INTERREG IVA 2 Mers Seas Zeeën Programme

SEFINS – Safeguarding the Environment From Invasive Non-native Species

Work Package 2 Report

Risk and optimal management measures for current INS and horizon species




Front cover photo credits: Arjan Gittenberger (top left; boat hull fouled with macroalgae), Jo Packet (top right; *Dreissena polymorpha*), Tim Adriaens (bottom left; *Cotula coronopifolia*), INBO (bottom right; the Scheldt and Antwerp harbour)

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This report is produced in the framework of the Cluster works, and coordinated by the INTERREG IV A 2 Seas Programme. This cluster is led by Norfolk County Council, and also gathers the partners CPIE Val d'Authie, ILVO, INBO and NVWA and the associate partners Het Zeeuwse Landschap, VLIZ, Eurisy, Natuurmonumenten, Bournemouth University, Suffolk County Council and CPIE Flandre Maritime.

SEFINS builds on the previous INTERREG projects RINSE and MEMO, which focused on invasive non-native species in the terrestrial and marine environment, respectively.

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Introduction

In comparison to terrestrial or marine environments, estuarine areas are much less well studied when it comes to introduced species. Nonetheless, estuaries in Western Europe are considered to be hubs of industrial, recreational and/or residential activities, while at the same time being of high ecological value. Combined, these elements are likely to facilitate the introduction of invasive non-native species (INS), with all of the above fields being potentially susceptible to their impacts.

Information and prevention are therefore the best ways to limit these risks and potential impacts. Thus, the SEFINS project aimed to implement tools and awareness actions, particularly to marina users, in order to consider this issue and thus reduce these impacts. This complements additional work on creating INS inventories and acquiring and sharing knowledge. In this report we showcase the different tools created by the SEFINS partnership and the actions implemented by the Partners to inform the target audience about INS.

Common definition of objectives and means

The main aim of SEFINS Phase 2 is to bring about a long-term improvement in the way INS are managed in estuaries across the 2 Seas area. This involves filling in the highest priority gaps in the current knowledge of INS in estuaries, as identified during SEFINS Phase 1, and creating a baseline of knowledge in the 2 Seas area. In order to work towards the achievement of this aim, three main objectives were outlined:

1) Complete and improve the availability and quality of data on the occurrence of INS in estuaries, and make it easier to share data across the 2 Seas area leading to a cross-border registry.

2) Identify the risks and optimal management measures for a number of existing and potential INS in estuaries across the 2 Seas.

3) Increase awareness of the impacts of INS in estuaries and the steps that can be taken to prevent their introduction and spread amongst key stakeholder groups (embedding a preventative approach) and assess the efficacy of the different approaches taken.

In order to meet this aim and deliver the project objectives, work packages were created in order to focus the efforts of the Cluster efficiently. Work Package 2 focuses on the risks and optimal management of current INS and horizon species across 2 Seas estuaries. This work package was subdivided into two stages:

- Develop and pilot cutting-edge tools for early detection of high risk INS in estuaries. A molecular ID method developed by the previous IVA 2 Seas project MEMO will be piloted on a collaborative cross-border scale in case studies across the four estuaries.

- Develop and improve management for invasive plants in estuaries. Partners will analyse risks, distributions and management options for the plant INS: Himalayan balsam, crassula, giant hogweed and buttonweed. Use of satellite imagery to map INS will be piloted in case studies.

This report summarizes the achievements and outputs of Work Package 2.

Cutting edge tools for early detection of high risk INS in Estuaries

1. Developing eDNA methods as an early warning system for estuarine INS

This work builds on the outputs of the previous IVA 2 Seas project MEMO, working in cross-border collaboration between ILVO (B) and NCC (UK) to develop and optimize an effective qualitative eDNA protocol for the detection of example INS in estuarine waters. NCC worked with Cefas (UK) as an external consultant on this project. Please view the separate report '*Developing eDNA methods as an early warning system for estuarine INS*' for a more detailed description of this work.

TECHNIQUE BACKGROUND

Traditional survey techniques for many INS rely on a combination of extensive sampling, physical examination and identification based on morphological features. This can be labour intensive, time consuming and very subjective. The use of environmental DNA (eDNA) represents an alternative approach to monitoring biodiversity and observing non-native species. Use of gene sequence data as taxonomic markers have successfully been used to identify many different species. The environmental DNA (eDNA) approach uses target nuclear and mitochondrial sequences to detect species without needing to observe, physically identify or capture individual animals. Instead, organisms are detected via their environmental secretions such as urine, faeces, mucus, saliva and epidermal cells. Any eDNA present within a water body will decompose and disappear within approximately 30 days, therefore detecting a species via its eDNA indicates it was recently present within the environment. This technique also offers several other advantages over traditional monitoring methods:

- Non-invasive method, minimizes the risks of disease transfer, stressing target species and negatively impacting a habitat;
- More sensitive and discriminatory than visual/traditional surveys methods;
- Early detection of species present in very low numbers;
- Less time consuming;
- Reduces taxonomic or observer bias;
- Detection and identification of rare, transient, difficult to find or cryptic species.

This approach could be used to supply an early warning system for horizon INS not yet present in a habitat or provide an effective means of tracking invasion and colonization. Currently eDNA is only widely used in the freshwater environment. Use of this technique in marine or estuarine habitats has yet to be fully developed and exploited. Using an eDNA approach in the marine environment is more difficult to a much higher ratio of water and biomass in seawater, in addition to increased currents, turbulence and salinity in comparison to freshwater environments.

BACKGROUND – SPECIES TESTED

eDNA analyses were performed using three species considered to be estuarine INS, which are present in some or all of the SEFINS case study estuaries.

- Pacific oyster *Crassostrea gigas*

Crassostrea gigas is native to Southeast Asia and Japan, but is now grown commercially across the world. This species was first imported to Europe when populations of the native flat oyster *Ostrea edulis* were almost lost in the harsh winter of 1962-63. It was initially thought that *C. gigas* would not be able to reproduce naturally in cold European winter water temperatures, however individuals

escaped from farms and were able to spawn and spread, leading to established wild populations. *C. gigas* is a strong competitive species, with a rapid growth rate, tolerance to changing environmental conditions and resistance to the oyster parasite *Bonamia ostreae* which affects *O. edulis*. Individuals settle in dense aggregations and exclude other intertidal species, or may form layered reefs which alter natural ecosystem state, threaten native oyster and mussel species and damage habitats. Pacific oyster shells are also very sharp, posing a hazard to human health when reefs form on previously accessible areas. Large populations of Pacific oyster are now found in the ports of Nieuwpoort, Oostende, Zeebrugge and Blankenberge, as well as in the Scheldt delta and the Wadden Sea. Within the Wash estuary it is unclear whether *C. gigas* is still present, as the population was known to have suffered from disease and only empty shells have been found in more recent surveys.

