Natural England Commissioned Report NECR244

An assessment of seed viability, germination and vegetative propagation requirements for *Nuphar pumila*

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Foreword

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.

Background

Least water-lily *Nuphar pumila* is known from over 100 sites in Scotland, but only from Cole Mere in England, although it formerly occurred in other meres in Shropshire.

Natural England commissioned this study of *Nuphar pumila,* under the Species Recovery Programme to gather information to improve its conservation status at Cole Mere SSSI, Shropshire and to develop and implement a Species Recovery Plan with the aim to maintain a self-sustaining population of N. pumila in England.

The work was commissioned in three parts:

- Commissioned report 243, to review the known ecology of *Nuphar pumila* and the population status at Cole Mere.
- This report to develop a seed and rhizome propagation protocol.
- Commissioned report 245, to assess the level of genetic variation present in English *N. pumila*; test whether English and *Scottish N. pumila* populations are genetically distinct from each other; and confirm the hybrid nature of samples identified as *N.* × *spenneriana*.

Natural England and others will use the findings to develop a plan to conserve England's only population of least water-lily, and in particular to manage the trees and the margins around Cole Mere.

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Key words - Nuphar pumila, Least Water-lily, ex-situ conservation, threatened, germination, propagation, seed viability, Tetrazolium test, cold stratification, rhizome, aquatic plant, vegetative, Millennium Seed Bank

Further information
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Royal Botanic Gardens Kew

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Report for Natural England

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June 2017

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Background

RBG Kew's UK Native Seed Hub was commissioned by Mags Cousins, Natural England to undertake this study as Part 2 of the Species Recovery Project: Develop a plan to conserve England's only population of *Nuphar pumila*, Least Water-lily (Lansdown, 2017 and Gargiulo, 2017).

The outputs of this work were to:

I. assess the viability of *N. pumila* seed from the single English population at Cole Mere;
II. establish a germination protocol for this species;
III. trial vegetative propagation by division of rhizomes;
IV. make recommendations regarding seed and vegetative propagation methodologies for establishing new populations of *N, pumila* in England;
V. establish an *ex-situ* population of English-origin *N. pumila* at RBG Kew.

This report addresses the first four outputs. All work was carried out by the UK Native Seed Hub at RBG Kew's Millennium Seed Bank, Wakehurst Place.

About the UK Native Seed Hub

The UK Native Seed Hub seeks to mobilise the seed collections, facilities, technical knowledge and scientific expertise of RBG Kew to support conservation and habitat restoration in the UK.

We provide plants and seed, technical services and research to help conservation practitioners overcome constraints to sourcing, producing and using native plant materials in the UK.

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1. Summary

Although fairly widespread in Scotland, Northern Europe and Asia, *Nuphar pumila* (Timm) DC., Least Water-lily, is considered Critically Endangered in England with its distribution restricted to a single site at Cole Mere, Shropshire (Stroh, 2014; Padgett, 2007; Preston and Croft, 1997). The isolation of this population has driven interest in its conservation and potential introduction to other sites, with both sexual reproduction by seed and clonal spread by rhizome fragments likely to play a part in establishing new plants and populations (Lansdown, 2017).

In 2016, seeds were taken from Cole Mere for study at the Millennium Seed Bank (MSB) to assess the viability of seeds produced by this population and to examine the factors necessary for germination. Viability tests have found that 92% of the seeds collected were viable. The experimental work carried out for this study has provided an effective germination protocol resulting in maximum germination of 96%. Large variations in germination levels between seed batches were also observed and this is discussed with particular reference to seed maturity and port-harvest handling for optimum germination.

Six rhizome fragments from Cole Mere were also sent to the nursery at Wakehurst Place in 2016, providing an opportunity to monitor their establishment in cultivation and trial further division of the rhizomes in spring 2017. Results to date suggest larger rhizome sections with a visible, healthy growth point are able to root and establish well, but that smaller sections and those without visible growth points are not. Division of rhizomes with more than one growing point appears to be feasible, and may occur spontaneously through the formation of natural breakage points within the rhizome.

Experience gained during the seed germination and vegetative propagation trials enables a simple comparison of the advantages and disadvantages of each technique. Plants produced using both methodologies are being retained as part of the living collections at Wakehurst Place and RBG Kew.

2. Seed Viability and Germination

Little is known about the germination and propagation requirements of *N. pumila* (Preston and Croft, 1997), however, the germination of other *Nuphar* species have been studied (Heslop-Harrison, 1955; Smits and Wetzels, 1986; Brock et al., 1989; Smits et al. 1989; Smits et al., 1990; Barrat-Segretain, 1996).

Low oxygen conditions have been found to stimulate germination of closely related *Nuphar lutea* seeds (Smits and Wetzels, 1986). This is likely to be a strategy to ensure that germination occurs at a suitable depth or under substrate. Smits et al. (1990) also found that germination will not take place under a depth of more than 6cm of sediment. The population of *N. pumila* at Cole Mere has been shown to be sensitive to tree shading and potentially secondary effects of leaf litter creating unsuitable conditions (Lansdown, 2017) so the influence of oxygen on germination may provide a useful insight into the limiting factors of seedling production.

Smits' study also found that seeds of *N. lutea* display physiological dormancy, preventing germination until conditions are favourable for seedling establishment. In many species displaying physiological dormancy, dormancy is lost during the winter months, allowing seed to germinate in spring.

There are differing opinions on the life cycle of *N. lutea* seeds. Smit et al. (1990) found that *N. lutea* has a transient seed bank with all viable seed germinating within the first year, whilst Heslop-Harrison (1955) and Brock et al. (1987) found that proportions of seed can remain viable in the seed bank to germinate in future years. In the case of *N. pumila*, the persistence of seed in the soil could inform monitoring of future conservation work and possibly allow short term *ex-situ* storage of seeds.

2.1 Methods

Collection

Two collections of *N. pumila* seed were made from Cole Mere, Shropshire, in the summer of 2016 - Collection A on 13th July and Collection B on 14th August. Collections were made following research into the phenology of the target species to avoid sampling immature seed, with fruit assessed for signs of maturity such as reduced firmness of fruit coat, reduced buoyancy in the water or observed breakdown of the carpel (Figure 1a). The most mature fruits were then harvested by hand (Figure 1b) and transported to the MSB in clear polythene bags containing water from the Mere.

