic invasive non-native species in the UK. A Natural England stakeholder workshop in 2022, which aimed to prioritise species for biological control, identified *E. canadensis* and *E. nuttallii* as potential candidates. Funding has been provided by Natural England to investigate the feasibility of biocontrol for these two species. This report presents the findings and outputs of this project.

Objectives

1. Prepare feasibility study into the potential for biological control of *Elodea canadensis* and *Elodea nuttallii*
2. Undertake initial natural enemy surveys in the UK and identify collected specimens
3. Assess suitability of MALDI-TOF technique for rapid identification of/distinction between both *Elodea* species and other selected aquatic plant species

Feasibility study for biocontrol of *Elodea canadensis* and *E. nuttallii*

Taxonomy and nomenclature

Elodea canadensis Michx., also known commonly as Canadian pondweed, is a submerged aquatic plant in the Hydrocharitaceae family. The closely related *Elodea nuttallii* (Planch.) H. St. John, 1920, is commonly known as Nuttall's waterweed. The classification of species in the genus *Elodea* has historically been cause for confusion, but the latest revision of the genus (Cook and Urmi-König, 1985) recognises five species of *Elodea*, all of them either endemic to South America (*E. potamogeton* (Bert) Espinosa; *E. callitrichoides* (Rich.) Caspary) or North America (*E. bifoliata* St. John; *E. canadensis*; *E. nuttallii)*.

Domain: Eukaryota
Kingdom: Plantae
Phylum: Spermatophyta
Subphylum: Angiospermae
Class: Monocotyledonae
Order: Hydrocharitales
Family: Hydrocharitaceae
Genus: *Elodea*

Introduction and distribution

Elodea canadensis and *E. nuttallii* were both introduced from North America into Europe as aquarium plants. *E. canadensis* is believed to have been introduced to the UK in the mid-1800s, and was first recorded in 1836 (Simpson, 1984). From here it spread eastwards throughout Western Europe, and it is now considered invasive across Europe, and Russia (Vinogradova and others, 2018), and is widespread and abundant in other parts of the world including New Zealand, south-eastern Australia, and South Africa. There are also records of its presence in a small number of other African and Asiatic countries (Duenas-Lopez, Popay & Dawson, 2018). It is widespread and common across much of the UK, apart from in the Highlands, Western Isles and Shetland (Figure 1A). Its spread has slowed to a certain extent, and is often replaced by *E. nuttallii* (Dadds, Bell & Wilson, 2007).

Similarly, *E. nuttallii* has spread across central and western Europe, but was introduced later than *E. canadensis*, in the mid-1900s. Records indicate its earliest introductions were to Belgium and the Netherlands, in 1939 and 1941 respectively (DAISIE, 2009), arriving in the UK in 1966 (McGavigan, 2017a). It is also considered invasive in Japan, where it was introduced in 1961 (Kadono, 2004). Even with its more recent appearance in Europe, the spread of *E. nuttallii* seems to have displaced *E. canadensis* from many well-established localities (Simpson, 1984; Barrat-Segretain, 2001).

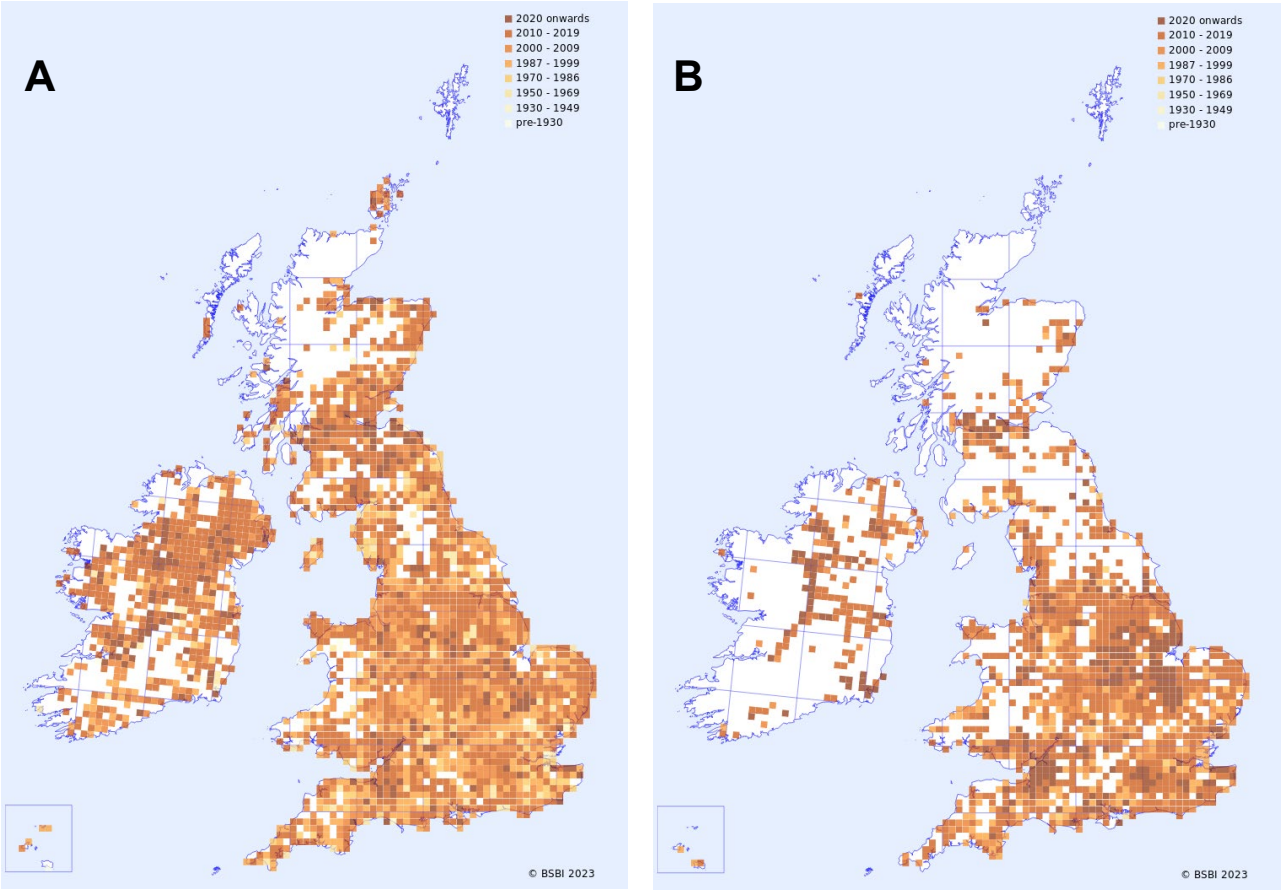

Figure 1. Distributions of *Elodea canadensis* (A) and *Elodea nuttallii* (B) in the UK (maps courtesy of the Botanical Society of Britain and Ireland, accessed April 2023)

Biology

Morphology

Both *E. canadensis* and *E. nuttallii* have very similar morphologies, along with other members of the Hydrocharitaceae family, including the closely related genera *Egeria*, *Lagarosiphon* and *Hydrilla* (Simpson, 1984). These similarities can make the morphological identification of *Elodea* species a challenge, compounded by their wide range of morphological variation (Simpson, 1988). They are submerged-root aquatic plants, with long, slender, freely branching stems up to 3 metres long (Dadds, Bell & Wilson, 2007). The leaves are pale green, strap-like and stalk-less, and are more or less equally spaced along the stem. While the leaves of *E. canadensis* are characterised by a flat elongate shape, those of *E. nuttallii* tend to have a twist of least half a turn along the length of the leaf blade (Duenas-Lopez, Popay & Dawson, 2018) (Figure 2).

Figure 2. Comparative morphology of *Elodea nuttallii* (left) and *Elodea canadensis* (right). Photo credit: Suzy Wood

Reproduction, physiology and phenology

Elodea canadensis and *E. nuttallii* are both dioecious plants, with male and female flowers on separate plants, which float on the water surface. In its native range, the male plants of *E. canadensis* are less common than female plants, while in Europe there have been no records of female plants since 1903 (Cook & Urmi-König, 1985). Reproduction is therefore solely vegetative, by fragments and overwintering stem tips/buds (turions). Likewise, the majority of *Elodea nuttallii* plants in Europe are female, except for one known male colony in Germany. In contrast, the invasive populations in Japan consist solely of male plants (Kunii, 1984).

Ecology

Both species of *Elodea* grow in a range of aquatic habitats, preferring slow-moving water, lakes, ponds and ditches. *Elodea canadensis* is common in nutrient rich/eutrophic inland freshwater bodies (McGavigan, 2017b) and is often associated with organic-rich muds.

The ability of *Elodea* species to reproduce from fragments and overwintering buds and to quickly develop new roots at the nodes allows them to establish rapidly in new habitats. Shoot elongation begins in spring as water temperatures reach 10°C, and ceases when the shoot reaches the water surface, after which a dense canopy is formed (Kunii, 1984). The plant material, which may have formed a large floating mat, then sinks when the water drops below 10°C, where it can then survive until the spring.

Populations of *E. nuttallii* in Europe have been shown to grow more vigorously than those in the native range (Thiebaut and De Nino, 2009), and the plant's high phenotypic plasticity allows it to enhance its performance, particularly in nutrient-rich environments, where it can outcompete *E. canadensis* (Simpson, 1990; Barrat-Segretain & Elger, 2004). Comparing the life-history traits of *E. canadensis* and *E. nuttallii* helps to explain this competitive success: *E. nuttallii* has a higher growth rate, is less sensitive to reduced light intensity, and has vegetative fragments with higher survival and regeneration abilities than *E. canadensis* (Barrat-Segretain and others, 2002; Barrat-Segretain, 2004; Angelstein & Schubert, 2009).

Impact

Economic

The dense submerged mats formed by both *Elodea* species are the main cause of negative impact in their invasive range. These mats prevent access to the water for navigation, recreation and infrastructure purposes. Water intake pipes blocked by *Elodea* can limit the use of water for industrial and domestic purposes (Sand-Jensen, 2000 in Josefsson 2011). Mats, sections and fragments of the plant can impede the drainage of waterways, exacerbating flooding, and limit the flow of water in irrigation channels, reducing the water supply to irrigation-fed crops, for example.

Ecological

In terms of environmental and ecological impact, again, it is the dense stands of plant material that cause problems. *Elodea* species can outcompete native plants for space, light and nutrients, sometimes to the point of complete displacement, thus reducing biodiversity (Josefsson & Andersson, 2001; Kadono, 2004). In a study of lakes in Northern Ireland, Kelly and others (2015) showed small but significant negative impacts of *E. nuttallii* invasion on freshwater plants, microalgae and invertebrates. *Elodea canadensis* in Finland has been found to alter invertebrate communities, with knock-on effects on fish predators (Kornijów and others, 2004). Recently, Piacente & Berg (2022) found that in Alaska (USA), where *E. canadensis* is invasive, the associated community structure of aquatic invertebrates differs to that of the native range in Illinois (USA); lower densities of macroinvertebrates in the shredder-herbivore functional feeding group were associated with *E. canadensis* in the invasive range, suggesting that its successful establishment in Alaska is likely facilitated by reduced herbivory.

Health and social

There are no recorded health impacts of either *Elodea* species within the invasive range. The large, dense mats do, however, have social impacts, adversely affect boating, fishing and recreational activities (Josefsson, 2011). Both species are listed as invasive non-native plants in Schedule 9 of the Wildlife and Countryside Act (England and Wales).

Management

Chemical

The aquatic environments in which these waterweeds exist limits the use of herbicides for their control in the UK.

Environmental

According to Newman & Duenas (2010a), shading of *E. canadensis* mats by either trees cover, floating plastic covers, or dyes in the water is an effective control method, although timing of this appears to be critical (Stockan &Fielding, 2017).

Manual/mechanical

Management advice for both *Elodea* species in the UK focuses primarily on physical removal by mechanical means. Recommendations for best practice, however, vary slightly between the two species. For *E. canadensis*, cutting in March using trailing knives dragged along the bottom of the waterbody is suggested to avoid the weed reaching peak biomass in the summer (Newman and Duenas, 2010a). For *E. nuttallii*, Newman and Duenas (2010b) recommend cutting before July. It is also possible to weaken the plant by repeated cutting during the summer months.