Figure 1: *C. gigas* ©Francis Kerckhof

- Japanese carpet shell *Ruditapes philippinarum*

Ruditapes philippinarum, sometimes known as Japanese carpet shell or Manila clam, is native to the Indo-Pacific region. Overfishing and poor yields of the native European clam *Ruditapes decussatus* led to the introduction of the Philippine clam into European waters. It was first imported to France and then to the UK, Portugal, Ireland, Spain and Italy (Humphreys et al., 2015). This species spread rapidly across Western Europe, invading the Oosterschelde and Veerse meer in the Netherlands. In Belgium, although *R. philippinarum* is grown commercially, distribution of the species in the wild is largely unknown although individuals have been observed in Zeebrugge and on beaches of the west coast near the 'Westhoek' in De Panne and around Koksijde. High densities of this claim can impact on the natural community composition of the seabed, however impacts at the densities typical of wild naturalized populations still need further study.

Figure 2: R. philippinarum © FAO

- Comb jelly *Mnemiopsis leidyi*

M. leidyi is native to the east coast of the Americas and is thought to have arrived in Europe via shipping, in ballast water. This ctenophore can tolerate a wide range of environmental conditions and has high growth and reproduction rates. It is a voracious predator, ingesting up to 10 times its own body weight per day and directly competes with fish larvae for food. It threatens fish stocks and is considered damaging to the structure and function of marine ecosystems. In the Black Sea, the invasion of *M. leidyi* contributed to the loss and economic collapse of pelagic fish populations. This combination of factors led to the inclusion of this species among the world's 100 worst invaders (www.issg.org/worst100_species.html).

M. leidyi was first recorded in northern Europe in 2005, in a harbour in northern France. It is now present the along the continental coasts of the Channel and North Sea, but has never been recorded in UK waters. The presence of *M. leidyi* in the 2 Seas area is concerning, as these waters contain some of the world's most important fishing grounds. The North Sea in particular is home to many commercially important fish stocks and hosts vital spawning and nursery grounds. Furthermore, mathematical modelling outputs from the previous IVa 2 Seas project MEMO suggest it is possible for *M. leidyi* populations on the continental coast to reach UK waters via natural current systems. Modelling data also suggest that conditions in the Wash estuary (on the east coast of the UK) are optimal for *M. leidyi* reproduction, due to a combination of high temperatures and food concentrations. This area is a protected European Marine Site of high ecological value and hosts multiple fisheries and aquaculture industries of great economic and cultural importance.

Figure 3*: M. leidyi* © Karl Van Ginderdeuren

AIMS OF THIS STUDY

This study aimed to build upon the outputs of MEMO, working in cross-border collaboration with RINSE Project Partner Norfolk County Council (NCC) to develop and optimize and effective qualitative eDNA protocol for the detection of some INS in estuarine waters. This technique was tested in exposure experiments for each of the three species.

PROTOCOL DEVELOPMENT (ILVO)

The first step in the development of an eDNA tool is the optimization of the molecular protocol. Most eDNA studies use polymerase chain reaction (PCR) to amplify a gene of interest within the mitochondrial genome in order to detect the presence or diversity of the target gene in a sample. This molecular marker is either cytochrome c oxidase subunit 1 (CO1) or cytochrome b (cytb), which are both species specific. After a PCR, DNA sequencing is conducted and a search is performed in a database of known sequences, such as that hosted by the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov), in order to identify the species.

- Primer development and PCR method

eDNA consists of small fragments of DNA (approximately 150 bp). The first step in this study was the development of the species specific primers needed to amplify a short fragment of DNA within the mitochondrial DNA. For each organism, species-specific molecular markers were developed which targeted a segment of the mtDNA (Table 1). Primers were designed using NCBI Primer-BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer specificity was tested by comparing the selected primer sequences to all previously published sequence data using the Basic Local Alignment Search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers were found to show good similarity with sequences available in NCBI for each of the three species of interest and no similarity with closely related species or other species likely to be present in the environment.

Table 1. Primer development

Name (direction)	Species	Sequence (5'-3')	Method, annealing temp.	Primer combination and amplicon size (bp)	Region
Rphil-EDNAcytb38F (Forward)	*Ruditapes phillipinarum*	[redacted]	PCR, 53°C	[redacted]	Cytochrome b gene
Rphil-EDNAcytb191R (Reverse)	*Ruditapes phillipinarum*	[redacted]			
Cgigas-EDNAcytb385F (Forward)	*Crassostrea gigas*	[redacted]	PCR, 50°C	[redacted]	Cytochrome b gene
Cgigas-EDNAcytb524R (Reverse)	*Crassostrea gigas*	[redacted]			
ML-EDNAcoi112F (Forward)	*Mnemiopsis leidyi*	[redacted]	PCR, 50°C	[redacted]	Cytochrome oxidase I gene
ML-EDNAcoi265R (Reverse)	*Mnemiopsis leidyi*	[redacted]			

The conventional PCR technique was optimised for the amplification using tissue samples. Amplification reactions (40 µL reaction volume) contained:

2 µL DNA
20 µL of 2X Taq Mastermix (Red Taq DNAPolymerase Mastermix 2.0 1.5 mM MgCl2, VWR)
10 µL RNAse free water (Gibco)
4 µL of primers Forward and Reverse (5 µM each)

Thermal cycle conditions were initial 95°C (10 min.) denaturation followed by 50 cycles of 95°C (45 sec.), X°C (45 sec., see Table 1), and 72°C (60 sec.). The reaction ended with an additional 7 min. extension at 72°C. As positive control, a sample of degraded tissue was included. A blank sample was included in each extraction as a negative control. PCR products were visualized using electrophoresis on 2% agarose gel. PCR products of tissue samples were sequenced in both directions by an independent laboratory (Macrogen, Amsterdam) for confirmation of the target species.

Both the primers and protocol worked well on tissue samples from all three species. PCR products were sequenced were sequenced and matched perfectly with all three target species.

- Water extraction

The second development stage tested the protocol for water extraction on seawater samples containing degraded DNA. Water was filtered over a 0.45 µm sterile filter (MO BIO Laboratories, Inc.) using a vacuum pump. DNA was extracted from the filters using protocol from the PowerWater DNA Isolation Kit (MO BIO Laboratories, Inc.). The extracted DNA was suspended in 100 µL elution buffer and concentration was determined using a NanoDrop spectrophotometer and a Quantus fluorimeter. The latter is more accurate for low DNA concentrations and was found to give better results.