Seed samples from the two collections were kept separate throughout the testing process. In total, 765 seeds were collected in Collection A and 441 in Collection B.

Figure 1a (l): Dispersal of *N. pumila* seeds from the fruit;

Figure 1b (r): Hand harvest of *N. pumila* from Cole Mere, July 2016

Post-harvest ripening

The fruit were kept in tap water in open containers at a controlled temperature of 15°C with water changed as necessary. Seeds were removed in batches for germination testing as they were naturally dispersed from the fruit. Fifteen batches were taken across the two collections, 8 from Collection A between July and October and 7 from Collection B between August and November (see Appendix A for full data).

The length of time batches spent in the after-ripening conditions varied according to the maturity of the fruit at the time of harvesting, from 2 days (batch B1) to 107 days (B7).

Tetrazolium test for seed viability

Tetrazolium (TZ) viability tests were carried out within two weeks of collection on random samples of 25 seeds from Collection A (8 days) and B (12 days).

Seeds (Figure 2a) were bisected laterally to remove a small proportion of the seed coat before immersing in a solution of 1% buffered 2,3,5-triphenyl tetrazolium chloride. Immersed seeds were then incubated in the dark for 48 hours at 30°C before being evaluated for staining, indicating respiring tissue, under a microscope (Figure 2b). Vital structures within the seed were evaluated based on staining intensity as fully stained (viable), unstained (non-viable) or partially stained (interpreted as viable or non-viable, depending on the intensity and location of staining).

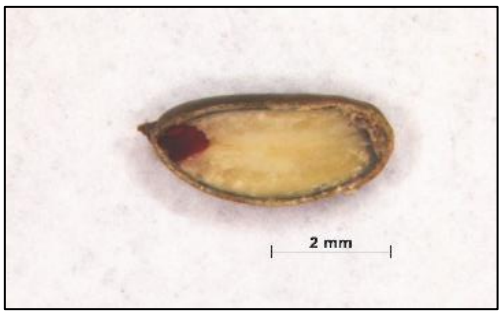


Figure 2a (l): *N. pumila* seed;

Figure 2b (r): Viable *N. pumila* seed following TZ test with embryo stained red.

Germination test

Germination testing was designed to develop a successful propagation protocol and investigate the influencing factors of aerobic/anaerobic conditions and the duration of post-harvest ripening.

To overcome suspected physiological dormancy, initial batches of ripe seeds were subject to a cold stratification of at least 56 days before transferring to the final germination temperature of 15°C in either anaerobic or aerobic conditions (Figure 3). Although not formally investigated, cold stratification lengths varied from 56 to 72 days. As anaerobic conditions were found to have little impact on germination, later batches of seed were tested in aerobic conditions alone.

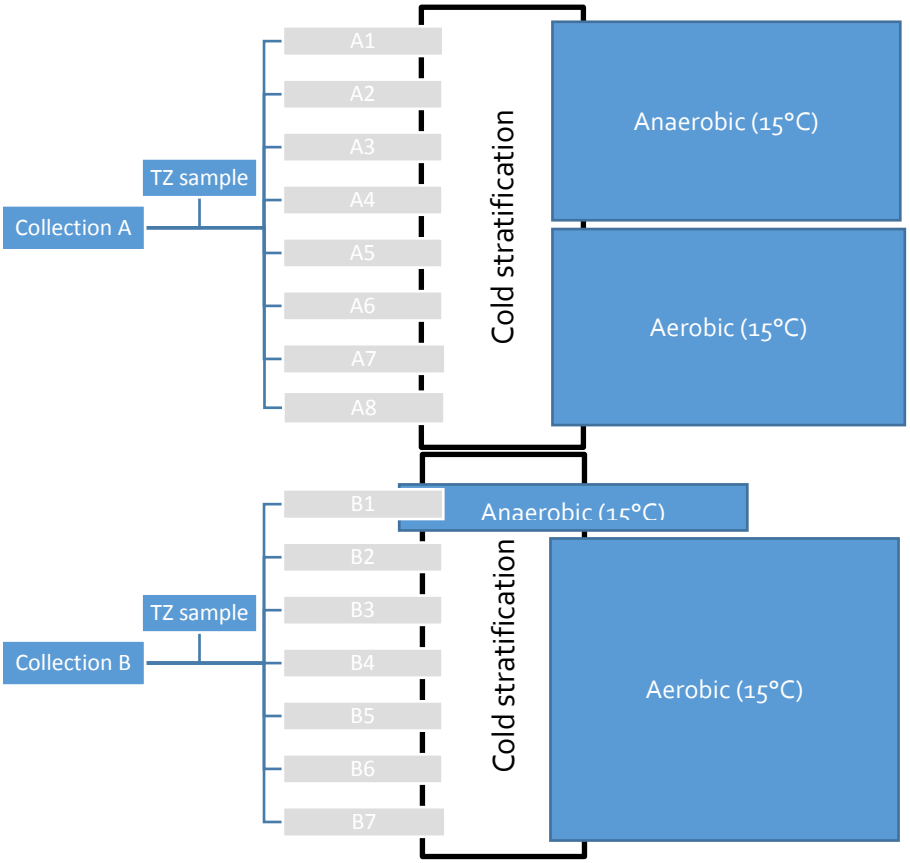


Figure 3: Experimental design to test effects of anaerobic conditions on germination. Each batch was subject to a cold stratification before being transferred to anaerobic or aerobic conditions

Germination tests were carried out using standard MSB protocols (Davies, Di Sacco and Newton, 2015). All germination tests were undertaken in distilled water, replaced as necessary throughout the testing process. Each batch was scored for germination approximately every 14 days. The criterion for germination was the emergence of >2mm of radicle. White florescent light was provided throughout testing with a 12 hour light/dark photoperiod.

When germination ceased, a cut test was performed on non-germinated seeds. Seeds were dissected under a microscope to examine their internal structure and assigned to one of four categories: fresh (assumed viable), mouldy (assumed non-viable), insect infested and empty. A Z-test was used to assess differences between seed viability and germination.