For both species, the cut plant material should be removed to avoid deoxygenation of the water; this can be left in small heaps away from the water's edge. Larger quantities should be taken off site for composting/incineration. The location itself also requires netting to retain propagules for the prevention of spread.

Any methods that require physical removal of plant material carry the constraints of time, access and disposal facilities.

Biological

Current biological control approaches for in the UK are based around the use of non-native, generalist fish species such as grass carp (*Ctenopharyngodon idella*), common carp (*Cyprinus carpio*) and rudd (*Scardinius erythrophthalmus*). Stockan & Fielding (2017) have reviewed studies on this method for both *E. canadensis* and *E. nuttallii*. In some cases up to a 100% reduction in coverage of *E. canadensis* was achieved using grass

carp (Mitzner, 1978; Maceina and others, 1992). Maceina and others (1992), however, reported increases in phosphate, phosphorus and ammonium after using herbivorous fish on *E. canadensis*. For *E. nuttallii*, expert opinion advises grass carp and common carp as effective control agents (Newman and Duenas, 2010b). In Germany, attempts have also been made to control *E. nuttallii* using rudd (Ruhrverband, 2009). A clear disadvantage of this approach is that without host-specific biocontrol agents, there is no guarantee that populations of native submerged macrophytes would not be equally reduced by the introduction of these fish.

Prospects for classical biological control in the UK

As opposed to the examples listed above, where generalist herbivores are used to reduce invasive plant growth, classical biological control (CBC) of invasive plants is the introduction of a host-specific natural enemy that can develop and sustain a population only on specific target plants and does not pose a threat to other species unintentionally. The intention is not to eradicate the target plant using biological control agents, but to use them to reduce the plant's vigour and growth to below an economic or ecological threshold. The following section of this report details natural enemies that have been found on *Elodea canadensis* and *E. nuttallii* and summarises the prospects for CBC of these two species.

Natural enemies

There are relatively few records of insect herbivory on *Elodea canadensis* and *E. nuttallii.* Much of the literature on these species cites the low preference for or avoidance of these waterweeds (in particular of *E. nuttallii*) by various invertebrates (Erhard and others. 2007; Redekop and others, 2018; Smirnov 1962, in Newman 1991). Both species are known to contain allelochemicals that allow the weeds to better compete against algae and cyanobacteria (Erhard & Gross 2006), and these are believed to be at least one of the factors responsible for low herbivory by insects (Erhard and others, 2007).

Nonetheless, larvae of the caddisfly *Phryganea grandis* have been cited as feeding on *Elodea* species (Smirnov 1962, in Newman 1991). Two generalist aquatic moths, one European (*Acentria ephemerella*) and one from New Zealand (*Hygraula nitens*), have been recorded feeding on *E. canadensis*, though in both cases this has not been preferential to other aquatic macrophytes (Erhard and others, 2007; Redekop and others, 2018, respectively). Host-range testing during US biological control research into the invasive South American waterweed *Egeria densa* has revealed that the stem-mining fly *Hydrellia egeriae* can complete its life-cycle on *E. canadensis* (Pratt and others, 2019). In their study on macroinvertebrates associated with *E. canadensis* in Illinois (native range), Piacente & Berg (2022) noted the presence of an unidentified Dolichopodidae (Diptera) within the stems and leaves of sampled plants, something that was not observed on *E. canadensis* in the invasive range, and which appears to be the first report of dolichopodids associated with *E. canadensis*. Two species of larval dolichopodids have been reported feeding on vascular bundles and fungi within petioles of *Eichhornia crassipes* (water

hyacinth) in Argentina (Hernandez and others, 2007), but further investigation would be required to understand whether the dolichopodids in *E. canadensis* are feeding/reproducing on the plant, or simply using it for structural support during development (Piacente & Berg, 2022). The lack of herbivores associated with *E. canadensis* in the invasive range of Alaska is likely to be the case in Europe too, and initial natural enemy surveys in the UK (see pages 15-18) support this.

Various literature sources mention fungal diseases or nematodes as likely causes for the rapid decline of *E. canadensis* during the first half of the 20th century in the UK and continental Europe (long before the introduction of *E. nuttallii*). However, such claims are never backed up with a reference to evidence-based research. Kowarik (2003), for example, claims that a fungus (*Fusarium* sp.) was identified in laboratory tests as damaging to *E. canadensis* citing Gollasch (2006), but unfortunately the source of this reference not available. Otherwise, there is only one other record of a fungus on *Elodea* (*E. canadensis*) from the USA, which is *Megachytrium westonii* recorded from New York (Farr & Rossman 2022). Zettler & Freeman (1972) cite in a paper assessing the potential of plant pathogens as biocontrols of aquatic weeds 'We have been unable to find reports of diseases affecting species of *Hydrilla*, *Egeria*, *Anacharis*, or *Elodea*, although we believe that diseases do affect these hydrocharitaceous plants.

Kowarik (2003) claims that some nematodes were identified in laboratory tests as damaging to *E. canadensis*, referring to a paper by Gerber & Smart (1987) which cannot be accessed. Other work of these authors generally refers to other aquatic species and their work is restricted to the USA, the natural range of *E. canadensis*. Although nematodes are known to damage aquatic macrophytes (Prejs 1986a, Prejs 1986b, Prejs 1987, Traunspurger 2002 [not listed in the reference list] in Podraza and others, 2008), control of *Elodea* plants from a reservoir in Germany didn't show any evidence of mining nematodes. Equally, rinsing of plant material didn't yield any nematodes living on *Elodea* plants (Podraza and others, 2008).

Studies on the cumulative feeding effect of generalist invertebrate herbivores on *Elodea nuttallii* in lakes across Europe have revealed a water snail (*Lymnaea peregra*), an isopod (*Asellus meridinanus*) and the larvae of a midge (*Endochironomus albipennis*) to be the primary consumers of *E. nuttallii* (Kornijów, 1996). However, this only contributed to an average 8% reduction in plant biomass.

Although both *Elodea* species have been identified as major weeds of aquatic habitats across Europe, so far, the prospect of classical biological control has not been explored. Indeed, there don't appear to have been any solid attempts to assess the occurrence of natural enemies in the native range of either *Elodea* species. Communication with a number of aquatic weed and biocontrol experts in North America, sought during this project, corroborates this.

Next steps

A potential next step for *Elodea* biocontrol comes from the research conducted by Pratt and others (2019), which revealed, under quarantine conditions, that the fly *Hydrellia egeriae* can complete its lifecycle on *Elodea canadensis* as well as on *Egeria densa* (the target species). *H. egeriae* developed at similar rates on both species under choice and no-choice tests, thus indicating that the fly may be a suitable candidate for biocontrol of *E. canadensis* in the UK. It should be noted, however, that *H. egeriae* originates from Argentina, where *E. canadensis* is not present. Closely related South American plant species *Elodea callatrichoides* and *Egeria natans*, though good hosts of the fly in laboratory tests, have never been observed as hosts in the field in Argentina (Fundación para el Estudio de Especies Invasivas, pers. comm., 24th March 2023). In South Africa, where *Hydrellia egeriae* has recently been released as a biocontrol agent against *Egeria densa*, a risk assessment was conducted to understand the physiological host range of the leaf-mining fly (Smith and others, 2019). This revealed that the non-target aquatic plant *Lagarosiphon major* was able to support larval development of the fly, but that the plant was not able to sustain a viable population for more than three generations. Further research with *Hydrellia egeriae* would be required to determine whether *Elodea canadensis* or indeed *Elodea nuttallii* could sustain a population of this fly species.

Further potential comes from expert opinion that suggests there may be *Hydrellia* species present in the native range of *Elodea canadensis* (USDA-ARS, pers. comm., 23rd March 2023), and possibly *E. nuttallii* too, in temperate North America. The overall consensus from researchers in North America who were contacted regarding the potential for biological control of *Elodea* species in the UK is that there are likely to be good biocontrol agents present in the native range, but that adequate efforts to find them have yet to be made. Several of these researchers will be present at the forthcoming XVI International Symposium on Biological Control of Weeds being held in Argentina in May 2023, which a number of CABI scientists will be attending. This will provide an opportunity to continue conversations on this topic and pursue potential collaboration.

The authors of this report recommend that, if current management practices are deemed insufficient for the control of *Elodea* species in the UK, the option of biological control should be explored further. With the presence of highly damaging natural enemies in this country unlikely (see section entitled “Natural enemy surveys in the UK” for more information), it is suggested that host-specific natural enemies from the native range are sought for the purposes of classical biological control. This would initially require field surveys to sites in North America where the plants are present, to search for signs of damage caused by invertebrates or pathogens, followed by species identifications, prioritisation, and testing for host-range specificity in UK quarantine facilities.

Natural enemy surveys in the UK

Background

With little available knowledge on the natural enemies of *Elodea canadensis* or *E. nuttallii*, both in the invasive and native range, CABI has been tasked with conducting surveys of populations of these waterweeds in the UK.

Sites

In total, seven sites known to contain either *E. canadensis* or *E. nuttallii* were surveyed, with a focus on south-east England. A total of 13 samples were collected during these surveys (Table 1).

Table 1. Details on samples of *Elodea canadensis* and *E. nuttallii* collected

Site number	Site name	Species	Location	Geographic co-ordinates	Date of collection
1	**Beaulieu River**	*Elodea canadensis*	Ashurst, New Forest	50.872172, -1.513378	07/07/22
2	**Burton Mill Pond**	*Elodea canadensis*	Petworth, West Sussex	50.953204, -0.607574	27/07/22
3	**Chilley Farm Road**	*Elodea nuttallii*	Pevensey, East Sussex	50.839275, 0.309722	27/07/22
4	**Horseshoe Lake**	*Elodea nuttallii*	Sandhurst, Berkshire	51.353159, -0.824466	14/08/22
5	**Jubilee River**	*Elodea nuttallii*	Slough, Berkshire	51.500632, -0.604651	30/08/22
6	**Jubilee River**	*Elodea nuttallii*	Slough, Berkshire	51.500753, -0.611068	30/08/22
7	**Jubilee River**	*Elodea nuttallii*	Slough, Berkshire	51.500674, -0.613145	30/08/22
8	**Jubilee River**	*Elodea nuttallii*	Slough, Berkshire	51.502969, -0.634090	30/08/22
9	**Jubilee River**	*Elodea nuttallii*	Slough, Berkshire	51.503186, -0.636365	30/08/22

Site number	Site name	Species	Location	Geographic co-ordinates	Date of collection
10	**Jubilee River**	*Elodea nuttallii*	Slough, Berkshire	51.503428, -0.639718	30/08/22
11	**Carter's Pit**	*Elodea nuttallii*	Rye, East Sussex	50.936350, 0.744732	27/09/22
12	**Rye Harbour Sewer**	*Elodea nuttallii*	Rye, East Sussex	50.939437, 0.749459	27/09/22
13	**Rye Harbour Sewer**	*Elodea canadensis*	Rye, East Sussex	50.939437, 0.749459	27/09/22

Sample assessments

On return from field surveys, the collected plant material was inspected for signs of damage and disease and the presence of invertebrates. Nematode extraction (Figure 2B) via water traps was carried out for subsamples of plants where any signs of decay were observed. Plants were then kept outdoors in containers filled with rain water.

Overall, the plant material collected was healthy, and with no major signs of decay, and no clear evidence of fungal or bacterial infection. Table 2 details the organisms that were found via microscopic inspection and extractions of the samples, and includes mostly free-living, non-associated invertebrates such as snails, leeches, copepods and protozoa (Figure 2C).

Table 2. Organisms found with samples of *Elodea canadensis* and *Elodea nuttallii*

Organism	Host/prey	Scientific name
Leech	Blood-feeders, or small invertebrates	Unknown
Snail	Detritus	*Physella acuta*
Flatworm	Small invertebrates, protozoa	*Turbellaria* species
Nematode (free-living)	Bacteria	Unknown
Water bug (Heteropteran nymph)	Plants	Family Coroxidae
Copepods	Plankton	Subclass Copepoda

Organism	Host/prey	Scientific name
Waterfleas	Plankton	Order Diplostraca (synonym Onychura)
Protozoan (free-living)	Bacteria	*Vorticella* species

Minor but widespread feeding damage on both *E. nuttallii* (Figure 2A) and *E. canadensis* (Figure 2D) collected from Rye Harbour Nature Reserve warranted further investigation. The damage, which appears to be single cells having been consumed, may have been caused by a sucking insect. Only a single Heteropteran bug nymph (Corixidae) was found in this sample, but it could be possible that many more were present before collection and had caused the damage previously. The free-living nematodes were also found with the sample but are not thought to be the cause of damage.