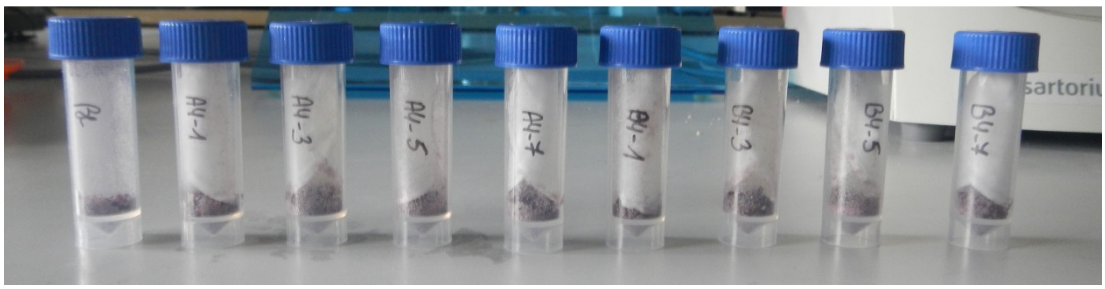

Figure 4: First step of water extraction. Filters in 5 ml tubes for bead beating.

- Exposure experiment

All DNA is vulnerable to degradation, however eDNA is particularly susceptible due to exposure to saline water, UV light and water currents. The final step in the eDNA protocol development was to test it on water samples exposed to the three different species for different amounts of time. Individuals of each species (0, 1, 5, and 10 per aquarium) were placed in tanks with approximately 15 L of filtered seawater. Four 50 ml samples were taken on days 1, 3, 5 and 7. For *M. leidyi*, 3 individuals were kept in a 10 L aquarium, with four 50 ml samples were taken on days 1, 4, 6 and 8. All filters were stored at -80°C until DNA extraction.

Figure 5: Exposure experiment.

This technique again worked well on degraded DNA samples in seawater tested during the exposure experiment). The primers and the PCR protocol developed were able to amplify DNA on all sampling days from the tanks containing 10 individuals of C. gigas and R. *philippinarum*. For *M. leidyi*, the primers and protocol were able to detect eDNA in water samples after 8 days of exposure to the species.

CONCLUSIONS AND RECOMMENDATIONS

This pilot study using a cutting edge DNA technique has shown it is possible to use a qualitative eDNA approach to detect species presence of INS in marine and estuarine water samples. This method can be used to discriminate between the presence and absence of each of the three species tested, even when they are only present in low densities such as in the early stages of invasion. This makes the eDNA protocol developed ideal for use as an early warning system for local areas and it should be tested for efficacy in 2 Seas estuaries.

2. Piloting eDNA monitoring as an early warning system in estuaries

This work builds on the outputs of the previous IVA 2 Seas project MEMO, working in cross-border collaboration between ILVO (B) and NCC (UK) to develop and optimize an effective qualitative eDNA protocol for the detection of example INS in estuarine waters. NCC worked with Cefas (UK) as an external consultant on this project. Please view the separate report '*Application of molecular techniques (eDNA) to detect Mnemiopsis leidyi in UK waters*' for a more detailed description of this work.

AIMS OF THIS STUDY

This study worked towards piloting the eDNA technique developed in the first part of this study in a 2 Seas case study estuary, in order to test its use as an INS early warning system for *M. leidyi* for the first time in marine/estuarine waters.

PROTOCOL DEVELOPMENT

The first step in applying eDNA to the natural environment is the optimization of the molecular protocol. A set of specific primers were used to target a region in the COI locus of *M. leidyi*. Conventional PCR and SYBR Green quantitative PCR (qPCR) techniques were optimised and compared for sensitivity in laboratory experiments.

- Primer specificity

Primers developed by ILVO (MnemiopsisF and MnemiopsisR) were tested against sequences available for *M. leidyi* at the National Centre for Biotechnology Information (NCBI) for specificity, using reference sequences from other ctenophores present in the same environment. Both were found to show 100% similarity with the sequences available in NCBI but none with the COI sequences from other ctenophores likely to be present in the environment. The specificity of these primers for *M. leidyi* was therefore confirmed.

- Technique sensitivity: PCR

Conventional PCR was performed with 2 sets of primers to amplify the partial and complete COI sequence, using different annealing temperatures. The optimal annealing temperature was determined to be 61^{o} C. The minimum detection level of *M. leidyi* using MemoCOI was found to be 2.6 fg of DNA.

- Technique sensitivity: qPCR

A calibration curve for qPCR was developed from a series of plasmid dilutions containing the COI sequence. The minimum detection level of *M. leidyi* using MemoCOI was found to be 0.1 fg of DNA

- Technique sensitivity: sample volume

Approximately 40 individuals of *M. leidyi* were placed in a 400 L circular aquarium filled with seawater. Water samples of different volumes (ranging from 100 ml to 1000 ml) were collected in triplicate at the inlet and outlet points. The samples were filtered through a membrane which was retained for DNA analysis. The amount of DNA extracted varied from 1.8 to 3.2 ng/µl for the inlet water and 2.6 to 4.3 ng/µl for the outlet water. There was no relationship between volume filtered and DNA recovery. All samples from the inlet water tested negative using the conventional PCR protocol regardless of the volume filtered. Positive PCR results were obtained after filtration of at least 250 ml of outlet water.

- Technique sensitivity: rate of DNA release by *M. leidyi*

18 beakers were filled with seawater and a single *M. leidyi* was placed in each. A 250 ml water sample was collected in triplicate 1, 6, 12, 18, 24 and 48 hours after adding the animal. Control water samples were collected from the same seawater supply but without the presence of *M. leidyi*. All water samples were filtered through a membrane which was retained for DNA analysis. The quantity of DNA detected was found to increase with time, with 6 times more DNA detected after 24 hours than after 1 hour. However, using conventional PCR, no DNA could be detected regardless of time, but using qPCR, DNA was detected at in each sample, and was shown to increase with time.

- Environmental testing of protocol

The Wash estuary is an embayment on the east coast of England, covering 615 km^2. Four stations within the Wash were regularly sampled between 2010 and 2014. Buoy (52°56.509, 00°19.104) is in an area permanently covered by water and the site least affected by human activity. In contrast, Toft (52°57.331, 00°08.115) and Wreck (52°52.500, 00°13.100) are both located above mussel or cockle beds. Stylemans (52°52.833, 00°23.512) is close to the mouth of the River Great Ouse and the international port of Kings Lynn, and therefore potentially the site most affected by human activity.