Germination percentages (G%) consider germinated seed (G) as a percentage of the number of seed sown (S) minus seed known to be infested (I) or empty (E):

$$G\% = G/(S-(I+E)) * 100$$

Germination rate (GR) is calculated as the mean time to germination where g is the number of seeds germinating on date a (b, c..), d is number of days from sowing on date a (b, c..) and G is total number of germinated seed:

$$GR = (\sum (g * d))/G$$

Statistical analysis was performed using Microsoft Excel (2013), GenStat (Version: 14.2.6297), Origins (Version: 9.0.0) and R Software (Version: 3.3.2). A Shapiro-Wilkes test was used to assess distribution of the data.

Aerobic/anaerobic germination conditions

Batches A1, A2, A3, A4 and B1 were placed in anaerobic conditions at 15°C following cold stratification, with remaining batches transferred to 15°C in standard closed vessels (Figure 3).

Anaerobic conditions were achieved by placing water filled containers into Anaerojars™ with AnaeroGen™ anaerobic generator sachets (Oxoid Ltd., Hampshire, England) in hermetically sealed flasks.

A Chi-square Test was used to assess differences in germination between those subject to anaerobic conditions and aerobic controls.

Variation between batches

Collection A provided eight batches of seed ranging in size from 173 to 14 seeds per batch. Collection B provided 7 batches ranging from 228 to 15. Ripening time for each batch varied from 2 to 107 days.

Full data is provided in Appendix A.

2.2 Results

Tetrazolium testing for seed viability

Tetrazolium testing indicated that 92% of the sample of 25 seeds taken from each collection (a subset of batches A1 and B1) were viable. Neither of these batches displayed high levels of germination, despite containing a high proportion of fresh and abnormal (showing signs of development but without radical emergence) seed. Potential reasons for this apparent discrepancy are included in the discussion below.

Germination tests

A marked difference was found in germination results between batches (Figure 4). The highest percentage germination observed was 96% for batch B4, followed by A4 and A5 at 81% and 82% respectively. Only batch B2 had no seeds germinate. In total, 441 seeds (37%) germinated across the two collections.

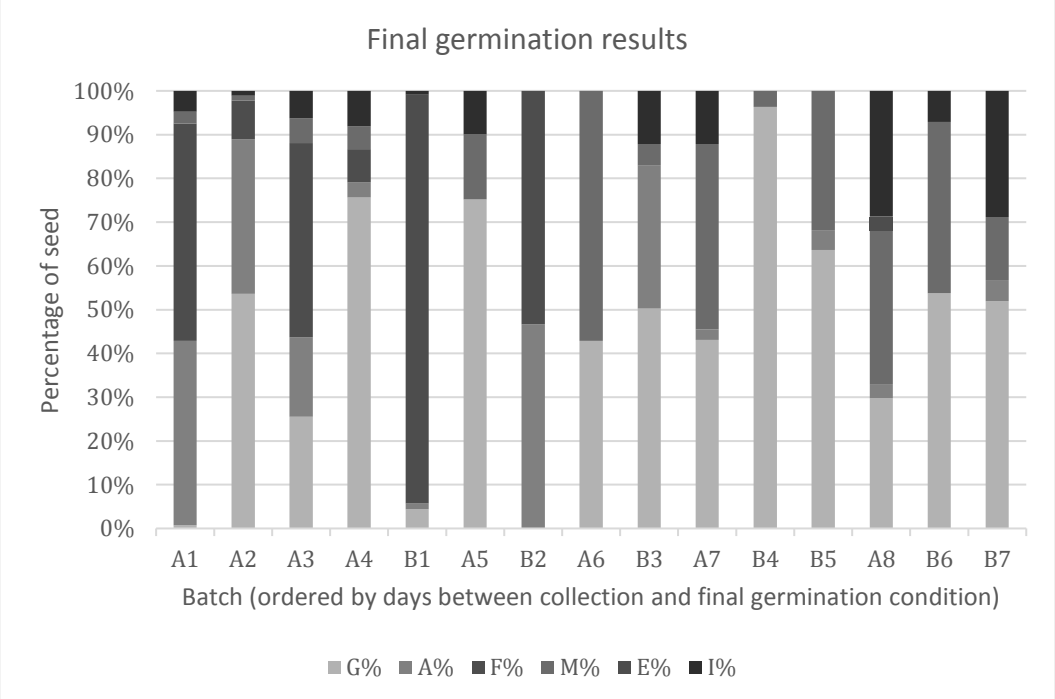


Figure 4: Results following the cut test showing percentages of germinated (G), abnormal (A), mouldy (M), empty (E) and infested (I) seed. Batches are ordered by the number of days between fruit collection and the move to the final germination conditions. This ranged from 58 days for batch A1 to 165 days for batch B7. For raw data see Appendix B.

Cut tests showed that 567 seeds were fresh or abnormal (showing signs of development but without radical emergence). Of these, 213 were from batch B1 which had a healthy embryo but a very 'chalky' endosperm with a very different texture to the other seeds noted as fresh during the cut test. In batch A4, five of the seeds classed as abnormal in the cut test had fully grown inside the seed coat.

Overall, 1008 out of 1206 seed (83%) were judged to be viable, having either germinated or found to be fresh or abnormal during the cut test.

There were significant differences between germination and viability within individual batches and collections, and between collections. For collection A, germination was 44% and viability was 87%. For collection B, germination was 32% and viability 94%. The earliest batches to be tested from each collection (A1, A2, A3, A4, B1, B2, B3) displayed significant differences between germination and viability, although later batches did not (see Appendix C). Overall, there was a significant difference between viability and germination within each collection as a whole, and when the two collections were pooled into a single sample. There was also a significant difference in germination between the two collections ($z=3.655$, $p>0.001$).

Mean time to germination was calculated as 70 days after transfer to the final germination temperature of 15°C, although again there was considerable variation within and between batches. Germination started as early as day 3 and as late as day 149 for batch B1. The mean germination rate was found to be longer for Collection B than Collection A (82 and 59 days respectively) largely due to the late spurt of germination by batch B1. Due to a data collection error, germination for batch B5 is assumed to be at a similar time to B6.