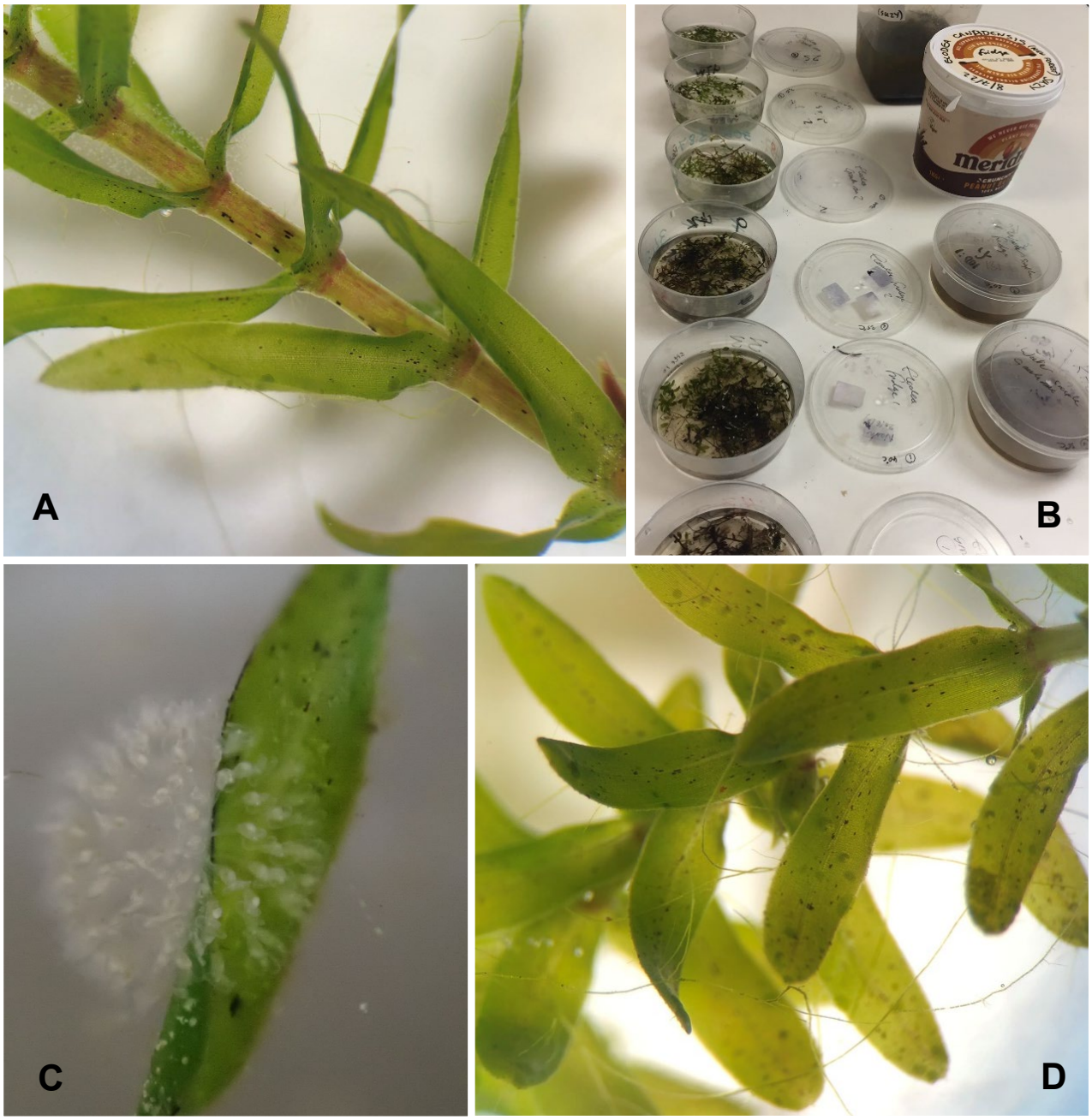

Figure 2. A) *Elodea nuttallii* sample with potential signs of insectivorous feeding; B) Samples being prepared for nematode extraction; C) Protozoan (likely *Vorticella* sp.) on *E. nuttallii*; D) *E. canadensis* sample with potential signs of insectivorous feeding.
Photo credit: Suzy Wood

Conclusions

Further efforts will be made in Spring/Summer 2023 to collect samples of *Elodea nuttallii* and *E. canadensis* across other parts of England, in order to build a better picture of the level of natural enemy damage on these species in this area of their invasive range. However, it appears unlikely that there will be any specific herbivores associated with either *Elodea* species in the UK, as was found to be the case with invasive *Elodea canadensis* in Alaska (Piacente & Berg, 2022).

Suitability of MALDI-TOF technique for *Elodea* species identification

As congeners, *Elodea canadensis* and *E. nuttallii* have very similar morphologies, and can also be easily mistaken for other invasive non-native aquatic weeds, particularly *Lagarosiphon major* and *Egeria densa*. Their visual similarities can make field identification – important for all management approaches – difficult. This objective aims to assess the suitability of MALDI-TOF, a mass spectrometry-based technique, for rapid identification of/distinction between both *Elodea* species and other selected aquatic plants species.

Appendix 1 of this report provides a manuscript-style write-up of the research.

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Appendices

Appendix 1

Rapid and inexpensive MALDI-TOF MS-based discrimination between the pondweeds *Elodea canadensis*, *Elodea nuttallii*, *Egeria* sp., and *Lagarosiphon major*

Michael A. Reeve, Lisa Offord and Suzy V. Wood

Abstract

Using a highly-simplified and inexpensive method for MALDI-TOF MS sample preparation that lyses cells by immersion in aqueous acetonitrile containing trifluoroacetic acid to selectively extract acid-soluble proteins, peptides, and other similarly-soluble basic cellular components (with extraction carried out in the presence of near-saturated and inexpensive-grade MALDI matrix), discrimination has been attempted between the visibly-similar pondweeds *Elodea canadensis*, *Elodea nuttallii*, *Egeria sp.*, and *Lagarosiphon major.* In part 1 (for *Elodea canadensis* and *Elodea nuttallii* sampled 04-10-2022), we have observed clear spectral separation with low variance between the average same-species Bruker score and the average different-species Bruker score, which is indicative of both good spectral difference between the two species (1.02 Bruker units between the average values) and good spectral reproducibility between replicates (+/-5.7% for the *Elodea canadensis* quadruplicates and +/-3.0% for the *Elodea nuttallii* quadruplicates). We therefore conclude that these two pondweeds are well suited to discrimination by means of MALDI-TOF MS using the method described and samples processed on 04-10-2022. In part 2 (for *Elodea canadensis*, *Elodea nuttallii*, *Egeria sp.*, and *Lagarosiphon major* sampled 29-03-2023), we observed good discrimination for the pondweeds *Elodea nuttallii*, *Egeria* sp., and *Lagarosiphon major* but not, as observed in part 1, *Elodea canadensis* using the samples processed on 29-03-2023, possible reasons for which are discussed.

Key words

Matrix-assisted laser-desorption and ionisation time-of-flight mass spectrometry, pondweeds, *Elodea canadensis*, *Elodea nuttallii*, *Egeria* sp., and *Lagarosiphon major*

Abbreviations used

MALDI-TOF MS (matrix-assisted laser-desorption and ionisation time-of-flight mass spectrometry)
HCCA (α-cyano-4-hydroxycinnamic acid)
TFA (trifluoroacetic acid)
EC (*Elodea canadensis*)
EN (*Elodea nuttallii*)
ES (*Egeria* sp.)

LM (*Lagarosiphon major*)

Introduction

The pondweeds *Elodea canadensis*, *Elodea nuttallii*, *Egeria* sp., and *Lagarosiphon major* are similar in appearance and therefore difficult to discriminate between. To this end, we have investigated the possibility of using matrix-assisted laser-desorption and ionisation time-of-flight mass spectrometry (MALDI-TOF MS) for this purpose.

MALDI-TOF MS is a powerful and versatile technique that employs the MALDI soft ionisation process[1] to prepare large proteins intact in the gas phase that carry predominantly a single positive charge[2]. When such charged proteins in the gas phase are accelerated by means of an electrical field along a tube held at high vacuum, their times-of-flight are proportional to the square root of their mass-over-charge ratios. Using this simple relationship, a mass spectrum can readily be generated for the protein components in a particular biological sample[3]. Mass spectra of a subset of the expressed proteome (most commonly the highly-expressed acid-soluble proteins, including many ribosomal proteins) are often employed for the characterisation and identification of biological samples[4]. Much of the development of MALDI-TOF MS for the identification and/or characterisation of biological samples has been shaped by human clinical microbiology, and a key driver has been the diagnosis of bacterial and yeast infections[4]. The role currently played by MALDI-TOF MS in human clinical microbiology is extensively reviewed by Clark *et al*.[3], together with much of the underlying theory, along with methods commonly employed for MALDI-TOF MS sample preparation. Further methods have also been developed for yeasts[5] and filamentous fungi[6], notably the 'full-extraction' protocols after the work of Cassagne *et al*.[7]. Reeve *et al.* have also developed a highly simplified and inexpensive method for MALDI-TOF MS sample preparation[8]. This method lyses cells by immersion (or maceration, for plant or insect material) in aqueous acetonitrile containing TFA to selectively extract acid-soluble proteins, peptides, and other similarly soluble basic cellular components. In the method, lysis and extraction are carried out in the presence of near-saturated and inexpensive-grade MALDI matrix. The resulting matrix-saturated lysate, which contains extracted acid-solubilised components, can then simply be dried down directly onto the MALDI-TOF MS sample plate and analysed.

In the current report, we have used this MALDI-TOF-MS as a rapid, efficient, and cost-effective method to attempt discrimination between the visibly similar pondweeds *Elodea canadensis*, *Elodea nuttallii*, *Egeria* sp., and *Lagarosiphon major*.

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Materials and methods

Reagents

≥99.8% ethanol, LC-MS-grade water, ≥ 98% (TLC-grade) α-cyano-4-hydroxycinnamic acid (HCCA) matrix, LC-MS-grade acetonitrile, and 99% ReagentPlus®-grade TFA were purchased from Sigma (Gillingham, UK).

MALDI-TOF MS sample preparation

Plant biomass from propagation in water was rinsed under tap water and roughly two 3 mm x 1 mm fragments were mixed with 50 µl of 11 mg/ml HCCA matrix in 65% (v/v) acetonitrile, 2.5% (v/v) TFA, and 32.5% (v/v) water and macerated with the blunt end of a plastic inoculating loop. One microlitre of the resulting crude lysates was then pipetted onto the Bruker sample plate, air dried, and loaded into the spectrometer. In part 1, visibly-green plant biomass was sampled for *Elodea canadensis* and *Elodea nuttallii* on 04-10-2022. In part 2, visibly-green plant biomass was sampled for *Elodea nuttallii*, *Egeria* sp., and *Lagarosiphon major*, and visibly-brown biomass was sampled for *Elodea canadensis* on 29-03-2023.

Mass spectrometry

Mass spectrometry covering the range 2 kDa to 20 kDa was carried out using a Bruker Microflex LT linear-mode instrument running the MALDI Biotyper 4.0 applications (Bruker Daltonik, Bremen, Germany), using a nitrogen laser at 337 nm, with 240 laser shots per sample, and an ion-source voltage of 19.98 kV. Calibration was carried out using the manufacturer's 'BTS' controls (E. coli proteins supplemented with ribonuclease A and myoglobin), using peaks with masses at 3,637.8; 5,096.8; 5,381.4; 6,255.4; 7,274.5; 10,300.2; 13,683.2, and 16,952.3 for calibration according to the manufacturer's instructions. Spectra were acquired using MALDI Biotyper RTC Version 4.0 (Build 19)

using the manufacturer's standard settings (Centroid peak-detection algorithm and TopHat baseline subtraction).