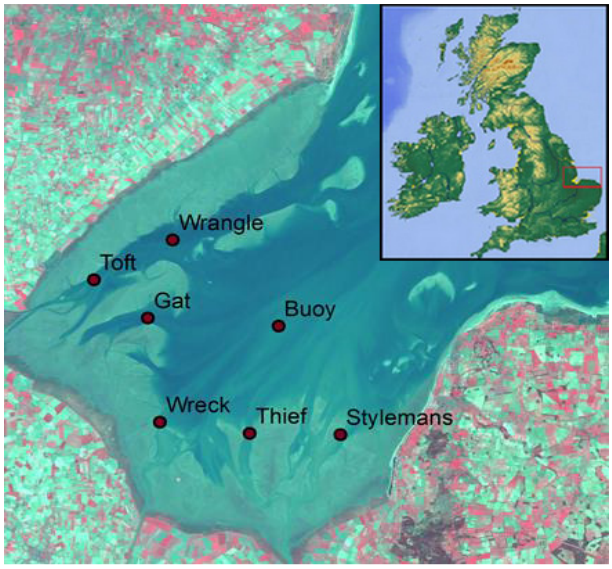

Figure 6: Sampling locations in the Wash

Applying conventional PCR protocol, we did not see any positive amplification in samples from 2010 to 2013. In contrast, samples from March, April, July and September 2014 showed a positive amplification for all stations. In October 2014, positive amplifications were seen at Buoy and Wreck. Samples were sequenced and the presence of *M. leidyi* in the Wash during 2014 was confirmed. The results were independently confirmed by a separate laboratory at ILVO (Belgium).

CONCLUSIONS AND RECOMMENDATIONS

This study showed *M. leidyi* was present in the Wash estuary during 2014. This is the first time this species has been detected in UK waters and confirms the results generated by MEMO which identified the Wash as a likely region for this ctenophore to settle and begin to spread. The presence of this ctenophore is likely to negatively impact both ecosystem function and economic activity in this area.
Once invasive species become established, it is difficult and costly to control and eradicate them. The Water Framework Directive (WFD) recommends eradication if it is feasible and cost effective. This must also be balanced against further damage to habitats and species through the removal process itself. The ability to detect INS in the first stage of invasion before they can be detected by traditional survey techniques provides a huge advantage in the eradication process. This work has demonstrated that the eDNA approach can be effectively applied to the very early detection of *M. leidyi* in coastal waters.

Previous successful studies using eDNA targeted aquatic or semi-aquatic freshwater species such as great crested newt. This pilot trial using eDNA to detect *M. leidyi* shows that it is possible to detect

very low quantities of DNA in seawater and consequently detect *M. leidyi* in the Wash even at low abundance. According to previous studies, persistence of eDNA in seawater (above detection thresholds) is only few days. This offers a further advantage of the technique in confirming the recent presence of a species.

There is great potential to use eDNA analysis in widespread standardised monitoring programmes. Although this technique uses cutting-edge technology, it is based on standardised molecular techniques and could be easily automated for wider stakeholder use in the future, through the development of dipsticks or similar devices. This would greatly reduce future monitoring and eradication costs for governments and offshore industries across the 2 Seas area, whilst assisting stakeholders to comply with EU Regulations and Directives.

3. Piloting novel monitoring approaches in 2 Seas estuaries

This work was commissioned by NVWA and conducted by NIOZ (external consultant) in cooperation with NCC, INBO and CPIE, in order to facilitate cross-border development of novel monitoring approaches across 2 Seas estuaries. Please view the separate reports '*Non-indigenous species inventory of estuarine intertidal areas: a comparison of estuaries and habitats using hard substrate transect methodology*' and '*SETL-plate inventory in estuaries in France, United Kingdom, Belgium and the Netherlands in April-June 2015*' for more detailed descriptions of this work.

BACKGROUND

The SEFINS cluster has identified a gap in estuarine INS knowledge and also in the monitoring efforts in place to identify the presence, developments, distributions and impacts of INS in 2 Seas estuaries. Hard substrates are the least investigated environment within the case study estuaries, as revealed by work conducted during Phase 1 and within Work Package 1 of Phase 2. However, hard substrates are likely to be the habitats that harbor a large proportion of INS. This is concerning as rapid expansions of hard substrates such as dams, dikes, foreshores, piles, machinery, boats and nets is currently ongoing in Western Europe. Natural areas of hard substrate are often rare. These artificial habitats represent an open niche waiting to be filled by opportunistic INS and are often close to sources of introduction, such as ports, marinas and aquaculture facilities. Furthermore, INS are often rapid colonizers of new (bare) substrate. It is therefore important to develop and implement early warning monitoring techniques to ensure any new INS arriving in an area can be tackled before they become established.

AIMS OF THIS STUDY

This study aimed to test low technology, cost effective approaches to INS monitoring of understudied hard substrates in estuaries, allowing information on INS presence and distribution at each estuary to be gathered and compared. Pilot studies testing two different approaches were conducted in estuaries across the 2 Seas area, including both Dutch and Belgian areas of the Scheldt, the Canche (France) and the Wash (UK). The methodology aimed to be widely applicable and easy to carry out with limited cost and effort, providing a useful tool for local stakeholders. These techniques built on earlier experiences and aimed to generate data which can be compared with inventories in other regions.

MONITORING

NVWA facilitated the SEFINS monitoring campaigns for invasive non-native species on hard substrates using SETL-plates and transect survey techniques. Neither of these methods had previously been applied to estuaries within the 2 Seas area. The sampling locations within each of the three estuaries were discussed at a SEFINS progress meeting (Ostend, 14 April 2015).

- Transect monitoring

This methodology was based on the internationally applied EMBOS (pan-European Marine Biodiversity Observatory System) protocol for hard substrate monitoring, designed to compare entire communities on a European scale in a standardized way. The EMBOS protocol was modified and refined to make it easier to perform, more suitable for detection of INS and maximize cost-effort efficiency. At each site, 2 transects were performed with 9 quadrats analyzed per transect. Each quadrat was inventoried for total and separate coverage of the 3D surface by flora and fauna.

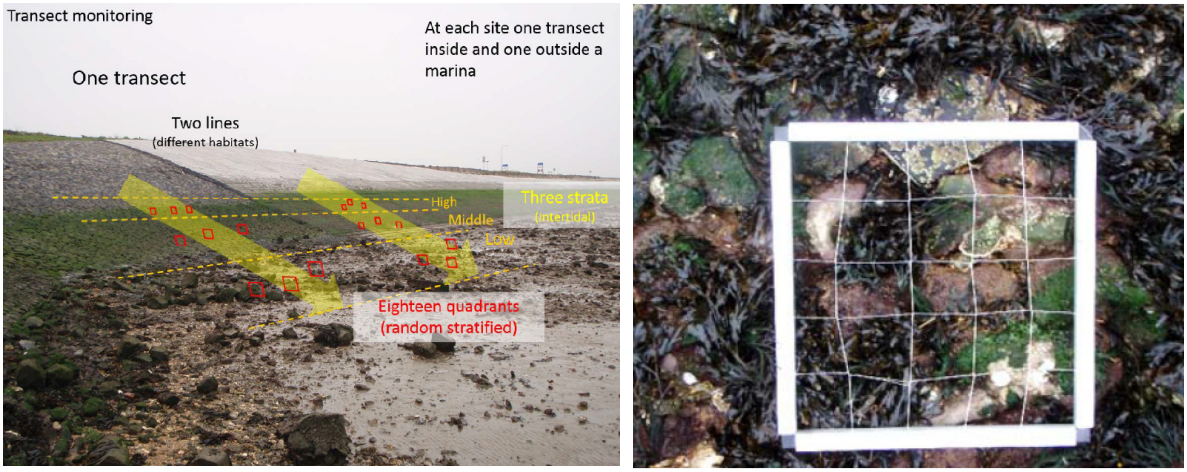

Figure 7. Example of a transect showing the ideal positioning of lines and strata and the random positioning of 18 quadrats and a randomly placed quadrat ready to be inventoried. The wiring helps to estimate percentage coverage of flora and fauna.