Post-harvest ripening

A moderate positive correlation was detected between post-harvest ripening and germination (Pearson's Correlation Coefficient, $r=0.424$, $p=0.88$), with peak germination observed for seeds spending 47 days in the post-harvest ripening conditions (Figure 5).

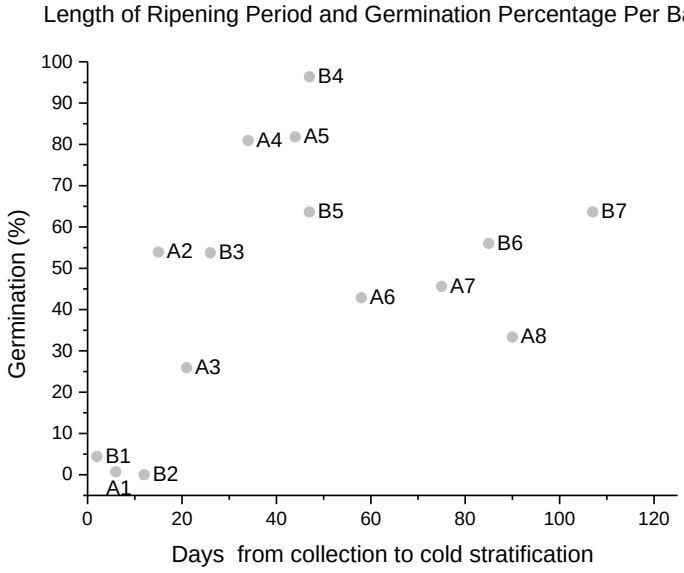


Figure 5: Germination results per batch with days from collection to start of cold stratification.

Seeds ripened for longer or shorter periods generally demonstrated lower germination levels, but the data did not follow a normal distribution ($W=0.95$, $p=0.5$) and no conclusive trends were observed.

Cold stratification

Germination was observed with cold stratification of varying lengths from 56 to 72 days. (Figure 6).

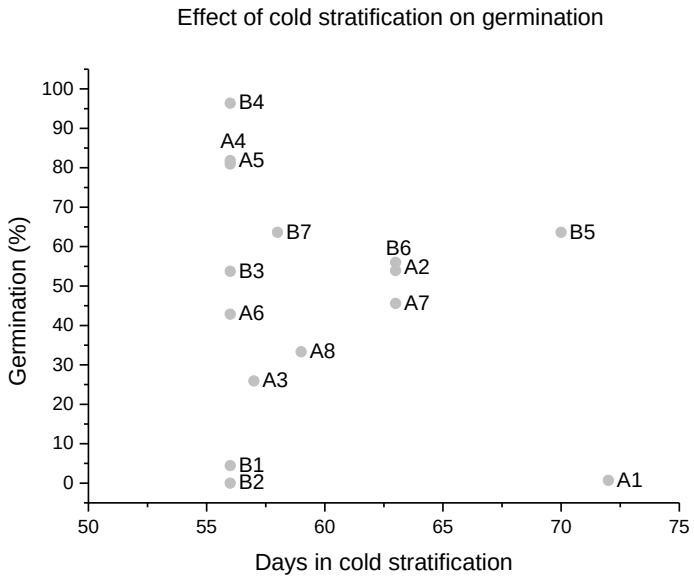


Figure 6: Observed effect of cold stratification on germination levels by batch.

Anaerobic/aerobic germination conditions

Batches A1, A2, A3, A4 and B1 were subject to anaerobic conditions during the final germination test and all other batches, aerobic conditions (Figure 3, with results in Figure 7). Batches placed in anaerobic conditions were found to have significantly lower germination ($\chi^2 = 83.28$, $p<0.001$) than those in aerobic conditions, although this is likely to be due to differences in the maturity of the seed batches rather than the test condition. Batches A4 (anaerobic) and A5 (aerobic) had very similar germination results of 80% and 81% respectively.

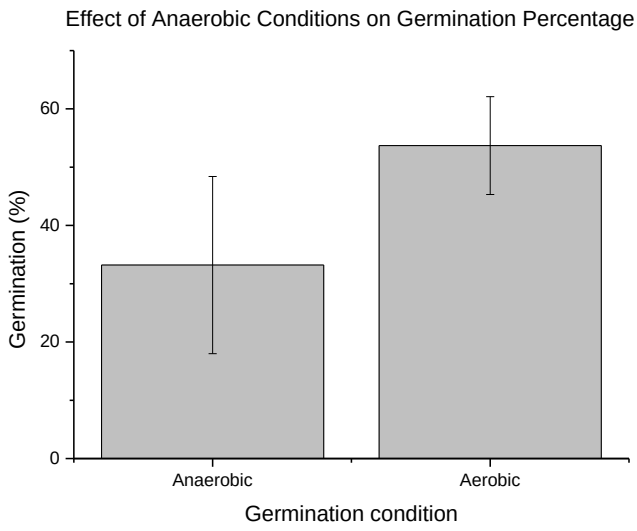


Figure 7: Observed effect of anaerobic conditions on germination percentage. Error bars show standard error (anaerobic SE=15.24, aerobic SE=8.36)

Further work and observations

A marked difference in seed coat colour was observed between batches ranging from green to brown with Collection B containing a larger proportion of brown seeds. As there was no obvious variation in quality between the different coloured seeds, this was not factored into the experimental design, but could be investigated in future work.

See Appendix B for all raw germination data.

2.3 Discussion

This study has found high levels of viability in the seeds of the Cole Mere population of *N. pumila* and has identified a successful germination protocol. Variation in germination between batches and relative numbers of fresh and abnormal seeds found at the cut testing stage suggest that seed maturity and post-harvest ripening may be the key factors to consider when collecting this species for propagation.

The low germination levels of batches dispersing earliest suggest an optimum post-harvest ripening time of around 6 weeks. This is supported by the similarity in the ripening periods between the batches with the highest germination, A4, A5 and B4 (74-96% germination after 34-47 days).