Spectral comparison

Database entries were made as single-spectra MSPs (Main Spectra) using the Bruker Online Client software suite (Version 4.0.19, Bruker Daltonik, Bremen, Germany), again using the manufacturer's standard settings. For spectral comparisons (performed between all samples in MSP data format), Bruker identification scores were derived using the standard Bruker algorithm. This first converts raw mass spectra into peak lists, which are then compared between spectra. Three separate values are computed: the number of peaks in the reference spectrum that have a closely matching partner in the test spectrum (value range 0-1), the number of peaks in the test spectrum that have a closely matching partner in the reference spectrum (value range 0-1), and the peak-height symmetry of the matching peaks (value range 0-1). The above three values are multiplied together and normalised to 1,000, and the base-10 logarithm is then taken to give the final Bruker score (range 0-3). Bruker scores of between 2.3 and 3.0 indicate very close relatedness, scores between 2.0 and 2.3 indicate close relatedness, and scores below 1.7 indicate low relatedness.

Results – part 1 (samples prepared 04-10-2022)

Figure 3 shows the MALDI-TOF MS reference spectra for the pondweeds *Elodea canadensis* and *Elodea nuttallii* with, from top to bottom, the *Elodea canadensis* quadruplicates EC-1, EC-2, EC-3, EC-4 and the *Elodea nuttallii* quadruplicates EN-1, EN-2, EN-3, and EN-4. Spectra are shown throughout as baseline-subtracted, smoothed, y-axis-autoscaled, and covering the mass range 2 kDa to 20 kDa.

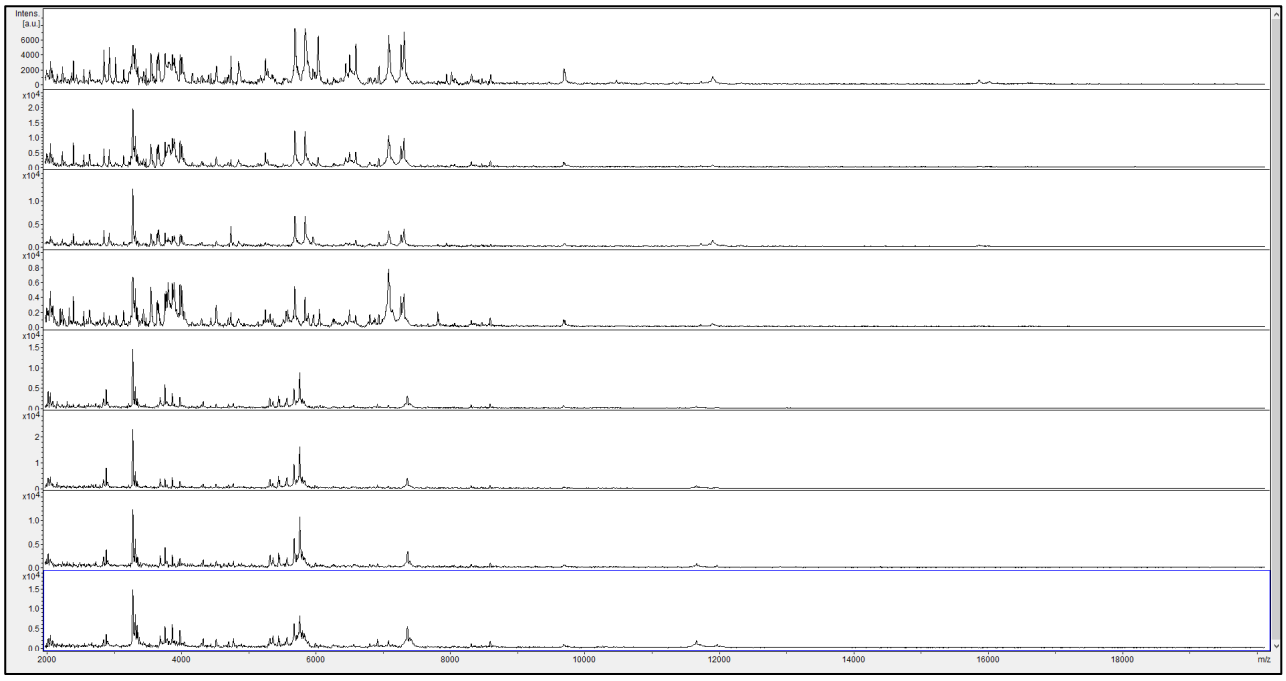

Figure 3. MALDI-TOF MS reference spectra for the pondweeds *Elodea canadensis* and *Elodea nuttallii* with, from top to bottom, the *Elodea canadensis* quadruplicates EC-1, EC-2, EC-3, EC-4 and the *Elodea nuttallii* quadruplicates EN-1, EN-2, EN-3, and EN-4.

Figure 3 shows reasonably good spectral reproducibility between the reference quadruplicates and visibly different spectra for *Elodea canadensis* and *Elodea nuttallii.*

Table 3 shows the spectral-comparison data for all comparisons between the reference spectra shown in Figure 3.

Table 3. Bruker-score spectral-comparison data for all comparisons between the reference spectra shown in Figure 3.

		Test spectrum							
		EC-1	EC-2	EC-3	EC-4	EN-1	EN-2	EN-3	EN-4
Reference spectrum	EC-1	3.00	2.46	2.28	2.29	0.85	1.20	1.35	1.16
	EC-2	2.46	3.00	2.23	2.55	1.44	1.48	1.60	1.49
	EC-3	2.28	2.23	3.00	2.20	1.11	1.33	1.31	1.30
	EC-4	2.29	2.55	2.20	3.00	1.41	1.56	1.63	1.41
	EN-1	0.65	1.47	1.11	1.45	3.00	2.44	2.39	2.44
	EN-2	1.20	1.49	1.33	1.56	2.44	3.00	2.45	2.50
	EN-3	1.35	1.60	1.31	1.55	2.39	2.45	3.00	2.28
	EN-4	1.16	1.49	1.31	1.41	2.44	2.50	2.28	3.00

Table 4 shows the average Bruker-score spectral-comparison data and standard deviations for all different-species comparisons and all same-species (but omitting perfect 3.00-score comparisons between identical-sample-spectra).

Table 4. Average Bruker-score spectral-comparison data and standard deviations for all same-species (omitting comparisons between identical-sample-spectra) and all different-species comparisons

	Average	Standard deviation
Same species	2.38	0.11
Different species	1.35	0.21

The data from Table 4 are shown graphically in Figure 4.

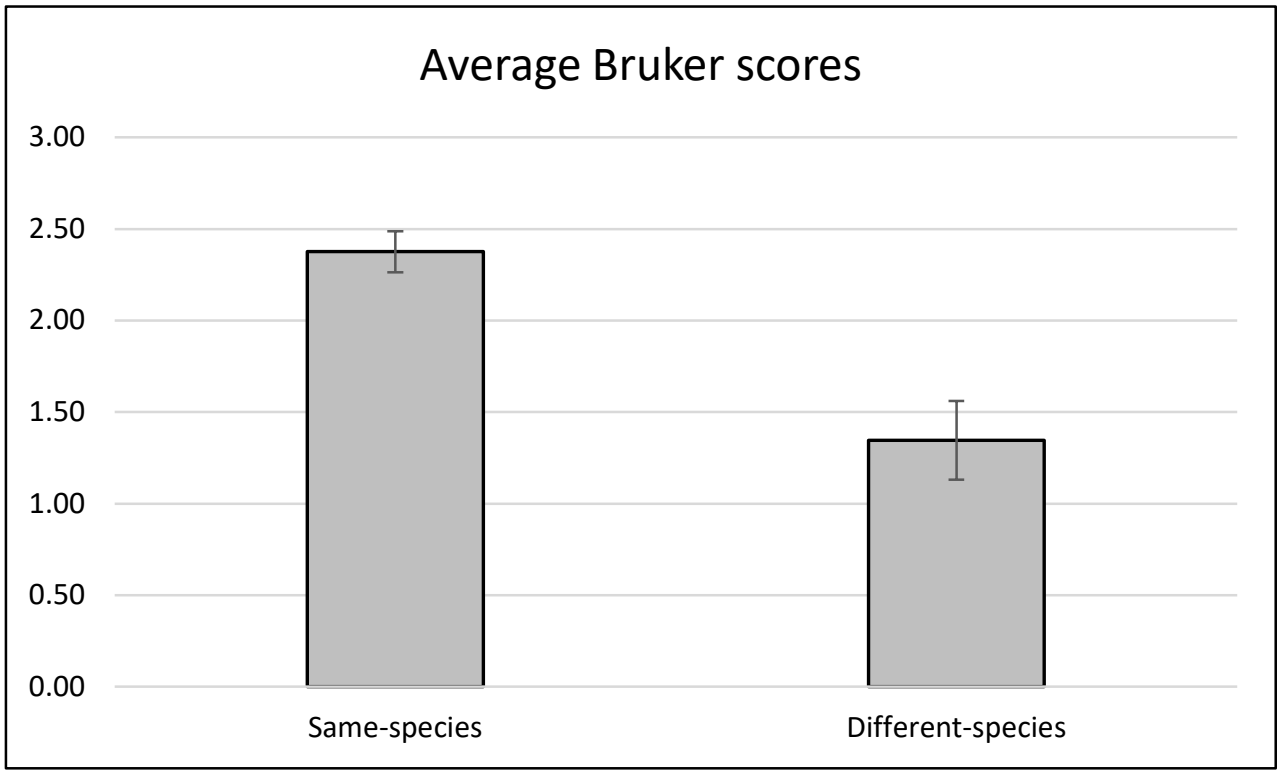

Figure 4. Average Bruker-score spectral-comparison data and standard deviations for all same-species (omitting comparisons between identical-sample-spectra) and all different-species comparisons

Figure 4 shows clear separation with low variance between the average same-species Bruker score and the average different-species Bruker scores, which is indicative of both good spectral difference between the two species (1.02 Bruker units between the average values in Figure 3) and good spectral reproducibility between the replicates (the average non-self spectral-comparison score for the quadruplicates EC-1 to EC-4 is 2.34 with a standard deviation of 0.13, giving a reproducibility of +/-5.7%, and the average non-self spectral-comparison score for the quadruplicates EN-1 to EN-4 is 2.42 with a standard deviation of 0.07, giving a reproducibility of +/-3.0%). These two pondweeds are therefore well suited to discrimination by means of MALDI-TOF MS using this method with the samples processed on 04-10-2022.

Results – part 2 (samples prepared 29-03-2023)

In part 2, samples of all four pondweeds were prepared as per the methods section and also diluted 10-fold in 11 mg/ml HCCA matrix in 65% (v/v) acetonitrile, 2.5% (v/v) TFA, and 32.5% (v/v) water due to the high levels of pigment visible in the undiluted samples (which can have an adverse impact on spectral quality).

Figure 5 shows the undiluted MALDI-TOF MS triplicate test spectra and triplicate reference spectra for the pondweed *Elodea canadensis*. Spectra are shown throughout as baseline-subtracted, smooth, y-axis-autoscaled, and covering the mass range 2 kDa to 20 kDa.