Transects in the Wash estuaries were performed by NIOZ with NCC and EIFCA. Transects in the Canche were performed by NIOZ with CPIE. Within the Scheldt estuary, three sites in the Dutch Western Scheldt at Breskens, Terneuzen and Hansweert were surveyed by NIOZ. A further two sites in the Belgian Zeeschelde at Doel and Wintam were surveyed by INBO. This collaboration between Partners, Associate Partners and Consultants ensured maximum transfer of knowledge and expertise to and from each local area. Transect monitoring focused on potential differences along the estuarine gradient and within and outside marinas in order to allow comparison with SETL-plate monitoring and to make the first steps towards estuary comparisons.

A total of 10 non-indigenous species were found out of the 126 species recorded during transect monitoring. Each of the 10 INS detected were present in the Scheldt estuary, however in both the Canche and the Wash, only 1 INS was found.

Overall, New Zealand barnacle *Austrominius modestus*, Japanese oyster *Crassostrea gigas* and Brush-clawed shore crab *Hemigrapsus takanoi* were most common INS with a wide distribution throughout a variety of habitats. The gammarid *Melita nitida* was found to extend from the mesohaline to the

entire polyhaline zone, which was previously unknown. It was also revealed that population expansions of *M. nitida* negatively impact populations of native *Melita palmata*. Similarly, *C. gigas* and *H. takanoi* were also found to strongly influence distributions of the native blue mussel *Mytilus edulis* and the common shore crab *Carcinus maenas*. Comparison of the results from the different estuaries indicates a movement of *C. maenas* to low salinity regions in the presence of *H. takanoi*. The orange striped anemone *Diadumene lineata* showed a significant eastwards range extension in the Western Schedlt, which was not expected as it was thought water column turbidity was too high. *A. modestus* was the only INS observed in both the Wash and the Canche estuary. This species was by far the dominant barnacle in each of the three case study estuaries. With the exception of the Japanese shore crab *Hemigrapsus sanguineus*, all other INS (quagga mussel *Dreissena bugensis*, zebra mussel *Dreissena polymorpha*, Chinese mitten crab *Eriocheir sinensis* and amphipod *Incisocalliope aestuarius*) were only observed in the Belgian part of the Scheldt.

One of the striking patterns emerging from this pilot study is the lower number of INS present inside marinas in comparison to outside, in accordance with higher species richness outside of marinas. This indicates that marinas are likely to be hotspots of INS introductions but that more INS eventually settle in the higher quality environments surrounding them.

As a result of this pilot study, the SEFINS Cluster therefore recommends establishment of transect monitoring combined with hard substrate habitat mapping to monitor INS presence and population developments. This should cover entire estuaries (e.g. the Scheldt estuary) with recurrent (once every few years) visits but limited yearly efforts. This type of monitoring program would be very valuable in supporting and evaluating the management of estuarine systems and could also function as an early-warning system, safeguarding local economies and resources against the arrival of new horizon species.

- Settlement (SETL) plates

SETL-plate methodology, including plate material, size and deployment depth was derived from studies on marine fouling communities in New Zealand, America and Europe. The technique was trialled in the Netherlands in 2006/2007 and since then has continued to be used as a monitoring coastal system, with plates checked for fouling species every three months. This technique has never been used in the other 2 Seas estuaries or to compare species composition on a cross-border basis.

SETL-plates consist of a 14 x 14 cm grey PVC plates attached to a brick which is tied to a rope (Figure 8). These are then attached to either floating or fixed structures (e.g. pontoons or harbour walls) and submersed 1 m below the water level (Figure 8). After 3 months, the plates are retrieved and photographed to document anything found to be growing on them. The photographs are then digitally divided into grids in order to analyse species presence/absence and abundance (Figure 9).

Figure 8. A SETL-plate and deployment of SETL-plates in the Wash estuary UK.

Figure 9. Overview photographs of 3 plates digitally divided into grids

SETL-plates were deployed for the first time at 9 sites in the UK, France and Belgium. A further 14 sites in the Netherlands were included, where SETL-plates have been deployed and checked for species almost continuously every 3 months since 2006.

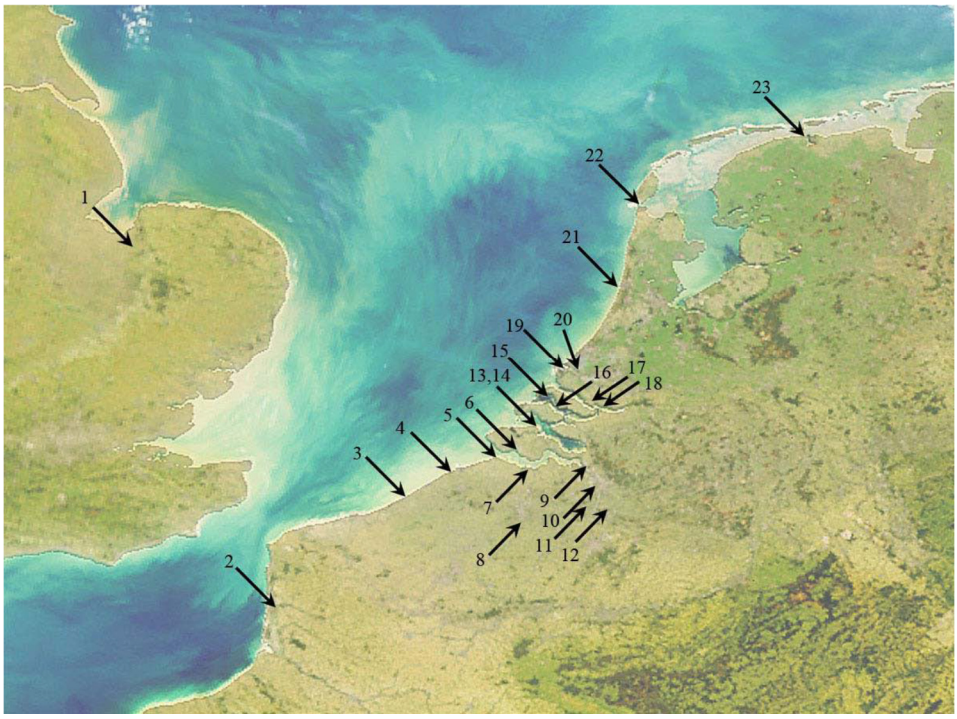

Figure 10. Sites along the NW European coast where SETL-plates have been deployed from March-April 2015 to June 2015.