It is also notable that the first batches to disperse seed from both collections (A1, B1 and B2) all showed the lowest germination during testing but also had the highest numbers of fresh seed and, in the case of A1 and B1, the highest numbers of abnormal seed. Further study is required to assess whether this is due to immaturity in the first batches or deeper dormancy in early-ripening seed. No fresh, ungerminated seeds were found in any of the samples with a ripening period in excess of 100 days, implying that

longer ripening may be detrimental for some seeds. It is also likely that these seeds were harvested prematurely and were not able to mature and survive once separated from the parent plant.

Although germination with cold stratification was not tested, the requirement for cold stratification is supported by the absence of germination during long post-harvest ripening periods of up to 107 days. These results support previous studies on closely related species (Smits, 1990) suggesting that seeds of *N. pumila* display physiological dormancy requiring a period of cold stratification before germination can occur. Eight weeks appears to be the optimum length of stratification, with longer periods observed to decrease germination, although results are likely to have been impacted by differences in seed maturity and the evidence is not conclusive.

Anaerobic conditions were expected to stimulate germination (Smits, 1990), but had no effect in this study. Although batches tested in anaerobic germination conditions were found to have significantly lower germination levels, differences in seed maturity are likely to have influenced these results more than the test condition. The very similar results for batches A4 and A5, with 81% germination in anaerobic conditions and 82% in aerobic conditions respectively, give further evidence to support this and suggest that the anaerobic conditions had no positive or negative effect on germination. This is a useful finding in terms of the *in-situ* conservation of this species, suggesting germination is not significantly inhibited by litter-induced hypoxia, although reduced light levels around the developing seedling may impact survival (see Section 4 below).

The mean viability of the two collections was not significantly different, suggesting successful collections can be made in both July and August. The maturity of individual fruits collected is likely to have varied for both collections. The sampling strategy (removing seeds as they were dispersed from the fruits) resulted in batches made up largely of seeds from single fruits. Differences in the maturity of each fruit at the point of collection could therefore result in the very varied performance of different batches seen in this study. It may also be possible that some fruits were harvested too early for the seeds to fully mature in the ripening conditions, producing batches of seed with little or no germination.

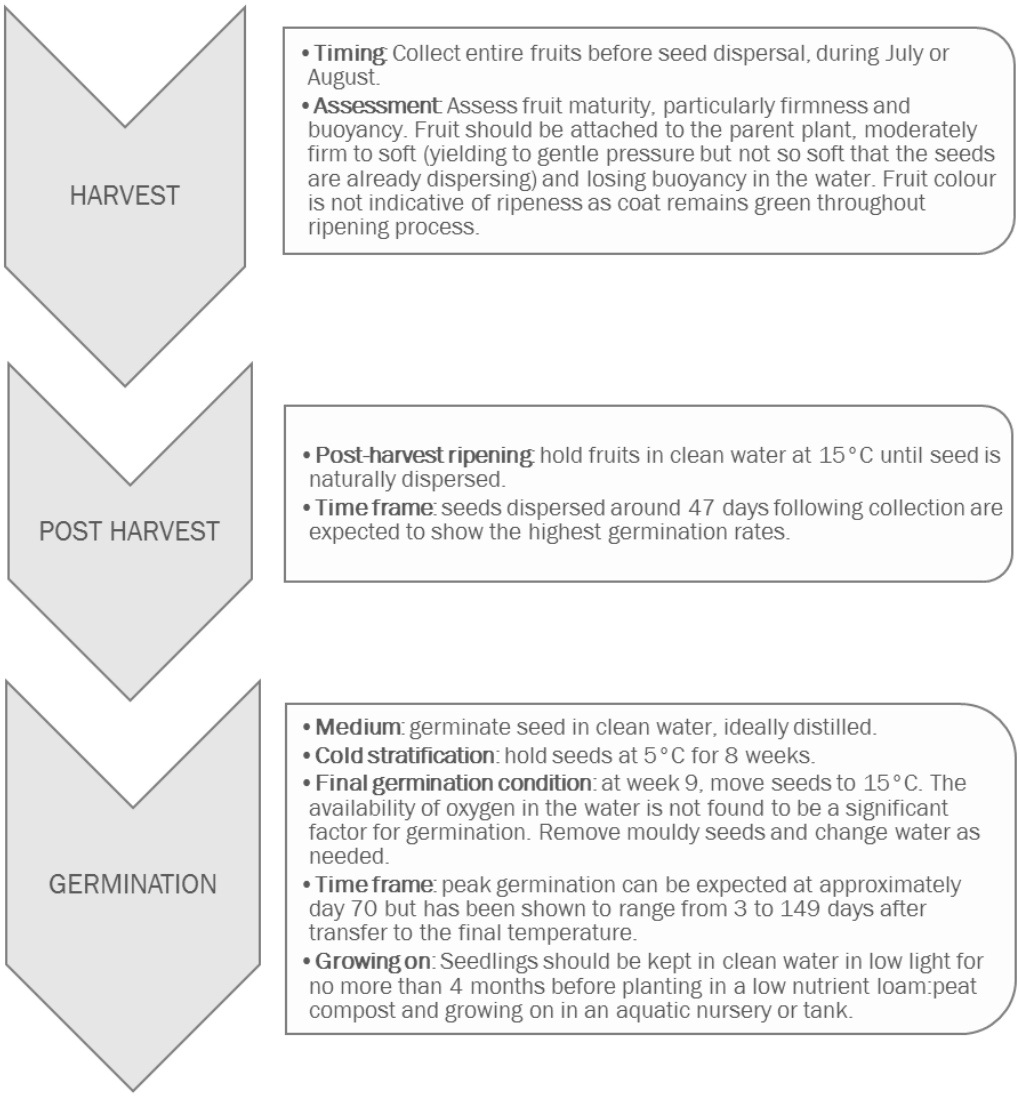
A limitation of the TZ test is the difficulty of distinguishing accurately between mature seed and immature seeds that are respiring, and therefore stain well, yet lack the capacity to germinate and form plants. An analysis of the relationship between seed colour, embryo size and germination could provide further insight but an in-depth assessment of these aspects was beyond the scope of this study. Aside from colour, no major difference in seed size or shape of seeds between batches was noted. This could be quantified by weighing samples or establishing the percentage gravimetric moisture content of seeds in a future collection.