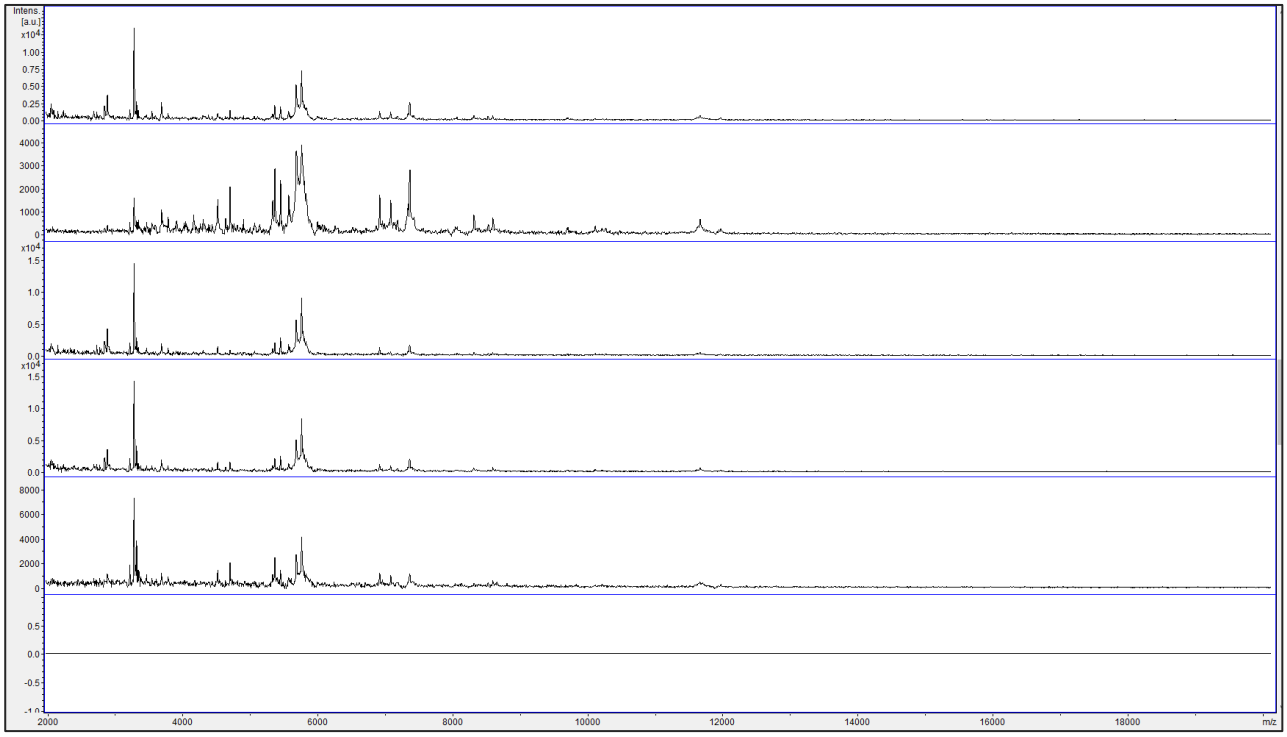

Figure 5. Undiluted MALDI-TOF MS test and reference spectra for the pondweed *Elodea canadensis* with, from top to bottom, the triplicate test spectra EC1, EC2, EC3, and the triplicate reference spectra EC4, EC5, EC6.

Figure 5 shows good spectral reproducibility between the *Elodea canadensis* samples EC1, EC3, EC4, and EC5, visible difference with sample EC2, and failure of EC6. The relatively high baseline noise for many of the spectra is indicative of weak spectral peaks from these samples.

Figure 6 shows the undiluted MALDI-TOF MS triplicate test spectra and triplicate reference spectra for the pond *Elodea nuttallii*.

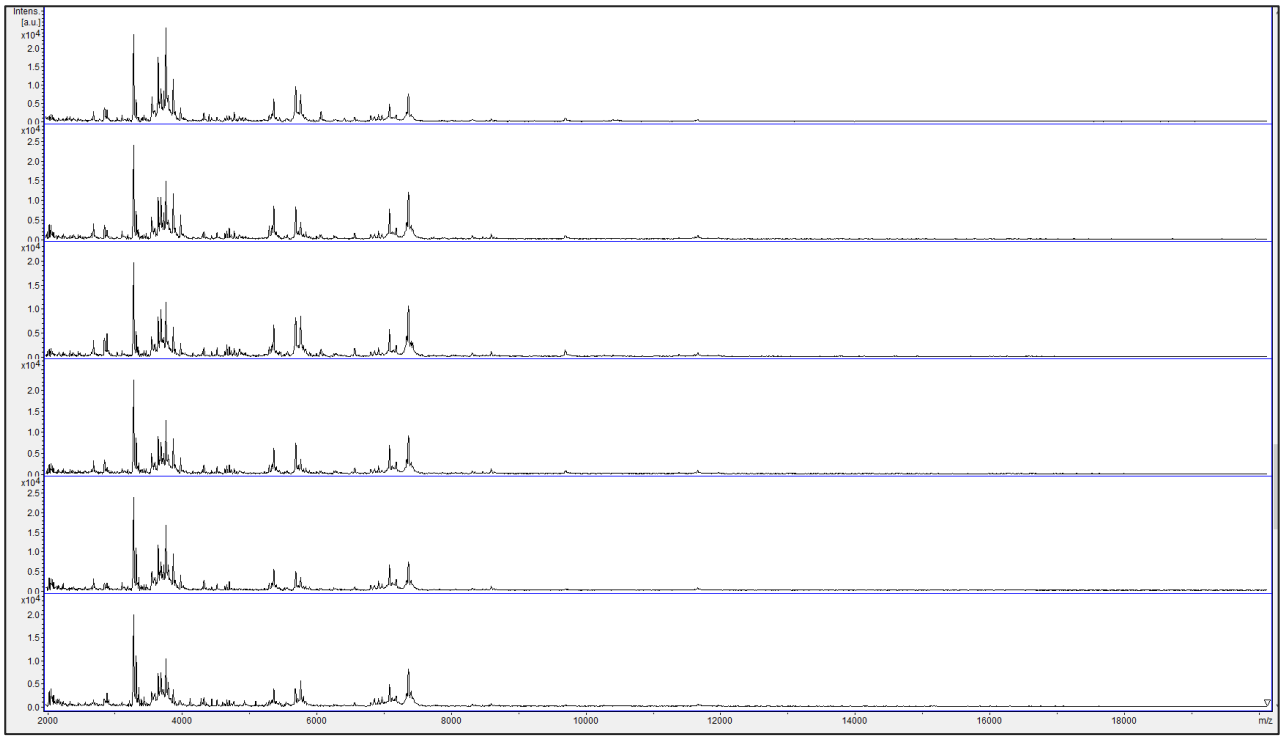

Figure 6. Undiluted MALDI-TOF MS test and reference spectra for the pondweed *Elodea nuttallii* with, from top to bottom, the triplicate test spectra EN1, EN2, and EN3, and the triplicate reference spectra EN4, EN5, and EN6.

Figure 6 shows good spectral reproducibility between the *Elodea nuttallii* test and reference triplicates.

Figure 7 shows the undiluted MALDI-TOF MS triplicate test spectra and triplicate reference spectra for the pondweed *Egeria* sp.

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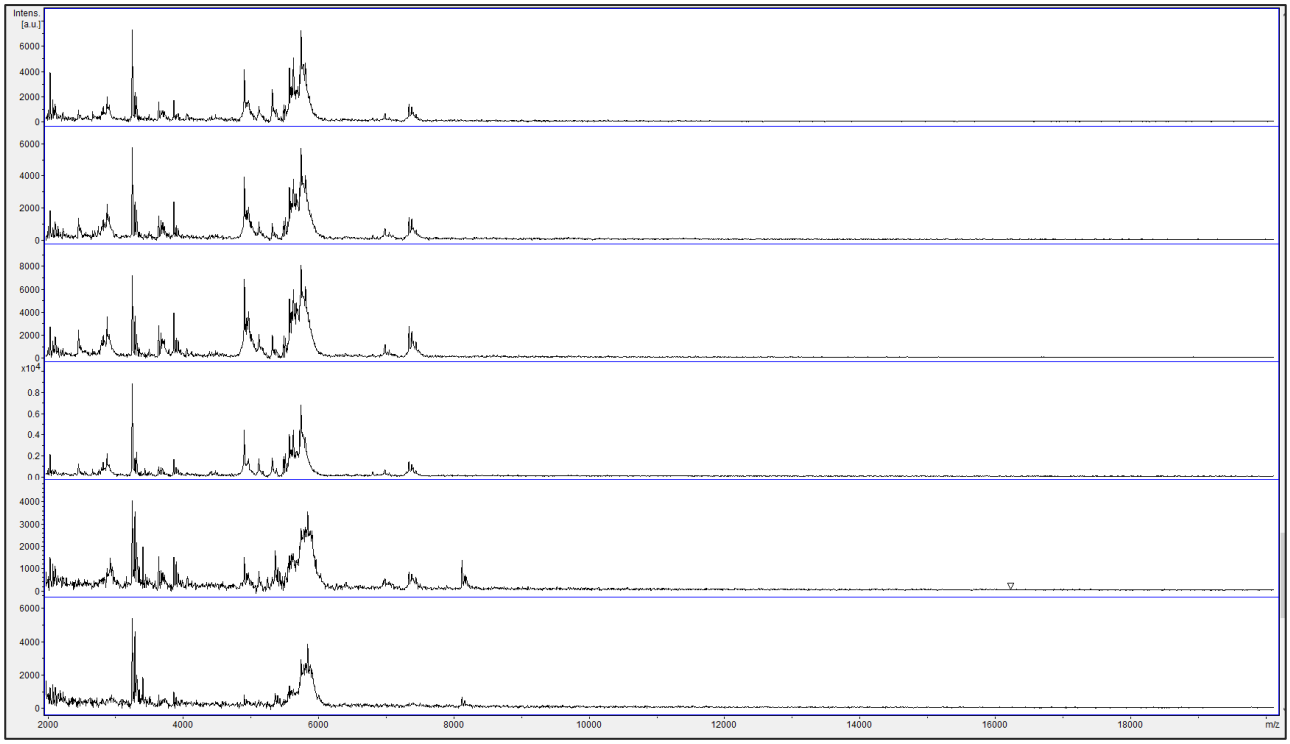

Figure 7. Undiluted MALDI-TOF MS test and reference spectra for the pondweed *Egeria* sp. with, from top to bottom, the triplicate test spectra ES1, ES2, and ES3, and the triplicate reference spectra ES4, ES5, and ES6.

Figure 7 shows reasonably good spectral reproducibility between the *Egeria* sp. test and reference triplicates.

Figure 8 shows the undiluted MALDI-TOF MS triplicate test spectra and triplicate reference spectra for the pondweed *Lagarosiphon major*.

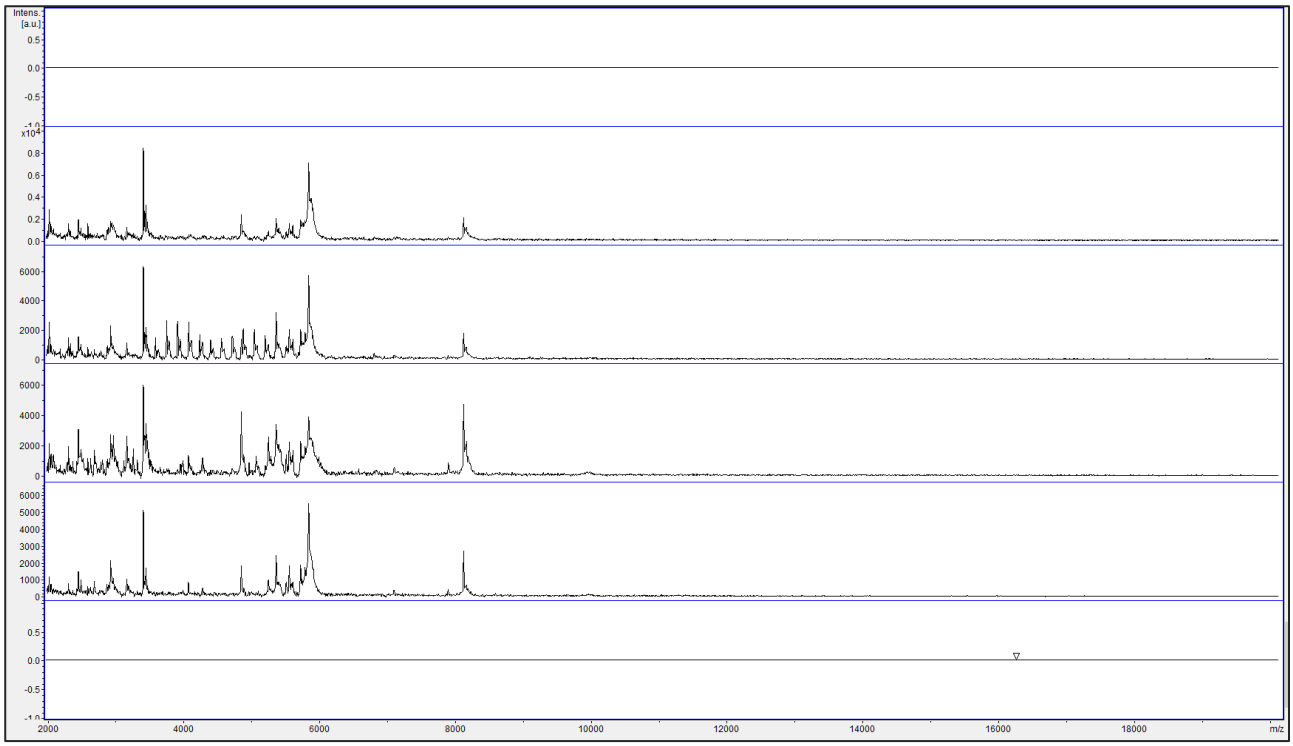

Figure 8. Undiluted MALDI-TOF MS test and reference spectra for the pondweed *Lagarosiphon major* with, from top to bottom, the triplicate test spectra LM1, LM2, and LM3, and the triplicate reference spectra LM4, LM5, LM6.