This deployment period of approximately three months was found to be unsuitable to record any fouling species in these more freshwater estuarine systems (i.e. with salinities < 1 ppt) as all plates across the 23 sites were still empty in June. At sites with higher salinities, the INS New Zealand barnacle *Austrominius modestus* was recorded in addition to a selection of native fouling species. Other non-native species recorded included the gastropod *Crepidula fornicata,* the ascidians *Molgula* cf *manhattensis* and *Botrylloides violaceus*, the amphipod *Jassa marmorata* and the barnacle *Amphibalanus improvises*.

The species communities recorded on each plate within a site were similar to each other, but different to the communities recorded at other sites. Comparison of the results of the 2015 survey with historical data from Dutch SETL-plates (which have been deployed for longer than 3 months) showed that a much higher number of INS is likely to be found if plates are deployed for longer time periods and checked at different times of year. This is true for both freshwater and more saline estuarine areas. The plates will be rechecked again in September to check whether a larger diversity of INS can be recorded by this technique.

From the data collected in this cross-border pilot study, it can be concluded that a three month deployment period is not sufficient to accurately assess communities of fouling species in Two Seas estuaries. For the SETL technique to become more successful in recording INS in Europe, it is recommended that plates are left submerged for longer than 3 months (ideally 6 months) and that deployment should occur over each of the four seasons of the year.

CONCLUSIONS AND RECOMMENDATIONS

Both of the hard substrate monitoring methodologies piloted in this Work Package appear to be highly complementary as only one INS (New Zealand barnacle) was detected by both techniques. The SETL-plate approach is an efficient and standardised way of detecting the presence of hard substrate related INS with larvae in the water column and is therefore particularly suited to detecting groups such as sea

squirts, barnacles and moss animals. Monitoring can be maintained for relatively little effort, although for optimal results it essential that plates in several successional stages are present and that they are submerged for sufficient time to cover spawning and settlement stages of species with planktonic life stages. They provide a good indication of competitive differences between species, invasiveness and INS fouling potential, whilst also allowing site/system comparison. Careful selections of deployment site will also result in monitoring representative of large areas of an estuary.

The transect monitoring approach is an efficient and standardised means of detecting and monitoring the actual distribution and abundance/dominance of intertidal hard substrate INS, such as seaweeds, crabs, shrimps, sea snails, bivalves and barnacles. Transect monitoring is ideal for identifying INS habitat, monitoring population developments, range extensions, competition and potential impacts of INS. This technique also allows comparisons of different sites/systems and potential upscaling to larger areas. As the methodology covers the range of available substrates and habitats, leading to an extensive list of species, efforts to maintain this type of monitoring are moderate. However, focusing on certain habitats or species of concern can greatly reduce this effort.
It is recommended to implement a monitoring program in Two Seas areas estuaries consisting of at least a limited number of SETL-plates (with recurrent refreshing of a few of them) at potential hot spots. These would function as an early warning, combined with a limited number of transects visited once every few years (rotation of sites) through dominant habitat types to monitor major shifts in developments, range extensions and impacts of NIS in these systems.

Develop and improve management for invasive plants in 2 Seas Estuaries

1. Distribution analysis of 5 invasive plant species in the Netherlands

NVWA contributed to an improved risk management for invasive plants in estuaries using expertise and performing field studies to record presence and abundance of targeted species, as well as providing management advice on trials to control the target species. This was performed in close collaboration with INBO and Dutch Associate Partners and demonstrated to local stakeholders during a Workshop. This work covered five invasive non-native plant species: buttonweed *Cotula coronopifolia*; crassula *Crassula helmsii*; giant hogweed *Heracleum mantegazzianum*; Himalayan balsam *Impatiens glandulifera* and water primrose *Ludwigia peploides*. These represent species that arrived in the Netherlands over 150 years ago, as well as those that have arrived more recently. Spread of these species in the Netherlands and in particular within the Two Seas region, is a more recent phenomenon. Himalayan balsam only arrived in the Two Seas region in 1955, whilst spread of giant hogweed only began after 1955.

Figure 11. Historic development of Himalayan balsam in the Netherlands. From top left to bottom right: 1991-1995; 1996-2000; 2001-2005; 2006-2010; 2011-2014.

The comparative study on the dynamic presence of Himalayan balsam in the Biesbosch area and the Scheldt estuary proved to be more complex than anticipated and will be continued beyond the scope of this project. Buttonweed was first recorded in the Two Seas area in 1985 and started to increase in 2005. For this species we also noted a shift from brackish and temporarily inundated areas to truly freshwater habitats. This new data will be published in a vegetation science paper in the near future and will be disseminated via the SEFINS webpage.