Further investigation would be beneficial to better understand the large range of results found in this study. The high numbers of fresh seed observed in the cut test could indicate that seeds of *N. pumila* do not all germinate in the first year and that a second cold stratification is required, as suggested for *N. lutea* (Heslop-Harrison, 1955; Brock et al., 1987). Handley and Davey (2005) also found secondary dormancy in the seed of

the native aquatic *Najas marina*, possibly to avoid germination in shortened growing seasons imposed by unusually long winters. This is a critical consideration for an annual like *N. marina* which depends on seed production for its persistence, but it may also be a strategy employed to enable the germination of a perennial such as *N. pumila* over more than one season.

2.4 Germination Protocol

A protocol for maximising germination of *N. pumila* is provided below.



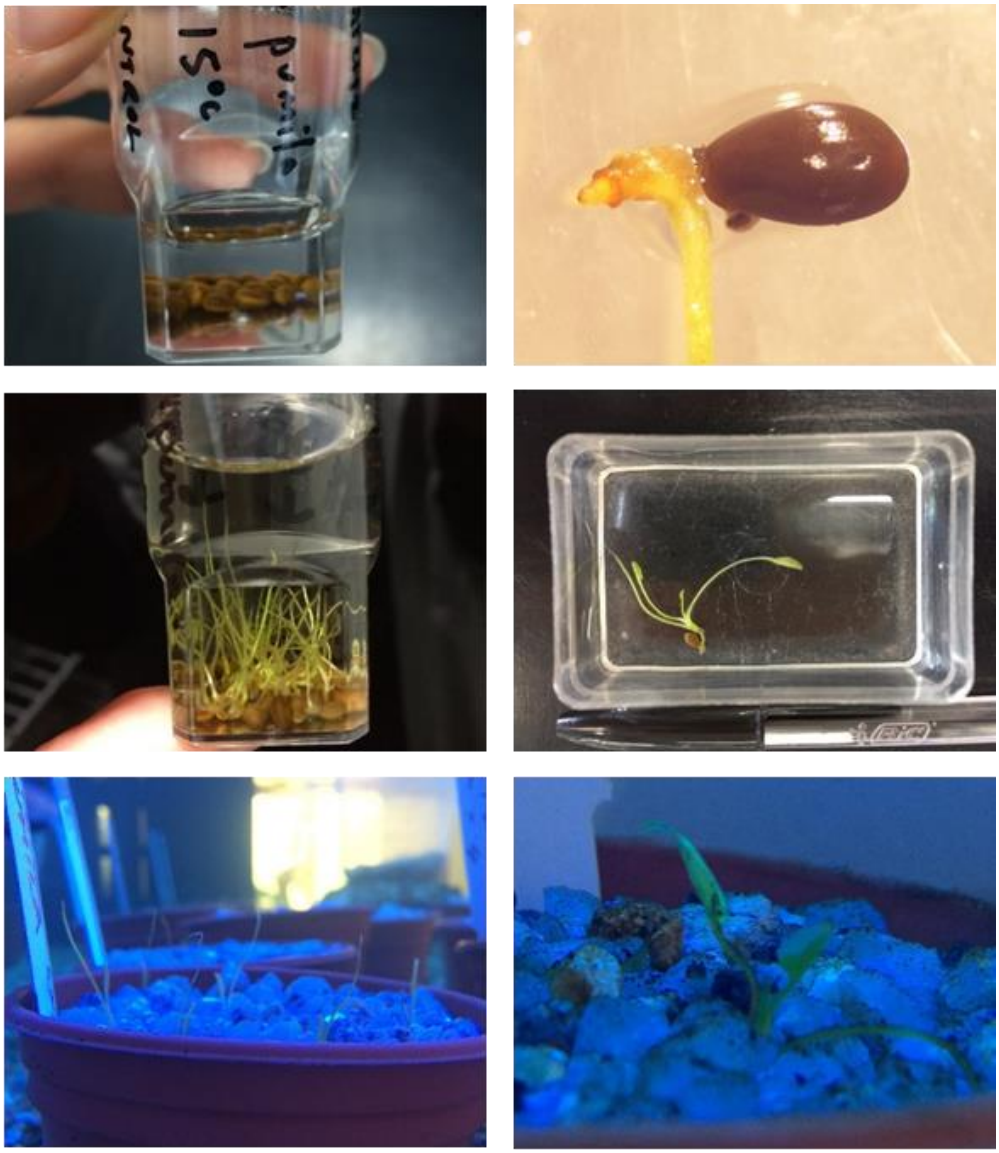


Figure 8: Top (l): Seed prepared for germination testing; (r) Seedling of Nuphar pumila: seed with radicle and coleoptile; Middle (l) seedlings; (r) seedling ready to be planted, approximate length 4cm; Bottom (l) seedlings in nursery (approx. 3 months since germination); (r) seedling in nursery (approx. 5 months since germination).

3. Seedling Establishment

Germinated seed remained in distilled water in the germination conditions (15°c, 12 hour light/dark photoperiod) for up to four weeks, allowing a healthy root and shoot to develop, approximately 4cm long (Figure 8). Seedlings were then pricked out into clean 9cm pots in a low-nutrient growing medium of equal parts fine sieved moss peat and sterilised loam. Pots were topped with washed grit to retain the growing medium and seedlings – fine grade Cornish (granite) grit was found to provide better anchorage for the seedlings than coarser Wessex grit.

Seedlings were submerged to a depth of 18cm in an aquarium filled with mains water and placed in various locations in the MSB glasshouses. High light levels and temperatures of 17-20°c in the glasshouse were associated with high seedling mortality and excessive algae in the aquarium, which was moved to a cooler passageway with indirect light. Early batches of seedlings were lost entirely - a chiller fitted to maintain a temperature of 15°c, together with water changes and regular cleaning of the tank, has controlled algae and promoted stronger seedling growth.

Supplementary lighting is provided for 12 hours a day using a blue Juwel Hillite fluorescent tube. Lighting is switched off for a few days when newly pricked-out seedlings are introduced to the aquarium, allowing them to become gradually accustomed to higher light levels outside the germination incubator.