Figure 8 shows poor specitral reproducibility between the *Lagarosiphon major* samples LM2, LM3, LM4, and LM5, and failure of samples LM1 and LM6.

Figure 9 shows for comparison the test-replicate-1 undiluted MALDI-TOF MS spectra for the pondweeds *Elodea candensis*, *Elodea nuttallii*, and *Egeria* sp., and the test-replicate-2 undiluted MALDI-TOF MS spectrum for the pondweed *Lagarosiphon major*.

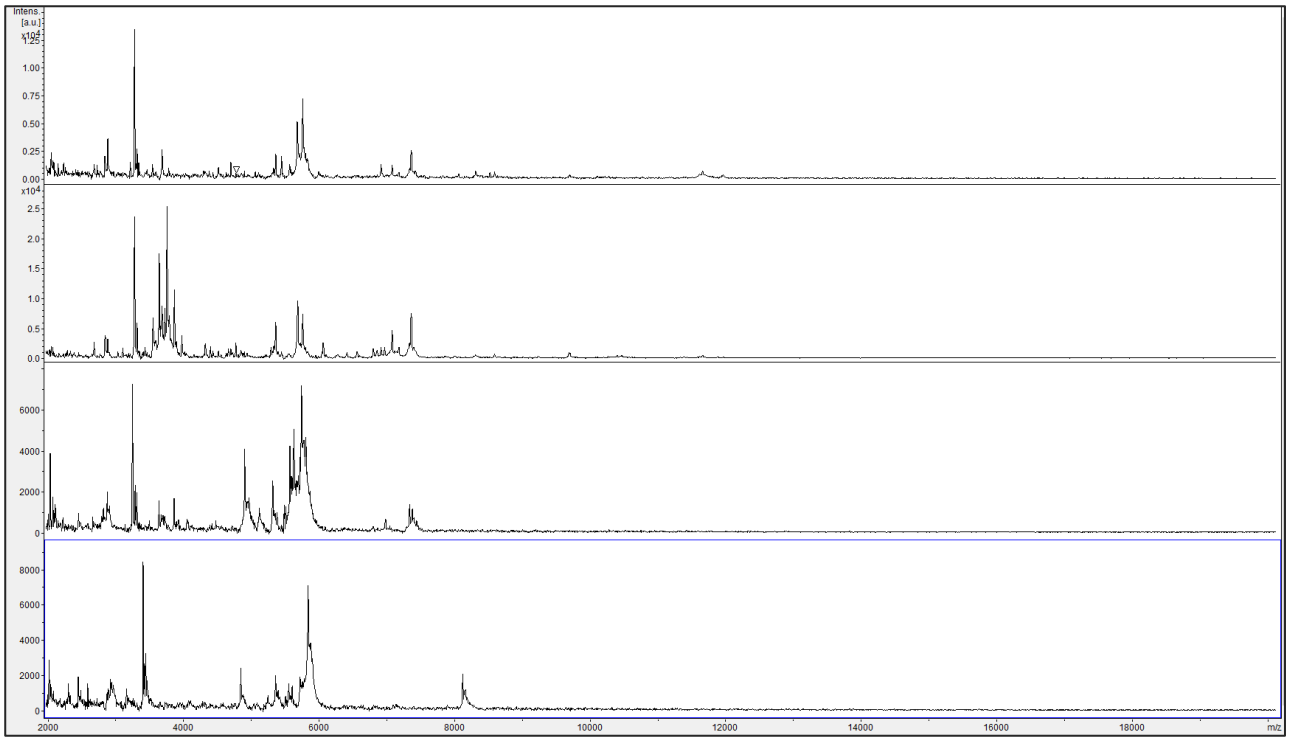

Figure 9. Test-replicate-1 undiluted MALDI-TOF MS spectra for the pondweeds *Elodea canadensis*, *Elodea nuttallii*, and *Egeria* sp., and the test-replicate-2 undiluted MALDI-TOF MS spectrum for the pondweed *Lagarosiphon major* with, from top to bottom, EC1, EN1, ES1, and LM2.

Figure 9 shows visibly different test-replicate-1 undiluted spectra for the pondweeds *Elodea canadensis*, *Elodea nuttallii*, and *Egeria* sp., and the test-replicate-2 undiluted spectrum for the pondweed *Lagarosiphon major*.

Figure 10 shows the diluted MALDI-TOF MS triplicate test spectra and triplicate reference spectra for the pondweed *Elodea candensis*.

Figure 10 shows good spectral reproducibility between the *Elodea canadensis* test and reference triplicates. The elevated baseline noise for the spectra is indicative of reasonably weak spectral peaks from these samples.

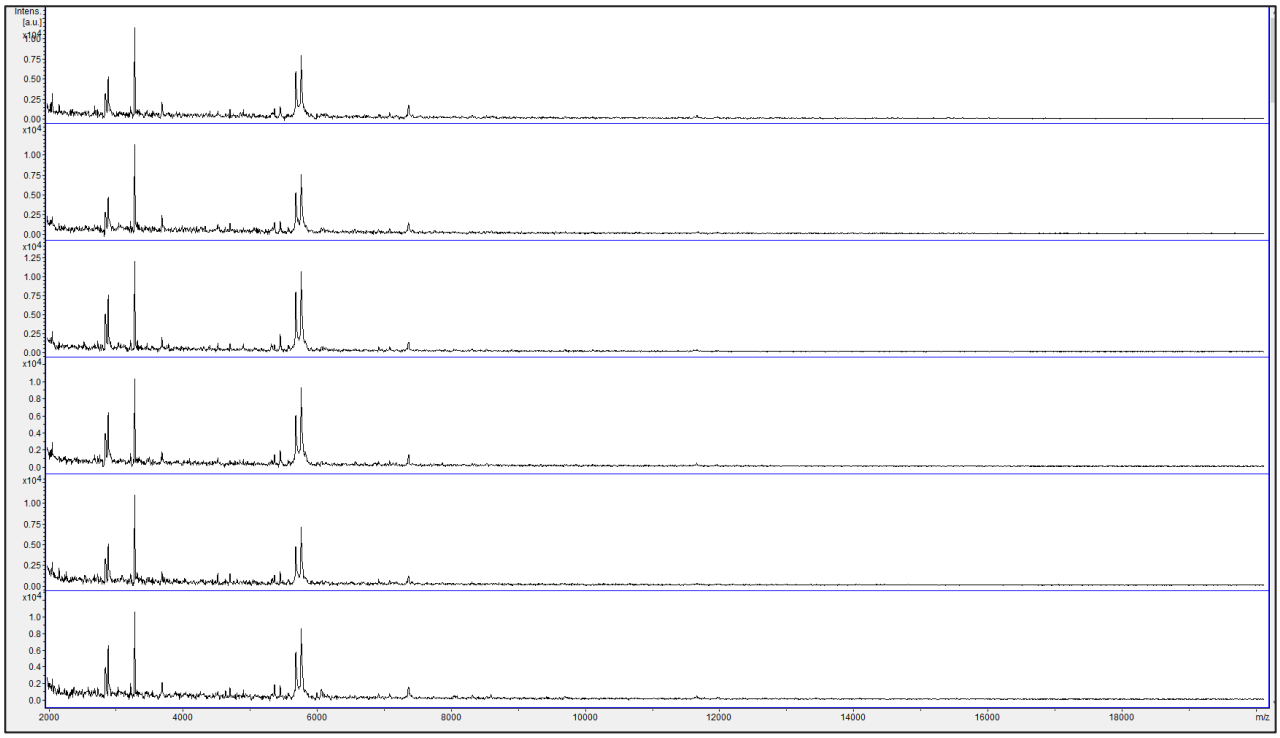

Figure 10. Diluted MALDI-TOF MS test and reference spectra for the pondweed *Elodea canadensis* with, from top to bottom, the triplicate test spectra DIL-EC1, DIL-EC2, and DIL-EC3, and the triplicate reference spectra DIL-EC4, DIL-EC5, and DIL-EC6.

Figure 11 shows the diluted MALDI-TOF MS triplicate test spectra and triplicate reference spectra for the pondweed *Elodea nuttallii*.

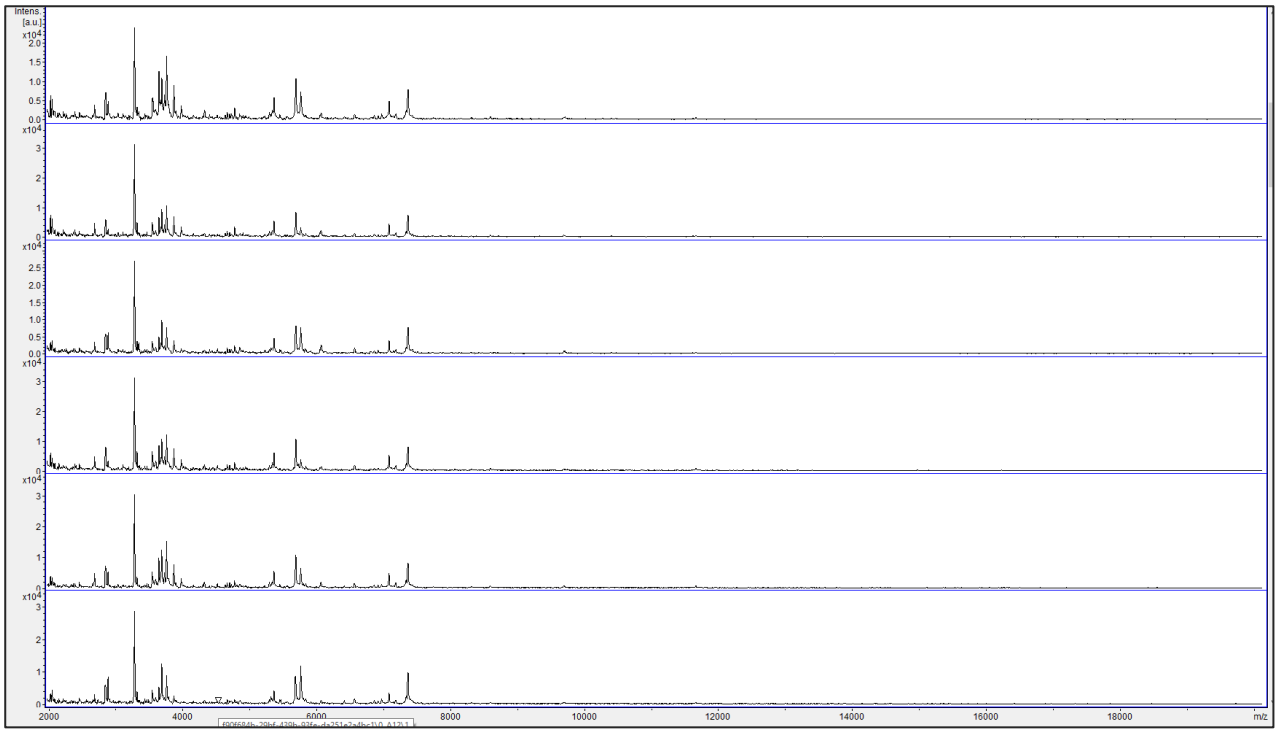

Figure 11. Diluted MALDI-TOF MS test and reference spectra for the pondweed *Elodea nuttallii* with, from top to bottom, the triplicate test spectra DIL-EN1, DIL-EN2, and DIL-EN3, and the triplicate reference spectra DIL-EN4, DIL-EN5, and DIL-EN6.

Figure 11 shows good spectral reproducibility between the *Elodea nuttallii* test and reference triplicates.

Figure 12 shows the diluted MALDI-TOF MS triplicate test spectra and triplicate reference spectra for the pondweed *Egeria* sp.