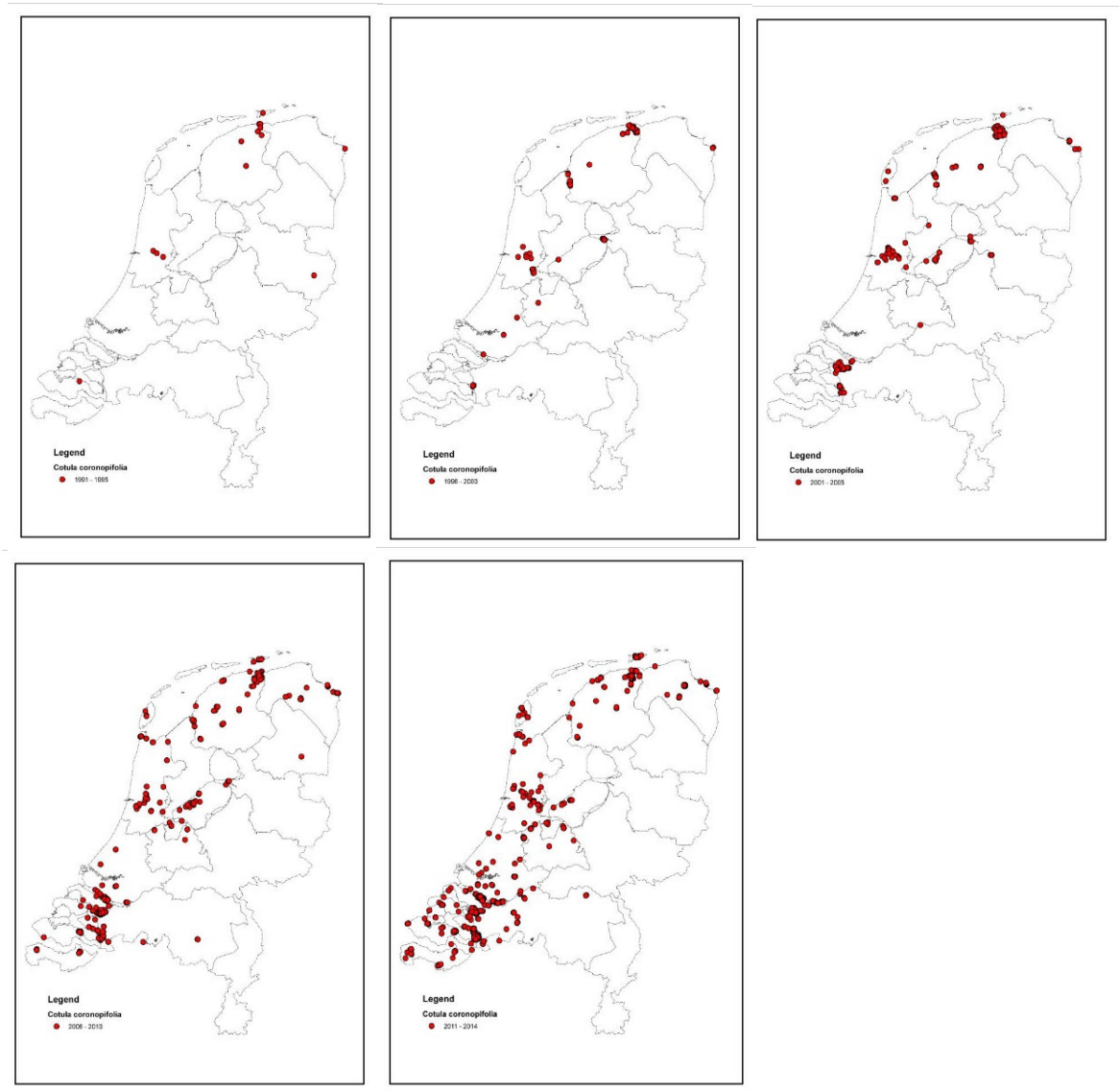

Figure 12. Historic development of Buttonweed in the Netherlands. From top left to bottom right: 1991-1995; 1996-2000; 2001-2005; 2006-2010; 2011-2014.

Crassula was first recorded in the Netherlands in 1995, spreading quickly across the Two Seas region in a range of habitats.

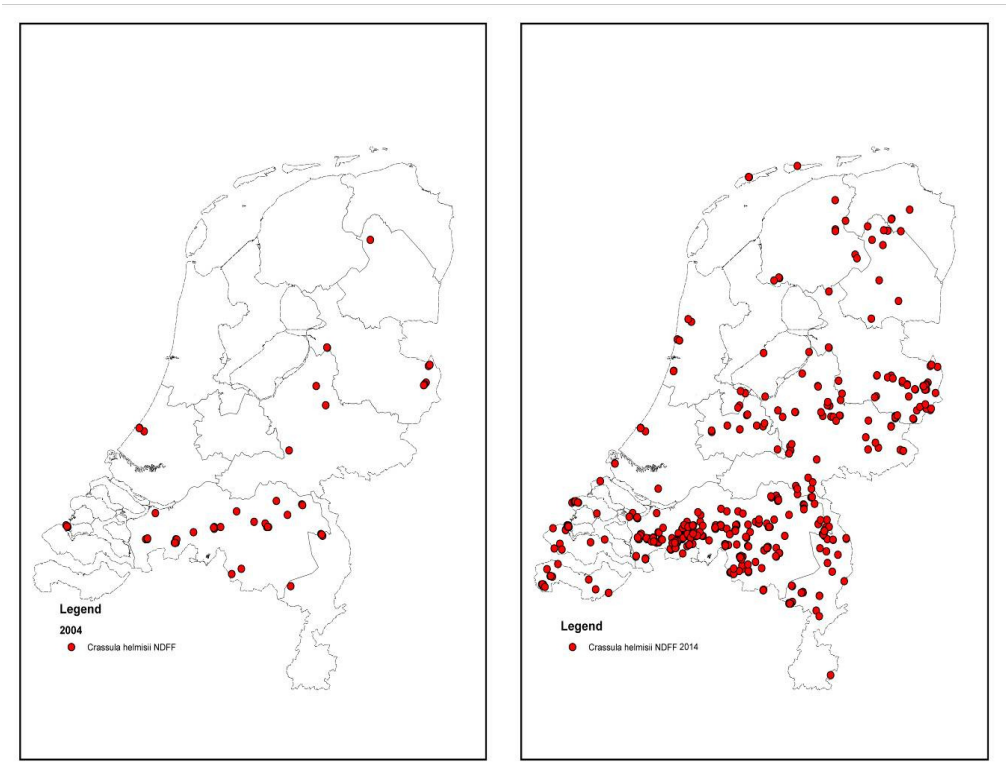

Figure 13. Historic development of Crassula in the Netherlands. From left to right: 2004; 2014.

Water primrose first appeared in 2012 and a range of management strategies are continuously being adapted by Natuurmonumenten and NVWA in an attempt to mitigate its presence at Tiengemeten.

2. The spatio-temporal dynamics of Himalayan balsam within the Scheldt Estuary

This work was conducted by INBO in association with NVWA and presented in the form of a poster.

Himalayan balsam is an annual plant species that is native to East Asia and was introduced into Europe as a garden ornamental. Since the mid-20th century, the species started to become widely established in the wild. Here, we use relevé data of 85 permanent plots from the tidal freshwater zone of the Scheldt and its tributaries to showcase the species' spatio-temporal trends, and try to explain its occurrence as a function of local habitat factors.

Himalayan balsam has become ever more ubiquitous in the freshwater zone over the last 20 years. The relevés show both the downstream colonization of the species, as well as the local expansion along the lower Scheldt's mid-course. It is the single most recorded plant species over all years, and now occurs in about 90% of the permanent plots.