10 healthy seedlings were growing in the aquarium at the time of writing. Once established, these seedlings will be transferred to larger growing tanks outdoors and used to establish living collections at RBG Kew and Wakehurst Place.

4. Vegetative Propagation

Vegetative reproduction by rhizome growth and fragmentation is likely to be the principal form of reproduction of *N. pumila* at Cole Mere, where large numbers of rhizome fragments have been observed floating on the surface (Lansdown, 2017). These fragments have not succeeded in establishing new populations, however, and may require *in-situ* intervention to physically root fragments in appropriate locations or *ex-situ* propagation and planting of established, container-grown plants.

4.1 Methodology

Rhizomes 1-4 were received by the Wakehurst Nursery in mid-July 2016. The rhizomes were 12 – 14cm long and 1–1.5cm in diameter. Rhizomes 1-3 had a single terminal growth point with healthy leaves, whilst Rhizome 4 had no visible growing point.

The rhizomes were planted in aquatic baskets (20cm x 20cm x 15cm) in a growing medium of equal parts coarse peat and loam, covered with a layer of coarse gravel (Wessex grit). Rhizomes were planted shallowly, with growing points protruding above the surface of the growing medium. The baskets were gently lowered into a large, translucent, white plastic tub filled with mains water and submerged to a depth of 60cm.

This tub remained outside in all weather conditions, with partial shading by the lid of the tub to inhibit algae growth.

Rhizomes 5-6 were received in mid-August 2016. Both fragments were substantially smaller at 6cm long and 1cm in diameter. Rhizome 5 had leaves growing from one end, whilst Rhizome 6 appeared to be a central section with no visible growing points.

These smaller rhizomes were potted into 9cm pots and submerged in a small translucent white plastic box with approximately 2cm of water above. This box was placed indoors in an unheated passageway with indirect light and protection from frost. Rapid algae growth was observed in March 2017, when the water was changed.

4.2 Results

Rhizome 6 - which had no visible growing point - rotted within a few days. Rhizome 5 survived with visible leaves until March – it was found to have rotted by early-April, possibly as a result of algae growth in the small plastic box.

Figure 9: Rhizome 1 prior to division in early April 2017.

Rhizome 1 produced two sets of new leaves in early-April, and was removed from the large outdoor tank and gently prised from the mud. A second growing point had formed at the opposite end of the rhizome, forming strong roots (Figure 9). It was easily detached from the parent rhizome because the point of attachment had rotted, a form of division that could readily take place in the wild. This ‘daughter’ rhizome remained small in mid-May, approximately 3cm long and 1cm in diameter, but healthy, with roots forming just below the leaves. The parent rhizome also appeared to be in good health.

Rhizomes 2 and 3 also showed signs of strong growth by mid-May, with new shoots developing just below the main growing point. Whilst these were too close to divide at the time of writing, it is possible the rhizome will elongate and the growing points become detachable or detach themselves – we will continue to monitor their growth and report to Natural England as necessary.

Rhizome 4, which did not have a visible growing point when it was potted, has not shown any signs of growth. Indeed, one end had rotted by mid-May – this rot was cut away and the remaining rhizome replanted and replaced in the tank.

4.3 Recommendations

Although small and time-restricted, this trial has yielded some useful findings.

- Rhizome fragments will root and establish once planted in a suitable growing media, provided they have visible growing points. Sections of rhizome without visible growing points do not appear to root and establish successfully.
- A low nutrient, organic-loamy growing medium analogous to the peat, mud and silty conditions of its natural growing sites (Lansdown, 2017) appears to produce good results in cultivation. Top dressing with grit retains the growing medium in the container and helps submerge the container in the tank.
- Cultivating larger sections of rhizome outdoors in ambient weather conditions was more successful than growing smaller sections in a protected environment.
- Diffuse but bright light appears to be essential for healthy growth of both rhizome fragments and seedlings, supporting the view that excessive depth, shading, leaf litter deposition and high water turbidity may inhibit the recruitment of new populations at Cole Mere.
- Once established, rhizome fragments produce new growing points which may detach themselves or be easily detached by hand.

We will continue to monitor the rhizomes over the summer of 2017, observing in particular whether new shoots detach themselves spontaneously or can be divided by hand. We will also seek to observe whether Rhizome 4 continues to decay or is able to develop a new shoot from the remaining healthy tissue.

5. Comparing Seed and Vegetative Propagation

Seed and rhizome growth and fragmentation both form part of the reproductive strategy of *N. pumila* in nature (Lansdown, 2017), and our trial suggests that both methodologies can be employed to produce new plants in cultivation. Neither is straightforward, however - a summary of the relative advantages and disadvantages of each approach is provided in Figure 10.

Seed	Rhizome Fragments
Systematic sampling from numerous individuals across the population is feasible.	Systematic sampling of rhizomes from numerous individuals may be more difficult.
Large numbers of propagules (several hundreds) may be collected each year.	Availability of propagules may be more restricted, although large numbers of naturally-produced rhizome fragments have been reported.
Conditions for natural seedling establishment do not exist currently at Cole Mere.	Rhizome fragments are likely to be the primary form of natural colonisation and reproduction and at Cole Mere, although suitable niches for new populations do not exist currently.
Seed has potentially high viability and germination in cultivation if collecting, after-ripening and germination protocols are followed.	Good establishment is possible in cultivation from large rhizome fragments with visible growing points.
After-ripening, germination and establishment of seedlings requires specialist horticultural skills and facilities.	Rhizome fragments are relatively simple to establish and maintain in cultivation.
Propagation and production of mature plants is currently untested, but is likely to be slow.	Production of mature plants is likely to be faster.
High seedling mortality is likely without careful control of temperature, light and algal growth.	Lower mortality (when large fragments with visible growing points are used) off-sets smaller numbers of founding propagules.
Seed-produced populations are likely to exhibit greater genetic diversity, although this may be impacted by selection during sampling and propagation.	Populations arising through vegetative propagation are likely to exhibit less genetic diversity. This may be mitigated by sampling from multiple individuals, ensuring clones of each are represented in the restored population.