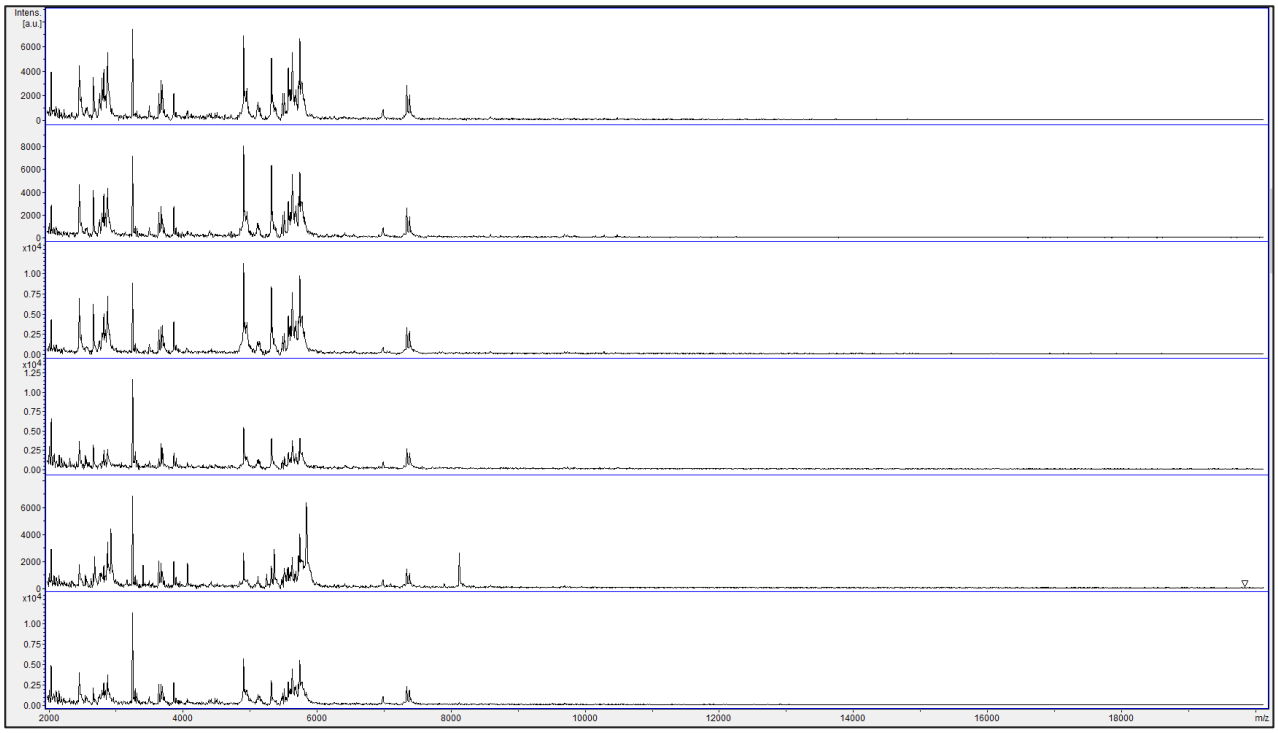

Figure 12. Diluted MALDI-TOF MS test and reference spectra for the pondweed *Egeria* sp. with, from top to bottom, the triplicate test spectra DIL-ES1, DIL-ES2, and DIL-ES3, and the triplicate reference spectra DIL-ES4, DIL-ES5, and DIL-ES6.

Figure 12 shows reasonably good spectral reproducibility between the *Egeria* sp. test and reference triplicates.

Figure 13 shows the diluted MALDI-TOF MS triplicate test spectra and triplicate reference spectra for the pondweed *Lagarosiphon major*.

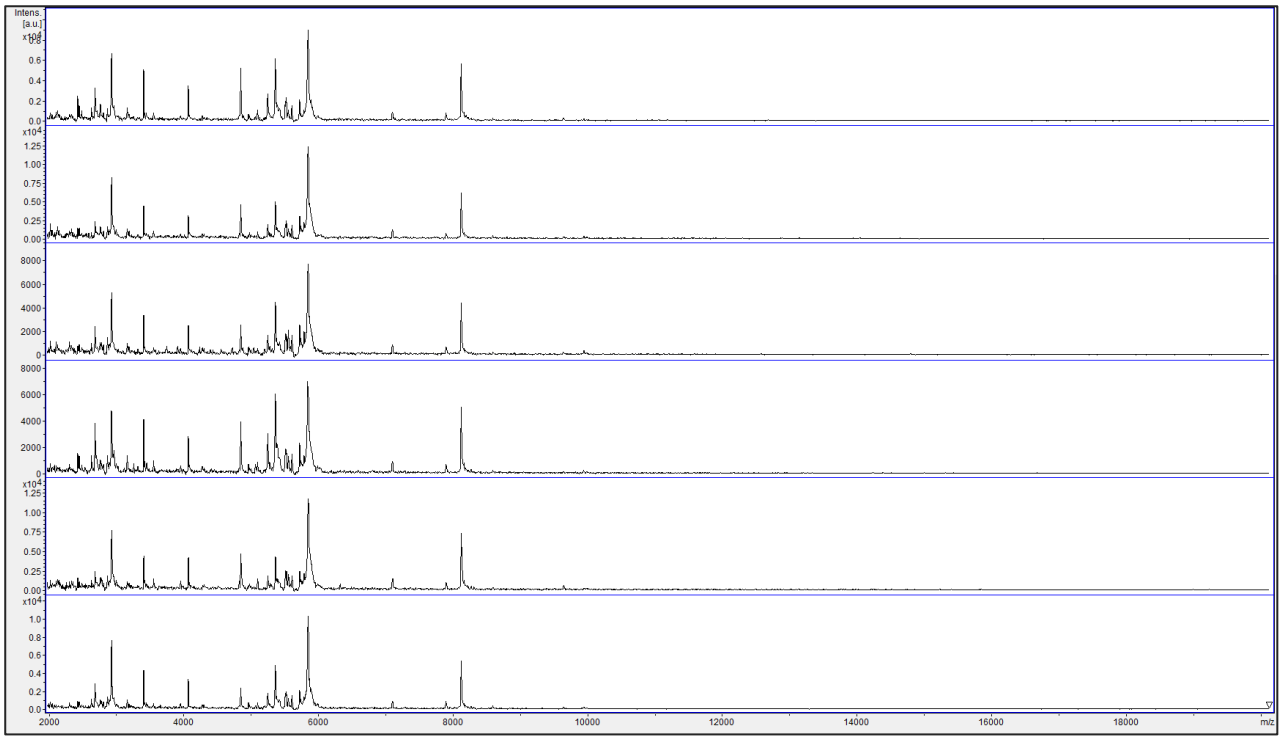

Figure 13. Diluted MALDI-TOF MS st and reference spectra for the pondweed *Lagarosiphon major* with, from top to bottom, the triplicate test spectra DIL-LM1, DIL-LM2, and DIL-LM3, and the triplicate reference spectra DIL-LM4, DIL-LM5, and DIL-LM6.

Figure 13 shows good spectral reproducibility between the *Lagarosiphon major* test and reference triplicates.

Figure 14 shows for comparison the test-replicate-1 diluted MALDI-TOF MS spectra for the pondweeds *Elodea candaensis*, *Elodea nuttallii*, *Egeria* sp., and *Lagarosiphon major*.

Figure 14 shows visibly different test-replicate-1 diluted spectra for *Elodea canadensis*, *Elodea nuttallii*, *Egeria sp.*, and *Lagarosiphon major*. The lower peak-to-baseline noise ratio for the *Elodea canadensis* spectrum suggests slightly weaker spectral peaks from this sample.

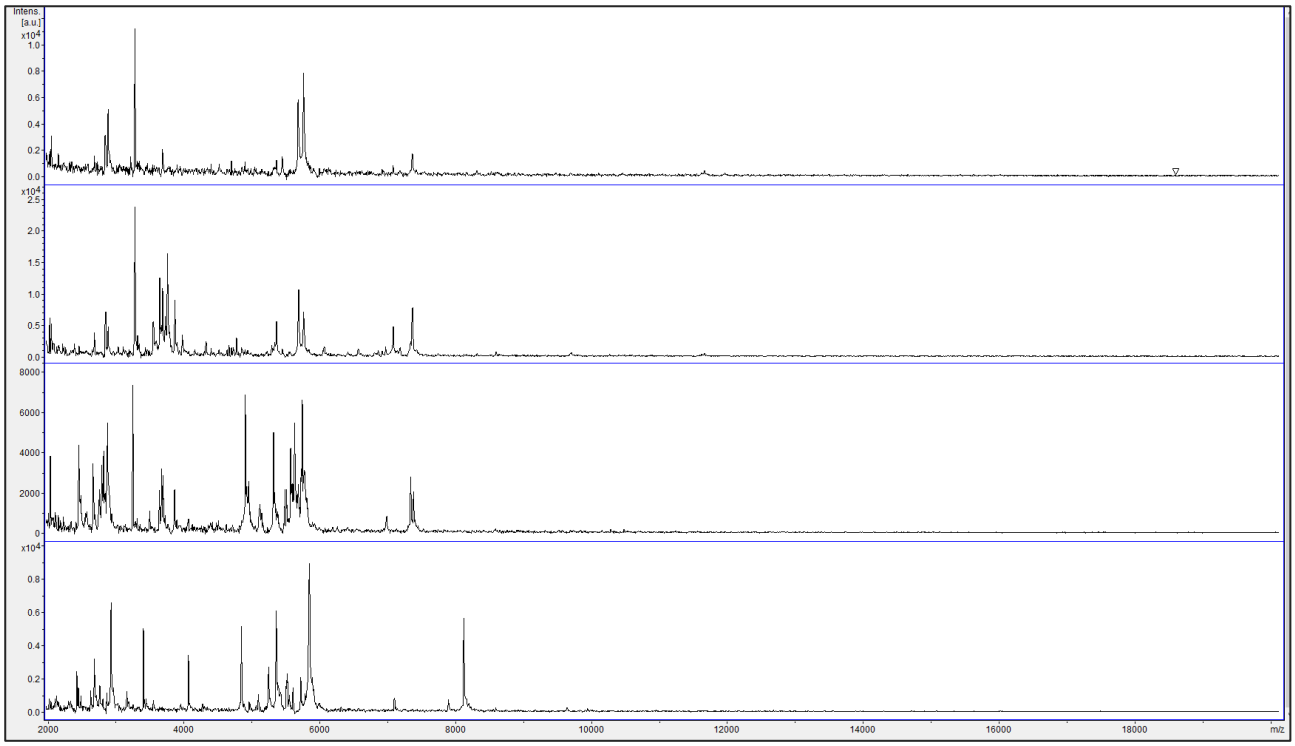

Figure 14. Test-replicate-1 diluted MALDI-TOF MS spectra for the pondweeds *Elodea canadensis*, *Elodea nuttallii*, *Egeria* sp., and *Lagarosiphon major* with, from top to bottom, DIL-EC1, DIL-EN1, DIL-ES1, and DIL-LM1.

Figures 10-14 show (as expected from the levels of residual plant pigment) higher-quality spectra for the diluted samples compared to Figures 5-9 for the undiluted samples. As a result, only the diluted samples were used for further spectral comparisons.

Table 5 shows the spectral-comparison data for all comparisons between the diluted test and reference spectra shown in Figures 10-13.

Table 5. Bruker-score spectral-comparison data for all comparisons between the diluted test and reference spectra shown in Figures 10-13.

		Test spectrum											
		DIL-EC1	DIL-EC2	DIL-EC3	DIL-EN1	DIL-EN2	DIL-EN3	DIL-ES1	DIL-ES2	DIL-ES3	DIL-LM1	DIL-LM2	DIL-LM3
Reference spectrum	DIL-EC4	1.69	1.75	1.58	1.77	1.72	1.74	0	0.64	0.54	0.58	0	0.37
	DIL-EC5	1.70	1.78	1.67	1.63	1.90	1.78	0	0.71	0.76	0.38	0.24	0.75
	DIL-EC6	1.59	1.60	1.81	1.62	1.54	1.62	0	0.42	0.33	0.05	0	0.41
	DIL-EN4	1.54	1.63	1.72	2.44	2.55	2.36	0	0.83	0	0.55	0.01	0
	DIL-EN5	1.40	1.72	1.63	2.47	2.58	2.42	0.48	1.07	0.87	0.04	0	0.36

		Test spectrum											
	DIL-EN6	1.48	1.67	1.91	2.47	2.44	2.45	0.13	0.34	0.20	0.32	0	0.19
	DIL-ES4	0.27	0.45	0.77	0.22	0.03	0.40	2.11	2.03	2.06	0	0	0.23
	DIL-ES5	0.56	0.53	0.77	0.72	0.35	0.19	1.73	1.97	2.04	1.53	1.60	1.58
	DIL-ES6	0.59	0.32	0	0	0	0	2.13	2.14	2.22	0	0.18	0
	DIL-LM4	0.22	0.46	0.02	0	0.33	0.28	0	0.55	0	2.17	1.98	2.10
	DIL-LM5	0.07	0.27	0.59	0.38	0	0	0	0	0.26	2.03	1.96	2.05
	DIL-LM6	0.81	0.24	0.33	0	0	0	0	0	0.39	2.38	2.19	2.19

Table 6 shows the replicate 4-6 averaged Bruker scores from Table 3 and Table 5 shows the replicate 4-6 Bruker-score standard deviations from Table 3.