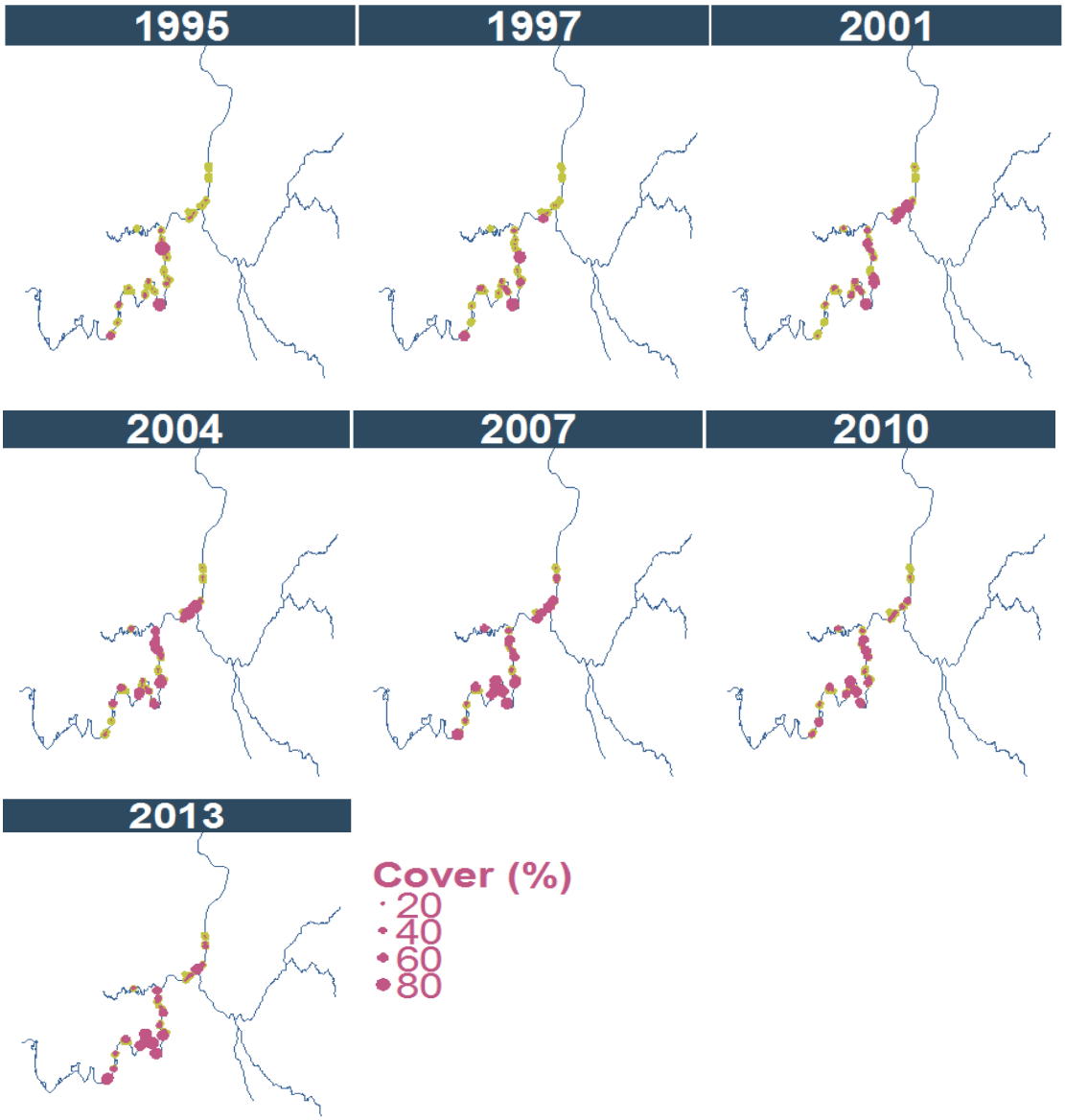

Figure 14. Location of the permanent plots along the Scheldt and Durme (in green, n=85), and the cover of *I. glandulifera* throug time (purple).

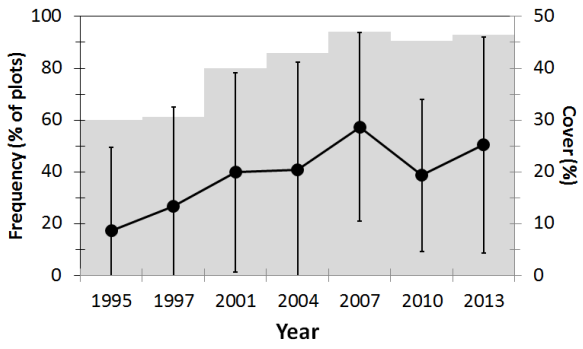

Figure 15. Frequency (bars) and mean cover (circles, ± st.dev.) of Himalayan balsam through time.

The species occurs in various types of vegetation, yet is found most optimally in roughened vegetation characteristic for later-successional stages, determined by sediment accretion and related hydrological change. The species' overall increase does not appear to be univocally linked to directional trends in the cover of the other dominant and ecologically related species *Phragmites australis*, *Urtica dioica* and *Epilobium hirsutum.* However, its marked increase between 2004 and 2007 did coincide with a drop in the cover of *P. australis* and *E. hirsutum*. It is unclear whether the observed trends represent mutual competition among species, patterns of succession (above), or both.

We tested for explanatory site factors by means of fuzzy set ordination, which is particularly suited for testing multivariate data against specific hypothesized relations. Of all hydrological variables included, the best results were found for inundation frequency as derived from topographical data. When looking into detail, Himalayan balsam tends to be typical for plots with a low inundation frequency or plots that become frequently inundated yet harbour a vegetation that is indicative for drier conditions. We suggest this is due to local drainage of the water received, with the species profiting from well-drained conditions. This latter finding corroborates the apparent observation that Himalayan balsam is relatively under-represented in newly established inundation areas that have a controlled reduced tide, and thus less extreme drainage conditions (cf. Sigma plan). Such restoration projects may thus prove particularly succesful in breaking local dominance of the species. As similar long-term plot vegetation data is available from tidal zones along the Dutch Meuse (e.g. The Biesbosch), these tests may become replicated as to further investigate the occurrence and effects of Himalayan balsam invasion in riverine habitats.

3. Do *Crassula helmsii* populations produce viable seeds in the Two Seas area?

This work was conducted by INBO in association with NVWA. For more details please view '*Invasive Crassula helmsii populations produce viable seeds throughout Western Europe*', a paper currently submitted to the Journal of Aquatic Invasions and which will subsequently be available on the SEFINS website.

The amphibious plant species *Crassula helmsii* is a widely established and still-spreading alien in various parts of Europe, where it is considered invasive as its dense swards stress the viability of local biota. The species was considered to exclusively reproduce through vegetative means, until *ex situ* germination was recorded from a single locality in Belgium.

We assessed whether this seed viability holds on a wider scale, by testing 16 populations from The Netherlands, Belgium, northern France and eastern England in a greenhouse germination experiment. Seedlings were observed from all populations but two, and from each of the five countries. Although most fruits were lacking seeds and the inferred germination percentages were overall low, germinable seed numbers are considerable given the high density of flowering stems. An *in situ* test revealed seeds to make it through normal winter conditions without signs of physical damage and with retention of germinability. Our results suggest that reproduction by seed is a relatively cryptic but widespread phenomenon throughout Western Europe. The persistency of seed banks requires further investigation. Nonetheless, these findings already challenge the efficacy of techniques currently applied in *C. helmsii* control.

Conclusion

The aims and actions of Work Package 2 listed in the application form were completed during the lifetime of the project, despite the short implementation time. However, some of the INS questions and issues tackled will require further work to continue to bridge the gaps in INS knowledge and management in estuaries. The Project Partners will continue to work together after the life of this project to build on the ground breaking work already carried out and ensure 2 Seas estuaries receive maximum benefits from the SEFINS Cluster.