Figure 10: Comparison of seed and vegetative propagation techniques for *N. pumila*

From a practical point of view, establishing new plants from rhizome fragments has proved the simplest and most reliable method, although the number of propagules available to trial this technique was small. Large rhizome fragments with well-developed growing points appear to contain the resources necessary to establish new plants relatively quickly, and may be more likely to succeed in a range of sub-optimal conditions than seedlings. Large numbers of seedlings were successfully germinated, but establishing these seedlings has been labour intensive, with a high mortality rate. This rate is likely to decline with experience and continued experiment – if a high survival rate could be achieved, the practical advantages of vegetative propagation would be reduced.

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Appendix A: Germination Conditions

Batch No.	Number of seed	Date to 5C	Cold strat days	Date to 15C	Days (collection to 5C)	Days (collection to final condition)	Final germination condition
A1	147	19-Jul	72	29-Sep	6	78	15°C/ anaerobic
A2	90	28-Jul	63	29-Sep	15	78	15°C / anaerobic
A3	173	03-Aug	57	29-Sep	21	78	15°C / anaerobic
A4	161	16-Aug	56	11-Oct	34	90	15°C / anaerobic
A5	228	26-Aug	56	21-Oct	44	100	15°C
A6	74	09-Sep	56	04-Nov	58	114	15°C
A7	15	26-Sep	74	28-Nov	75	138	15°C
A8	14	11-Oct	59	09-Dec	90	149	15°C
B1	77	16-Aug	56	11-Oct	2	58	15°C / Anaerobic
B2	78	26-Aug	56	21-Oct	12	68	15°C
B3	55	09-Sep	56	04-Nov	26	82	15°C
B4	22	30-Sep	56	25-Nov	47	103	15°C
B5	28	30-Sep	70	09-Dec	47	117	15°C
B6	27	07-Nov	63	09-Jan	85	148	15°C
B7	17	29-Nov	58	26-Jan	107	165	15°C

Appendix B: Number and dates of seed germinated and final germination percentage for all batches of *N. pumila* tested at the MSB from 13th July 2016 to the 24th January 2017. Numbers of fresh (F) mouldy (M), infested (I) and abnormal (A) seeds are included.

Batch	#seed	28-Oct	15-Nov	28-Nov	09-Dec	09-Jan	24-Jan	07-Feb	16-Feb	09-Mar	27-Mar	15-May	22-May	F	M	E	I	A	G	G %
A1	147	1	0	0	0	0	0	0	0	0	0	0	END	73	4	0	7	62	1	0.7
A2	90	46	2	0	0	0	0	0	0	0	0	END		8	1	0	1	32	48	53.9
A3	173	25	16	0	1	0	0	0	0	0	0	END		78	10	0	11	32	42	25.9
A4	161	0	105	0	14	0	0	0	0	0	0	END		13	9	0	14	6	119	81.0
A5	74	0	45	8	0	0	1	0	0	0	0	END		0	12	0	8	0	54	81.8
A6	14		0	6	0	0	0	0	0	0	0	END		0	8	0	0	0	6	42.9
A7	78					30	1	0	0	0	0	END		0	35	0	10	2	31	45.6
A8	28					6	0	END						0	11	1	9	1	6	33.3
B1	228	0	0	0	0	0	0	0	0	7	3	END		213	0	0	2	3	10	4.4
B2	15	0	0	0	0	0	0	0	0	0	0	END		8	0	0	0	7	0	0.0
B3	77	0	19	0	6	11	0	0	0	0	0	END		0	4	0	10	27	36	53.7
B4	55			0	0	53	0	0	0	END				0	2	0	0	0	53	96.4
B5	22					0	0	0	0	0	14	END		0	7	0	0	1	14	63.6
B6	27						12	2	0	END				0	11	0	2	0	14	56.0
B7	17							5	1	1	END			0	3	0	6	1	7	63.6

Appendix C: Z-test results comparing observed germination and viability by batch, collection and across both collections.

Batch	Cumulative Total Viable	Cumulative Total seeds	Viablity Proportion	Cumulative Total Germinated	Cumulative Total seeds	Germinated Proportion	Pooled Proportion	Absolute Difference in Proportion	Lower CI	Upper CI	Z value	2-tailed p	Sig
A1	136	140	0.97	1	140	0.01	0.49	0.96	0.93	1.00	16.14	0.00	Yes
A2	88	89	0.99	48	89	0.54	0.76	0.45	0.34	0.56	7.06	0.00	Yes
A3	152	162	0.94	42	162	0.26	0.60	0.68	0.60	0.76	12.47	0.00	Yes
A4	138	147	0.94	119	147	0.81	0.87	0.13	0.05	0.20	3.34	0.00	Yes
A5	54	66	0.82	54	66	0.82	0.82	0.00	-0.13	0.13	0.00	0.50	No
A6	6	14	0.43	6	14	0.43	0.43	0.00	-0.37	0.37	0.00	0.50	No
A7	33	68	0.49	31	68	0.46	0.47	0.03	-0.14	0.20	0.34	0.37	No
A8	7	18	0.39	6	17	0.35	0.37	0.04	-0.28	0.36	0.22	0.41	No
B1	226	226	1.00	10	226	0.04	0.52	0.96	0.93	0.98	20.34	0.00	Yes
B2	15	15	1.00	0	15	0.00	0.50	1.00	1.00	1.00	5.48	0.00	Yes
B3	63	67	0.94	36	67	0.54	0.74	0.40	0.27	0.54	5.31	0.00	Yes
B4	53	55	0.96	53	55	0.96	0.96	0.00	-0.07	0.07	0.00	0.50	No
B5	15	22	0.68	14	22	0.64	0.66	0.05	-0.23	0.33	0.32	0.38	No
B6	14	25	0.56	14	25	0.56	0.56	0.00	-0.28	0.28	0.00	0.50	No
B7	8	11	0.73	7	11	0.64	0.68	0.09	-0.30	0.48	0.46	0.32	No
Collection													
A & B	1008	1125	0.90	441	1124	0.39	0.64	0.50	0.47	0.54	24.95	0.00	Yes
A	614	704	0.87	307	703	0.44	0.65	0.44	0.39	0.48	17.18	0.00	Yes
B	394	421	0.94	134	421	0.32	0.63	0.62	0.57	0.67	18.53	0.00	Yes