Table 6. Replicate 4-6 averaged Bruker scores from Table 5.

		Test spectra											
		DIL-EC1	DIL-EC2	DIL-EC3	DIL-EN1	DIL-EN2	DIL-EN3	DIL-ES1	DIL-ES2	DIL-ES3	DIL-LM1	DIL-LM2	DIL-LM3
Reference spectra	DIL-EC	1.66	1.71	1.69	1.67	1.72	1.71	0.00	0.59	0.54	0.34	0.08	0.51
	DIL-EN	1.47	1.67	1.75	2.46	2.52	2.41	0.20	0.75	0.36	0.30	0.00	0.18
	DIL-ES	0.47	0.43	0.51	0.31	0.13	0.20	1.99	2.05	2.11	0.51	0.59	0.60
	DIL-LM	0.37	0.32	0.31	0.13	0.11	0.09	0.00	0.18	0.22	2.19	2.04	2.11

Table 7. Replicate 4-6 Bruker-score standard deviations from Table 5.

		Test spectra											
		DIL-EC1	DIL-EC2	DIL-EC3	DIL-EN1	DIL-EN2	DIL-EN3	DIL-ES1	DIL-ES2	DIL-ES3	DIL-LM1	DIL-LM2	DIL-LM3
Reference spectra	DIL-EC	0.06	0.10	0.12	0.08	0.18	0.08	0.00	0.15	0.22	0.27	0.14	0.21
	DIL-EN	0.07	0.05	0.14	0.02	0.07	0.05	0.25	0.37	0.46	0.26	0.01	0.18
	DIL-ES	0.18	0.11	0.44	0.37	0.19	0.20	0.23	0.09	0.10	0.88	0.88	0.85

		Test spectra											
	DIL-LM	0.39	0.12	0.29	0.22	0.19	0.16	0.00	0.32	0.20	0.18	0.13	0.07

The data from Table 4 and Table 5 are shown graphically in Figure 13.

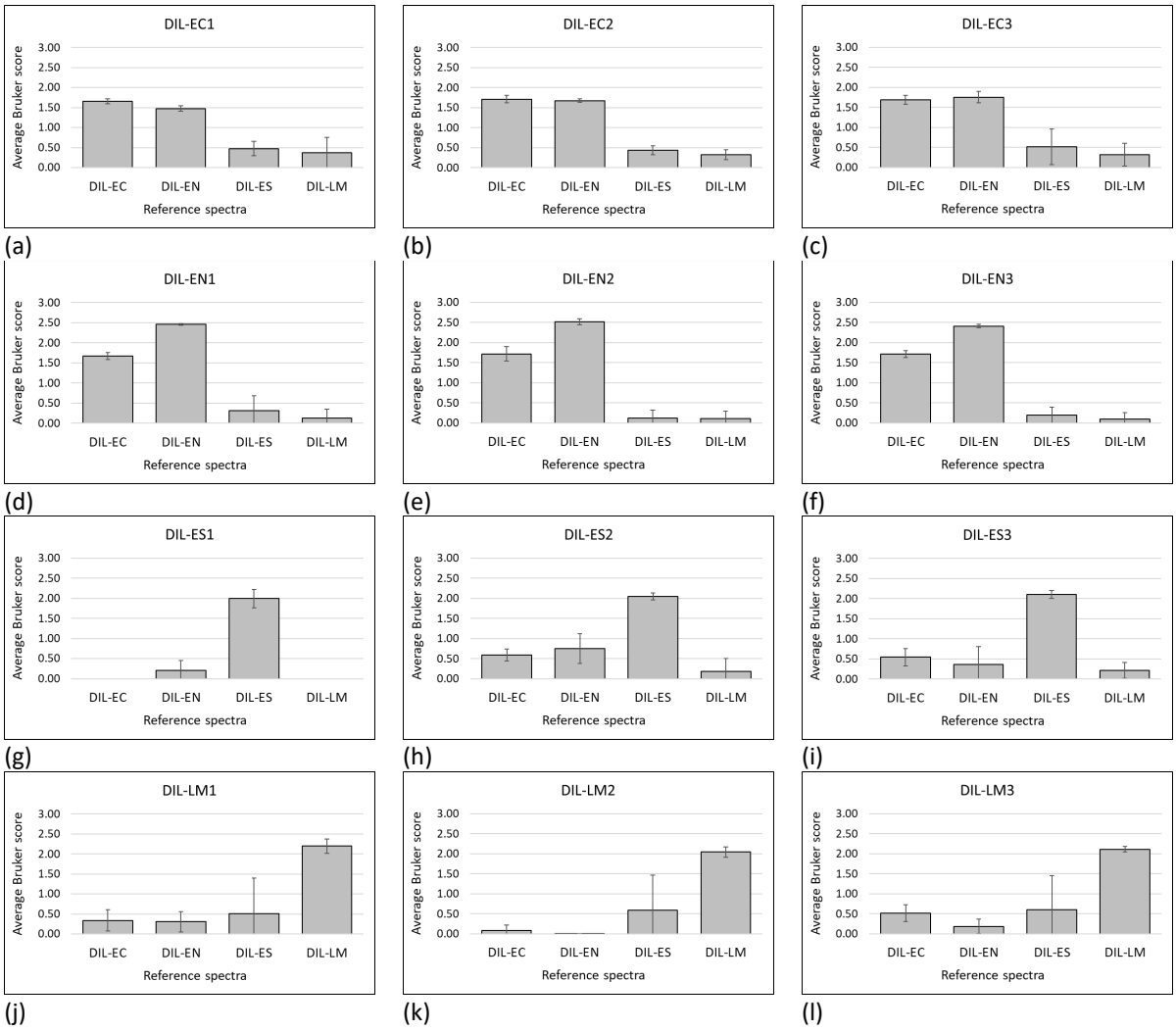

Figure 15. Replicate 4-6 averaged Bruker scores and standard deviations from Tables 3, 4 and 5.

Figure 15 shows good discrimination for the pondweeds *Elodea nuttallii* (all three test triplicates correctly and clearly discriminated), *Egeria sp.* (all three test triplicates correctly and clearly discriminated), and *Lagarosiphon major* (all three test triplicates correctly and clearly discriminated) but not, as observed in part 1, for *Elodea canadensis* (one test triplicates correctly and clearly discriminated, one test triplicates correctly but not clearly discriminated, and one test triplicate incorrectly discriminated).

Discussion and conclusions

Using a highly-simplified and inexpensive method for MALDI-TOF MS sample preparation that lyses cells by immersion in aqueous acetonitrile containing trifluoroacetic acid to selectively extract acid-soluble proteins, peptides, and other similarly-soluble basic cellular components (with extraction carried out in the presence of near-saturated and

inexpensive-grade MALDI matrix), discrimination has been attempted in part 1 between the visibly-similar pondweeds *Elodea canadensis* and *Elodea nuttallii.* We have observed clear spectral separation with low variance between the average same-species Bruker score and the average different-species Bruker score, which is indicative of both good spectral difference between the two species (1.02 Bruker units between the average values) and good spectral reproducibility between replicates (+/-5.7% for the *Elodea canadensis* quadruplicates and +/-3.0% for the *Elodea nuttallii* quadruplicates). We therefore conclude that these two pondweeds are well suited to discrimination by means of MALDI-TOF MS using the method described and samples prepared on 04-10-2022. In part 2 (for *Elodea canadensis*, *Elodea nuttallii*, *Egeria sp.*, and *Lagarosiphon major*), we observed good discrimination for the pondweeds *Elodea nuttallii*, *Egeria* sp., and *Lagarosiphon major* but not, as observed in part 1, *Elodea canadensis* using the samples processed on 29-03-2023, possible reasons for which are likely to be linked to the poor visible condition of the *Elodea canadensis* material. It is possible that the less-complex spectra resulting from this (see Figures 8 and 12) limit the Bruker scoring. In support of this hypothesis, the Table 5 within-species spectral comparisons are shown below (Figure 16), where the scores for *Elodea canadensis* are noticeably lower than for the other species in this study.

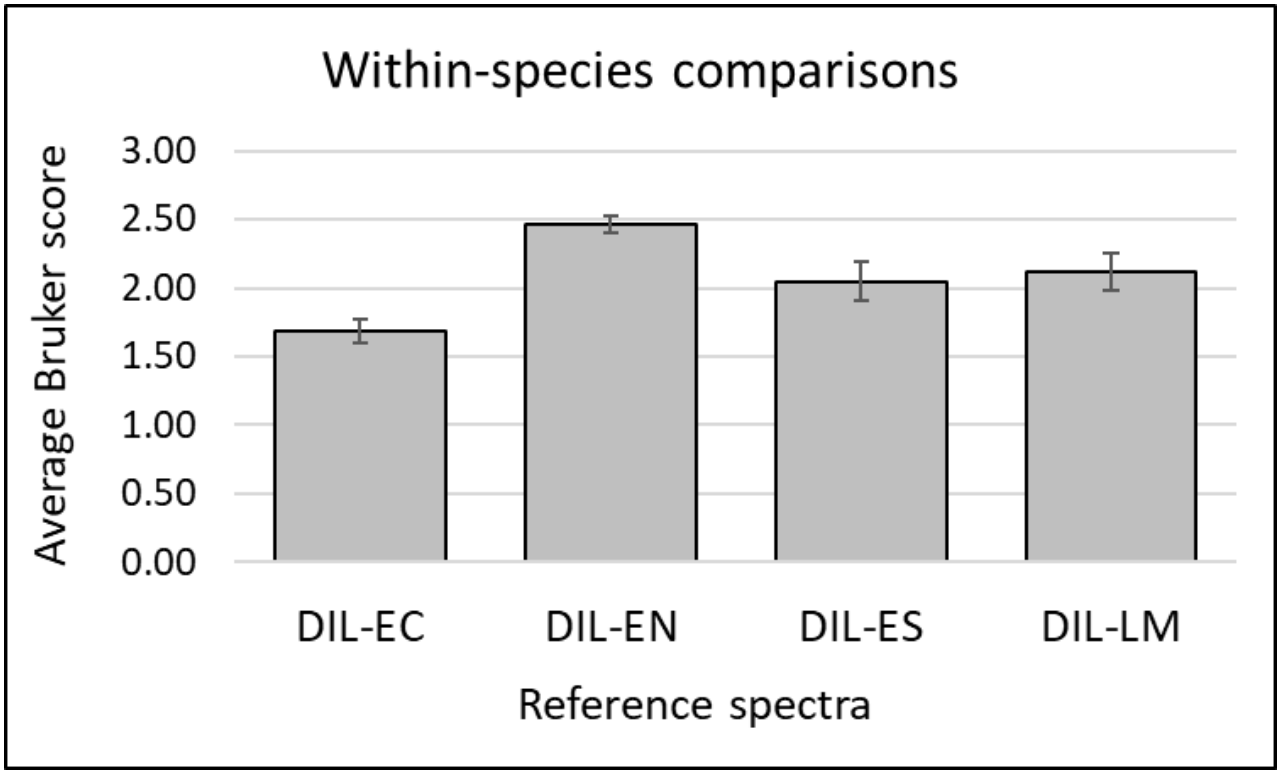

Figure 16. Within-species spectral comparison Bruker scores and standard deviations from Table 5.

Future work

While the experiments conducted in this study required fresh plant samples, this technique has the potential to be adapted to a more user-friendly method that would be more suited to situations where plant material cannot be processed immediately or kept fresh for long periods (i.e. during field surveys). If the wider application of this technique was identified

as a need by stakeholders (i.e. site managers, ecologists etc.), the authors would recommend this as a next step.

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